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Prediction of antibiotic mass flows in urban catchments and their environmental prioritization

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Abstract

Urban emissions of antibiotics into the environment have the potential to adversely affect terrestrial and aquatic organisms. Developed standardized test methods allow the quantification of the resulting ecotoxicological risk, which strongly relies on a comprehensive situation analysis by predicting or measuring a representative antibiotic concentration of interest. Predicting the input loads of antibiotics to wastewater treatment plants using secondary input data (e.g. prescriptions) is a reasonable method if no analytical data is available. The absence of such data poses the question of an aquired reasonable sample quantity to capture local seasonal differences in prescriptions as well as flow conditions within the catchment area. Both, the theoretical and measurement based determination of environmental concentrations have been scarcely verified in practice. Hence, high resolution prescription data in combination with an extensive monitoring campaign at the wastewater treatment plant Dresden-Kaditz (WWTP) were used as a basis to evaluate the reliability of predicting and measuring urban antibiotic emissions.

As expected, the recovery of antibiotic input loads strongly varies among substances. The group of macrolides as well as sulfamethoxazole and trimethoprim were almost fully recovered whereas nearly all substances of the beta-lactam family exhibit high elimination rates during the wastewater transport in the sewer system. Yet other antibiotics (e.g. fluoroquinolones) show distinct fluctuations through the year, which was not obvious from relatively constant prescriptions. The latter substances are an example that available data are not *per se* sufficient to predict the actual release into the environment which, in certain cases, emphasizes the necessity of adequate measuring campaings. The extensive data pool of this study was hence used to calculate the necessary number of samples to determine a representative annual mean load to the WWTP. Based on the applied approach, a minimum number of 20 to 40 samples per year is proposed to reasonably estimate a representative annual input load of antibiotics and other micropollutants. Regarding the WWTP, the mass flow analysis revealed that macrolides, clindamycin/clindamycin-sulfoxide and trimethoprim were mainly released with the effluent, while penicillins, cephalosporins as well as sulfamethoxazole were partly degraded in the studied WWTP. Levofloxacin and ciprofloxacin are the only antibiotics under investigation with a significant mass fraction bound to primary, excess and digested sludge. In this context, the sludge concentrations are considered to be highly inconsistent which leads to questionable results. It remains unclear whether the inconsistencies are due to insufficiencies in sampling and/or analytical determination or if the fluctuations can be considered reasonable for digesters.

Subsequently, verified antibiotic loads were evaluated regarding their ecotoxicological effects in the aquatic environment. Two approaches were applied (1) to address the ecological impact on individual trophic levels algae, daphnia and fish, and (2) to assess the possible synergistic potential of antibiotic combinations. Ciprofloxacin, levofloxacin and the group of cephalosporins showed to significantly affect the aquatic environment. They either have the highest impact on (one

of) the lowest trophic level(s) or disproportionately increase the ecotoxicological risk due to their synergistic characteristics. In this regard, the deficiencies regarding the input prediction of these antibiotics is of particular concern. The underestimation of such critical mass flow conditions weakens the approach of assessing environmental risks on the basis of secondary data like prescriptions. Hence, efforts must be made to further develop the projection model by improving the quality of secondary data, identifying additional emitters and understanding possible retention and degradation dynamics of antibiotics within the sewer system.

Keywords: antibiotics, input prediction, mass flow analysis, risk assessment, synergism

Zusammenfassung

In der Humanmedizin eingesetzte Antibiotika werden im menschlichen Körper nicht vollständig metabolisiert und gelangen über die Ausscheidungen in das kommunale Abwasser. In der Kläranlage erfolgt nur eine unvollständige Elimination dieser Stoffe, so dass der Kläranlagenablauf einen Hot Spot für Antibiotikaemissionen in die Umwelt darstellt. Das induzierte ökotoxikologische Risiko kann anhand standardisierter Testverfahren und allgemein anerkannter Bewertungsansätze für Einzelsubstanzen abgeschätzt werden. Erfolgt jedoch die Betrachtung von Antibiotikagemischen, wie es für den gereinigten Ablauf einer Kläranlage sinnvoll ist, sind aufgrund zumeist unspezifischer Wirkmechanismen und dem Mangel an repräsentativen Daten eine Reihe von Vereinfachungen und Annahmen zu treffen. Es besteht in der Folge die Gefahr einer Unterschätzung des durch Substanzgemische hervorgerufenen ökotoxikologischen Risikos. Eine vielversprechende Möglichkeit den Entscheidungsprozess über mögliche Vermeidungs- und Eliminationsmaßnahmen zu unterstützen besteht in der Priorisierung von Antibiotika entsprechend ihres Effektpotentials. Hierbei sind Substanzen zu identifizieren, die den größten Einfluss auf die Nahrungskette im Gewässer bzw. das höchste (negative) Synergiepotential mit anderen Substanzen aufweisen. Die Verringerung dieser Substanzen führt zu einer hohen ökologischen Effektivität und Effizienz der eingesetzten Mittel.

Wie im Fall des klassischen Bewertungsansatzes, ist auch für den Priorisierungsansatz eine umfängliche und zuverlässige Situationsanalyse die Grundvoraussetzung für verwertbare Ergebnisse. Die Situationsanalyse beruht auf der analytischen Bestimmung bzw. der Abschätzung von emittierten Antibiotikafrachten zur Berechnung von repräsentativen Umweltkonzentrationen. Analytisch ermittelte Umweltkonzentrationen vieler Antibiotika weisen aufgrund saisonaler Verschreibungsmuster eine hohe zeitliche und räumliche Variabilität auf. Die für eine adäquate Erfassung der Situation notwendigen Messkampagnen sind kostenintensiv, wobei die tatsächlich notwendige Häufigkeit der Probenahme von zumeist nicht hinreichend bekannten substanzspezifischen Informationen, wie der chemischen Stabilität im Rohabwasser und der saisonal beeinflussten Applikation, abhängt. Alternativ können Antibiotikaeinträge in die Kanalisation anhand von Verschreibungsdaten abgeschätzt und mit Hilfe von Stoffflussanalysen (SFA) zur ökotoxikologischen Bewertung herangezogen werden. Eine vom Umfang befriedigende, direkte Gegenüberstellung von prognostizierten und analytisch ermittelten Frachten ist bisher jedoch nicht erfolgt, so dass die Verifizierung dieses Ansatzes noch aussteht. Für den Fall einer bestehenden Verschreibungspflicht für Antibiotika besitzen Verschreibungsdaten eine vergleichsweise hohe zeitliche und räumliche Informationsgüte. In Verbindung mit einer an diese Datenqualität angepassten Messkampagne, ergibt sich die Möglichkeit einer detaillierten SFA mit substanzspezifischer Bewertung der Eignung des Prognoseansatzes.

Die am Beispiel der Stadt Dresden durchgeführte Bewertung des Prognoseansatzes fußt auf einer 15-monatigen Messkampagne und den für das Einzugsgebiet der Zentralkläranlage Dresden-Kaditz verfügbaren Verschreibungsdaten der AOK PLUS. Erwartungsgemäß ergibt der Abgleich von erwarteten und analytisch ermittelten Frachten eine starke Variation der für den Zulauf der Kläranlage ermittelten Wiederfindungsdaten verschiedener Substanzen. Die analytisch ermittelten Frachten von Sulfamethoxazol, Trimethoprim sowie der Gruppe der Makrolid-Antibiotika entsprechen nahezu den prognostizierten Mengen. Die Beta-Laktam-Antibiotika unterliegen bereits während des Abwassertransports einer umfänglichen, zumeist biologisch bedingten, Elimination, was zu hohen Unterbefunden im Zulauf der Kläranlage führt. Andere Substanzen hingegen (z.B. Fluorchinolone) weisen messtechnisch eine signifikante Jahresdynamik auf, die aufgrund der weitgehend konstanten Verschreibung in dieser Ausprägung nicht zu erwarten ist. Die Auswertung zuletzt genannter Substanzen zeigt deutlich, dass die Nutzung von Verschreibungsdaten nicht per se ausreicht, um die Emission von Antibiotika (und anderer Pharmazeutika) sowie die sich daraus ergebenden Umweltkonzentrationen mit ausreichender Sicherheit prognostizieren zu können. Für eine nachgelagerte ökotoxikologische Bewertung ist in diesen Fällen die Durchführung von Messungen unumgänglich. Zur effizienten Planung derartiger Kampagnen wurde der umfassende Datenpool dieser Studie hinsichtlich der erforderlichen Probenanzahl zur Bestimmung einer repräsentativen mittleren Jahresfracht ausgewertet. Es ergibt sich ein Minimum von 20 bis 40 homogen über das Jahr verteilten Proben, um die jährlich in die Kläranlage eingetragene Fracht an Antibiotika bzw. anderer Mikroschadstoffe mit ausreichender Sicherheit abschätzen zu können.

Im Rahmen der SFA in der Kläranlage Dresden-Kaditz wird deutlich, dass Makrolide, Clindamycin und dessen Humanmetabolit Clindamycin-Sulfoxid sowie Trimethoprim in der nahezu keiner Elimination unterliegen, wohingegen Penizilline, Cefalosporine und auch Sulfamethoxazol teilweise bis vollständig abgebaut werden. Mit Levofloxacin und Ciprofloxacin handelt es sich um die einzigen untersuchten Antibiotika, welche zu einem signifikanten Massenanteil an Primär-, Überschuss- und Faulschlamm gebunden vorgefunden werden. Aufgrund der hohen Relevanz dieses Eliminationspfades für die zuvor genannten Antibiotika bedarf die Beobachtung von z. T. widersprüchlichen Schwankungen einer kritischen Betrachtung der Ergebnisse. Es ist nicht abschließend geklärt, ob die beobachteten Fluktuationen auf eine unzureichende Qualität der Probenahme und/oder der Analytik zurückzuführen sind oder sich die Schwankungen in einem für Faulbehälter tolerierbaren Bereich befinden.

Im Anschluss an die verifizierten Antibiotikaemissionen erfolgte die Priorisierung der betrachteten Antibiotika nach ihrem ökotoxikologischen Effektpotential. Zum einen wurde der ökologische Einfluss auf verschiedene, die Nahrungskette bildende trophische Ebenen (Alge, Daphnie, Fisch) untersucht. In Anlehnung an die humanmedizinische Kombinationstherapie erfolgte im zweiten Ansatz die

Beurteilung der Antibiotika hinsichtlich ihres möglichen Potentials zur Verstärkung von negativen Effekten durch das gleichzeitige Auftreten mit anderen Substanzen. Für Ciprofloxacin, Levofloxacin und die Gruppen der Makrolide und Cefalosporine konnten signifikante Beeinträchtigungen der aquatischen Umwelt nachgewiesen werden. Diese Stoffe und Stoffgruppen führten im Rahmen der untersuchten Substanzen entweder zur höchsten Schadwirkung gegenüber der niedrigsten trophischen Ebene oder besitzen das höchste Synergiepotential in Kombination mit anderen Substanzen.

Die Auswertung der SFA bestätigt die grundsätzliche Eignung der Verschreibungsdaten sowie des entwickelten Prognosemodells zur Vorhersage von Antibiotikaemissionen im urbanen Raum. Die Stoffflussanalyse stellt somit ein strategisches, im Vergleich zur Messung kostengünstiges Instrument zur Identifikation von Hot Spots der Antibiotikaemission dar und erleichtert die Entscheidungsfindung für monetär aufwendige Reduktionsmaßnahmen am Ort der Entstehung oder in der Kläranlage (z.B. 4. Reinigungsstufe). Die Vorgehensweise zur Priorisierung von Substanzen hinsichtlich ihres ökotoxikologischen Effektpotentials eignet sich sehr gut, Antibiotika mit dem höchsten Schadpotential zu identifizieren. Die Verschneidung der Kenntnis dieser Substanzen mit den Ergebnissen der SFA macht deutlich, dass mit Ausnahme der Makrolide, alle ökotoxikologisch priorisierten Antibiotika eine mangelhafte Prognosefähigkeit aufweisen. Die unvollständige Abbildung kritischer Stoffströme, wie z.B. Frachtspitzen, führt insbesondere im Fall der ökotoxikologisch priorisierten Substanzen zu einer Minderung der Aussagekraft des auf Verschreibungsdaten beruhenden Prognoseansatzes. An diesem Punkt ist in zukünftigen Betrachtungen anzusetzen, um die Qualität von Verschreibungsdaten zu verbessern, potentiell nicht erfasste Emittenten in die Betrachtungen einzubeziehen, sowie die Dynamik der Rückhalte- und Eliminationsprozesse in der Kanalisation adäquat beschreiben zu können. Die ergänzende Betrachtung weiterer Anlagentechnologien (z.B. Festbettreaktoren) kann zur Bestätigung der am Beispiel der Kläranlage Dresden-Kaditz gewonnenen Ergebnisse beitragen bzw. Unterschiede bei der Elimination von Antibiotika das Potential, die Problematik der Antibiotika und anderer Mikroschadstoffe bereits während der Planung von Abwasseranlagen berücksichtigen zu können.

Schlagwörter: Antibiotika, Zulaufprognose, Stoffflussanalyse, Risikobewertung, Synergie

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Introduction

Introduction

SCOPE

Many infectious diseases are treated or prevented by using antibiotics, which are an important group of pharmaceuticals in today's human and veterinary medicine. Although there is no real alternative to substitute their specific effect regarding bacterial infections, the extensive use of antibiotics must be related to two major concerns. First, they are known to promote the emergence of resistances and studies suggest an increase of resistance-related deaths in Europe from 23'000 in 2015 to 400'000 in 2050, if no action is taken (Meyer 2015). Hence, the increasingly compromised ability to treat infectious diseases is considered to be the strongest concern of human future (BIO 2013). Reasons for the vast spread of resistances can be found in poor hygiene and prescribing practice, self-medication in countries with freely available antibiotics, non-compliance as well as a lack of uniform European, not to mention international, regulations (WHO 2015b). According to current knowledge, increasing consumption of antibiotics proportionally affects the probability of clinical resistances (ECDC/EFSA/EMA 2015, Rodriguez-Mozaz et al. 2015). As a consequence, the World Health Organization (WHO) has brought the draft "global action plan on antimicrobial resistance" into being to prospectively ensure the successful treatment and prevention of infectious diseases (WHO 2015a). The German implementation was accordingly executed in form of the German antimicrobial resistance strategy DART 2020 (BMG/BMEL/BMBF 2015) which underlines the urgency of this topic.

On the other hand, urban emissions of antibiotics into the environment have the potential to adversely affect terrestrial and aquatic organisms (Kümmerer 2009). In contrast to the environmental hazard caused by resistances, for which no adequate assessment approach presently exists (Berendonk et al. 2015), standardized test methods have been developed recently to quantify the ecotoxicological risk resulting from antibiotics (EU 2003). Their adequate application strongly relies on a comprehensive situation analysis by predicting or measuring a representative antibiotic concentration of interest (Predicted or Measured Environmental Concentration – PEC or MEC) as well as the estimation of a specific antibiotic concentration below which the exposition is not expected to cause adverse effects (Predicted No-Effect Concentration – PNEC). PNEC values are determined on the basis of toxicological endpoints covering a variety of sensitive species to protect community function (EU 2003). In many cases only a limited number of toxicity studies are available, since the market introduction of most antibiotics was during the 20th century, years before the "Guideline on the environmental risk assessment of medicinal products for human use" was put into action (EMA 2006). The resulting limited scientific validity of data sets is accounted for by using empirically derived assessment factors, which depend on the number and relevance of tested organisms as well as acute or chronic test durations (EU 2003). In this context, the concept of "concentration addition" (also termed "dose addition")

allows the transformation of multiple toxicity values of single antibiotics into an overall mixture toxicity called risk or hazard index RIadd or Hadd, respectively (EPA 2000). Even though this concept is based on the idea of similar modes of action (Berenbaum 1985), it also leads to reasonable predictions, irrespective of the specific pharmacological action of substances present in the mixture (Altenburger et al. 1996).

Regarding the situation analysis, measured environmental concentrations are varying in time and space (Ternes and Joss 2007), limiting the significance of local samplings for entire water bodies, not to mention catchment areas. A reasonable sample quantity is acquired to capture local seasonal differences in prescriptions (Mühlbauer 2014) as well as flow conditions, which leads to time and budgetary constraints. The mass flow analysis (MFA) within a defined system represents a promising tool as a basis for risk control regarding antibiotics, if coupled with quality criteria like PNEC values (Chevre et al. 2013, Chevre et al. 2011). The knowledge on the combination of mass flow and quality criteria allows the identification of critical flow conditions in order to take appropriate action. The estimation of antibiotic loads entering the system can be carried out using secondary data like sales (Bendz et al. 2005), productions (Choi et al. 2008) and prescriptions (Gobel et al. 2005). Data quality strongly depends on its primary purpose of generation and regional input predictions are often limited by insufficient temporal and spatial resolution of data. These limitations were shown to be crucial regarding antibiotic prescriptions (GERMAP 2012, Li and Zhang 2011, Mühlbauer 2014), but rarely discussed or questioned in past environmental risk assessment (ERA) studies (Coutu et al. 2012).

After medical application, antibiotics are partly eliminated in the human body, the sewer system, the wastewater treatment plant (WWTP) as well as the aquatic environment. Human excretion values are based on pharmacological studies, but have been rarely validated in practice (Ghosh et al. 2009). Degradation and sorption are the main processes influencing antibiotic loads during the wastewater transport, which strongly affects the mass flow of antibiotics and corresponding recovery of expected loads at the WWTP input. A direct comparison between predicted and measured antibiotic input loads has been scarcely performed in practice (Bendz et al. 2005, Ghosh et al. 2009, Gobel et al. 2005). Resulting recovery values are partly disproportionate and likely to be linked to a very limited number of samples. So far, it can be stated that the pending verification of the approach's applicability and the associated suitability of load estimation as a prediction tool must be questioned. In this context, the question on the necessary sample quantity to reasonably determine characteristic input loads of WWTP arises.

The majority of WWTP are designed to purposefully eliminate organic compounds as well as, depending on WWTP size and legislation, the nutrients nitrogen and phosphorus. In most countries the comprehensive introduction of an advan-

ced treatment step to eliminate micropollutants in WWTP, i.e. oxidation and adsorption processes, is not required under national legislation. Hence, the conventional treatment process applying nitrogen and phosphorus removal is state of the art and representative for the current situation regarding antibiotic removal in WWTP. The results of previous studies regarding the removal efficiency of various antibiotics ranged from complete elimination to significant production (Luo et al. 2014), whereby differences can be partly related to changing conditions originating from WWTP operation as well as flow and load variations. In this context, the term "operational parameters" will be understood to include typical WWTP parameters, which are not subject to any control (e.g. temperature). In particular, operation parameters like solid retention time SRT (Suarez et al. 2012, Vieno et al. 2007), hydraulic retention time HRT (Guerra et al. 2014, MacLeod and Wong 2010) as well as plant design (Guerra et al. 2014, Larcher and Yargeau 2012, Suarez et al. 2010) and seasons (Gracia-Lor et al. 2012, Guerra et al. 2014, Kwon and Rodriguez 2014, Zhang et al. 2015) were shown to possibly influence the removal process. Moreover, the re-transformation of metabolites to their parent substances was discussed (Goebel et al. 2007), which also affects the actual calculation of removal rates. Apart from this aspects, in many cases negative removal values are considered to be the result of an insufficient sampling method (Majewsky et al. 2011) and longer sampling campaigns are required to achieve reliable results (Gobel et al. 2005).

GOALS AND STRUCTURE

The main objective of this work comprises the realization and verification of MFA for fate characterization of antibiotics in an urban wastewater system, in combination with risk assessment approaches to identify the most toxic and unpredictable representatives. The timeliness of emerging contaminants in urban wastewater systems has led to numerous publications aiming for answers regarding fate and ecological impact of antibiotics. The majority of conclusions is based on very few samples, without considering specific inflow and catchment characteristics of the respective WWTP. These essential restrictions led in particular to the ambition of critically evaluating results concerning their reliability and significance (data quantity and quality).

The catchment area of the WWTP Dresden-Kaditz and the receiving stream Elbe constitute the study area of the present work. A total of 14 antibiotics as well as one human metabolite, which were chosen on the basis of their amount of consumption, are investigated.

The thesis is composed of four articles covering the following three compartments and according objectives:

Table 1: Structure and objectives.

		Objective	Reference
Compartment	1. Catchment and sewer system	Prediction and verification of input loads	Marx, C., Mühlbauer, V., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V. (2015). **Representative input load of antibiotics to WWTPs: Predictive accuracy and determination of a required sampling quantity** *Water Research 76, pp. 19-32.*
	2. Wastewater treatment plant	Mass flow analysis WWTP	Marx, C., Günther, N., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V. (2015). **Mass flow of antibiotics in a wastewater treatment plant focusing on removal variations due to operational parameters** *Science of The Total Environment 538, pp. 779-788*
	3. Environment	Prioritization of risk-driving antibiotics	Marx C., Mühlbauer V., Krebs P., Kuehn V. (2015). **Species-related risk assessment of antibiotics using the probability distribution of long-term toxicity data as weighting function: a case study** *Stochastic Environmental Research and Risk Assessment 29, pp. 2073-2085* Marx, C., Mühlbauer, V., Krebs, P., Kuehn, V. (2015). **Environmental risk assessment of antibiotics including synergistic and antagonistic combination effects** *Science of The Total Environment 524-525, pp. 269–279*

Each article provides a detailed characterization of boundary conditions and possible sources for uncertainties and errors. Combining the results of all investigated compartments leads to an integrative evaluation of antibiotic's impact on the environment and further provides evidence on the suitability of secondary data to perform environmental risk assessment. The subsequent identification of high-impact substances constitutes the basis for sophisticated risk management strategies resulting in high ecological effectiveness.

APPROACH AND METHODS

1. Compartment: Catchment and sewer system

The prediction model for the input of antibiotics into the sewer system is based on prescriptions provided by the statutory health insurance company AOK PLUS and several hospital pharmacies. The model considers the projection of available prescription data to the entire population of the catchment area as well as the prediction of actual and future prescriptions, based on prescribed amounts from past years. Outpatient data are available on a weekly basis but do not provide information on the treatment initiation, the application period or the patient's compliance. In connection with low resolution inpatient prescription data, the evaluation on a monthly basis is regarded as more expedient for a comparison between predicted and measured input loads to the WWTP. The available outpatient prescriptions represent about 41 % of Dresden's total population, whereby an identical prescription practice was assumed for patients insured under the remaining statutory health insurance schemes (about 49 %). This assumption is also justified regarding privately insured patients (about 10 %) due to their corresponding nationwide share in antibiotic preparations (Wild 2015). Subsequent to the projection, the prediction approach is based on the evaluation of past prescription data and consists of two steps. First, the estimation of the annual drug consumption based on their development of the last few years, and second, the determination of a characteristic seasonal trend to calculate respective monthly drug loads. In connection with the antibiotic-specific excretion rate, the input load into the sewer system of the catchment area can be estimated.

Hereafter, estimated input loads were compared with analytically determined input loads at the WWTP. The WWTP monitoring was carried out for 15 months, taking daily 24h-composite samples. Intermediate degradation processes during the residence time in the sewers were taken into consideration carrying out laboratory experiments. Discharge losses from combined sewer overflows were estimated to about 10 % of the annual load (Marx and Kuehn 2015) but not quantitatively considered due to their irregular, event-based occurrence. To address the significance and reliability of the findings, both the predicted and measured values were evaluated with respect to uncertainties deriving from the prediction model and analytics.

2. Compartment: Mass flow analysis WWTP

In contrast to the monthly evaluation of input loads, the detection of possible operational influences on the removal of antibiotics calls for a substantiated approach. In- and output loads considerably fluctuate within the period of one month, which does not permit their comparison with moderately changing parameters like solid retention time and temperature. Hence, the removal from the liquid phase was determined from cumulative in- and output loads within time series of gapless in- and effluent data sets. Operational parameters under investi-

gation were averaged correspondingly for each time series to detect possible dependencies. Additionally, antibiotics were analytically determined in primary, excess and digested sludge to estimate the removal of antibiotics by adsorption as well as to assess their fate during the anaerobic sludge treatment. The applied analytical method was specifically developed to determine antibiotic concentrations from matrices with high solids content.

The mass flow analysis was established normalizing antibiotic loads from the effluent and the digested sludge to the diurnal input load. The difference between 100 % and both output streams hereby indicates a degradation or production of the respective compound, depending on a positive or negative outcome, respectively. In this context, degradation is understood as the loss of the antibiotic's original molecule structure by biotic or abiotic processes. So-formed transformation products can have similar properties, compared to their parent substances, but are not further taken into consideration regarding the actual mass flow analysis.

3. Compartment: Prioritization of risk-driving antibiotics

The study focuses on risk assessment of antibiotics in the liquid phase. It was carried out on the basis of prescription data applying excretion and elimination values, which have been validated by the results of the first two studied compartments. The dilution of the WWTP effluent entering the receiving stream Elbe is usually estimated assuming complete mixing. Nevertheless, limited mixing processes are the reason for expanded effluent plumes in the receiving streams (Jirka et al. 2004) whose antibiotic concentrations are likely to remain higher than the complete mixing calculation suggests. In order to cover the two concepts, the ERA was carried out for both, complete mixing and undiluted effluent plume. In addition, the scope of this study focuses on single antibiotics (no transformation products), neither considering elimination processes in surface water bodies like sorption, hydrolysis and photolysis nor the existing preload from upstream settlements.

The determination of specific PNEC values was primarily carried out according to EU guidelines (EU 2003), based on an intense literature review covering long-term toxicity data of all available tested species. The lowest available toxicity thresholds for considered standard organisms (algae, daphnia, fish) was merged with the species sensitivity distribution of all available species to express the risk probability towards each species level. The most hazardous antibiotics to the lowest species level additionally weaken higher-tier organisms, in terms of food supply, and can be considered as potential environmental risk-driving substances. Synergistic and antagonistic effects were assessed applying the interaction-based hazard index (EPA 2000) which is based on binary interaction experiments. The subsequently performed sensitivity analysis provides evidence on the relevance of available studies as well as tested concentration ranges.

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CHAPTER 2

1. COMPARTMENT: CATCHMENT AND SEWER SYSTEM

Representative input load of antibiotics to WWTPs: Predictive accuracy and determination of a required sampling quantity

Representative input load of antibiotics to WWTPs: Predictive accuracy and determination of a required sampling quantity

Marx, C., Mühlbauer, V., Schubert, S., Oertel, R., Ahnert, M., Krebs, P., Kuehn, V.

ABSTRACT

Predicting the input loads of antibiotics to wastewater treatment plants (WWTP) using certain input data (e.g. prescriptions) is a reasonable method if no analytical data is available. Besides the spatiotemporal uncertainties of the projection itself, only a few studies exist to confirm the suitability of required excretion data from literature. Prescription data with a comparatively high resolution and a sampling campaign covering 15 months were used to answer the question of applicability of the prediction approach. As a result, macrolides, sulfamethoxazole and trimethoprim were almost fully recovered close to 100 % of the expected input loads. Nearly all substances of the beta-lactam family exhibit high elimination rates during the wastewater transport in the sewer system with a low recovery rate at the WWTP. The measured input loads of cefuroxime, ciprofloxacin and levofloxacin fluctuated greatly through the year which was not obvious from relatively constant prescribed amounts. The latter substances are an example that available data are not *per se* sufficient to monitor the actual release into the environment. Furthermore, the extensive data pool of this study was used to calculate the necessary number of samples to determine a representative annual mean load to the WWTP. For antibiotics with low seasonality and low input scattering a minimum of about 10 samples is required. In the case of antibiotics exhibiting fluctuating input loads 20 to 30 evenly distributed samples are necessary for a representative input determination. As a high level estimate, a minimum number of 20 to 40 samples per year is proposed to reasonably estimate a representative annual input load of antibiotics and other micropollutants.

KEYWORDS

Antibiotics, recovery rate, prescription data, sample quantity, wastewater treatment plant

INTRODUCTION

Input loads of antibiotics into the sewer system and the environment are often estimated using information on nationwide productions, sales or prescribed amounts (Bendz et al. 2005, Choi et al. 2008, Gobel et al. 2005). If applying this approximation method, it has to be taken into account that the catchments of wastewater treatment plants (WWTP) can vary significantly in amounts of actually administered antibiotics due to socio-economic reasons. Li and Zhang carried out an evaluation of prescription data of antibiotics from different districts in Hong Kong and stated that a breakdown of district- or nation-wide prescriptions to single catchments is not acceptable to sufficiently estimate input loads (Li and Zhang 2011). In addition to local variations regarding drug administration further uncertainties arise through the necessary use of substance-specific excretion rates. Those pharmacokinetic information are based on only a few pharmacological studies and have been rarely validated in practice (Ghosh et al. 2009). One of the few studies comparing predicted and measured influent was carried out for trimethoprim at the Kälby WWTP in Sweden (Bendz et al. 2005). As a result the theoretical load was almost 5 times higher than the measured value. Reasons for the discrepancy were not provided. At a Swiss WWTP about 50 % and 75 % of predicted sulfamethoxazole and trimethoprim loads were determined, respectively, taking a sum of 8 samples in 2002 (March) and 2003 (February, November) (Gobel et al. 2005). Investigated macrolides azithromycin, clarithromycin and roxithromycin were fully recovered by the measurement. Those investigations provide important information on the reliability of applied extrapolation methods for load determination but also raise the question of an appropriate sample quantity in this field of investigations. For example, grab samples and short-term composite samples (2h, 4h etc.) were shown to be not suitable for input load characterization due to high fluctuations within one day (Coutu et al. 2013, Li and Zhang 2011). In this context, most of the previous investigations with the aim of determining antibiotic input loads used a comparatively low number of input samples. Zhou et al. used each 2 hr time-integrated samples in two consecutive days to perform a mass balance analysis of two WWTP in South China during May and November 2010 (Zhou et al. 2013). Plosz et al. (2010) and Gao et al. (2012) used 6 h-, 8 h- and 24 h-composite samples (three of each type) to investigate input load dynamics and evaluate the contributing processes for pharmaceutical removal, respectively (Gao et al. 2012, Plosz et al. 2010). Four 24 h- composite samples were used to investigate the elimination pathways of antibiotic loads in a Chinese WWTP (Li et al. 2013). A total of eight 24 h-composite (flow proportional) samples were taken to determine the mean input load to the WWTP Kloten-Opfikon, near Zurich, Switzerland (Gobel et al. 2005). By far the highest number of 24 h-mixed composite samples was taken to examine temporal dynamics of antibiotics in the WWTP Lausanne, Switzerland. A total of 84 samples were evenly distributed over the year to capture the seasonality of antibiotics (Coutu et al. 2013).

The current state of knowledge poses two main questions regarding the determination of the representative input load of antibiotics to WWTP:

1) Is the extrapolation of suitable input data (production, sales or prescriptions) in combination with the excretion rate sufficiently accurate to estimate the input loads to WWTPs?
2) What is the appropriate number of samples to determine the characteristic input loads of WWTP, in case alternative input data are not available?

In order to provide answers to the above questions an intensive monitoring program was carried out at the WWTP Dresden-Kaditz taking daily samples over a period of 15 months. The determined input loads were compared to predicted values based on in- and outpatient prescription data for the catchment area of the WWTP. This comprehensive information give insights into load fluctuations of antibiotics at WWTP inflows and establish a solid basis to evaluate excretion rates from literature. Proceeding from the pool of high resolution data a substance specific estimation on the minimal number of samples which satisfactorily describes the annual mean load was carried out.

MATERIALS AND METHODS

Ambulant and clinical prescription data

A total of 14 antibiotics were investigated covering the following classes: macrolides (azithromycin, clarithromycin, roxithromycin), tetracyclines (doxycycline), cephalosporins (cefuroxime, cefotaxime), sulfonamides (sulfamethoxazole + trimethoprim), lincosamide (clindamycin), penicillins (penicillin V, piperacillin, amoxicillin) and fluoroquinolones (ciprofloxacin, levofloxacin).

The projection model is based on in- and outpatient prescription data. Outpatient prescription data were provided by the AOK PLUS, the largest statutory health insurance company in the district of Saxony, Germany. About 41 % of the population in Dresden is insured by the AOK PLUS while the remaining part is being held by other statutory and private health insurance companies. Ambulant data are available weekly for 2005 – 2012. Inpatient prescriptions were available for three major hospitals covering about 65 % of hospital beds in the catchment area of the WWTP Dresden-Kaditz. Hospital prescription data are available for 2011 and 2012 on a quarterly or monthly basis, depending on the institution. The projection factor of stationary prescriptions to the entire catchment area was calculated to 100%/65% = 1.54. Comparing the amounts of in- and outpatient prescribed amounts it can be stated that most antibiotics are predominantly prescribed in the ambulant sector. The projected amounts of all hospitals constitute less than 30 % of the total prescriptions (= sum of in- and outpatient loads) in the catchment area (see Figure 1). Levofloxacin (40 %), cefuroxime (80 %), piperacillin (100 %) and cefotaxime (100 %) are exceptional cases and hospitals are considered to be the main contributors to the overall administration, regarding those substances.

In most cases hospital antibiotics are constantly applied throughout the year. The standard deviations of mentioned high-impact substances are less than 17 % and justify the assumption of an even (constant) administration in hospitals. The prescription characteristic of remaining antibiotics with higher standard deviations SD (roxithromycin: 77 %, amoxicillin: 61 %, clarithromycin: 49 %, doxycycline: 42 %, azithromycin: 38 %, ciprofloxacin: 27 %, clindamycin: 25 %) will also be assumed to be evenly distributed over the year since no repeating seasonal pattern is evident and their impact on the total input to the catchment area is negligible.

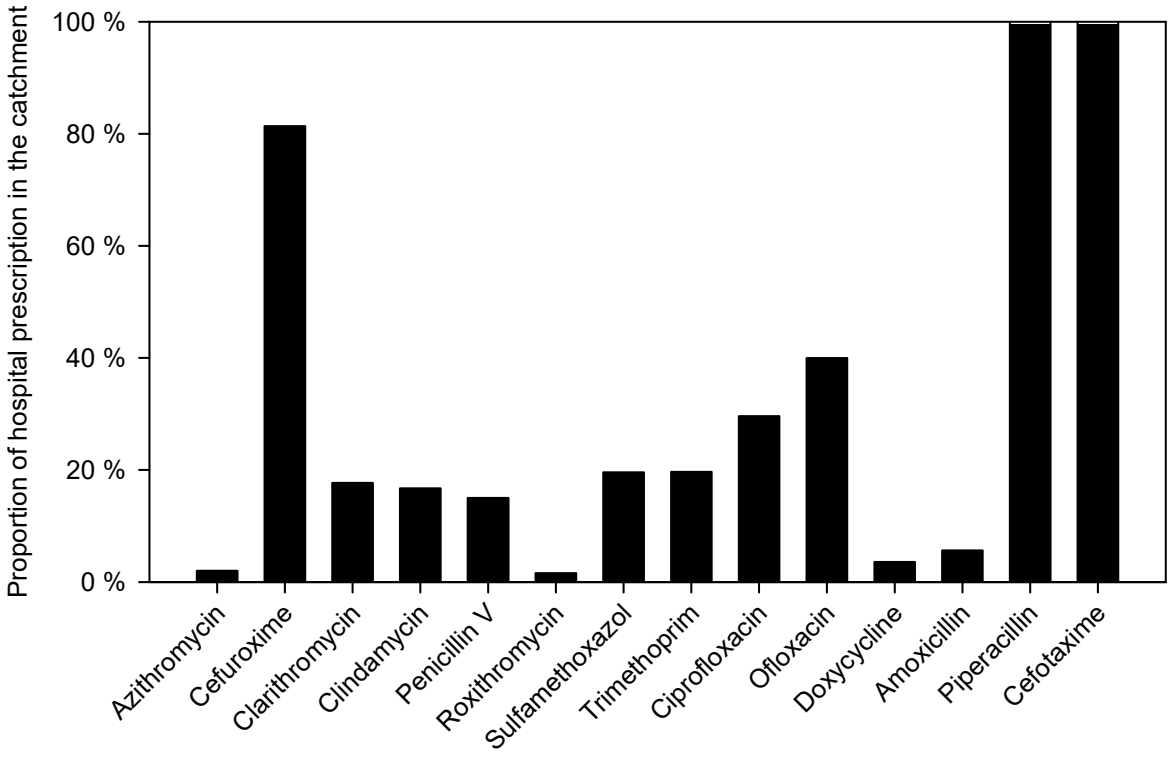

Figure 1: Proportion of antibiotics administered in the three major hospitals in Dresden in 2011 and 2012, referred to the total catchment's input

Ambulant macrolide prescriptions follow a distinct seasonality throughout the year. During the months of January and February prescriptions are more than double compared to the summer season (May to August). Sulfamethoxazole, trimethoprim, penicillin V and levofloxacin show a seasonal influence. Nonetheless, in relation to annual average the differences between summer (80 %) and winter (120 %) are not as profound as for macrolides. In the ambulant sector clindamycin, doxycycline and ciprofloxacin are evenly administered throughout the year. In the case of cefuroxime the seasonality developed during the period under review. During the years 2005 and 2006 no seasonality was observed. In subsequent years, differences between winter and summer started to increase. In relation to the annual mean, 115 % and 80 % of cefuroxime were prescribed in winter and summer 2012, respectively, which amounts a gain of about 50 %. This significant development is interesting but carries no weight considering the vast amount of plainly prescribed cefuroxime in hospitals.

Projection and prognosis model of influent loads

The WWTP Dresden-Kaditz treats the wastewater of five cities (Dresden, Freital, Heidenau, Pirna and eastern Radebeul) and some bordering municipalities. The current loading is about 650000 PE, of which around 80 % are living in Dresden. The WWTP has a design capacity of 740000 PE. The catchment area has a size of about 9 400 km² with no known industries producing or applying significant

amounts of antibiotics (e.g. industrial husbandry). The sewer system consists of about 800 km of combined sewers, around 450 km of separate sewerage system and 350 km for stormwater runoff. Due to the significant amount of combined sewers (nearly 50 %) the loss of antibiotic loads in case of a stormwater induced discharge of mixed wastewater (wastewater + stormwater) must be kept in mind. These events are usually of very short duration and do not influence the prediction at normal conditions, i.e. dry weather.

It is assumed that AOK-insured people constitute a representative portion of the entire catchment area of the WWTP. Concluding, an extrapolation using the insurance ratio of 41 % is regarded to be justified. The share of privately insured people (about 10 %) will not be considered independently, due to a lack of information regarding a specific prescription practice. An equal prescription behavior will be assumed for patients of private and statutory health insurance companies. The variety of hospital prescriptions depends on the number and type of clinical stations and can vary significantly between institutions. A detailed differentiation between the available hospitals, based on existing stations and corresponding antibiotic usage was not possible from the available data set. Hence, the extrapolation of clinical prescriptions to the catchment area was carried out using the 65 % coverage of hospital beds. The following equation 1 sums up the assumptions made to calculate the total antibiotic input to the catchment area of Dresden Kaditz. $P_{AB,a,AOK}$ and $P_{AB,a,hospital}$ represent the ambulant (AOK) and hospital prescriptions, respectively.

$$I_{AB,proj,a} = \frac{P_{AB,a,AOK}}{41\,\%} + \frac{P_{AB,a,hospital}}{65\,\%} \qquad \text{(Equation 1)}$$

(for a = 2005 .. 2012)

$$I_{AB,pred,a+1} = I_{AB,proj,a} - \frac{I_{AB,proj,a-y-1} - I_{AB,proj,a}}{y} \qquad \text{(Equation 2)}$$

(for a = 2004+y .. 2012 and y = 2, 3, 4)

The monitoring program at the WWTP Dresden-Kaditz was predominantly carried out in 2013. While corresponding prescription data are not available for this year, it is necessary to extend the projected input of antibiotics to predict the prescriptions for 2013. The prediction of absolute monthly input loads of 2013 was carried out following a two-stage procedure. First, the prediction of monthly changes can be reasonably assumed on the basis of a relative seasonality within the year. As outlined in 2.1, the seasonal characteristics of most antibiotics, except cefuroxime, annually repeat with nearly identical pattern. This characteristic

becomes even more apparent normalizing each month to the corresponding annual mean. Hence, the normalized seasonal pattern of 2005 – 2012 is averaged (see Figure 2B) and will be referred to as 'relative seasonality'. The corresponding monthly standard deviation will be subsequently used for quantifying the uncertainty (see equation 3) of applying a recurring seasonality (see 3.1). The average uncertainty of seasonality of each antibiotic was calculated using equation 4 (see Figure 3). As the second step, neglecting the monthly variations, the prognosis of the annual quantity of administered antibiotics is carried out using the mean consumption change of the previous 2, 3 and 4 years. The procedure originates from the Euler's forward method and was adopted according to equation 2. The three approaches using the trend prediction according to the prior 2, 3 and 4 years were tested for their degree of uncertainty, in order to determine the one with the lowest derivation between predicted and actual annual quantity. For this purpose, Euler's forward method was applied on the entire dataset using y = 2, 3 and 4. In general, the maximal derivations using either one of the prognosis approaches were nearly similar (supplementary material Figure S2, annex 1). At least the trend prognosis of the annual consumption change using the previous 3 years showed slightly better results (especially for sulfamethoxazole and trimethoprim) and was ultimately used for the prediction of administered antibiotics in 2013 (see Figure 2A and equation 2 applying y=3). As a result, the specific mean input load of 2013 can be predicted considering the region-specific consumption development. The uncertainty of each antibiotic will be expressed using the maximal deviation from the application of equation 2 (y=3) for the years 2008 – 2012 using equation 5. The results are summarized in supplementary material Figure S2 (annex 1). Finally, combining the information of the mean annual input load and the relative seasonality gives a solid forecast for absolute monthly input loads for 2013.

$$U_{AB,season,m} = \sigma\left\{\frac{I_{AB,real,a,m}}{I_{AB,real,a}}\right\} \qquad \text{(Equation 3)}$$

for m = Jan .. Dec, and a = 2005 .. 2012

$$U_{AB,season} = \frac{\sum_{m=Jan}^{Dec} U_{AB,season,m}}{12} \qquad \text{(Equation 4)}$$

$$U_{AB,trend} = \max\left\{\frac{\left|I_{AB,pred,a} - I_{AB,real,a}\right|}{I_{AB,real,a}}\right\} \qquad \text{(Equation 5)}$$

for a = 2008 .. 2012

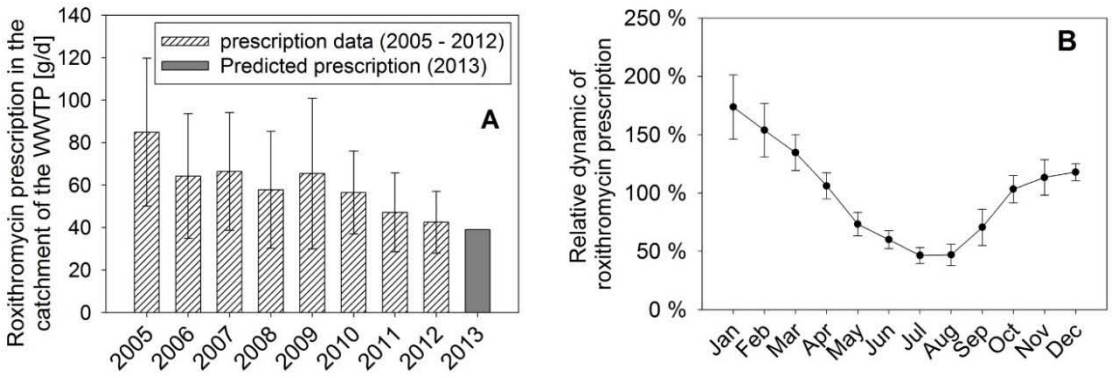

Figure 2-A: annual prescription loads of roxithromycin from 2005 – 2012 (hatched bars) and the prognosis for 2013 (grey bar); 2-B: mean relative seasonality of roxithromycin for the time period between 2005 – 2012 (standard deviation represented by error bars)

Excretion rate of antibiotics

A crucial link between drug prescription and its recovery at the inflow of a WWTP is the human excretion rate of the parent drug and its behavior within the sewer system. A lack of reliable data must be stated since the accurate excretion rate of a new drug plays a minor role in permission requirements for a commercial launch. Hence, pharmaceutical companies do not share the interest of providing profound information on human elimination pathways and their individual products in detail. Bibliographical references contain information on total excretion including metabolites, oral or parenteral administration as well as excretion rates specifically referring to urine, faeces or a combination of both. Due to this variety in data type and range an intensive literature search was carried out in order to minimize uncertainty and back up applied data. A summary of excretion rates is given in Table 1.

Table 1: Excretion rate of antibiotics under investigation

Antibiotic	Excretion rate (E_{AB})
Cefuroxime	42.8 – 57.0 % (ODDB 2014)
Clarithromycin	60.0 – 78.1[1] % (Abbott 2006. Hirsch et al. 1999)
Azithromycin	67.4[2] % (Sandoz 2009)
Roxithromycin	47.8[3] – 60.0 % (Hirsch et al. 1999. Sanofi-Aventis 2009)
Clindamycin	10 – 35 % (Pharma 2012b. Still et al. 2006)
Amoxicillin	60 – 85 % (Aktories et al. 2009. Martindale 1993)
Penicillin V	29 - 43 % (Pharma 2012c)
Ciprofloxacin	40.0 – 69.7 % (Kümmerer et al. 2000. Pharma 2012a)
Levofloxacin	74.9 – 85.9 % (Wagenlehner et al. 2006)
Sulfamethoxazole	15 – 25[4] % (Hirsch et al. 1999. Martindale 1993)
Doxycycline	22 – 70 % (Hirsch et al. 1999, Pharma 2008)
Trimethoprim	40 – 60 % (HSDB 2014, Martindale 1993)
Piperacillin	60 – 95 % (Aktories et al. 2009, Fresenius 2012b)
Cefotaxime	40 – 60 % (Fresenius 2012a)

The reliability of excretion data cannot be assessed from the information provided in literature. Hence, the uncertainty regarding the determined range of excretion values will be calculated using equation 6, expressing the relative deviation of the range limits to the range's mean.

$$U_{AB,excretion} = \frac{E_{AB,max} - E_{AB,min}}{E_{AB,max} + E_{AB,min}} \qquad \text{(Equation 6)}$$

Monitoring program and analytical methods

The monitoring at the influent of the WWTP Dresden-Kaditz was carried out from October 2012 until December 2013. All 14 antibiotics were analyzed daily in flow proportional 24h-composite samples. Samples taken were stored at 4°C using brown glass flasks for a maximum time period of 7 days until transport and analysis in the lab. In June 2013 the inflow measurement was interrupted due to flood water in the receiving stream, River Elbe. The inflow of the WWTP was maxed out and measuring equipment was put out of service as a precautionary measure. Since the 24h-sampler was operational most of that time, the inflow hydrograph could be restored using the incoming ammonia concentration (see supplementary material Figure S1, annex 1).

[1] Using equation S1 (annex 1) with $f_{A,AB}$ = 59.9 % and $f_{M,AB}$ = 36.6 %
[2] Using equation S1 (annex 1) with $f_{A,AB}$ = 37 % and $f_{M,AB}$ = 88 %
[3] Elimination of 37.8 % through feces + 10 % through urine
[4] Using equation S1 (annex 1) with $f_{A,AB}$ = 100 % and $f_{M,AB}$ = 75 – 85 %

In the laboratory, composite wastewater samples were immediately prepared for the antibiotic analyses and a method developed by Rossmann et al. (2014) was used to quantify the selected antibiotics and determine their stability in diverse wastewater during a maximum storage of 7 days (Rossmann et al. 2014). Briefly, a 50-mL aliquot of homogeneous wastewater was mixed with 0.8 mg/mL EDTA-Na2 (Ethylenediaminetetraacetic acid disodium salt dehydrate, ACS reagents; Sigma, St. Louis, MO, USA) and filtered through a glass fibre filter (< 0.9 μm; WICOM, Heppenheim, Germany). With formic acid (LC-MS grade; Sigma, St. Louis, MO, USA) the sample was adjusted to a pH of 3.5 (± 0.2). Then, 2.5 mL of prepared wastewater were extracted by Solid Phase Extraction (SPE) onto a 30 mg Oasis HLB Vac Cartidge (Waters, Milford, MA, USA) using the Gilson Automatic Sample Processor ASPEC XL (Middleton, WI, USA). The extracts were analyzed by a LC-MS/MS system. The chromatographic separation was performed with a Synergi 4μ HydroRP 80A, 150 x 2.0 mm (Phenomenex, Aschaffenburg, Germany) column with a Security Guard C18, 4 mm x 2 mm i. D. (Phenomenex, Aschaffenburg, Germany) and secondly, with a 100 mm x 3 mm Nucleoshell HILIC 2.7 μm column (Machery-Nagel, Düren, Germany) for the antibiotics amoxicillin, ciprofloxacin, doxycycline and levofloxacin. For the detection, an API 4000 tandem mass spectrometer (ABSciex, Framingham MA, USA) was equipped with an electrospray interface (ESI) in the multiple reaction monitoring mode (MRM).

The maximal analytical errors resulting from spiking diluted urine with 100, 1000 and 5000 ng/l are 14.6 % (amoxicillin), 13.5 % (levofloxacin, 12.0 (cefuroxime, penicillin V), 11.5 % (piperacillin), 10.6 % (ciprofloxacin), 9.8 % (roxithromycin), 9.7 % (cefotaxime), 9.6 % (sulfamethoxazole), 9.3 % (clarithromycin, doxycycline), 7.3 % (azithromycin, trimethoprim) and 5.9 % (clindamycin). The influent matrix showed to be very different compared to diluted urine and quantification was realized using standard addition of 1000 ng/l and 5000 ng/l (only cefuroxime, amoxicillin and doxycycline), respectively (Rossmann et al. 2014).

Laboratory investigation on the stability in raw wastewater

Laboratory experiments were conducted in order to determine antibiotic behavior in raw wastewater which, along with excretion, is a second crucial link between drug prescription and its recovery at the inflow of WWTPs. The sewer system is a very heterogeneous system and oxygen conditions along the flow path are difficult to describe. In order to assess different elimination characteristics the experiments were conducted at both, anaerobic (oxygen < 0.1 mg/l) and aerobic (oxygen > 2.0 mg/l) conditions. The experiments were carried out in the dark at 10 and 20 °C. The temperatures were kept constant using a water bath. Aerobic and anaerobic conditions were established by introducing air or nitrogen, respectively. 2 ml of the stock solution containing 1 μg/ml of each antibiotic were added to 2 L glass flasks in order to raise the antibiotic concentration by 1000 ng/l. The mean residence time of wastewater in the sewer systems until it reaches the WWTP can

be estimated to about 12 h. Accordingly, in order to describe the stability of antibiotics within this time horizon samples were taken before the addition of 1000 ng/l as well as after 15 min and 12 h. Values determined for 10 and 20 °C were averaged to address the different temperature conditions throughout the year. The recovery rate was determined using equation 7. The corresponding error was calculated according to linear error propagation using equation 8.

$$R_t = \frac{c_t}{c_0 + 1000\,^{ng}/_{l}} \qquad \text{(Equation 7)}$$

$$E_t = \sqrt{\left(\frac{1}{c_0 + 1000\,^{ng}/_{l}}\right)^2 \cdot \sigma_t^{\,2} + \left(\frac{c_t}{\left(c_0 + 1000\,^{ng}/_{l}\right)^2}\right)^2 \cdot \sigma_0^{\,2}} \qquad \text{(Equation 8)}$$

With t being the concentration after 15 min (initial recovery) and 12 h.

RESULTS AND DISCUSSION

Uncertainties of predicted input loads

The recovery rate of projected antibiotic inputs involves a set of uncertainties which are depicted as three levels of grey in Figure 3 and Figure 5 and will be presented in the following. The regarded uncertainties were added and related to the mean monthly input load to depict the entire range of values predicted by this approach. The internal band (darkest grey) represents the variation of excretion values found in literature Table 1 and was calculated using equation 6. On average, absolute numbers of excretion data deviate between 10 and 20 %. In case of doxycycline and piperacillin the variation exhibits the largest spread of all antibiotics, whereas only one literature value was available for azithromycin. Based on the relative uncertainty of the mean excretion, clindamycin and doxycycline exhibit the largest deviation of about 50 % (see Figure 3).

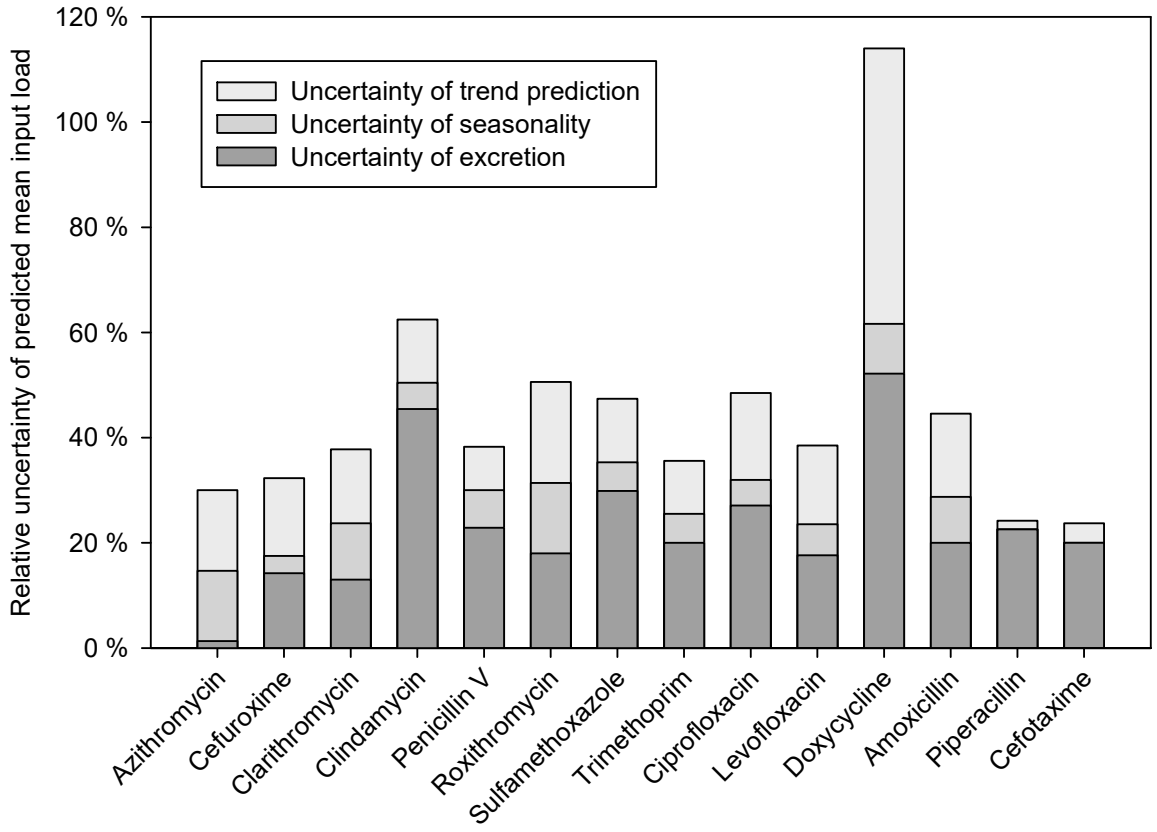

Figure 3: Composition of uncertainty regarding the predicted input loads based on prescription data (deviation due to seasonality was averaged over the year)

The mid band of grey (see Figure 3 and Figure 5) represents the uncertainty of the relative seasonality (fluctuations) from 2005 – 2012 and was calculated using equations 3 and 4, respectively. The standard deviation percentage (SD) of each month is a measure of how reliable the application of an averaged seasonal trend is. The

highest mean SD between 11 – 13 % were determined for all macrolides (see Figure 4). Macrolides have the most pronounced seasonality and exhibit peak values between January and March. Irrespective of the seasonality itself, the peak values cannot be specifically related to certain (illness-) events and seem to occur randomly, which makes a solid forecast difficult. Without taking macrolides into account, the average uncertainty due to seasonal fluctuations of antibiotics amounts to about 6 %.

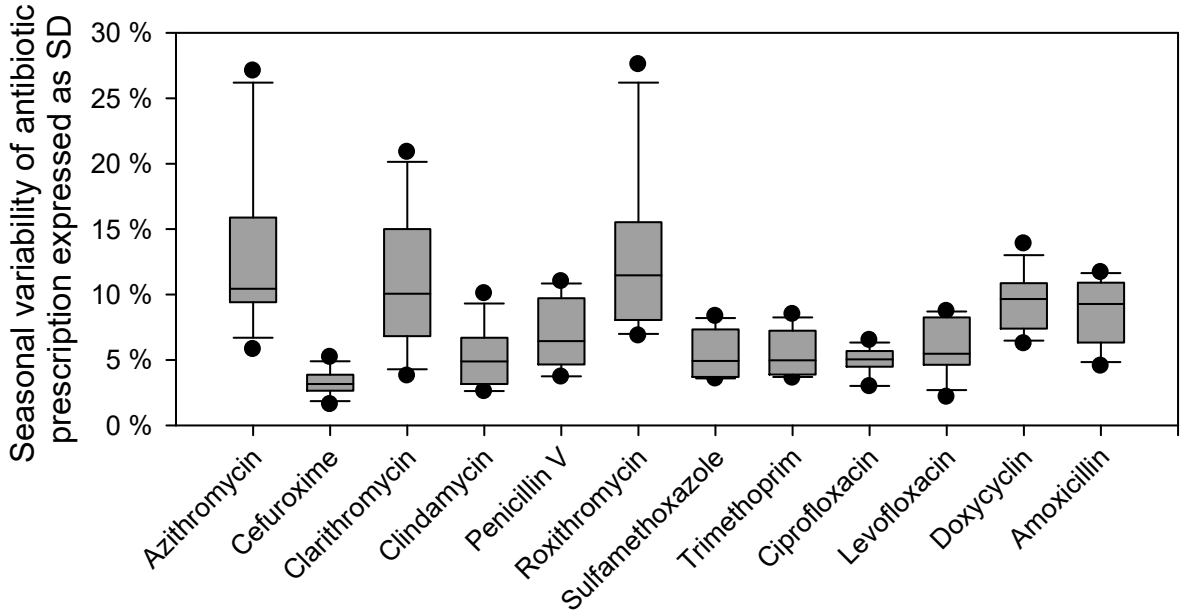

Figure 4: Monthly SD of the mean relative seasonality for each antibiotic (n = 12 months (Jan – Dec), SD of each month calculated from 8 years; area of boxplots represents the range of 25 % to 75 % percentile, whiskers illustrate 10 to 90 % percentiles of all data, outliers are depicted as dots)

The external band (lightest grey in Figure 3 and Figure 5) addresses the maximum uncertainty of the applied annual trend prediction. Therefore, the trend was calculated for 2008 to 2012 and compared with the corresponding actual prescription. The maximum relative discrepancy of the five years was calculated according to equation 5 and is considered to be well suited to act as indicator of the prognosis's reliability (see 3-year trend prediction in supplementary material Figure S2, annex 1). Except for doxycycline, all substances under investigation have a maximum trend uncertainty of 10 to 20 %. Doxycycline shows a continuously decreasing prescription from 2005 – 2008 and 2010 – 2012. In 2009 the prescription increased above the value of 2005 which is the reason for the vast uncertainty of 52 %.

Figure 3 summarizes the addressed deviations of expected input loads to the WWTP. Except for clindamycin and doxycycline, total deviations of all substances vary between 30 and 50 %. The strongly diverging excretion values of clindamycin and doxycycline, as well as the above presented trend uncertainty of the latter, lead

to a comparatively high overall uncertainty of 62 % and 113 %, respectively. The percentages are referred to the mean predicted input load derived from the projection and forecast model.

Seasonal recovery rate of antibiotics

Figure 5 illustrates the predicted (grey-leveled band) and measured (box plots) input loads of antibiotics. The black solid line characterizes the mean WWTP-inflow. In the following, the discussion of recovery rates for each antibiotic is divided into sub-sections, regarding the mode of antibiotic application, measured input characteristic and characteristic properties of antibiotics under investigation.

Sulfamethoxazole and trimethoprim

As sulfamethoxazole and trimethoprim are nearly exclusively administered together, mono-preparation of trimethoprim can be neglected. Measured data of trimethoprim widely correspond with the lower limit of the predicted input range. An average recovery of 76 % was yielded whereas about 67 % of all data points can be found within the forecasted area (see Figure 5B). The seasonal characteristic of trimethoprim exhibits a more distinctive variation (2.5-fold increase in January and February) than supposed from prescription data. This variation of measured input loads was also observed at the Vidy WWTP (220 000 inhabitants and several hospitals), in Lausanne, Switzerland, where the gain was in the range of a 3-fold increase (Coutu et al. 2013). Comparing the two studies, similar specific input loads of 21.6 – 64.8 µg/(PE*d) (Coutu et al. 2013) and 30.8 – 76.9 µg/(PE*d) (present study) were determined. The lower PE-loads in Switzerland were expected since antibiotic consumption in Switzerland is about 30 % lower than in Germany (Filippini et al. 2006). The range of measured input loads in five Swedish WWTP of 29 – 450 µg/(E*d) is too large to be compared with results from this study (Lindberg et al. 2005).

Measured sulfamethoxazole data fit well into the predicted input band from December 2012 to October 2013 (see Figure 5A). At the end of 2012 and 2013 significantly higher values were detected which cannot be explained by the prediction model. The unexpected increase could neither be verified using the sulfamethoxazole/trimethoprim-ratio which should be constant in raw wastewater throughout the year. Göbel et al. also used the information of the substance ratio in human medicine for verification purposes of the analytical measurements, neglecting differing excretion ratios of both substances (Gobel et al. 2005). Without taking the specific ratio of the excreted parent substance into account, the results of this estimation are misleading and the verification must be questioned. Considering the prescription and excretion data a theoretical ratio in the range of 1.25 to 3.13 is expected to be found in wastewater (administration ratio S/T = 5/1; excretion sulfamethoxazole (S): (0,15 – 0,25) x 5; excretion trimethoprim (T): (0,40 – 0,60) x 1; expected wastewater ratio: 0,75/0,6 – 1,25/0,40 = 1,25 – 3,13). This ratio was not observed during months with elevated sulfamethoxazole loads, which puts those measurements into question. No plausible explanation could be

found for such high sulfamethoxazole loads and corresponding data were excluded from further data analysis. Neglecting implausible data, a mean recovery of 85 % was yielded and 85 % of all data points are to be found within the predicted input range. Determined influent loads of 58.0 – 190.3 μg/(E*d) agree well with results from Sweden (46 – 210 μg/(E*d)) (Lindberg et al. 2005) and Australia (72 – 100 μg/(E*d)) (Watkinson et al. 2007).

Azithromycin, clarithromycin and roxithromycin

All three macrolides have a strong seasonal prescription characteristic which correlates with the monitoring data at the WWTP inflow. Clarithromycin and roxithromycin are to be found at the lower limit of the expected range and partly below it (see Figure 5D and E, respectively). It can be noticed, that, based on prescription data, the measured winter peak is shifted back by one month for all three macrolides. In case of clarithromycin and roxithromycin this is partly explained by the duration of administration of about 10 – 14 days, which prolongs the period of time between prescription and excretion. Azithromycin is usually administered for 3 – 5 days which is not sufficient to describe this discrepancy (see Figure 5C). Nevertheless, azithromycin correlates well with the predicted input load but shows significantly higher variation within one month, compared to clarithromycin and roxithromycin. The higher scatter might lead to a distortion of the actual input load which coincidently results in a backwards shifted peak of the monitoring data.

It can be noticed that all macrolides exhibit a load peak during the stormwater event in June 2013. Due to the protonation of the basic dimethylamino group macrolides are mainly positively charged at neutral pH (Gobel et al. 2005) which may lead to an adsorption to the negatively charged wastewater particles via cation exchange. The stormwater-induced load peak might be an indication of the remobilization of sewer sediment and/or adsorbed substances, respectively. A load peak was also observed for trimethoprim which has low to moderate adsorption capability (Straub 2013). In contrast, sulfamethoxazole is negatively charged at neutral pH and adsorption to particle matter might be insignificant (Tambosi et al. 2010) which, based on the absent load peak in June 2013, supports the assumption. The stormwater event in June 2013 was the only time a significant increase of input loads could be put into relation to an elevated rainwater-induced WWTP inflow, respectively. Regarding the load peak it must be also kept in mind that load losses through the discharge of mixed water (combined sewer system) are quite likely which diminishes the measured load at the WWTP. Hence, the load increase at the WWTP constitutes only the net amount of antibiotics which were remobilized in the sewer systems.

The mean recovery rates of azithromycin, clarithromycin and roxithromycin were determined as 110%, 58 % and 76 %, respectively, whereas 30 %, 43 % and 53 % of available data are to be found within the predicted input band. The macrolides covered in the present study were also quantified in the input of 2 Swiss WWTP

(Gobel et al. 2005). Except for azithromycin the recovery values remain below the expectations referring to results from Gobel et al. (each around 100 %) which might be due to further interferences like the sampling method and/or the analytical error (see 3.3). Regardless of the recovery rate, the daily input load of azithromycin from study (25.8 – 155.3 μg/(E*d)) is comparable to results from other investigations: 45 – 101 μg/(E*d) (Gobel et al. 2005); 118.9 – 207.3 μg/(E*d)) (Ghosh et al. 2009). More than 800 μg/(E*d) of clarithromycin were determined Japanese WWTPs which is vastly exceeding the load of this (45.9 – 260.0 μg/(E*d)) and other studies (59 – 160 μg/(E*d) (Gobel et al. 2005); 22.9 – 210.4 μg/(E*d) (McArdell et al. 2003)). The Input loads of roxithromycin amount between 8.4 and 49.9 μg/(E*d) which is comparable to other investigations: 5.0 – 19.3 μg/(E*d) (McArdell et al. 2003); 4.7 – 31.0 μg/(E*d) (Li and Zhang 2011); 20.8 – 71.3 μg/(E*d) (Ghosh et al. 2009).

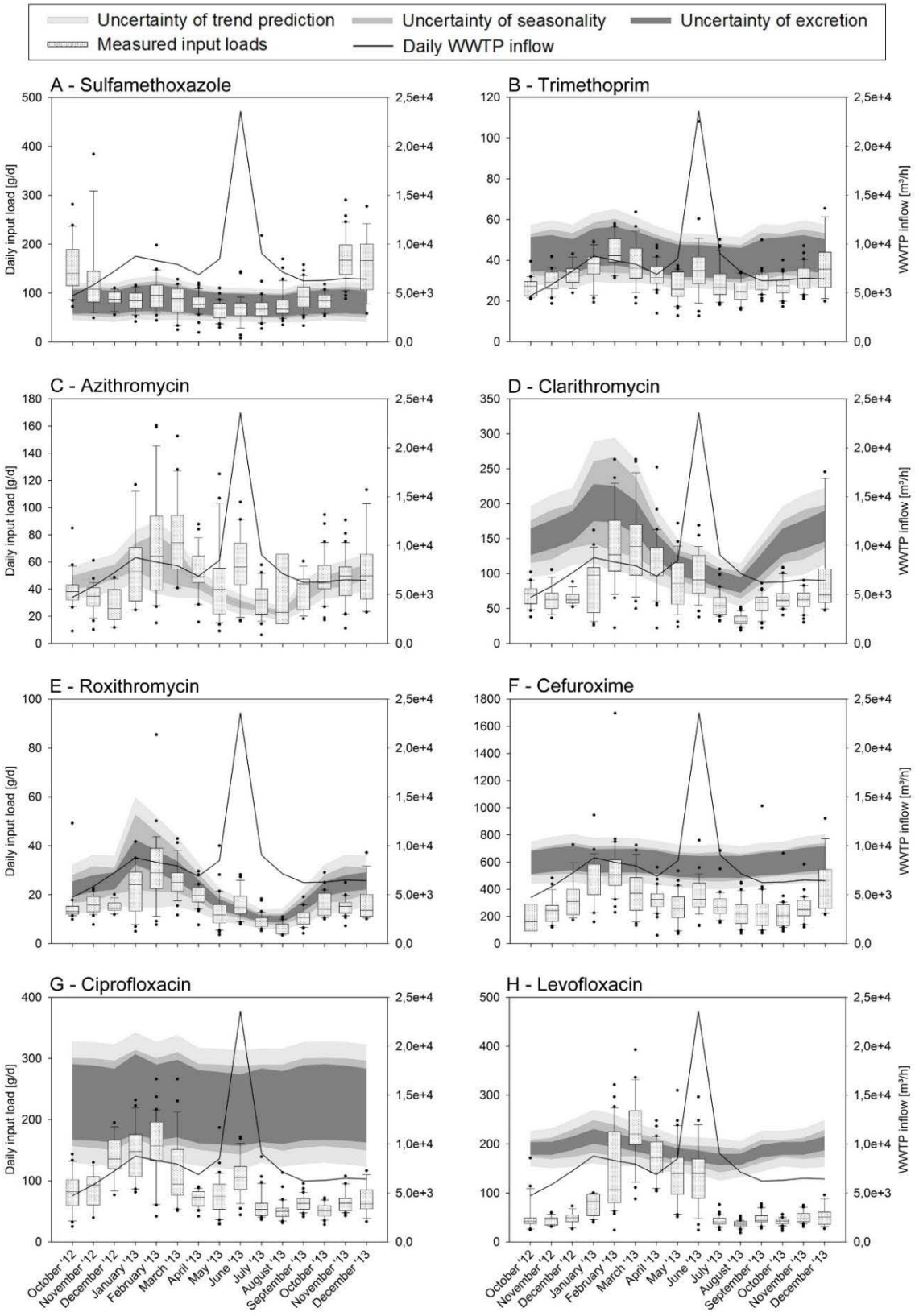

Figure 5: Predicted (band of three levels of grey) and measured input loads of antibiotics (area of boxplots represents the range of 25 % to 75 % percentile, whiskers illustrate 10 to 90 % percentiles of all data, outliers are depicted as dots); the monthly mean of the daily WWTP inflow is illustrated as solid line

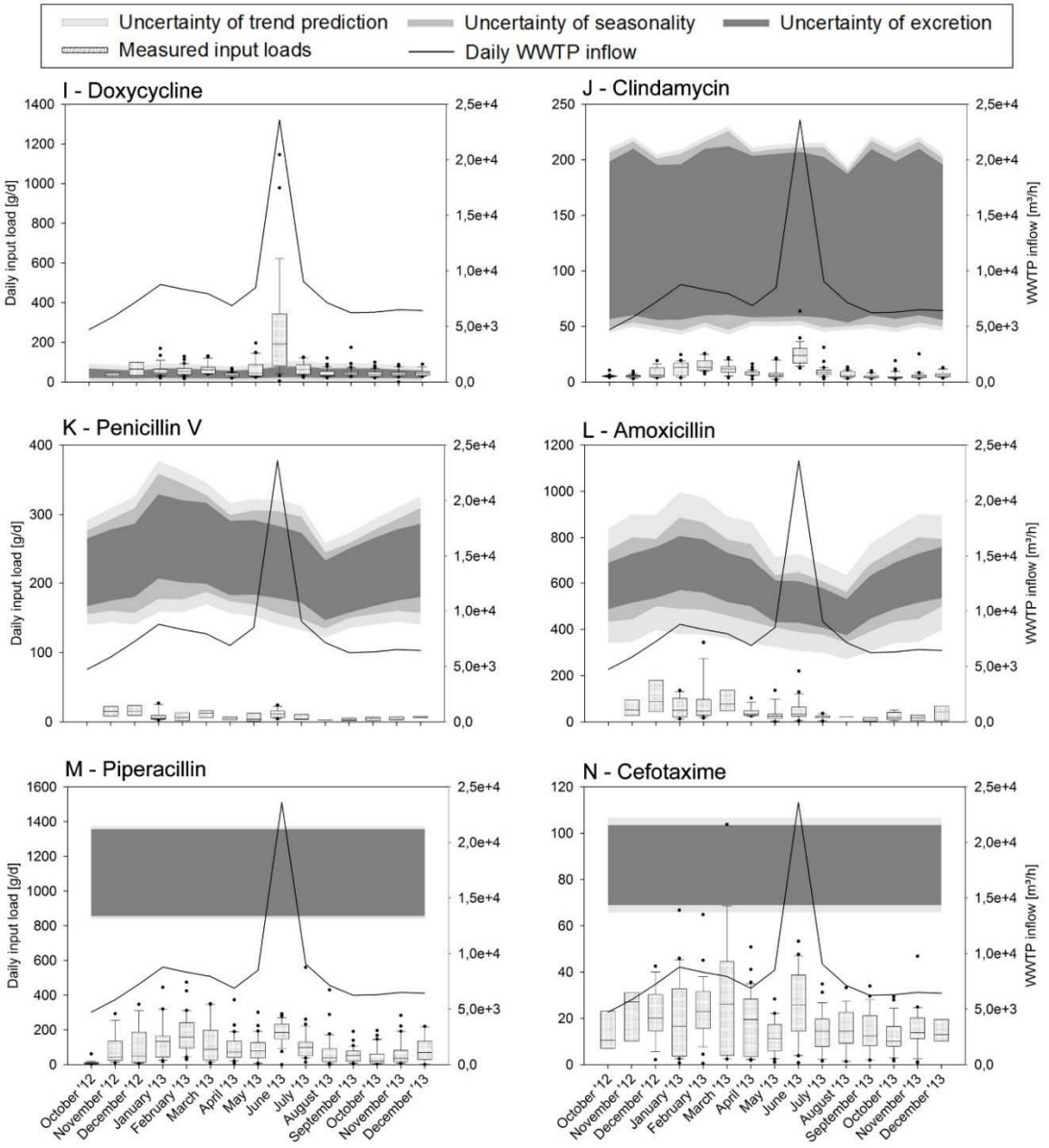

Figure 5 (continued): Predicted (band of three levels of grey) and measured input loads of antibiotics (area of boxplots represents the range of 25 % to 75 % percentile, whiskers illustrate 10 to 90 % percentiles of all data, outliers are depicted as dots); the monthly mean of the daily WWTP inflow is illustrated as solid line

Cefuroxime, ciprofloxacin, levofloxacin, doxycycline

Cefuroxime, ciprofloxacin, doxycycline and levofloxacin are examples of an unsystematic connection between constant prescriptions and unexpected high fluctuation in measured loads. Interestingly, the increase of ciprofloxacin and cefuroxime corresponds to the seasonality of macrolides from December to February (see Figure 5F and G) unlike prescriptions would suggest. Levofloxacin belongs to the same antibiotic group of fluoroquinolones like ciprofloxacin but has a prolonged peak from February to June (see Figure 5H). This is surprising since similar

medical application (same antibiotic group) suggests similar input characteristics at the WWTP input. On the other hand, nearly during the entire year doxycycline meets predicted input loads but shows a 4 to 5-fold increase during the stormwater event in June 2013 (see Figure 5I). A possible explanation is the fact that tetracyclines have complexing properties (Pamreddy et al. 2013) which suggest a delay during the wastewater transport in sewer systems at dry weather. Stormwater might subsequently lead to remobilization of sewer sediment and/or re-dissolution of doxycycline from the sewer particles, respectively, which in turn leads to higher input loads at the WWTP. Nevertheless, based on total predicted input loads doxycycline is heavily underestimated, unlike the other antibiotics of this group. Hence, it was questioned, if a load peak in the range of approximately 3 additional input months is acceptable, in terms of adsorption capacity of suspended solids (SS) in raw wastewater. As a result, additional measurements comparing the inlet and outlet of the preliminary settling tank (n = 31, separation efficiency of SS: 60 %) did not show significant load removal ($p = 0.1654$) during the settling process, suggesting only a minor adsorption to SS. Hence, it seems implausible that the additional 22 t SS (+ 40 %) entering the WWTP during the stormwater event contributed to the load peak of doxycycline. During the flood event, the infiltration of ground- and river water holds a share in the prolonged inflow increase. There are no data available to determine whether a significant concentrations in the infiltration water could have contributed to the load peak at the WWTP or if only dilution took place. According to the plants efficiency regarding doxycycline removal of about 76 % (n = 300, median = 100 % removal, own data) no significant doxycycline emissions into the environment are expected and concentrations in river- or groundwater are assumed to be comparatively low. Hence, it is rather unlikely that the input load of the WWTP was influenced by natural water bodies. Furthermore, it is difficult to determine how an erroneous analytical method could have affected the load calculation. Assuming a reliable analysis method, re-dissolution of doxycycline from sewer particles is favored in this context but needs further investigations to be supported.

The strongly fluctuating input values of ciprofloxacin and levofloxacin (Ofloxacin and levofloxacin are racemats and analytically considered as one substance) were also observed in a WWTP in Switzerland. In the present study, ciprofloxacin loads elevate from about 50 g/d up to more 200 g/d which corresponds well with the 4 – 5-fold increase determined in Switzerland (Coutu et al. 2013). The results for the input loads of ciprofloxacin (59.9 – 273.6 µg/(E*d)) show to be similar to other investigations: 27.5 – 171.7 µg/(E*d) (Ghosh et al. 2009); 259 µg/(E*d) (Castiglioni et al. 2006); 49.6 – 104.3 µg/(E*d) (Karthikeyan and Meyer 2006); 200 – 300 µg/(E*d) (Li and Zhang 2011). Levofloxacin's input load increases from 50 g/d to about 250 g/d and is comparable to the 5-fold increase at Vidy WWTP (Coutu et al. 2013). The determined specific values for levofloxacin of this study (47.7 – 364.6 µg/(E*d)) are in the range of comparable investigations: 189.5 – 395.3 µg/(E*d) (Ghosh et al. 2009); 360 µg/(E*d) (Castiglioni et al. 2006). Cefuroxime and doxycycline exhibited specific input loads of

183.3 – 273.6 µg/(E*d) and 39.2 – 174.1 µg/(E*d), respectively. A second increase at the end of the year, as it was observed in the Swiss WWTP, could not be reproduced in the present study. The mean recovery rates of 53 %, 41 %, 155 % and 49 % were determined for cefuroxime, ciprofloxacin, doxycycline and levofloxacin, respectively, whereas 18 %, 18 %, 86 % and 15 % of the measured data are to be found within the predicted input band.

Amoxicillin, clindamycin, cefotaxime, piperacillin and penicillin V

The antibiotics amoxicillin, clindamycin, cefotaxime, piperacillin and penicillin V are significantly overestimated by input projection and exhibit unsatisfying recovery results. Except for clindamycin, all antibiotics of this sub-section belong to the beta-lactam family (Figure 5K – N) and are prone to biodegradation processes in raw wastewater. As for pre-degradation in raw wastewater (see 3.3), out of 4 investigated beta-lactams three decreased to a significant degree within 12 h. Due to analytical problems no data were obtained for penicillin V. However, given the fact that penicillin is well known for its instability in biological systems (Watkinson et al. 2009) the lack of data can be omitted in this case. In summary, low recovery results were expected and verified for beta-lactams and were within the applied projection model. Mean recovery values during the monitoring program were determined to 3 % (penicillin V), 8% (amoxicillin), 9 % (piperacillin) and 22 % (cefotaxime). Cefotaxime was the only substance with 3 (1 %) measuring values within the predicted area, the remaining beta-lactams did not hit the target area. The corresponding measured input loads amount to 9.4 – 222.6 µg/(E*d) (amoxicillin), 2.9 – 66.0 µg/(E*d) (cefotaxime), 9.0 – 445.5 µg/(E*d) (piperacillin) and 1.3 – 35.5 µg/(E*d) (penicillin V). Due to the expected inconsistency of the measured values (e.g. biodegradation) a comparison to other investigations is not promising to be carried out.

In case of clindamycin (see Figure 5J), the uncertainties of the projection model, or rather the excretion rate, lead to a comparatively large range of expected input loads. Oddly enough, incoming quantities are still below the projected values (mean: 7%) and only 16 % of determined loads (5.1 – 36.3 µg/(E*d)) are within predicted area. At Vidy WWTP per capita values of 2.2 – 4.4 µg/(PE*d) (Coutu et al. 2013) are significantly lower compared to the present study (7.7 – 30.7 µg/(PE*d)). Furthermore, during the stormwater event in June 2013 increased input loads were determined. For the time being, due to the lack of information regarding the fate of clindamycin, the elevated values during stormwater must be attributable to both remobilization and degradation processes as a consequence of a reduced retention time in the sewer system (see next paragraph).

Recovery and degradation in raw wastewater

In the laboratory experiments on the stability in raw wastewater (see 2.5) it was found, that expected values (raw sample + 1000 ng/l) of many antibiotics were not completely recovered 15 min after their addition to wastewater. This was unexpected since both dissolved and adsorbed antibiotics were equally included in the

analytical method. In order to further address the initial loss, the ratio of measured value and expected value has been termed "initial recovery". It raises the question as to whether the difference between expected value and initial recovery was due to a spontaneous decay subsequent to entering the wastewater matrix or due to an insufficient sampling procedure and analytical method. Even the use of deuterated antibiotics like trimethoprim d9 and ciprofloxacin d6 resulted in an initial "disappearance" of 20 and 40 – 60 %, respectively. Furthermore, sampling as well as transport, storage time and conditions prior to analytical extraction might be a source of inconsistent results in investigations on the antibiotic stability in raw wastewater. A final explanation could not be found on the basis of the present data set. Nevertheless, even if the analytical methodology failed to include certain fractions of antibiotics, an integrative view of WWTP results and pre-degradation data is key to evaluating the recovery of prescription data, since both were determined using the same methodology. Determined mean recovery rates derived from the WWTP were compared with the range of recovered antibiotics after addition (initial recovery) and 12 h sewer residence time, respectively, referring to the expected value (see Figure 6). The two latter values were illustrated using bands with two levels of grey, referring to anaerobic (dark) and aerobic (light) results. The corresponding errors are depicted as dark and light grey with lower opacity.

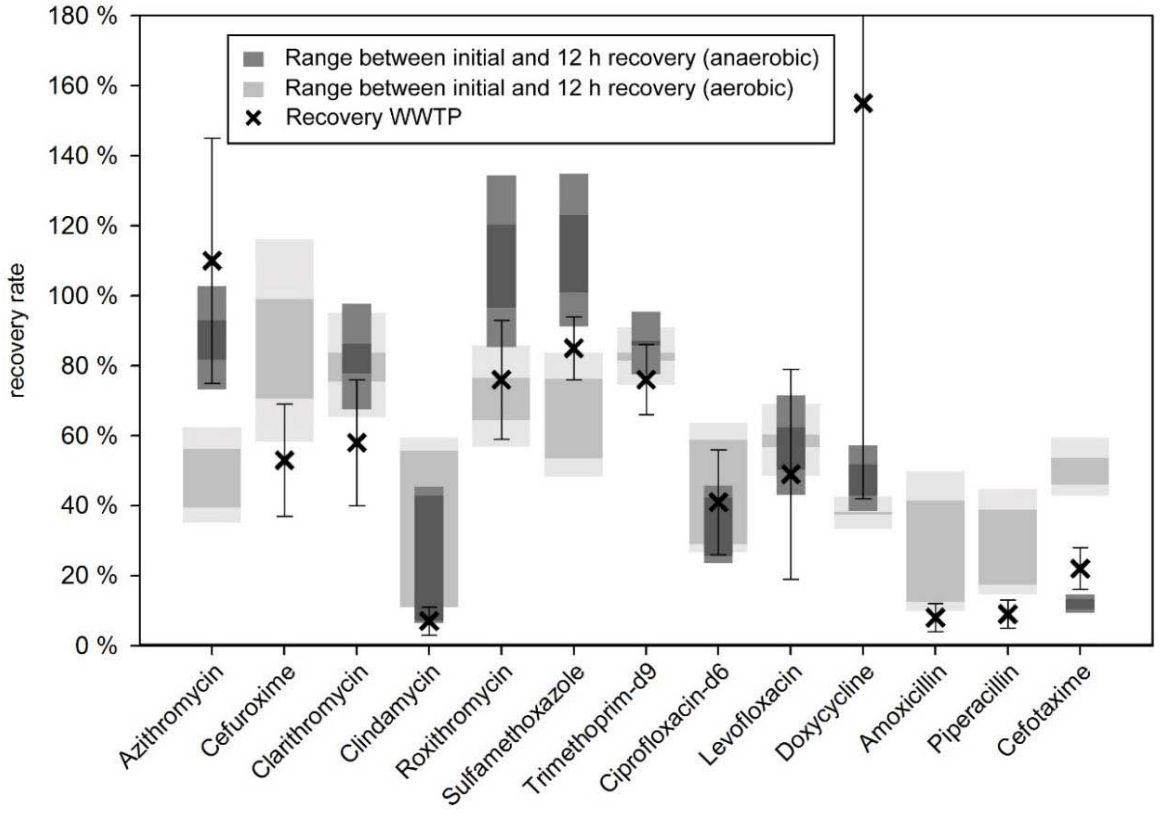

Figure 6: Recovery of antibiotics in raw wastewater on a laboratory scale at anaerobic and aerobic conditions compared with the mean WWTP input (standard deviation is illustrated by error bars)

Clarithromycin, clindamycin, trimethoprim, ciprofloxacin and levofloxacin show similar elimination characteristics under aerobic and anaerobic conditions. Azithromycin, roxithromycin, sulfamethoxazole and doxycycline are eliminated to a lesser extent under reducing conditions, while cefotaxime behaves vice versa. Cefuroxime, amoxicillin and piperacillin could not be evaluated under anaerobic conditions due to analytical problems. Neglecting the initial recovery and taking the band width as indicator of degradability azithromycin, clarithromycin, roxithromycin (only under aerobic conditions), trimethoprim, ciprofloxacin (only under anaerobic conditions), levofloxacin and doxycycline are stable in raw wastewater exhibiting less than 20 % reduction within 12 h. All beta-lactams, clindamycin, roxithromycin (only under anaerobic conditions), sulfamethoxazole and ciprofloxacin (only under aerobic conditions) were reduced by more than 20 %.

It is noted that the majority of data points from the WWTP recovery are within or close to both, anaerobic and aerobic recovery ranges (see Figure 6). To some extent cefuroxime can be found below the expected recovery in wastewater under aerobic conditions. Apart from analytical errors the discrepancy could be due to a misleading projection approach. Among all outpatient antibiotics under investigation cefuroxime exhibited the highest prescription gain between 2005 and 2012 of which about 80 % is attributed to hospitals (see Figure 1). This might be an indication that projecting hospital prescription using the number of hospital beds in the catchment is not sufficient to accurately determine the input to the WWTP. A detailed differentiation between medical stations and corresponding prescriptions may be expedient rather than blurring the data by putting total hospital prescription into relation to institution's bed capacity.

Strangely, roxithromycin recovery at the WWTP is closer to the results of aerobic wastewater conditions while recovery values of azithromycin and cefotaxime seem only to fit if wastewater was mainly anaerobic. The oxygen introduced into wastewater during its passage through the sewer system is assumed to be low, compared to oxygen consumption. Hence, anaerobic conditions are supposed to be predominating, although not solely.

Except for doxycycline, the projection and prediction model of input loads can be considered to be verified by the pre-degradation experiments in raw wastewater. The recovery rates determined from the monitoring data are within or close to the values of the laboratory results which provide information on the overall recovery and pre-degradation of antibiotics during the residence time of wastewater in the sewer system. Furthermore, the verification of the projection model also supports the use of the excretion data from medical literature to predict environmental concentrations using production, sales and prescription data.

Significance of determined input loads depending on sample quantity

Previous studies already demonstrated the insignificance of grab samples, in terms of load characterization of WWTPs (Loganathan et al. 2009), due to major input fluctuations within one day (Coutu et al. 2013, Li and Zhang 2011). Hence, the

use of composite samples using auto-samplers is the favoured alternative. In this context, the solid data pool of this study will be used to estimate a necessary number of samples to approximate a representative annual input load of antibiotics. It is assumed that the average load of this study is representative for the actual input load to the WWTP Dresden-Kaditz, excluding the data during the time period of the flood event (01.06. – 19.06.2013). Sample values were randomly drawn from the entire data set of each and every antibiotic using a uniform distribution without replacement. The number of drawings was varied between one and the maximum amount of available monitoring values (see supplementary material Table S1, annex 1). Each drawing range (1 sample, 2 samples, 3 samples etc.) was repeated 500 times. All repetitions within the drawing ranges were averaged to determine the corresponding mean of the theoretical sampling campaign. Preliminary calculations carried out to assess the impact of repetitions showed that 500 replicates were sufficient to minimize the deviation of necessary sample numbers to a value below 10 % (see supplementary material Figure S3). From supplementary material Figure S4 it can be seen that higher sample quantities result in lower deviations from the annual mean, in terms of uncertainty. Hence, uncertainty was related to an appropriate number of samples for 95 % of all repetitions with 2.5 % and 97.5 % being the lower and upper quantiles. As a result, outliers were excluded and the practicability of results was increased. Figure 7 summarizes the relationship between deviation and sample quantity for all antibiotics. For comparison reasons the results were normalized to one year (365 days) due to different quantities of monitoring values (see supplementary material Table S1, annex 1).

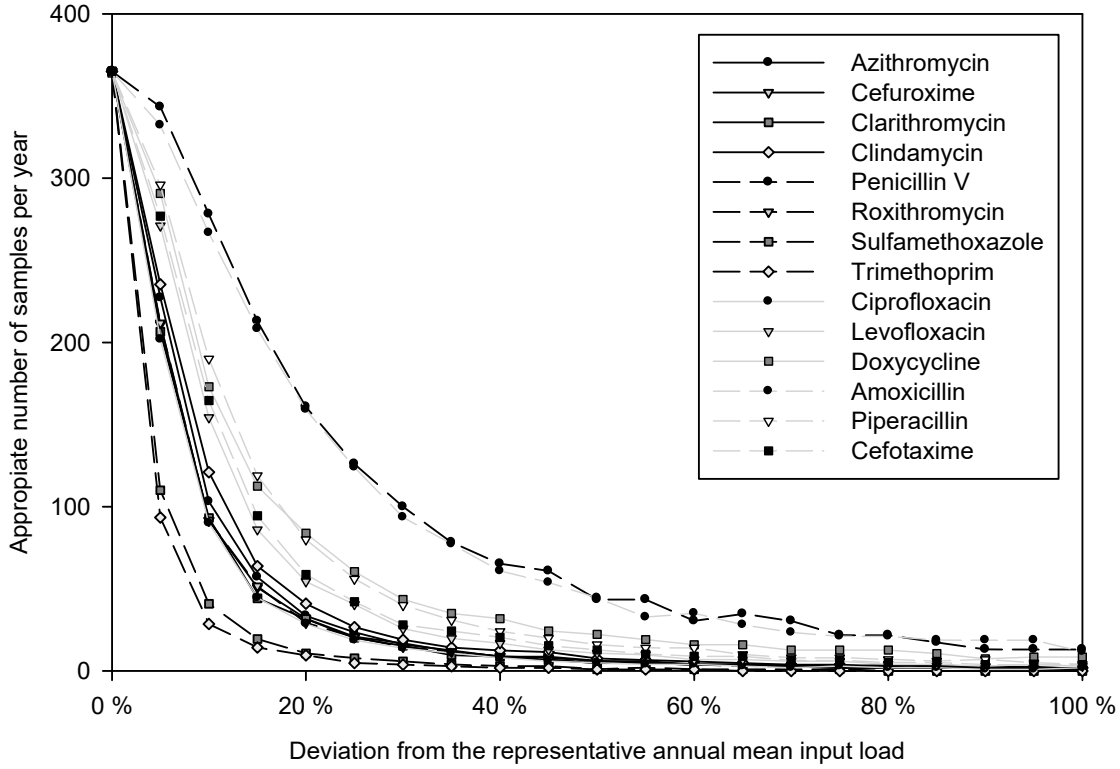

Figure 7: Number of necessary samples per year depending on the theoretical deviation (uncertainty) of the representative annual mean input load

All antibiotics were evaluated for a deviation (or uncertainty) of 20 %, which is supposed to be close to reasonable considering the significant expense of any resulting scope of investigations. In the following, the given number of samples are considered to be evenly distributed throughout the year. Trimethoprim and sulfamethoxazole, the latter excluding implausible months (see 3.2), have been shown to be the most reliable measurements. Fluctuations of measured input loads have in general been small which results in an appropriate measurement quantity of 10 samples per year. In contrast, 160 samples must be taken to reliably determine the characteristic input load of penicillin and amoxicillin. The former has a lower detection frequency than most antibiotics which makes an increase of sampling incidence necessary. The seasonality of the macrolides azithromycin, clarithromycin and roxithromycin is comparable with the fluctuations measured for clindamycin, cefuroxime and ciprofloxacin, in terms of uncertainty. A number of 30 - 40 samples per year are necessary to determine the characteristic input load of those antibiotics. Doxycycline and levofloxacin as well as the inpatient antibiotics piperacillin and cefotaxime exhibit slightly higher fluctuations of input loads and 55 - 80 samples are required to calculate the mean annual input load, accepting an uncertainty of 20 %.

Table 2: Uncertainty of measured input loads of antibiotics applying the relationship between sample quantity and uncertainty determined in this study

Literature (sample quantity)	Plosz et al. (2010) (3 samples)	Gao et al. (2012) (3 samples)	Li and Zhang (2011) (4 samples)	Gao et al. (2005) (8 samples)	Coutu et al. (2013) (84 samples)
Ciprofloxacin	65%	-	55%	-	10%
Trimethoprim	35%	-	-	20%	5%
Sulfamethoxazole	40%	-	35%	25%	-
Doxycycline	-	> 100 %	-	-	-
Levofloxacin	-	-	80%	-	15%
Roxithromycin	-	-	60%	40%	-
Azithromycin	-	-	60%	45%	-
Clarithromycin	-	-	60%	40%	-
Clindamycin	-	-	70%	50%	15%

The influencing factors for the uncertainty, and therefore the number of necessary samples, are manifold. These factors are potentially attributed to the length and structure of the sewer system, catchment size, location and operation of the auto-sampler (representative sample from completely mixed inflow), sample storage (temperature, duration) and the applied analytical method. Among those, the storage of samples and the analytical method are mainly the only factors that can be classified and used for comparison purposes between studies. Hence, it is proposed that the link between load characterization of antibiotics (and other anthropogenic substances related with wastewater) and the appropriate number of samples can be generalized. Accordingly, monitoring of 20 to 40 samples, evenly distributed throughout the year, is recommended depending on whether the substance under investigation is seasonally influenced or not. In summary, the results clearly show that most monitoring programs rely on far too few samples. Similar findings at Vidy WWTP, Switzerland, result from an appropriate number of samples. The deviation of the characteristic loads is within the range of 5 % (trimethoprim) and 15 % (clindamycin and levofloxacin) and covers relevant fluctuation throughout the year (Coutu et al. 2013).

Table 2 summarizes the uncertainty of measured input loads from other studies applying the information provided in Figure 7. It can be concluded that the results of most studies carry a significant error of more than 50 % due to the small number of samples taken. This seriously diminishes the significance of the presented results. Hence, for future sampling campaigns regarding antibiotics and other pharmaceuticals a sufficient sample quantity should be provided to minimize the error and increase the scientific value of the investigation.

CONCLUSION

For most antibiotics the applied projection and prediction model in combination with excretion ratios from literature could be verified by loads determined during the long-term monitoring at the WWTP. Additionally, laboratory experiments provided information on the pre-degradation in raw wastewater which further improved the understanding of the occurring processes during the wastewater transport. Some antibiotics like ciprofloxacin or levofloxacin behaved unexpected over the time period under investigation. According to the prescription data the comparatively low variations were not confirmed by the analytical data. It is unclear at which point the projection model is afflicted with errors. Hospital data might constitute an input path which cannot be sufficiently projected on the basis of previous time periods or the number of hospital beds. Further simplifications like an assumed equality of prescription between private and statutory insurants, the non-compliance in the use of prescription drugs (Grosso et al. 2012) or the use of antibiotics in veterinary medicine, respectively, might also contribute to the discrepancy between expected and measured values. In case of doxycycline the measured values during the stormwater event play a crucial role regarding overall recovery. A quantitative assessment of the above-mentioned is not possible within the frame of the present results.

On average, the analytical error of < 15 % is 2 – 3 times lower than the uncertainty of predicted input loads (see Figure 3). Hence, the analytical method is regarded to be sufficiently reliable for input measurements. On the basis of the extensive data pool of this study, calculations were carried out to evaluate the validity of monitoring programs based on the number of samples. Taking antibiotics with low seasonality and low input scattering like trimethoprim and sulfamethoxazole a minimum of about 10 samples is required to calculate a representative annual mean load. For antibiotics exhibiting fluctuating input loads, e.g. due to seasonality, 30 to 40 evenly distributed samples are necessary for a representative input determination with a deviation of about 20 %. Hence, it is proposed that a minimum number of 20 to 40 samples should certainly be taken to reasonably estimate a representative input load. The main findings of the study can be summarized as follows:

- Estimation of antibiotic input loads on the basis of up-to-date prescription data is provides results with an average uncertainty of 30 – 50 %
- The analytical error of current analytical methods using LC-MS/MS is sufficiently low for the quantification of antibiotic input loads
- A minimum sample quantity of 20 – 40 samples is recommended for the reliable determination of antibiotic input loads.

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CHAPTER 3

2. COMPARTMENT: WASTEWATER TREATMENT PLANT

Mass flow of antibiotics in a wastewater treatment plant focusing on removal variations due to operational parameters

Mass flow of antibiotics in a wastewater treatment plant focusing on removal variations due to operational parameters

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ABSTRACT

Wastewater treatment plants (WWTPs) are not designed to purposefully eliminate antibiotics and therefore many previous investigations have been carried out to assess their fate in biological wastewater treatment processes. In order to consolidate previous findings regarding influencing factors like the solid and hydraulic retention time an intensive monitoring was carried out in a municipal WWTP in Germany. Over a period of 12 months daily samples were taken from the in- and effluent as well as diverse sludge streams. The 14 selected antibiotics and one metabolite cover the following classes: cephalosporins, diaminopyrimidines, fluoroquinolones, lincosamide, macrolides, penicillins, sulfonamides and tetracyclines.

Out of the 15 investigated substances, the removal of only clindamycin and ciprofloxacin show significant correlations to SRT, temperature, HRT and nitrogen removal. The dependency of clindamycin's removal could be related to the significant negative removal (i.e. production) of clindamycin in the treatment process and was corrected using the human metabolite clindamycinsulfoxide. The average elimination was adjusted from -225 % to 3 % which suggests that clindamycin can be considered as an inert substance during the wastewater treatment process. Based on the presented data, the mass flow analysis revealed that macrolides, clindamycin/clindamycin-sulfoxide and trimethoprim were mainly released with the effluent, while penicillins, cephalosporins as well as sulfamethoxazole were partly degraded in the studied WWTP. Furthermore, levofloxacin and ciprofloxacin are the only antibiotics under investigation with a significant mass fraction bound to primary, excess and digested sludge. Nevertheless, the sludge concentrations are highly inconsistent which leads to questionable results. It remains unclear whether the inconsistencies are due to insufficiencies in sampling and/or analytical determination or if the fluctuations can be considered reasonable for digesters. Hence, future investigations have to address antibiotic's temporal dynamics during the sludge treatment to decide whether or not the widely reported standard deviations of sludge concentrations reflect realistic fluctuations.

KEYWORDS

Wastewater treatment, sludge, antibiotics, elimination, removal, mass flow analysis

INTRODUCTION

The elimination of antibiotics from municipal wastewater receives great scientific and political attention due to their associated potential risk to the environment and human health. Recently, macrolide antibiotics were added to the European watch list of substances for Union-wide monitoring (EU, 2015) which underlines the timeliness of antibiotic's release from urban areas into the environment. Wastewater treatment plants (WWTPs) are originally designed to reduce organic compounds, nitrogen and phosphorus from municipal and industrial wastewaters. Hence, previous investigations focusing on the fate of introduced antibiotics in WWTPs resulted in a heterogeneous picture regarding the span of removal efficiencies of certain substances (Luo et al., 2014), while significant systematic differences in removal rates between various substances could be detected. The differences between studies were partly associated to differing operational regimes of the WWTP like solid retention time (SRT) (Clara et al., 2005; Suarez et al., 2012; Vieno et al., 2007), hydraulic retention time (HRT) (Guerra et al., 2014; MacLeod and Wong, 2010) and the plant design itself, e.g. nitrification-denitrification and microbial community of the activated sludge system (Guerra et al., 2014; Larcher and Yargeau, 2012; Suarez et al., 2010). Other studies investigated the differences of removal efficiencies between seasons (Gracia-Lor et al., 2012; Guerra et al., 2014; Kwon and Rodriguez, 2014; Zhang et al., 2015). Many of the above mentioned investigations also reported negative elimination rates, which are interpreted as a production of antibiotics during the biological treatment process. Despite the fact that the re-transformation of metabolites to their parent substances has been previously proposed (Goebel et al., 2007) many of the negative values are likely to be the result of an insufficient sampling method (Majewsky et al., 2011a) and longer sampling campaigns are required to achieve reliable results (Gobel et al., 2005).

In addition to the aqueous phase, it is advantageous if mass flow studies also involve sludge loads to fully understand the fate of antibiotics during wastewater treatment (Guerra et al., 2014; Kasprzyk-Hordern et al., 2009). A few studies are available providing information on concentrations of antibiotics in both the liquid and the solids phases. The reason why studies providing an integrative view on all removal pathways are scarce is presumably mainly due to the high efforts necessary to carry out substance analyses with complex matrices like sludge (Jelic et al., 2011). Most investigations performing a mass flow analysis rely on a very limited number of liquid samples (4 (Yan et al., 2014a; Yan et al., 2014b; Zhou et al., 2013a); 6 (Gobel et al., 2005; Guerra et al., 2014; Zhang et al., 2013)) and of sludge samples (1 (Zhang et al., 2013); 2 (Zhou et al., 2013a); 3 (Zuccato et al., 2010)) per WWTP. Despite the limited number of samples the studies mentioned above provide valuable initial information on antibiotic removal pathways. Nevertheless, due to the expected influence of the plant design, operational parameters etc. on the removal of antibiotics in combination with low sampling quantities, the significance of results from previous mass flow analyses needs to be improved.

An intensive sampling of one single WWTP can provide detailed information on the removal mechanism at different operational conditions. Due to diversity in plant design, operational regime and wastewater composition such results cannot be adequately gathered from monitoring campaigns targeting multiple plants.

In this study, the WWTP Dresden-Kaditz was monitored over a period of 12 months taking daily in- and effluent samples. Additional sludge samples from the in- and output of the digesters were taken to include the adsorbed fraction of antibiotics into the mass flow analysis. The high data quantity in connection with routine operational data was used as a basis to investigate the influence of WWTP operation on the removal efficiency of antibiotics. The subsequently performed mass flow analysis will be used as a tool to describe the fractioning of antibiotics after the wastewater and sludge treatment.

MATERIALS AND METHODS

Catchment area and WWTP operation

The WWTP Dresden-Kaditz treats the wastewater of five cities (Dresden, Freital, Heidenau, Pirna and eastern Radebeul) and some bordering municipalities in Saxony, Germany. The WWTP has a design capacity of 740,000 PE with a current communal loading according to Table 1. The catchment area has a size of about 9400 km² with no known industries producing or applying significant amounts of antibiotics (e.g. intensive livestock husbandry). The sewer system consists of about 800 km of combined sewers and around 800 km of separate systems, consisting of some 450 km of sewerage system and 350 km of stormwater sewers. The wastewater is being mechanically treated by four coarse screens of 65 mm, three fine screens of 15 mm, a grit chamber and primary clarifiers with a total volume of 4800 m³. The plant was designed for biological nitrogen removal and chemical precipitation of phosphorus with a total treatment tank volume of 112,000 m³ of which up to 60 % can be operated without aeration. The secondary circular settling tanks have a total surface area of 10,920 m³. The anaerobic digesters are operated at 37 °C, have a total volume of 21,000 m³, a current hydraulic retention time of about 19 days and a loading rate of about 2.9 to 3.2 kgCOD/(m³*d). The total suspended solid (TSS) reduction in the digester amounts to 37 % and around 20,000 m³ of biogas is produced per day. The process diagram of the WWTP Dresden-Kaditz including all relevant wastewater and sludge flows is provided in Figure 1.

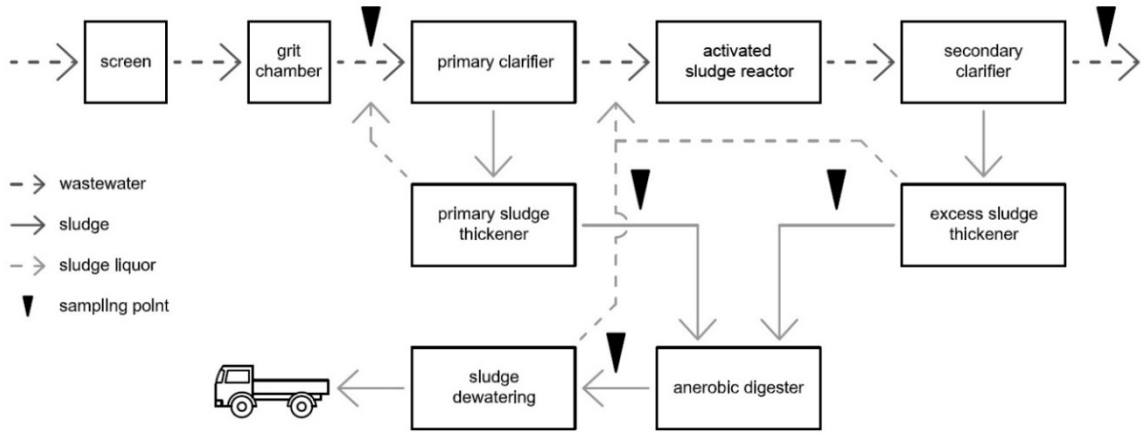

Figure 1: Process diagram of the WWTP Dresden-Kaditz with sampling points of the wastewater and sludge streams

Due to the legal framework regarding the elimination of total inorganic nitrogen, denitrification is carried out from May to October (see Table 1). Consequently, from November to April the WWTP is being operated at predominant aerobic conditions to keep nitrifiers within the system and to ensure the reliable start-up

of N-removal in April. During summer the focus of operation is on guaranteeing complete nitrification and denitrification. Accordingly, the TSS in the treatment tank is operated at about 3 g/L. During winter, insufficient settling characteristics of the activated sludge (higher sludge indices) require a lowering of the TSS in the activated sludge tank to meet the targeted sludge volume of about 400 mL/L. Thus, the failure probability of the secondary clarifiers is minimized.

Considering the monitoring period of 12 months from January to December 2013, the three resulting operation periods which are characterized by the presence and absence of denitrification are further characterized in Table 1. The input loads and pH-values are comparable due to low industrial contribution from the catchment area. In June 2013 (time period II) a flood event decisively influenced the WWTP inflow which results in a comparatively high standard deviation (SD) of the hydraulic retention time (HRT). The inflow temperatures deviate between time periods according to seasonality and the NO3-N effluent concentration is reflecting the operational regime regarding N-removal.

Table 1: Characteristic information of the three time periods under investigation (mean ± standard deviation)

Operation period	Winter 01.01. - 30.04.2013	Summer 01.05. - 31.10.2013	Winter 01.11. - 31.12.2013
Input load COD [kg/d]	83329 ± 10417	81881 ± 21458	94651 ± 20067
Input load TKN [kg/d]	9514 ± 571	8904 ± 1,444	10936 ± 2,372
Input load total P [kg/d]	1320 ± 99	1402 ± 299	1648 ± 379
Inflow temperature [°C]	11.9 ± 1.0	15.9 ± 2.0	14.3 ± 1.6
Inflow pH [-]	7.8 ± 0.1	7.8 ± 0.2	7.8 ± 0.1
HRT [d]	0.82 ± 0.22	0.65 ± 0.56	1.09 ± 0.21
SRT [d]	13.1 ± 0.5	18.9 ± 1.2	18.5 ± 0.9
Effluent NO_3-N [mg/l]	20.1 ± 5.3	5.5 ± 1.7	16.5 ± 3.2

Sample collection and analytical methods

The monitoring campaign at the WWTP Dresden-Kaditz was carried out from January until December 2013. The 24h volume proportional composite samples from the in- and effluent (see Figure 1) were taken daily starting at 8:00 a.m using brown glass flasks. The automatic sampler (Endress+Hauser ASP Station 2000) takes a sample volume of 25 ml per 480 m³ inflow and is equipped with 12 and 24 bottles for influent and effluent sampling, respectively. The total sample volume during dry weather is about 250 – 300 ml/h. Samples were stored at 4°C using brown glass flasks for a maximum time period of 7 days until transport and analysis in the lab. From a previous investigation using the same influent data set (Marx et al., 2015) it can be concluded, that refrigeration was sufficient to preserve the antibiotics during the storage time. In June 2013, the inflow measurement was

out of order due to the flood event caused by the river Elbe. The respective inflow hydrograph was reconstructed using the ammonia concentration. Details are described in Marx et al. (2015).

A total of 14 sludge samples were taken as grab samples during 2013 (summer) and 2014 (winter) to cover different seasons of the year. Primary sludge (PS), excess sludge (ES) and digested sludge (DS) were separately sampled using provided sampling taps and analyzed in order to assess the fate of antibiotics during the anaerobic sludge treatment. PS, ES, and DS were collected after the PS and ES thickeners and from the effluent of the digester (see Figure 1), respectively. A summary of the determined sludge concentrations is provided in

Table 3. The analysis parameters and according regulations of in- and effluent and sludge samples are summarized in the supplementary material S1.

A total of 14 antibiotics and one human metabolite were investigated covering the following classes: macrolides (AZI – azithromycin, CLA – clarithromycin, ROX – roxithromycin), lincosamide clindamycin (CLI) and its metabolite clindamycin-sulfoxide (CLI-S)), tetracyclines (DOX – doxycycline), cephalosporins (CEF – cefuroxime, CEFO – cefotaxime), sulfonamides (SUL – sulfamethoxazole), diaminopyrimidines (TRI – trimethoprim), penicillins (PEN – penicillin V, PIP – piperacillin, AMO – amoxicillin) and fluoroquinolones (CIP – ciprofloxacin, LEV – levofloxacin). The in- and effluent samples were analyzed by SPE and LC-MS/MS according to a method developed by Rossmann et al. (2014). Briefly, wastewater samples were spiked with Na2EDTA, centrifuged and filtered through glass fibre filters. Then, samples were adjusted to a pH of 3.5 using formic acid and spiked with a standard addition of 1000 ng/L. For SPE, 30 mg Water Oasis HLB cartridges were used. For the following analysis by LC-MS/MS, a Synergi HydroRP (Phenomenex, Aschaffenburg, Germany) and a Nucleoshell HILIC column (Machery-Nagel, Düren, Germany) were used for separation.

Sewage sludge samples were analyzed using ultrasonic associated extraction (USE) as additional extraction step before following the above mentioned SPE and LC-MS/MS method (Rossmann et al., 2014). USE was applied as it was described in other studies for extraction of sewage sludge, agricultural soil or other biosolids (Ho et al., 2012; Martin et al., 2010). In detail, approximately 0.2 to 1.0 g of homogenized, dried sludge (25 °C, 4 d) was accurately weighed into 50 mL centrifuge tubes. For the spiking of sludge, working standard solutions of 1 and 10 µg/mL were weekly prepared and stored at 4°C. For USE, a mixture of Acetonitril, 0.1 M EDTA and McIlvaine buffer (pH 4.5) was used. 5 mL of the extraction buffer (ACN:EDTA-McIlvaine Buffer, 50:50) were added to the spiked sludge, vortex mixed for 30 s, placed into a ultrasonic bath for 5 min and then centrifuged at 6000 rpm for 6 min. Supernatant was collected into a separate tube. The settled sludge was extracted by USE twice more and 0.5 mL of the combined supernatant was completely reduced by gently air stream and then diluted with 5 mL of purified water. Then, samples as well as the liquid fraction of the sludge were analyzed by

SPE onto a 60 mg HLB cartridges (Water Oasis, USA) followed by LC-MS/MS as described in Rossmann et al. (2014) under use the above mentioned columns for separation. The liquid fraction of the sludge was determined to correct the sludge concentrations. After the centrifugation step about 80 % of water remains in the sample before it is dried in the oven. Hence, the sludge concentration has to be reduced by the dissolved mass of antibiotics to calculate the actual sludge specific sludge loading. The liquid fraction of most antibiotics was determined to be below 15 % (see supplementary material S2).

Determination of elimination rates from the liquid phase

The varying HRT as well as internal mixing processes and devices in WWTP reactors significantly increase the uncertainty of removal rates based on 24h mixed samples. Shifts between calculated in- and output loads lead to a high scatter of calculated removal efficiencies as being found in some studies (e.g. trimethoprim: -88 to 85 % (Verlicchi et al., 2012)). For large catchment areas and long sewer residence times broadly prescribed pharmaceuticals exhibit a low load variance between consecutive days and the concentration of inert substances with low sorption potential is almost exclusively influenced by the WWTP inflow, i.e. inversely proportional to the daily inflow rate. Although the measured variance cannot *per se* be attributed to the sampling strategy and inflow dynamics it still complicates the estimation of a sufficiently reliable removal rate. Furthermore, it might mislead subsequent investigations regarding influences on the removal rate of antibiotics depending on plant operation alternatives. In order to minimize this uncertainty the elimination rate of antibiotics was determined from the cumulative in- and output loads within time series of gapless in- and effluent data sets. A minimum of seven consecutive daily in- and output loads was chosen in order to cover every day of the week at least once. A summary of all identified time series is given in supplementary material S3 (annex 2). The actual elimination rate of each time series was determined using linear regression (see $slope_{AB,i}$ in equation 1) and is shown as the gradient of the approximated lines which are exemplarily shown in Figure 2. The index "i" refers to the numbering of the time series. Four antibiotics were chosen to demonstrate the variety of curve slopes from moderate elimination (SUL and LEV) to inert behavior (TRI and ROX), where the measurements actually yielded slightly negative elimination.

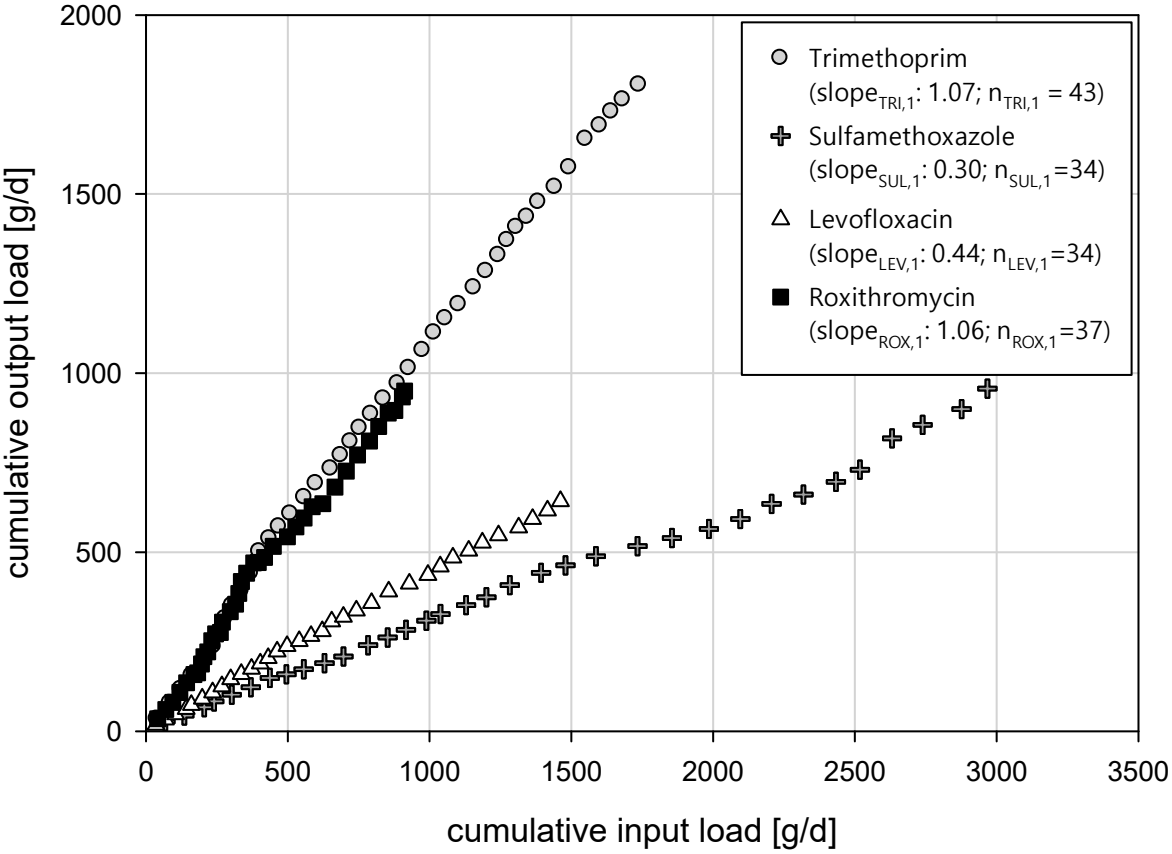

Figure 2: Comparison of cumulative in- and output loads of trimethoprim, sulfamethoxazole, levofloxacin and roxithromycin (the index "1" in "slope$_{AB,1}$" and "n$_{AB,1}$" refers to the number of the depicted time series according to supplementary material S3)

$$R_{AB,liquid} = 1 - slope_{AB,i} \quad [\%] \quad \text{(Equation 1)}$$

Subsequently, corresponding operational parameters were averaged for each identified time series to investigate the dependency between elimination efficiency and operational parameters using the t-test on the < 0.05 significance level. The operational parameter under investigation were chosen on the basis of previous investigations. The temperature (Golovko et al., 2014; Suarez et al., 2012; Zhang et al., 2015) as well as HRT and SRT (Kwon and Rodriguez, 2014; Verlicchi et al., 2012) are among the most discussed influencing parameters and will also be a subject of discussion in this study. Furthermore, nitrification and denitrification (Gobel et al., 2005; Suarez et al., 2010; Suarez et al., 2012) as well as iron concentration (Polesel et al., 2015a) were shown to have an influence on the removal of certain antibiotics. The calculation of normalized mass flows is provided in supplementary material S4 (annex 2).

RESULTS AND DISCUSSION

Elimination from the liquid phase

Figure 3 presents the mean removal efficiencies from the liquid phase ($R_{AB,liquid}$) based on all identified time series for each antibiotic. As expected, CEFO (Gulkowska et al., 2008; Li and Zhang, 2011), CIP (Gao et al., 2012a; Singer et al., 2014; Zhou et al., 2013a; Zuccato et al., 2010) and SUL (Li and Zhang, 2011; Sahar et al., 2011) are partly removed from the liquid phase while the macrolides AZI (Morasch et al., 2010; Yan et al., 2014b), CLA (Golovko et al., 2014; Morasch et al., 2010) and ROX (Li and Zhang, 2011; Yan et al., 2014a) as well as TRI (Golovko et al., 2014) and CLI-S (no comparable studies) show low (< 20 %) or no removal. Apart from AZI the determined mean removal efficiency of all substances are consolidated by 6 to 10 gapless time series covering different seasons of the year. Hence, they can be considered to be highly representative for the treatment plant under investigation. The comparison of cumulative in- and output loads results in high coefficients of determination covering time series of up to 44 days without missing values (see supplementary material S3, annex 2). On average, each time series is consolidated with 21 days providing a high reliability of the determined values. CEFO, DOX and AMO were eliminated to values below the detection limit most of the time under investigation and were only sporadically released with the effluent. Due to the unsystematic behavior regarding their release from the WWTP those antibiotics will be discussed apart from the ubiquitous antibiotics presented in Figure 3. PEN was only occasionally detected in the influent and completely removed in the WWTP. Accordingly, no further evaluation will be carried out in this context.

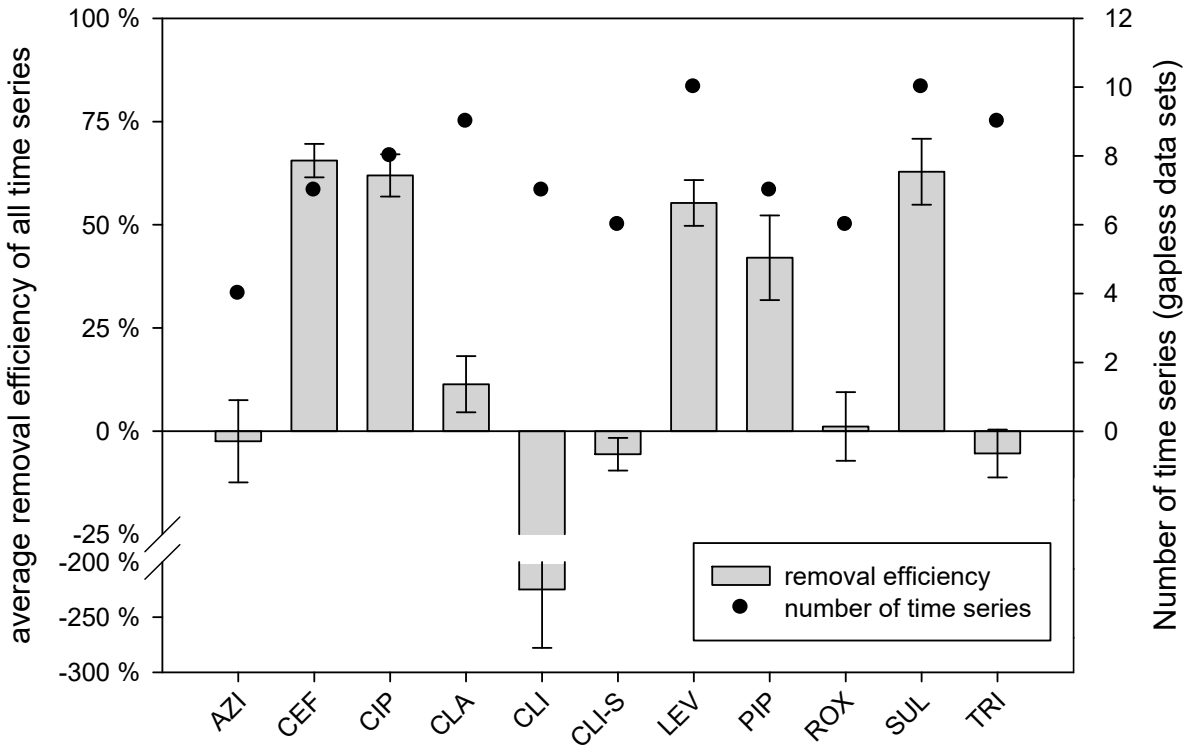

Figure 3: Mean elimination and corresponding number of available time series (bars represent the mean and whisker the standard deviation of all time series)

As stated above, operational parameters were shown to have an effect on the removal efficiency of antibiotics. For example, higher temperatures enhance microbial activity and possibly the biotransformation of certain substances. On the other hand, adsorption decreases with rising temperatures. SRT can be interpreted as a measure of microbial diversity of the activated sludge and a positive correlation to the removal efficiency is expectable. In general, the variation of determined removal efficiencies of all studied antibiotics is rather low and thus no significant dependencies to operational parameters are expected. The applied linear regression model confirmed this expectation for most antibiotics (see Table 2). Only CLI and CIP exhibit high correlation coefficients for some operational parameters at the 5 % significance level. Hereby, CLI is of special interest due to its "production" during the treatment process, compared to the input load, which has been already reported elsewhere (Alexy et al., 2006; Morasch et al., 2010; Oertel et al., 2013). According to Marx et al. (2015), CLI is constantly prescribed over the year but shows strongly fluctuating input loads (SD = 80 %, n=312), which can also be observed for other easily degradable antibiotics like PEN (SD = 110 %, n = 71) and PIP (SD = 99 %, n = 297). In connection with the negative and inflow-dependent elimination in the WWTP (see supplementary material S5) a pre-transformation of CLI to a so far unidentified transformation product (CLI-TP) is proposed to occur during the transport in the sewer system. In this context, the term transformation is used since the negative elimination during the wastewater

treatment indicates a re-transformation of CLI-TP to the parent substance CLI. Furthermore, CLI-S seems to be a promising indicator to correct measured input loads of CLI. The metabolite is jointly excreted with CLI, exhibits a low variation of input loads (SD = 27 %, n = 210), which agrees with a constant prescription pattern, and showed to be inert during the wastewater treatment (Figure 3). CLI-S does not seem to re-transform to CLI and is thus also considered inert in sewers. According to human pharmacokinetics, about 85 % of the taken drug is metabolized by the liver (Holly and Stevens, 1997) of which 90 % are excreted as CLI-S (Wynalda et al., 2003). Hence, around 75 % of the taken CLI will be excreted as CLI-S. On the other hand, 10 to 35 % of CLI is supposed to be excreted unchanged (Pharma, 2012; Still et al., 2006), which is partly contradictory in connection with the excretion of CLI-S. For consistency reasons the excretion of CLI is therefore assumed to be between 10 and 25 % which results in an expected excretion ratio of CLI-S/CLI between 3.0 and 7.5. Since no conclusive information are available on the share of other human metabolites, e.g. N-dimethyl-clindamycin, those substances will be considered negligible in this context.

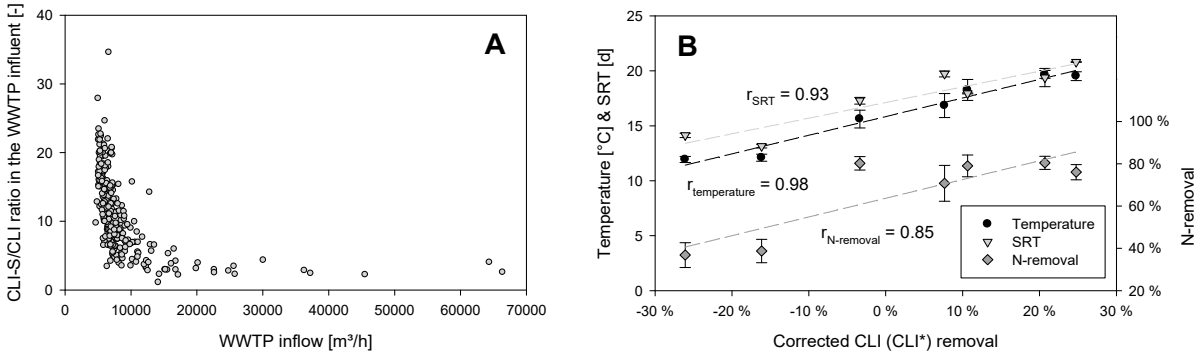

Figure 4-A: Dependency between measured CLI-S/CLI-ratio and WWTP inflow; 4-B: correlation of corrected CLI (CLI*) removal with SRT, temperature and N-removal (whisker represent the SD of corresponding time series)

Due to the ideal tracer characteristics of CLI-S and the proposed pre-transformation of CLI the CLI-S/CLI ratio should be close to the theoretical excretion ratio at low sewer residence times or high WWTP inflows, of which both are of equivalent use. A study focusing on hospital wastewater determined a low removal efficiency but no production for clindamycin (Kovalova et al., 2012). These results support the above thesis since the residence time between excretion and WWTP inlet was very short and therefore, pre-transformation processes in the sewer are minimized and can be assumed to be close to zero. From Figure 4-A it can be seen that in the present study, above an inflow of 15.000 m³/h the measured CLI-S/CLI-ratio approaches a value of 3.3 ± 1.0 (n = 22), which meets the lower limit of the theoretical excretion ratio of 3.0. This finding supports the assumption of an in-sewer pre-transformation of CLI and furthermore provides evidence that the analytical methods used for both substances are reliable. The calculated ratios

from Figure 4-A were subsequently used to determine the theoretical amount of CLI-TP entering the WWTP (see equation 2). Proposing a complete re-transformation into CLI during the treatment process the input loads of CLIi and CLI-TPi were summed up and compared to effluent loads CLIe (see equation 3). The resulting removal efficiency (CLI*) of 3 % ± 17 % closes the gap between in- and output load and points to the conclusion that CLI is not prone to elimination processes during the wastewater treatment. Correlating the corrected removal efficiencies CLI* with operational parameters (n = 7) reveals that applying the proposed re-transformation model leads to an insignificant dependency to WWTP inflow (r = -0.17) and iron concentration (r = 0.23) but strengthens the correlations to temperature (r = 0.98), SRT (r = 0.93) and N-removal (r = 0.85) (see Figure 4-B). It should be noted that the variability of removal must be addressed to both elimination of CLI and re-transformation of CLI-TP. No further differentiation can be made on the basis of the available information.

$$CLI - TP_i = \frac{CLI - S_i}{3.3} - CLI_i \qquad \text{[ng/L]} \qquad \text{(Equation 2)}$$

$$CLI^* = \left(1 - \frac{CLI - TP_i + CLI_i}{CLI_e}\right) \times 100\% \qquad \text{[\%]} \qquad \text{(Equation 3)}$$

The apparent positive cross correlation between temperature and SRT (r = 0.93) is partly due to the change of operational regimes of the WWTP Dresden-Kaditz, switching from predominantly aerated conditions in winter to denitrification in summer. For the identification of factors influencing the removal of clindamycin, this operational change is rather hindering because no precise information can be provided whether SRT, temperature or denitrification is responsible for the varied removal efficiency. Furthermore, due to enforced denitrification along with higher SRT in the summer period the relationship between CLI removal and N-removal seems to be a spurious correlation rather than a causal relationship. As a summary it can be stated that temperature and/or SRT have an influence on the analytically determined removal rate of CLI, whereby a differentiation between both factors is not possible on the basis of the available data. Moreover, the lack of detailed information on the so far unidentified transformation product CLI-TP does not allow to differentiate whether the dependency to temperature and/or SRT actually refers to the re-transformation of CLI-TP or the removal of CLI itself.

Table 2: Correlation coefficients between removal efficiency of antibiotics and operational parameters of the WWTP Dresden-Kaditz (asterisks indicate correlations on the < 0.05 significance level)

Antibiotic	HRT	Temperature	PH	SRT	Iron conc.	N-removal
AMO	0.00	0.00	0.00	0.00	0.00	0.00
AZI	0.16	-0.66	-0.48	-0.80	-0.75	0.75
CEFO	-0.68	0.83	-0.07	0.95	0.04	0.99*
CEF	0.00	0.03	-0.29	0.09	-0.08	0.50
CIP	-0.09	0.79*	-0.30	0.77*	-0.15	0.79*
CLA	0.28	0.15	-0.03	0.17	0.16	0.41
CLI	0.80*	-0.85*	0.06	-0.77*	-0.84*	-0.45
CLI-S	0.28	0.40	0.15	0.67	-0.21	-0.46
DOX	0.00	0.00	0.00	0.00	0.00	0.00
LEV	-0.13	-0.15	-0.25	-0.24	0.48	0.07
PEN	0.00	0.00	0.00	0.00	0.00	0.00
PIP	0.39	0.39	0.05	0.56	-0.60	0.43
ROX	0.69	0.02	0.64	0.19	0.18	-0.53
SUL	-0.48	0.21	-0.17	0.08	-0.28	0.19
TRI	0.55	-0.30	0.46	0.04	-0.26	-0.38

Similar to CLI, the removal of CIP is also significantly correlated to treatment temperature, SRT and N-removal ($p < 0.05$, see Figure 5) but is not influenced by the HRT. As in the case of CLI or CLI-TP, respectively, the temperature and SRT cannot be clearly evaluated independently from each other and removal must be considered to be influenced by either one or both parameters. In this context, in a study from Finland where 12 WWTP were investigated, no obvious dependency between SRT and CIP removal was found (Vieno et al., 2007). Similarly, CIP removal was similar during winter (January to March, 60 % removal) and summer (June to September, 63 %) in a study of six Italian WWTP (Castiglioni et al., 2006). Comparing activated sludge processes with biological nitrogen removal and simple nitrification also exhibits similar removal capacities with 86 % ($n = 7$) and 79 % ($n = 11$) elimination of the total CIP input load, respectively (Vieno et al., 2007). In summary, it can be concluded that none of the presented findings regarding influential parameters are directly confirmed by other investigations. Accepting the results from Finland leads to the conclusion that temperature is the driving force for the observed removal fluctuations. On the other hand, the SRT becomes the main influencing factor if relying on the results from Italy. The inconsistency between the present findings and the studies from Finland and Italy might be explained by the applied approaches of considering one single WWTP (this study) and the integrative evaluation of multiple plants (Castiglioni et al., 2006; Vieno et

al., 2007). The diversity of plant configurations and catchment characteristics might blur the results and complicate comparability between results. Hence, further investigations are needed to understand the actual mechanism of the identified influences in order to control and take advantage of the remaining removal "gap" between 54 and 68 % (see Figure 5).

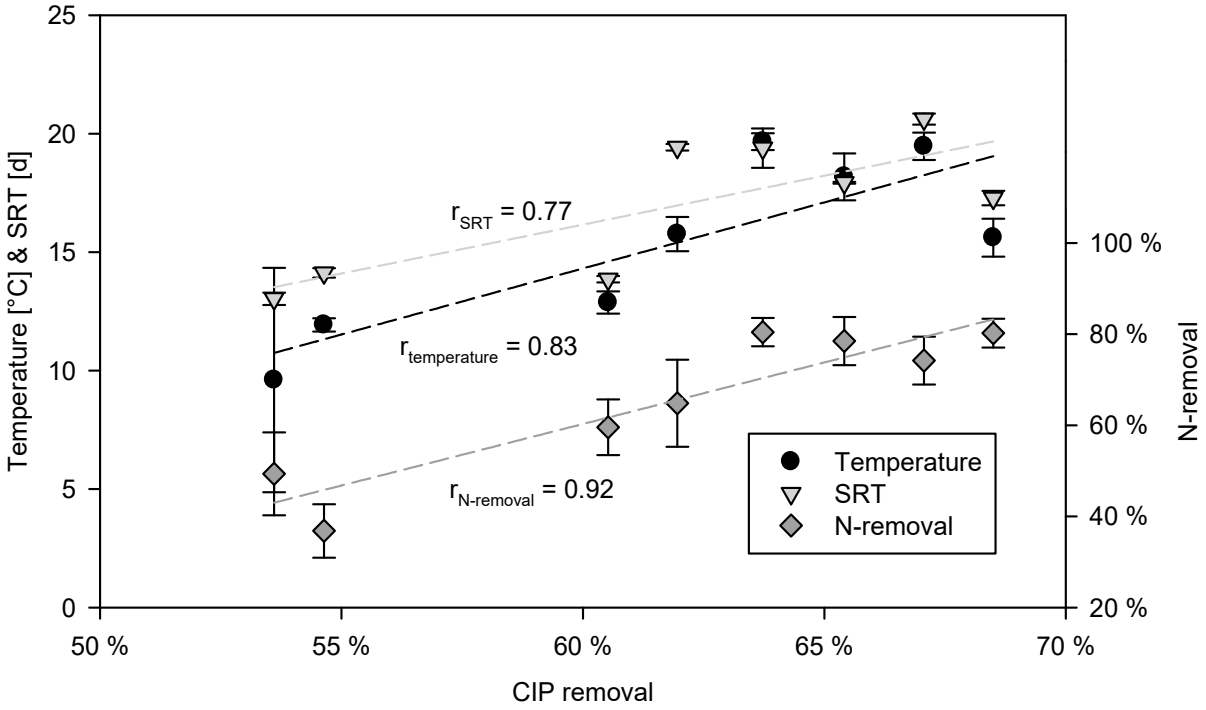

Figure 5: Correlation of CIP removal with SRT, temperature and N-removal (whisker represent the SD of corresponding time series)

CIP is known to strongly adsorb to activated sludge, a process that is supposed to be highly pH-dependent (Kuemmerer, 2009). This suggests that pH should actually be responsible for the changing removal efficiencies. Nevertheless, according to Table 2, the pH is of subordinate importance which becomes plausible taking into account a close-to-constant pH-value in the biological wastewater treatment process of Dresden-Kaditz (6.63 ± 0.06, n = 235). The maximum adsorption to aerobic and anoxic sludge occurs between pH 6 and pH 8 with no significant intermediate variation (Zhou et al., 2013b), which indicates that pH does not significantly influence the removal efficiency of CIP during conventional wastewater treatment. The optimal operation pH for microorganisms in the biological treatment systems lies between 6.5 and 8.5 (Junkins et al., 1983; Metcalf and Eddy, 1972) which implies that pH will mostly be found within this range and is generally of minor importance regarding the variability of CIP removal in most WWTP applying the conventional activated sludge process. Nevertheless, the negligible pH-range in the present investigation does not allow a solid conclusion with statistical significance. Previous findings demonstrate an enhancement of CIP removal induced by the addition of iron salts (Polesel et al., 2015a). The study

was carried out with activated sludge of 3.0 gTSS/L applying an iron concentration of 20 mgFe^{3+}/L (Polesel et al., 2015a) which is comparable to the conditions at the WWTP Dresden-Kaditz (2.88 - 3.05 gTSS/L, 4 -17 mgFe^{3+}/L). Despite the similarity of boundary conditions the reported dependency between CIP removal and iron addition is not supported for the time period under investigation (see Table 2).

The removal of LEV has been reported to be highly inconsistent and ranges from negative (Yan et al., 2014a; Zhang et al., 2015) to moderate (Li and Zhang, 2011; Zhang et al., 2013) up to an almost complete elimination (Singer et al., 2014). In this study the removal rate was determined to 55 ± 5 % and is consolidated by 10 time series. Hereby, the adsorption to sludge seems to be the main removal route (see also 3.3), since biodegradation is considered to be of low significance (Kim et al., 2005). Other parameters like temperature (Castiglioni et al., 2006), SRT and denitrification (Vieno et al., 2007) were shown to have no influence on the removal of LEV, which can be supported by the results of this study (see Table 2).

AZI, CLA and ROX as well as TRI were found to be poorly removed during the treatment process with no significant dependencies on either one of the investigated parameters. The results on SRT and temperature for ROX and TRI are supported by studies from Suarez et al (2012) who also reported a significant decrease of ROX removal due to denitrification (Suarez et al., 2010; Suarez et al., 2012). The latter has been investigated on a lab-scale, but cannot be confirmed from present data of a large-scale WWTP (see Table 2). In an extensive monitoring, CLA and TRI removal resulted to be seasonally influenced (Golovko et al., 2014), which is not supported by neither the present nor other studies (Castiglioni et al., 2006; Suarez et al., 2012).

In previous studies, the removal of SUL ranges from below 10 % (Zuccato et al., 2010) up to 90 % (Gracia-Lor et al., 2012), where temperature, the microbial community of the activated sludge system, the sampling strategy as well as the SRT are considered to influence the overall efficiency of this process (Kwon and Rodriguez, 2014; Larcher and Yargeau, 2012; Suarez et al., 2012). Then again there is evidence that neither temperature, SRT, HRT nor plant configuration, in terms of denitrification, significantly contribute to the variance of SUL reduction (Goebel et al., 2007; Suarez et al., 2010) which is analogous to the results from the present study. This variety of differing results leads to the conclusion that the knowledge on the fate of this antibiotic remains rather incomplete. In this context, the possibility of N4-acetyl-sulfamethoxazole being re-transformed to SUL, a hypothesis which is based on the reported re-transformation of N4- acetylsulfamethazine to its parent substance, was introduced by Gobel (2005), still remains unproved. On the other hand, the good correlation between predicted and measured WWTP input loads of SUL (Marx et al., 2015) in connection with the constant removal efficiency from 10 time series rather rejects this explanation of a re-transformable

metabolite being the reason for inconsistent removal rates. The monitoring of human metabolites in combination with their parent substances is an important part in mass flow analyses but it should be focused on sampling procedures and differences in the microbial communities of the activated sludge in order to understand the reported variability of SUL removal (Larcher and Yargeau, 2012; Majewsky et al., 2011b).

CEFO was detected on an irregular basis in the in- and effluent with a detection frequency of 71 and 14 %, respectively, and resulted in a generally high removal efficiency (100 % in 3 out of 4 time series with 13 to 15 values each, see supplementary material S3). During the first quarter in 2013 one time series of 14 days exhibited low temperature and low SRT with an elimination of approximately 42 %. It remains unclear why elimination was incomplete during that time because complete elimination could be observed in the fourth quarter with similar operation conditions. On the basis of this very limited evidence it must be concluded, that temperature and SRT do not significantly influence the removal of CEFO during wastewater treatment.

DOX was detected in 70 and 25 % out of all in- and effluent samples, respectively. The data suggest complete removal (i.e. effluent concentration were below the detection limit) for 65 % of the samples. For the remaining 35 % effluent concentration could be detected, but no pattern was found and release from WWTP seems rather incidentally. This observation is supported by results with variable removal efficiencies determined within other studies (Lindberg et al., 2005; Singer et al., 2014). AMO was detected in the effluent only once during the entire monitoring and is considered to be completely removed. Accordingly, seasonal differences as observed by Castiglioni et al. (2006) cannot be confirmed.

Adsorption to the sludge phase and degradation during the anaerobic treatment

Apart from the WWTP effluent, the withdrawal of PS, ES and ultimately DS is the second major removal route for antibiotics from the water phase in WWTPs. Accordingly, considering sludge loads are inevitable to perform a reliable mass flow analysis.

Table 3 presents the analytically determined concentrations of antibiotics in the main sludge streams of the WWTP Dresden-Kaditz. All macrolides show to have a minor adsorption potential which has been reported elsewhere (Jelic et al., 2012; Jelic et al., 2011; Peng et al., 2011; Zhang et al., 2013). It can be noted for macrolides that PS exhibits the highest adsorption capacity per gram TSS. The antibiotic CLI is poorly found in either type of sludge which is supported by results from Ohio with 10 to 20 μg/kg TSS (Spongberg and Witter, 2008). The low values of the metabolite CLI-S are similar to CLI but cannot be confirmed due to the lack of respective studies described in literature. TRI was reported with sludge concentrations between 8 and 140 μg/kg TSS (Gobel et al., 2005; Guerra et al., 2014;

Jelic et al., 2011) which is similar to the values of this study. The DOX concentrations of ES and PS are about two times lower (ES = 313 µg/kg TSS; PS = 762 µg/kg TSS) than those found in investigations by Gao et al. (Gao et al., 2012b).

SUL concentration is somewhat higher in PS, whereas ES as well as DS exhibit an adsorption capability which is about 1/3 lower. In contrast to other antibiotics under investigation the determined values are well above most comparable studies which reported concentrations in the range between 27 and 88 µg/kg TSS (Gao et al., 2012b; Gobel et al., 2005). Merely the DS concentrations in two Spanish WWTPs (112 and 178 µg/kg TSS (Nieto et al., 2010)) are comparable to results found in this study. As reported elsewhere, CIP and LEV are highly affine to either type of sludge (Gao et al., 2012a; Guerra et al., 2014; Martin et al., 2015; Peng et al., 2011) and maximum sludge concentrations were determined up to 2256 and 1296 µg/kg TSS, respectively. The high affinity of fluoroquinolones to sludge is supposed to be triggered by electrostatic interactions resulting from positively charged locations of these substances (Stevens-Garmon et al., 2011). The antibiotics CEFO, CEF, PEN, PIP and AMO could not be detected in any sludge samples.

Table 3: Concentrations of antibiotics in primary (PS), excess (ES) and digested sludge (DS) in ng/g (mean ± SD; number of values in brackets, n.d. – not detected)

Name	Abbreviation	Primary sludge ($c_{AB,PS}$) [µg/kg TSS]	Excess sludge ($c_{AB,ES}$) [µg/kg TSS]	Digested sludge ($c_{AB,DS}$) [µg/kg TSS]
Amoxicillin	AMO	n.d.	n.d.	n.d.
Azithromycin	AZI	111 ± 58 (9)	43 ± 32 (12)	87 ± 46 (14)
Cefotaxime	CEFO	n.d.	n.d.	n.d.
Cefuroxime	CEF	n.d.	n.d.	n.d.
Ciprofloxacin	CIP	1878 ± 965 (10)	1451 ± 731 (12)	2145 ± 1425 (12)
Clarithromycin	CLA	47 ± 24 (10)	12 ± 5 (11)	9 ± 4 (5)
Clindamycin	CLI	10 ± 4 (9)	18 ± 6 (12)	18 ± 6 (13)
Clindamycin-Sulfoxid	CLI-S	18 ± 7 (8)	12 ± 5 (9)	12 ± 7 (11)
Doxycycline	DOX	220 ± 177 (10)	90 ± 87 (12)	193 ± 202 (14)
Levofloxacine	LEV	631 ± 297 (10)	1142 ± 395 (12)	1296 ± 852 (13)
Penicillin V	PEN	n.d.	n.d.	n.d.
Piperacillin	PIP	n.d.	n.d.	n.d.
Roxithromycin	ROX	14 ± 6 (9)	4 ± 2 (8)	6 ± 3 (5)
Sulfamethoxazole	SUL	292 ± 343 (7)	213 ± 202 (12)	196 ± 146 (5)
Trimethoprim	TRI	34 ± 14 (9)	69 ± 54 (10)	66 ± 62 (7)

The mass flows from the input (PS + ES) to the output (DS) of the digester were determined to validate the calculated sludge loadings and to characterize the fate of antibiotics during the anaerobic treatment (see Table 3 and supplementary material S6, annex 2). On average, most sludge-borne antibiotics decrease only marginally during the anaerobic sludge treatment, whereas CLA, CLI-S, ROX and CIP

are reduced by more than 50 % (see supplementary material S6, annex 2). Taking the corresponding SD of $R_{AB,digester}$ into account it must be concluded that the reliability of most results is not sufficiently high to draw firm conclusions. In case of AZI, CLI, TRI, DOX and LEV the standard deviation exceeds 40 % and makes a statement on the fate during the anaerobic treatment highly questionable. In contrast, a decrease of CLA, CLI-S, ROX, SUL and CIP during the anaerobic treatment is assumed to be very likely. The purpose of performing the mass flow analysis of the digester is not merely to determine precise elimination rates but to give an idea on the behavior of antibiotics in the anaerobic treatment and to draw attention to the instance of highly inconsistent sludge concentrations. The SD of sludge concentrations can be found in the range of 50 (e.g. CLA and ROX) and even >100 % (e.g. SUL and DOX) of the mean value. These deviations have already been reported elsewhere (Guerra et al., 2014; Martin et al., 2015; Peng et al., 2011) without discussing it in further detail.

Possible reasons for this inconsistency are manifold and can be theoretically addressed to input fluctuations, sorption and desorption processes, hydrolysis and degradation processes as well as sampling, sample storage/preparation and the analytical method. Input fluctuations directly affect the antibiotic concentration in primary sludge but have minor influence on excess and digested sludge. The high SRT in aeration and digestion tanks buffers load peaks and sludge concentration changes slowly over time. On the other hand, hydrolysis, degradation, sorption and desorption occur on a faster time scale, but are expected to be at a close-to-constant rate in case the operation regime is not changed. Hence, the reason for the high deviations of sludge concentrations cannot be found on the basis of the available data or the understanding of WWTP operations. From the analytical point of view, the complex matrix composition of sludge (high TSS, impurities like high ammonia concentrations in DS) requires elaborate extraction and purification methods. Still, the extensive sorption of analytes can lead to very low recovery rates (Heidler and Halden, 2008), which demonstrates the limitations of such methods. A promising approach to evaluate the reliability of sludge concentrations is to perform mass flow analyses across key units in the sludge streams, e.g. digesters and dewatering units. Nonetheless, at the present there is a lack of such peer-reviewed literature (including this one) providing a sufficient number of sludge samples with an adequate resolution to distinguish between real load fluctuations and an erroneous analytical determination. Hence, it is highly recommended that future studies dealing with sludge-borne antibiotics focus more closely on this type of data plausibilization.

WWTP mass flow analysis

The mass flow analysis combines all information on the removal routes of the investigated antibiotics and CLI-S. As demonstrated in Figure 6 most antibiotics have a low sorption to the digested sludge and either pass through the plant unhindered or are prone to degradation. The macrolides AZI, CLA and ROX are mostly inert with only a small fraction adsorbing to the sludge. The load of AZI

slightly increased by about 12 %, CLA was degraded by 11 % and ROX was fully recovered not showing any production nor degradation. Considering the re-transformation model for CLI (CLI*, see 3.1) leads to the conclusion that the introduced (excreted) load is not affected by the urban sanitary system and completely discharged with the effluent. About 13 % of the total CLI* input adsorbed to the sludge. The human metabolite CLI-S and the antibiotic TRI behave similarly with high mass fractions found in the effluent and a low amount found in the sludge. The mass flow analyses of both substances result in a low overall production of about 6 and 12 % during the treatment process, respectively, which is in all probability due to the uncertainty of the mass flow analysis.

PIP and SUL are absent in the sludge and the difference between in- and output load of about 40 and 60 %, respectively, can be attributed to degradation. It is not clear why degradation of SUL or PIP stopped at these points, an observation which can also be found in other studies (Golovko et al., 2014; Singer et al., 2014; Suarez et al., 2012). The cephalosporins CEF and CEFO were exclusively found in the effluent and the degradation was determined to 66 and 86 %, respectively.

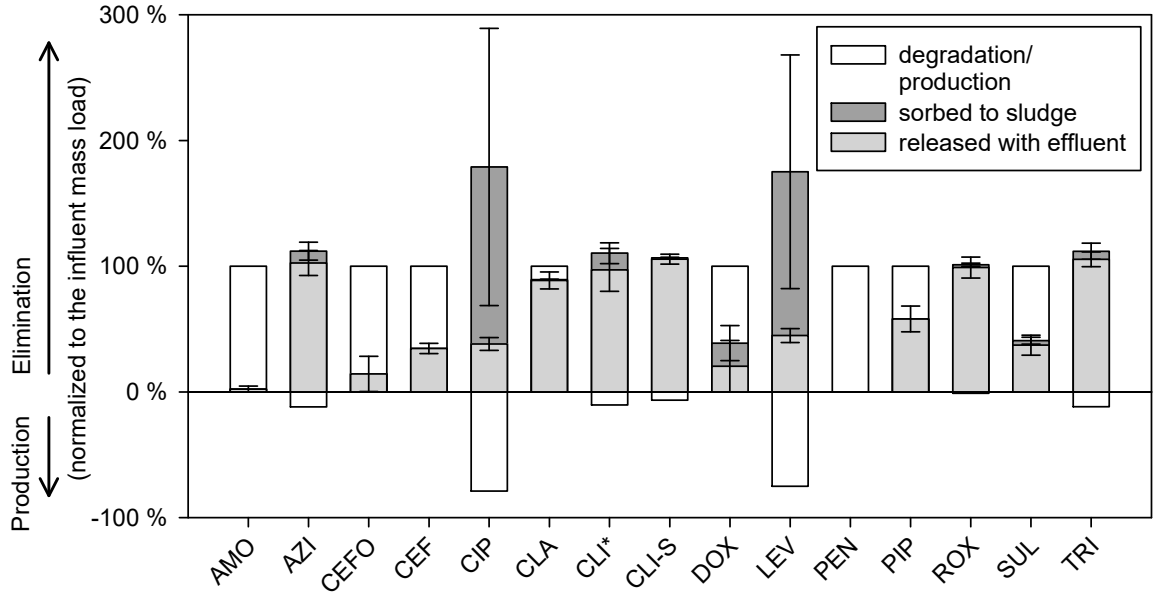

Figure 6: Fate of antibiotics during wastewater treatment

As already discussed, CIP and LEV have high sorption propensities and the sludge path is of major importance regarding their overall removal. The material flow analyses of CIP and LEV reveal a production of 79 % and 75 %, respectively. On average, 141 % of CIP's input load is found in the sludge which is highly questionable and not in accordance with most of the data from other studies (Lindberg et al., 2006; Petrie et al., 2014; Zhou et al., 2013b). The average sludge fraction of LEV determined in this study (130 %) is also difficult to bring into context with other literature results ranging from 9.35 (Yan et al., 2014b) to 69 % (Zhou et al.,

2013a). Nevertheless, similar "questionable" results have been reported from one Canadian WWTP with 245 % (LEV) and 480 % (CIP) of the median input load being found in the digested sludge (Guerra et al., 2014). A possible error source for the deviating results might be the determination of input loads which constitutes the basis of the mass flow approach. A recent study estimated that 37 % of influent CIP was sorbed onto particulates (Polesel et al., 2015b) with a chance of not being completely desorbed during the sample preparation of the applied analytical method. Making use of this theoretically resulting correction factor of 0.63 reduces the CIP production, originating from the results of the mass flow analysis, from 79 to 12 %. This outcome seems to be within the realms of possibility but does not consider the moderate removal (≥ 33%) during the anaerobic sludge treatment (see supplementary material S6, annex 2). The solid fraction in the effluent was estimated by multiplying the mean TSS-content of 12.9 mgTSS/l (SD = 3.7 mgTSS/l, n = 333) with the mean CIP concentration determined for ES. As a result, 18.7 ngCIP/l is emitted with the effluent which constitutes a theoretical increase of about 11 %, compared to the mean effluent concentration of 164 ng/l (SD = 101 ng/l, n = 328). This result corresponds very well to previous observations (also 11 %) using a pilot scale activated sludge process (Petrie et al., 2014). In case of LEV the load increase in the effluent amounts about 7 %, which is 2/3 lower than determined by Petrie et al.

CONCLUSIONS

The significance of the determined removal efficacy of antibiotics and other micropollutants is strongly limited by uncertainties deriving from sampling and analytics. Apart from the complex water and sludge matrices, particularly the sampling of consecutive in- and effluent samples is very difficult to accomplish in mixed systems like WWTP. In this context, quantifying antibiotic mass loads is the only way to evaluate the analytically determined concentrations in the liquid and solid phase in order to increase the reliability of results.

The use of gapless time series of at least 7 consecutive days is very well suited to provide reliable information on the removal of antibiotics from the liquid phase. The so-determined loads from the in- and output of the investigated WWTP are costly and time-consuming but minimize spurious influences like HRT as well as diurnal and other short-term load fluctuations. Furthermore, the comparison of different time series and their respective operating parameters is very promising to understand removal mechanisms and to identify options for operational intervention. In the majority of present cases operational parameters do not significantly influence the removal rate during the biological treatment process. Solely the elimination of CIP and CLI seems to depend on either SRT, temperature or a combination of them. Hereby it must be noted that the standard deviation of the removal rate of 18 % (CLI) and 5 % (CIP) are too low to draw conclusions.

Except for CIP and LEV, the mass flows of antibiotics under investigation can be sufficiently described by sampling the liquid phase, since the adsorbed fraction is negligible compared to the corresponding influent load. On the other hand, only inadequate information is available on the actual fate of CIP and LEV. Based on the evaluation of gapless times series, the removal from the liquid phase is considered to be reliable. In contrast, the integration of corresponding sludge loads leads to highly uncertain results The above considerations regarding the possibility of an incomplete analytical coverage of particulate-bound antibiotics (see 3.3) only partially explain these discrepancies and illustrate the need for additional explanatory approaches. Regarding the sampling strategy of the present investigation, the sludge sampling was partly carried out in 2014 and does not correspond to the monitoring campaign in 2013. Although the prescription behavior, which directly affects input and sludge loads of the WWTP, is not expected to undergo excessive changes within this time frame, an insufficient sampling strategy has likewise to be considered as possible error source. At the present there is also a lack of studies regarding the short- and long-term variability of sludge loads during sludge treatment as it was presented in this study. Therefore, it remains unclear whether the inconsistent results are due to insufficiencies in sampling and/or analytical determination or if they can be considered to be reasonable fluctuations. The focus of future investigations should hence be laid on antibiotic's temporal dynamics during the sludge treatment.

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CHAPTER 4

3. COMPARTMENT: ENVIRONMENT

Species-related risk assessment of antibiotics using the probability distribution of long-term toxicity data as weighting function – a case study

Species-related risk assessment of antibiotics using the probability distribution of long-term toxicity data as weighting function – a case study

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ABSTRACT

Urban areas are among the main sources which release antibiotics into the environment. The fate of antibiotics during their passage through the human body, the sewer system and the waste water treatment processes can be estimated and used for ecological risk assessment. The present approach deals with the possibility of addressing the ecological impact on individual trophic levels using a probability function to attenuate the classical PNEC approach. The species sensitivity distribution (SSD) is based on available long-term toxicity data and was fitted using the Hill-equation. The species-related toxicity threshold was merged with the slope characteristics gathered from SSD to express the risk probability of each species level. The results for algae and crustaceans show that azithromycin, clarithromycin and ciprofloxacin contribute the highest risk portions to the risk index (RI). The determined RI for fish was found to be below the threshold value of 1 and thus no risk is expected for this species.

KEYWORDS

Antibiotics, sensitivity distribution, risk assessment, prescription data

INTRODUCTION

Chemical substances which are released into the aquatic environment carry the potential to cause harm to a variety of organisms and compartments (Cao et al. 2009, Guo et al. 2013). The impairment of a few established organisms in natural ecosystems can severely disrupt the sensitive food web which requires a proper characterisation and control of the according substances. In this context, the European Commission offers a widely accepted guideline (Technical Guidance Document on Risk Assessment - TGD) on how to assess the environmental risk of chemicals in e.g. water bodies (EU 2003). This guideline is based on the identification of Predicted No-Effect Concentration (PNEC), which is put into relation with a Predicted or Measured Environmental Concentration (PEC or MEC) to define the resulting risk (Fahd et al. 2014). According to the TGD high risk is defined as PEC/PNEC ratios above 1, while the Swedish Environmental Classification of Pharmaceuticals considers 10 as threshold ratio for a substance provoking high risk to the environment (fass.se 2014). The difference is equivocal and leads to a drawback of the risk rating system. There are no procedures proposed on how risk values above 1 (hereinafter referring to the ranking system of the TGD) are supposed to be dealt with. Ranking the risk-substances according to the species level which they actually affect is a promising adjustment of the widely accepted methodology of chemical risk assessment and will be proposed in this work.

Considering a reliable determination of PNEC, the procedure allows a good estimation of the overall risk to water bodies. The risk linearly depends on PEC which is a necessary assumption, but does not reflect the typical dose-effect-relationship (Cao et al. 2009) for organisms. Usually a logarithmic sigmoidal shaped function is applied to express the relationship in this context (Chevre et al. 2008). Using the linear instead of the sigmoidal dependency between risk and substance concentration most probably leads to a vast overestimation of risk since it is not ecologically justified. Hence, the present approach will evaluate the use of the species sensitivity distribution (SSD), proposed by TGD (EU 2003), to approximate a sigmoidal shape as correction function for the calculated risk. With the aid of the Hill coefficients DH and m, substances with a lack of toxicity information can be linked to a corresponding dose-effect relationship, or SSD, according to their mode of action and potency. This procedure allows the trophic-level-based risk assessment for those substances which significantly contribute to the overall risk, in terms of emitted amounts, but offer little or no information on ecotoxicology.

Antibiotics were chosen to demonstrate the concept of the proposed risk assessment approach. This group of pharmaceuticals is of great scientific and environmental interest due to their resistance-promoting potential and ecotoxic effects. At the present, there are some concerns, that the release and spread of antibiotic resistant genes affect the therapeutic potential of antibiotics against human pathogens (Zhang et al. 2009), which greatly decreases their operational area. The crux regarding these concerns is that no reliable methods exist to assess the risk of

antibiotic resistances at present, which complicates the evaluation of implications that antibiotic resistances in the environment have for public health. Many investigations have been carried out to gain knowledge on the occurrence and dissemination of antibiotic resistances. However, the current available information on resistances in the environment is still very limited and more data are needed to better understand their development and selection processes (Rizzo et al. 2013). Investigations on resistances against heavy metals and antibiotics show that both types often occur simultaneously (Yamina et al. 2012). Furthermore, heavy metals co-select for antibiotic resistances and hence directly influence their development (Seiler and Berendonk 2012). The introduction of the minimal selection concentration (MSC), which can be several orders of magnitude lower than concentrations causing observable effects to organisms, gives evidence that the selective pressure under very low antibiotic concentrations is high enough to permanently maintain resistances in the ecosystem (Gullberg et al. 2014). This effect progresses with increasing number of compounds (e.g. heavy metals, herbicides) added to the system.

On the other hand, the extensive use of antibiotics has also posed the question of the risk they provoke in environmental compartments from the chemical point of view. Considering the standard species algae, crustacean and fish, the former is assumed to be the most sensitive in the aquatic food chain, causing the highest effect if being stunt by toxic substances. Comparing toxicological investigations of antibiotics regarding the three species mentioned above, differences according to their sensitivity ranking can be observed. For instance, crustaceans are more susceptible to clarithromycin than algae, on the other hand fish proved to be the most sensible species in presence of trimethoprim (see supplementary material S1, annex 3). The evaluation of these differences can therefore help to prioritize substances more detailed than it is possible using the standard PNEC-approach (see supplementary material S2, annex 3).

MATERIALS AND METHODS

Concept

The TGD suggests to perform a SSD to determine the most probabilistic PNEC value. The SSD includes all available long-term toxicity data[5] on different taxonomic groups of organisms. It is assumed that they follow a theoretical distribution function and that each group of organisms tested in the laboratory constitutes a random sample of this distribution. Assuming an adequate amount of data, the PNEC is defined as the 5 percentile of the SSD. An assessment factor of 1 to 5 is applied and accounts for further uncertainties, e.g. the diversity and representativeness of the included organisms or the adequate coverage of sensible life stages

[5] NOEC (No Observable Effect Concentration), LOEC (Lowest Observable Effect Concentration), EC_{10} (Effect Concentration causing 10 % effect) and LC_{10} (Lethal Concentration causing a lethal effect of 10 %) values were combined in this study to extend the applicable data set

of the organisms. Additionally, the TGD provides guidance on the minimal requirements for SSD in terms of number of taxonomic groups and available NOEC as well as information how multiple data on species are to be considered. Those information state general principles on how to perform extrapolation techniques but are hardly applicable to antibiotics. The majority of substances neither have the variety of taxonomic groups nor the amount of data available to carry out a "proper" risk assessment, in terms of a SSD. Nevertheless, the guideline is widely accepted and offers valuable information on how to deal with available data sets. Hence, the present approach will comply with the basic principles of the guideline.

The general idea is that the SSD specifies the probability of the PNEC to be accurate, depending on the available data. This distribution is substance-specific and hence provides a measure on how sensitive organisms respond to concentration changes (mode of action). This characteristic will be used to transfer the specific dose-effect relationship to the three classical species algae, crustaceans and fish. Depending on their minimal NOEC value the SSD distribution is going to be adjusted in order to describe each effect probability individually. Subsequently, the probability is used to weight the calculated risk quotient, according to its reliability. A summary of the steps to species-related risk assessment is given in Figure 1.

The SSDs will be fitted using the HILL-equation (equation 1). The parameter *m* defines the curve's slope and is specific for the relevant mode of action. This assumption was confirmed for herbicides and pharmaceuticals with similar mode of action (Chevre et al. 2006, Chevre et al. 2008) and hence can be transferred to the group of antibiotics. DH expresses the substance potency and is defined as the concentration where PNEC-probability amounts to 50 % (see Chèvre at al. 2006 and supplementary material S3 for equation conversion). The PEC was calculated as outlined in 2.3.3.

The determination of all SSD was not carried out for antibiotics with less than 3 available effect concentrations, instead the SSD character of other antibiotics with the same mode of action was adopted. This approximation was necessary for azithromycin, clindamycin, roxithromycin and doxycycline which share the same mode of action with clarithromycin (block of protein biosynthesis) whereas the former also share the same point of action. Cefuroxime was the only cephalosporine with toxicity data and provides the slope characteristics for cefaclor, cefadroxil and cefixime. Penicilline V belongs to the same beta-lactam group as amoxicillin and will be described by the latter. All antibiotics investigated in this study are summarized in Table 1.

$$f_{AB} = \frac{1}{\left(\frac{D_H}{PEC_{AB}}\right)^m + 1} \qquad \text{(Equation 1)}$$

The parameter *m* (also referred to as slope) has significant impact on the share in risk an antibiotic (AB) contributes. Low data availability might lead to wrong conclusions of slopes, a drawback which needs to be addressed to. Therefore, the influence of *m* was evaluated by globally setting different *m* values from 0.1 – 3.0 to all substances (hereinafter addressed to as SSD_m), neglecting the SSD determined for each substance. The global use of *m* implies identical modes of action for every antibiotic class. This assumption can be accepted at this point since sufficient data do not exist to prove the opposite for environmental organisms.

In this approach the distribution characteristic determined from all species is assumed to be valid for single species, too. Even if the use of individual values for m, which defines the shape of the characteristic distribution, seems to be the appropriate procedure to characterize the risk on the species level, it is not practicable in this case. The factor influences the curve progression and describes the organism's sensitivity to the substance. The higher the chosen or approximated *m* is, the steeper the curve becomes. Comparing two dose-effect distributions, one with low potency and flat curve progression (high DH, low m) and the other having the reverse characteristics, the low potency curve exhibits higher probability values at lower concentrations and exceeds the high potency distribution, in terms of calculated effect probability. This instance may be right for some antibiotics, but cannot be assumed in the first place without having the adequate data available. For this reason identical parameter *m* are applied to all species level under investigation.

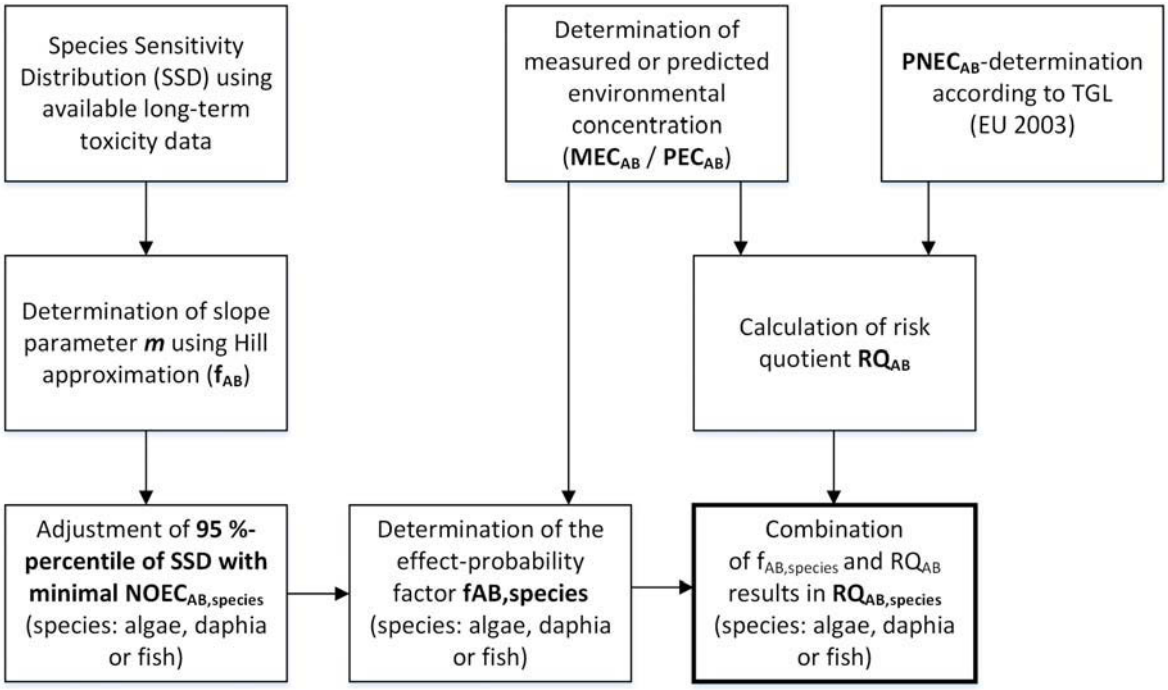

Figure 1: Flowchart of the proposed methodology using the probability distribution of long-term toxicity data as weighting function for environmental risk assessment on the species level for single substances

The overall risk quotient (RQ) will be calculated using the wastewater treatment plant's effluent concentration of each antibiotic (PEC), which was generated from prescription data (see 0). Subsequently, the RQ is weighted using the effect-probability factor ($f_{AB,species}$) arising from SSD adjustment (see 2.2 and equation 2), according to the considered species and antibiotic (equation 4). For the calculation of the risk index (RI) the method of concentration addition is applied (equation 3) (EPA 2000). The RI will serve as standardization method to calculate the relative impact of single antibiotics on the overall risk of the species level. As a result, this procedure, complying with TGD guidelines, extends the generally accepted risk assessment methodology to a species-based risk ranking within the aquatic ecosystem.

$$f_{AB,species} = \frac{1}{\left(\frac{1-0.95}{0.95}\right) \cdot \left(\frac{NOEC_{AB,species}}{PEC_{AB}}\right)^{m} + 1} \qquad \text{(Equation 2)}$$

$$RI_{species} = \sum RQ_{AB,species} \qquad \text{(Equation 3)}$$

PNEC values applied in equation 4 were taken from literature and compared with the results from the SSD of each antibiotic. A brief overview regarding the selection of PNEC is presented in the supplementary material S4.

Derivation of species-based risk assessment

Only for a few substances an adequate data set is available to reasonably carry out a SSD. Sulfamethoxazole is a well investigated compound for which reason thus it was chosen to exemplify the involved steps of species-based risk assessment.

At first, the Hill curve is fitted to the data points of all available species to calculate the dose-probability as well as the potency of each substance (see Figure 2). The next step involves merging the minimal effect concentration of each species with the slope characteristics gathered from SSD (and SSD_m). According to the 5 % percentile for PNEC-estimation, the 95 % percentile of SSD_{SUL} is adjusted to the corresponding minimal $NOEC_{AB,species}$ value of each species (= 5 %-ile of Hill curve, dashed grey curve in Figure 2). However, at this concentration the $RQ_{AB,species}$ nearly equals the RQ determined from classical PNEC calculation. From this point, reduced concentrations lead to lower $f_{AB,species}$ and attenuated $RQ_{AB,species}$.

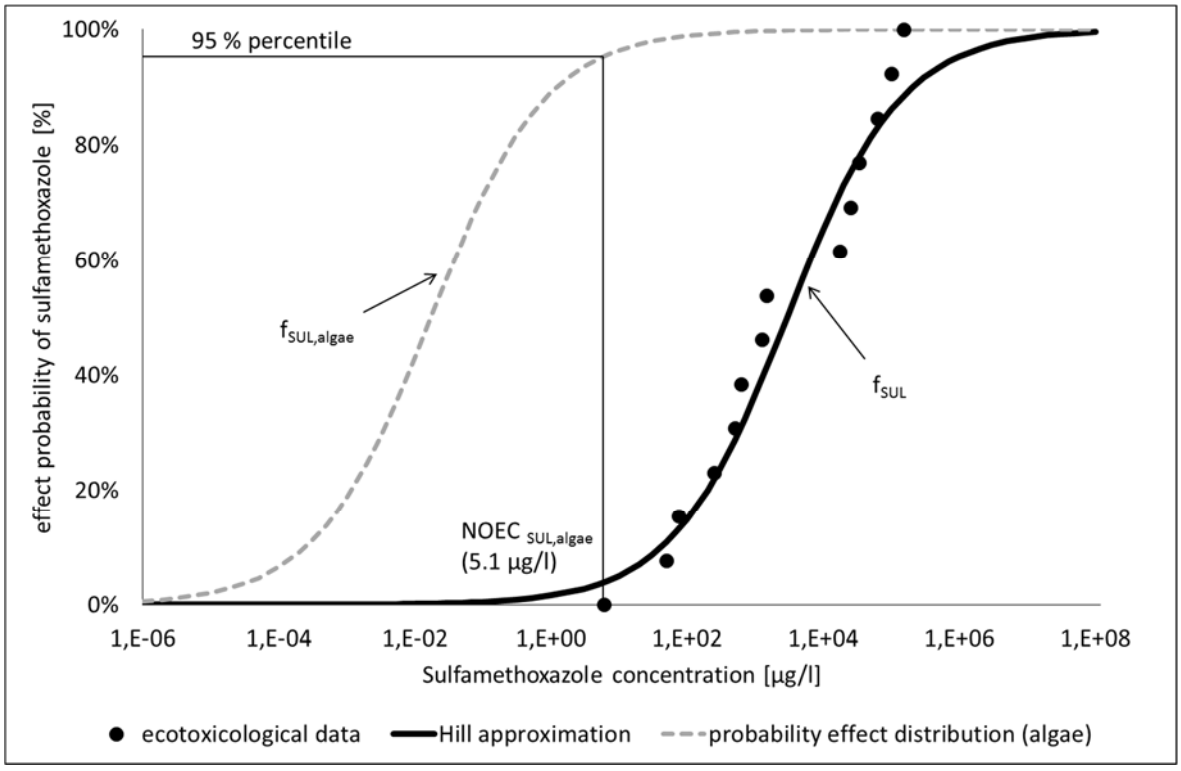

Figure 2: Example for fitting the SSD to Hill-equation using sulfamethoxazole (SUL) as an example; the shifted probability distribution was calculated using equation 2. Considering the individual potency of each species, the formula is applied to the calculated effluent concentration in order to determine the probabilistic factor for each RQ.

$$RQ_{AB,species} = \frac{PEC_{AB}}{PNEC_{AB}} \cdot f_{AB,species} \qquad \text{(Equation 4)}$$

It is assumed, that the probability of each species to be affected by an antibiotic depends on the mode of action and hence is identical within one group of antibiotics (for groups see Table 1. Therefore, missing species-related NOEC-values were replenished using the probability distribution of the antibiotic in question in combination with a NOEC-probability of another, better investigated antibiotic within the same group. For example, the NOEC probability for algae of ciprofloxacin (3.00 µg/l) constitutes 24 % which in turn assigns an NOEC probability for algae of 84.96 µg/l for levofloxacin, regarding its specific probability distribution (Table 3).

No crustacean and fish data are available for antibiotics belonging to the group of fluoroquinolones. Hence, the probability of crustacean and fish to be affected by levofloxacin and ciprofloxacin was divided into three and set to 67 and 100 %, respectively. This implies that fish (highest effect concentration) are more tolerant to fluoroquinolones than crustaceans, which in turn are more tolerant than algae (lowest effect concentration). Yielded effect concentrations are given in Table 3.

Input data and scope of the study

Substances

Prescription data of antibiotics from 2005 – 2011 were provided by the statutory health insurance company AOK PLUS which insures about 41 % of the people living in Dresden, Germany. The dataset covers only ambulant drug prescription. Additionally, data on the consumption of antibiotics were provided by the three major hospitals covering about 65 % of hospital beds available in the catchment area of the wastewater treatment plant (WWTP). In Table 1 the 15 most prescribed antibiotics in Dresden which were chosen for this study are listed. Ofloxacin and levofloxacin are racemats and considered as one substance.

Table 1: Prescription data of investigated antibiotics

Antibiotic	Therapeutic class	Prescription in WWTP catchment (2005 – 2010) [kg/a]	Share in group	Total group coverage
Cefadroxil	Cephalosporine (1. generation)	10 ± 3	3 %	91 %
Cefuroxime	Cephalosporine (2. generation)	302 ± 58	78 %	
Cefaclor	Cephalosporine (2. generation)	25 ± 8	6 %	
Cefixime	Cephalosporine (3. generation)	14 ± 5	4 %	
Clarithromycin	Macrolide	85 ± 26	23 %	82 %
Azithromycin	Macrolide	24 ± 9	6 %	
Roxithromycin	Macrolide	24 ± 11	6 %	
Clindamycin	Lincosamide	176 ± 45	47 %	
Amoxicillin	Penicillin	243 ± 41	40 %	88 %
Penicillin V	Penicillin	286 ± 39	48 %	
Ciprofloxacin	Fluoroquinolone	140 ± 9	55 %	86 %
Levofloxacin	Fluoroquinolone	80 ± 10	31 %	
Sulfamethoxazole	Sulfonamide	207 ± 27	100 %	

Doxycyclin	Tetracycline	44 ± 8	82 %
Trimethoprim	Diaminopyrimi-dine	42 ± 6	98 %

The sewer system of the WWTP Dresden-Kaditz has a length of 1700 km and collects the wastewater of five cities (Dresden, Freital, Heidenau, Pirna and eastern Radebeul) and some bordering municipalities. Presently, the wastewater of approximately 650000 inhabitants is being treated, whereof 80 % live in Dresden. It is assumed that the prescription data of AOK-insured people in Dresden can be extrapolated to the entire catchment area of the WWTP and to all respective inhabitants. The theory of antibiotic person equivalents was examined comparing the calculated amount of the total catchment area with the bordering municipalities of the catchment area. The ratio of five investigated antibiotics with seasonal and non-seasonal influence yielded 15 to 30 % of the total input to the total catchment area (see Figure 3). To cover the entire catchment area, the mean share of the five presented antibiotics (24 %) will be used for extrapolation purposes of remaining antibiotics.

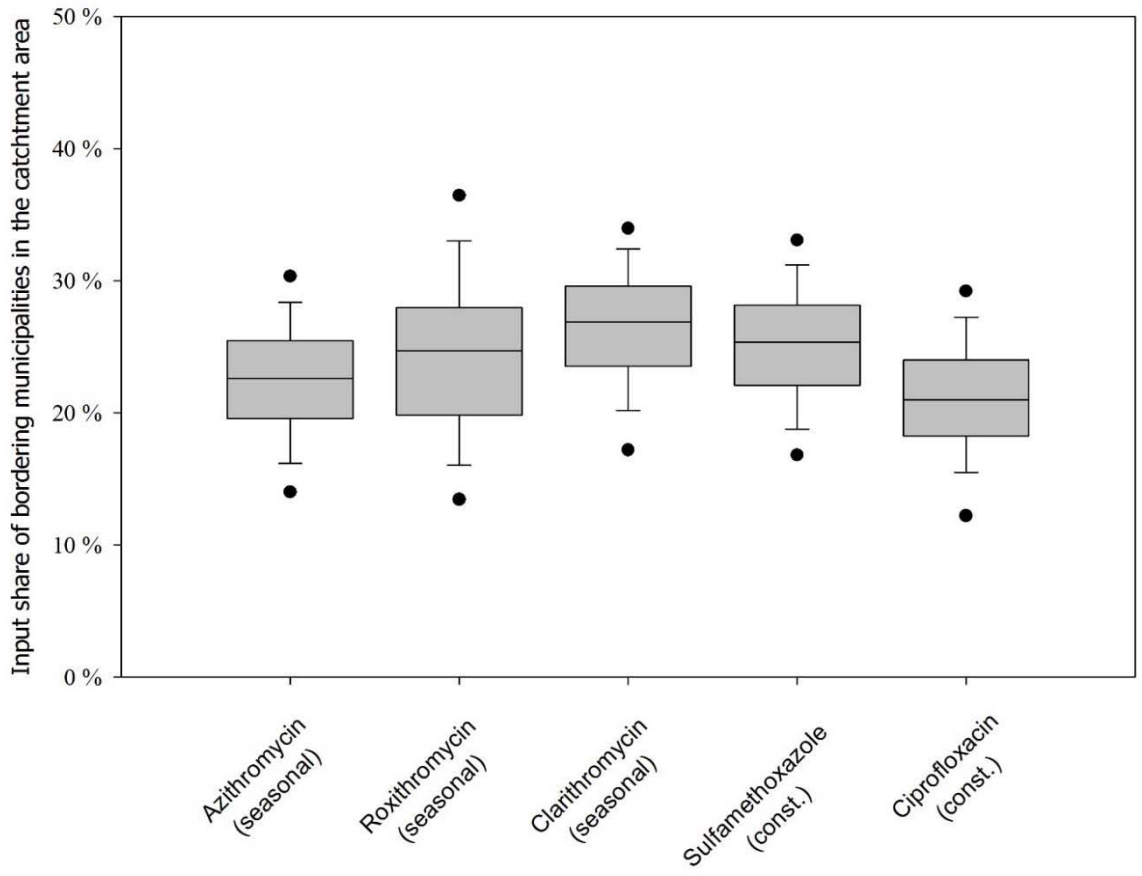

Figure 3: Bordering municipalities' input share in the entire catchment area of the WWTP (data set: weekly prescription from 2005 – 2011)

Furthermore, it is accepted that insurants of private health companies, which hold a share of 10 % of the total inhabitants, receive the same type and amount of antibiotics as those being statutorily insured. The approximated net number of commuters of about 40 000 (6 % of the catchment's population) will not be considered since antibiotic taking usually involves a sick certificate.

Scope of the case study

Estimating the input load of antibiotics into the aquatic environment using prescription or production data is an accepted method for the environmental risk assessment of chemicals (Besse and Garric 2008, Ortiz de Garcia et al. 2013). Hence, this methodology was applied to estimate the environmental hazard induced by antibiotics, including their specific human excretion information and elimination rate during wastewater treatment (see Table 2).

Apart from that stated above, there is a variety of processes and factors in natural water bodies that have a potential influence on the assessment of antibiotics and can lead to different risk characteristics than determined by the general methodology. For differentiation purposes an overview of possible interaction processes will be briefly presented in the following in order to define the boundary conditions for the case study under investigation.

After their release into natural water bodies antibiotics are prone to a variety of processes which lead to a change of concentration and hence a change of toxicity. Depending on the actual situation the mixing of the effluent and the receiving stream usually results in lower antibiotic concentrations. Regarding the exemplifying case under investigation, the mean daily low flow of the river Elbe was determined to 102 m^3/s (DWSO 2014) and indicates a mean dilution of the wastewater effluent (1.8 m^3/s) of about 1/57, if complete mixing is assumed. In contrast, due to the slow transverse mixing between the effluent and the receiving river a dilution of the former is not achieved for several flow kilometers and substance concentrations remain higher than the complete mixing calculation suggests. The level of transverse mixing depends on the receiving stream's characteristics (width, flow velocity, degree of meandering) and an estimation is difficult to carry out without applying appropriate mixing zone models (Jirka et al. 2004).

Despite the fact that the main part of antibiotics with favorable adsorption characteristics are eliminated during wastewater treatment (excess sludge), sediment-water interactions (sorption) still contribute to an alteration of antibiotic concentrations in natural water bodies (Zhou and Broodbank 2014). Besides, the processes hydrolysis and photolysis are considered as further elimination pathways, leading to transformation or complete mineralization. Hereby, temperature, pH and inorganic matter in the receiving river play a crucial role concerning the influence that sorption (Gu and Karthikeyan 2008, Zhang et al. 2014), hydrolysis (Bialk-Bielinska et al. 2012, Kang et al. 2012, Mitchell et al. 2014) and photolysis

(Kümmerer 2009, Sirtori et al. 2012, Wang and Lin 2012) have regarding the overall elimination of antibiotics. In case of incomplete mineralization, transformation products technically become a part of the risk assessment. Nevertheless, the classical risk assessment only addresses the toxicity of the parent compounds under investigation, neglecting transformation products from technical (wastewater treatment) or natural (e.g. photolysis) elimination of antibiotics. Considering the fact that some transformation products can exhibit a higher toxicity compared to their parent substance (El Najjar et al. 2013, Iskender et al. 2007, Sirtori et al. 2012, Wang and Lin 2012) makes the necessity of including those substances in the risk assessment evident. However, the high number of possible and partly unknown compounds causes the inclusion of transformation products to be *per se* incomplete and hence difficult to carry out and evaluate.

As shown above, the dilution and elimination in natural water bodies as well as the transformation pathways are difficult to assess in a complete manner due to the variety of substances, processes and changing conditions (pH, inorganic matter etc.) along the flow path. In order to demonstrate the concept of the proposed methodology the scope of this study focuses on the single parent substances (no transformation products, no synergistic effects from antibiotic mixtures) in the urban catchment and the respective WWTP. The manifold processes in the receiving river Elbe, which cannot be adequately included without using appropriate hydrological and material flow models, will not be considered.

Antibiotic's fate within the urban catchment and the WWTP

As described in the previous section, the case study focuses on the antibiotic flow starting with the consumption and ending at the outlet of the WWTP. After the consumption a partial elimination of the parent substance takes place during its passage through the human body. They are partly adsorbed by the gastric mucosa and/or gut and may be prone to metabolizing processes. The content that was not adsorbed is assumed to be excreted unchanged. The excreted ratio of the parent compound (E_{AB}) can be calculated using equation 5, where $f_{A,AB}$ characterises antibiotic-dependent the adsorption ratio in the human body. The parameter $f_{M,AB}$ determines the share of the respective antibiotic in the adsorbed fraction that is prone to metabolism, which in turn decreases the amount of the parent substance in the faeces. The necessary information are usually provided by the pharmaceutical industry (see Table 2).

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$$E_{AB} = (1 - f_{A,AB}) + f_{A,AB} \cdot (1 - f_{M,AB}) \qquad \text{(Equation 5)}$$

Many anthropogenic substances pass through the sewer system and WWTP before they are released into the environment. During their passage they are prone to adsorption and degradation processes which differ significantly among substances (Liu et al. 2013). In case of antibiotics, most of the penicillins are easily

biodegradable due to cleavage of the beta-lactam ring and rarely found in the effluent of WWTP (Watkinson et al. 2007). Cephalosporines, being a sub-group of the latter, show higher persistence in raw wastewater and the treatment process. The macrolides azithromycin, clarithromycin and roxithromycin show highly variable elimination rates from -45 to 55 % (Goebel et al. 2007). In order to consider the uncertainties regarding macrolide's reduction in WWTP, the elimination rate is thus set to 0 % for this study. Tetracyclines and fluoroquinolones have strong adsorption capabilities. Their main removal route is expected to be excess sludge removal (Golet et al. 2003) which influences the fate in sewer systems as well. There is no evidence that tetracyclines are biodegradable (Kim et al. 2005). Sulfonamides are partly removed in waste water treatment whereas sorption to sludge is irrelevant (Watkinson et al. 2007, Yang et al. 2005). A summary of the excretion and elimination rates applied in this study can be seen in Table 2.

Table 2: Excretion and elimination rate of antibiotics under investigation

Antibiotic	Excretion rate	Elimination during wastewater treatment
Amoxicillin	60 – 85 % (mean: 72.5 %) (Aktories et al. 2009, Martindale 1993)	60 – 85 % (mean: 72.5 %) (Aktories et al. 2009, Martindale 1993)
Azithromycin	99 %[6] (Watkinson et al. 2009)	99 % (Watkinson et al. 2009)
Cefaclor	67.4 % (Sandoz 2009)	67.4 % (Sandoz 2009)
Cefadroxil	0 % (Goebel et al. 2007)	0 % (Goebel et al. 2007)
Cefixime	53 % (Lode et al. 1979)	53 % (Lode et al. 1979)
Cefuroxime	100 % (Watkinson et al. 2007) (used in study: 99 %)	100 % (Watkinson et al. 2007) (used in study: 99 %)
Ciprofloxacin	88 % (Pfeffer et al. 1977)	88 % (Pfeffer et al. 1977)
Clarithromycin	50 % (assumption, no data available)	50 % (assumption, no data available)
Clindamycin	18 % (Brittain et al. 1985)	18 % (Brittain et al. 1985)
Doxycyclin	50 % (assumption, no data available)	50 % (assumption, no data available)
Levofloxacin	50 % (ODDB 2014)	50 % (ODDB 2014)
Penicillin V	60 % (unpublished data)	60 % (unpublished data)
Roxithromycin	40 % (urine), 15 % (faeces) (sum: 55 %) (Aktories et al. 2009, Vancebryan et al. 1990)	40 % (urine), 15 % (faeces) (sum: 55 %) (Aktories et al. 2009, Vancebryan et al. 1990)
Sulfamethoxazole	66 % (Li and Zhang 2011)	66 % (Li and Zhang 2011)
Trimethoprim	60 % (Hirsch et al. 1999)	60 % (Hirsch et al. 1999)

Considering the elimination pathways before the substance's discharge into the receiving river the PEC can be calculated using the following equation:

[6] Using equation 5 with $f_{A,AB}$ = 37 % and $f_{M,AB}$ = 88 %

$$PEC_{AB} = \frac{\left(\frac{P_{AOK,Dresden}}{0.41 \cdot (1.00 - 0.24)} + \frac{P_{hospitals,Dresden}}{0.65} \right) \cdot (1 - \eta_{Elimination})}{Q_{WWTP}} \qquad \text{(Equation 6)}$$

with the ambulant prescription data of Dresden ($P_{AOK,Dresden}$), the available hospital prescription ($P_{hospitals,Dresden}$), the daily WWTP outflow of Dresden-Kaditz (Q_{WWTP}), the elimination during the wastewater treatment ($\eta_{Elimination}$), the share of insured inhabitants (41 %), the share of bordering municipalities (24 %) and the share of hospital beds covered by the hospital prescription (65 %). In the region of Dresden agriculture plays a minor role and antibiotic inputs originating from veterinary use are not expected.

RESULTS AND DISCUSSION

Species-related toxicity thresholds

In Table 3, toxicity threshold values related to algae, crustacean and fish are listed for the antibiotics under investigation. The values are either taken from literature (see supplementary material S1, annex 3) or estimated from the substitutes approach. The most potent antibiotics regarding toxicity towards algae are amoxicillin and penicillin V with effect concentrations of 1.17 µg/l. Furthermore, ciprofloxacin (3.00 µg/l), the macrolides azithromycin, clarithromycin and roxithromycin (11.54 – 25.00 µg/l) as well as sulfamethoxazole (5.90 µg/l) are also to be considered to provoke effects on the algae level. The species of crustaceans show to be responsive to azithromycin and clarithromycin (4.40 and 4.70 µg/l) plus ciprofloxacin, clindamycin, doxycycline, roxithromycin and sulfamethoxazole (30.20 – 250.00µg/l). The most effective antibiotics regarding fish toxicity are trimethoprim (157.00 µg/l) and levofloxacin (937.13 µg/l). Considering the results above macrolides and ciprofloxacin are the most potent substances regarding their effect on the lower trophic levels in this study.

Table 3: Summary of the fitting parameters and toxicity threshold for each species

Antibiotic	D_H [µg/l]	slope (m) [-]	species-related toxicity threshold		
			Algae [µg/l]	Crustacean [µg/l]	Fish [µg/l]
Amoxicillin	222250	0.87	1.17	2300000	182700
Azithromycin	35[7]	1.18[3]	11.54	4.4	84000
Cefaclor	201658[4]	1.74[4]	76000[8]	83100[4]	1000000[4]
Cefadroxil	201658[4]	1.74[4]	76000[4]	831000[4]	1000000[4]
Cefixim	201658[4]	1.74[4]	76000[4]	831000[4]	1000000[4]
Cefuroxime	201658	1.74	76000	831000	1000000
Ciprofloxacin	13	0.78	3	30.2	4535.06
Clarithromycin	35	1.18	11.54	4.7	1000000
Clindamycin	256[5]	1.83[5]	81.1[3]	42.07[3]	329020.05[3]
Doxycyclin	256[9]	1.83[5]	81.1[3]	42.07[3]	329020.05[3]
Levofloxacin	137	2.39	84.96[11]	181.17	937.13
Penicillin V	222250[10]	0.87[6]	1.17[6]	2300000[6]	182700[6]
Roxithromycin	477	2.48	25	183.88	62383.17
Sulfamethoxazole	1945	0.54	5.9	250	33848.57
Trimethoprim	11394	0.87	3100	13000	157

Antibiotics are purposefully designed to decrease the abundance of bacteria in the human body. Hence, it is not surprising that some toxicological investigations using environmental bacteria as test organisms also show considerable effects caused by some antimicrobial agents. Levofloxacin is being effective towards vibrio fisheri at 1.99 µg/l and ciprofloxacin affects pseudomonas putida at 4.90 µg/l.

[7] Adopted from clarithromycin
[8] Adopted from cefuroxime
[9] Adopted from azithromycin and clarithromycin by averaging
[10] Adopted from amoxicillin
[11] Adopted from ciprofloxacin using probability distribution

Hartmann et al. (1998) used a genotoxicity assay to investigate the effects of clarithromycin onto bacteria and determined a LOEC of 5.00 µg/l. Bacteria showed a similar sensitivity against toxicity like algae, however, differences in potency ranking can be seen. Unfortunately, the available data sets are not sufficient to carry out a separate bacteria-related risk assessment for the entire spectrum of antibiotics which are covered in this study. At this juncture, it has to be assumed that bacteria, which should be the most susceptible organisms, show similar effects like algae, even if few toxicological investigations seem to suggest otherwise.

Evaluation of risk distribution using individual slopes

The RIalgae nearly completely fails to fulfil the quality criteria of < 1. Only at the beginning of 2006, a RI_{algae} below 1 can be determined (see supplementary material S5, annex 3). Concluding from calculated values, a permanent risk on the algae level emanates from antibiotics in the effluent of WWTP. Taking a closer look onto the contributing substances, the following conclusions can be drawn. The antibiotics showing the highest risk potency do not significantly contribute to the overall risk for algae originating from the WWTPs effluent. Beta-lactams are well biodegradable and the expected effluent concentration of 0.02 – 0.03 µg/l is rather low. Their contribution to the RIalgae is about 1 to 2 %. The effluent concentration of ciprofloxacin is about 0.42 µg/l and causes the highest risk share in RIalgae of about 59 % ($RQ_{CIP,algae}$ = 2.84). Azithromycin and clarithromycin have a contribution to the total risk of 15 % each. Sulfamethoxazole accounts for a minor share of around 6 %, only.

It is proposed to define the substances which contribute more than 80 – 90 % to the overall risk of the most sensitive species, in this case algae, as first-order risk substances. Algae play a crucial role in aquatic ecosystems and many edible species are being consumed by zooplankton (e.g. crustaceans), which in turn are the basis for fish ranking higher in the food chain. In order to prioritize measures to reduce the substance-induced risk on aquatic ecosystems the proposed ranking gives advice on which substances should be focused on. Accordingly, ciprofloxacin, azithromycin and clarithromycin constitute first-order risk antibiotics, whereas ciprofloxacin clearly provokes the highest risk among them.

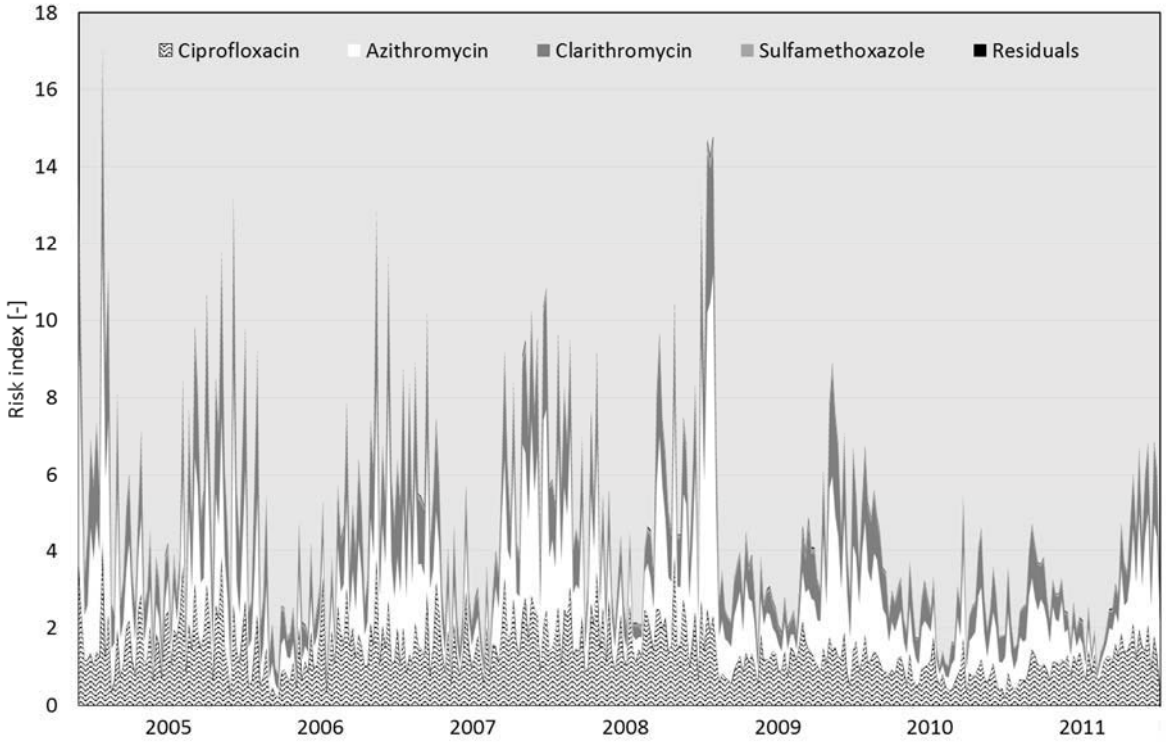

Figure 4: Risk index of the species crustacean ($RI_{crustacean}$) – remaining RQ_{AB} are summed up and displayed as residuals

It can be seen that risk indices for crustaceans ($RI_{crustacean}$) is nearly as high as RI_{algae} (on average 92 % of RI_{algae}, R2 = 0.95, $p < 0.01$, see Figure 5 and S5, annex 3), which is surprising since a significantly higher sensibility for algae was expected. Still, the composition of RIcrustacean is somewhat different (see Figure 4) from that of RI_{algae}. As expected from the species-related thresholds, azithromycin (35 %), along with ciprofloxacin (35 %) hold the highest share in $RI_{crustacean}$. The RQ of clarithromycin constitutes on average 1.20 and amounts 26 % of $RI_{crustacean}$. Additionally, sulfamethoxazole contributes an average $RQ_{SUL,crustacean}$ of 0.12 which, depending on the season, corresponds to a proportion between 1 and 6 % of RI-crustacean. The substances which induce the risk to crustaceans are the same as those for algae, even though their proportions are different. Crustaceans are more susceptible to macrolides which in turn compensates for the higher sensitivity of RI_{algae} to ciprofloxacin. The RI_{fish} amounts up to 0.20 (see supplementary material S6, annex 3) and consists mainly of RQ_{CIP} and RQ_{SUL}. The index does not exceed the threshold value of 1 which leads to the conclusion of an acceptable risk for fish.

Using the proposed sigmoidal probability distribution rather than a linear relationship between concentration and estimated risk leads to a mean attenuation of the classic PNEC-approach ($RI_{classic}$) of about 70 % for algae and crustaceans. The attenuation for RI_{fish} is even higher and reduces the risk index below the threshold value of 1. The relationship between the species-related and the classical RI are shown in Figure 5.

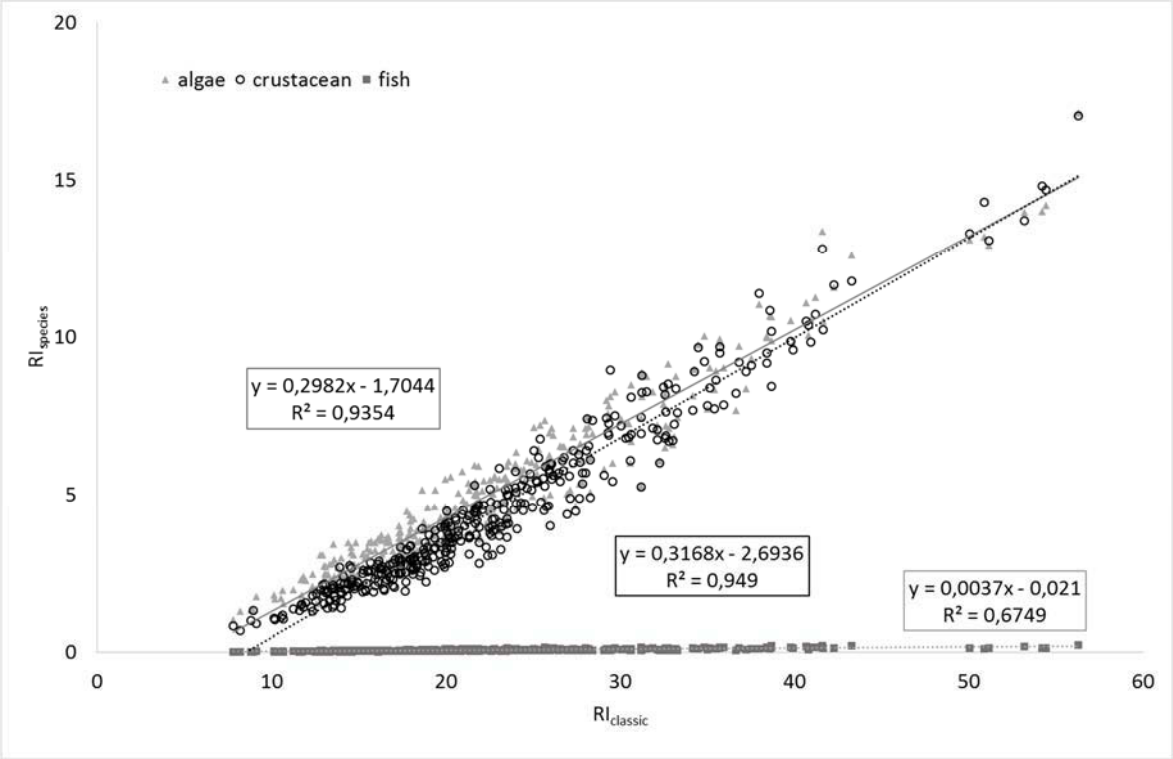

Figure 5: Species-related $RI_{species}$ versus classical PNEC-derived $RI_{classic}$

Evaluation of risk distribution using global slopes

The probability distributions used in this approach are influenced by the number and quantity of investigated species. Owing to circumstances of randomly investigated organisms an unequal distribution of available data regarding the trophic levels is possible. In order to overcome, or assess, these uncertainties and evaluate the robustness of the present approach, the parameter *m* of the Hill equation was set constant for all antibiotics and globally diversified to estimate its influence on risk proportions, depending on its dimension.

In general, a similar distribution pattern can be observed for algae and crustaceans, compared to the approach using individual slopes. Regarding algae, ciprofloxacin becomes massively dominant above a slope of 0.5 and reaches a share in total risk of over 90 % at a slope of 3 (see Figure 6A). Azithromycin and clarithromycin contribute a minor part to $RI_{m,algae}$, which complies with the results of individual distribution curves for antibiotics.

Azithromycin and clarithromycin show to be the most potent antibiotics for crustaceans (see Figure 6B). Below a slope of 1.5 the former contributes the largest risk proportion. Increasing *m* up to 3 causes the influence of clarithromycin to rise further, while azithromycin becomes less important. Compared with the approach using individual slopes, ciprofloxacin affects the risk to a lesser extent.

Evaluating the risk proportion of fish (see Figure 6C), it becomes apparent that levofloxacin and trimethoprim dominate the determined risk. Ciprofloxacin, which contributed between 70 and 90 % to the overall RI_{fish} using individual slopes, reaches a proportion of around 30 % at m = 0.5 and is further decreasing with higher m-values. The reason for the observed differences are to be found in the comparatively low species-related toxicity thresholds of levofloxacin and trimethoprim. Ciprofloxacin's potency is lower by factors 5 (levofloxacin) and 28 (trimethoprim) which leads to minor RQ_{CIP} at identical m-values.

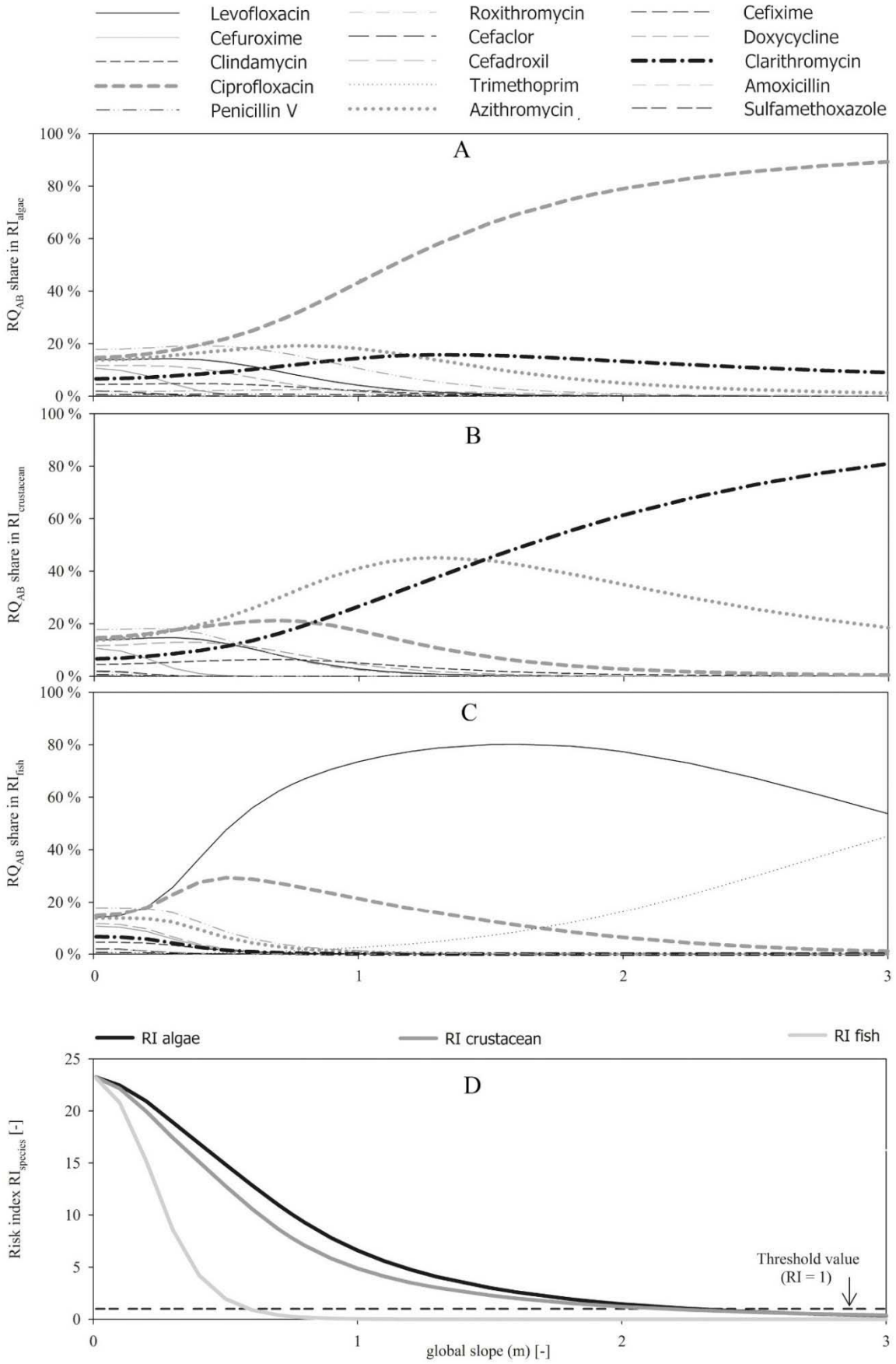

Figure 6: Risk proportions using global *m*-values (A – algae; B – crustacean; C – fish; D – influence on $RI_{species}$)

The slope *m* also has a stake in the dimension of RI_{algae}, $RI_{crustacean}$ and RI_{fish}. A slope of zero is almost matching the average $RI_{classic}$ (24.54), only reduced by a factor of 0.95 (setting *m* to zero in equation 2 and Figure 6D). High slope values describe a faster response to concentration changes, which increases the effect probability considerably. But this is only the case, if calculated effluent concentrations are close or above the species-related toxicity thresholds. Substantially lower effluent concentrations lead to probability factors close to zero and, subsequently, to decreasing RI. RI_{fish} hits the threshold value of 1 at a slope of around 0.6, RI_{algae} and $RI_{crustacean}$ reach the threshold line at around 2.3 and 2.0, respectively (see Figure 6D). Due to the interpretation that antibiotics do not constitute a risk to the environment above these *m*-values, a further investigation of the risk proportions will not be necessary. Chevre et al. (2008) studied the sensitivity distribution of herbicides, organophosphates and beta-blockers. The lowest slope calculated from toxicity data of the pharmaceutical group beta-blockers was 0.6 which is assumed to represent the lower limit of this investigation, too (see supplementary material S3 for formula conversion, annex 3). Consequently, the assessed risk towards fish is low which corresponds well with the approach applying individual slopes. Within the range of reasonable *m*-values the risk assessed for the species algae and crustaceans is high and shows similar risk distributions of antibiotics, independently of the approach used.

CONCLUSIONS AND SUMMARY

The introduced approach addresses two drawbacks of the PNEC-based risk assessment. Firstly, the linear relationship between concentration and effect can be corrected using the probability distribution as weighting function, also referred to as SSD. The resulting attenuated risk quotient RQ reflects the quality of the data set as well as the effect range which is defined through the variety of test organisms. Secondly, a differentiated risk assessment on the species level delivers insight into the real impact potential of antibiotics. It was shown, that algae are the most sensitive among the investigated species, while environmental bacteria might prove to be even more susceptible to antibiotics. The latter need to be intensively investigated in the future in order provide a reliable data basis and to give a justified answer to this question.

The combined approach of using a sigmoidal probability distribution on the species level leads to strongly attenuated risk indices. RI_{algae} and $RI_{crustacean}$ are 70 % lower than the RI determined using the classical PNEC approach. RI_{fish} was even decreased below the threshold value and thus does not indicate a risk for this species.

The application of both global and individual *m*-values is suitable to estimate the contribution to the overall risk from each substance. The first-order priority substances, i.e. those that hold the highest share in the overall risk at the most sensitive species-level in the food chain, can be identified using either global or individual

slopes for the probability distribution. The knowledge of first-order priority substances is a solid step into bottom-up risk management from the chemical's point of view, concentrating on the substances which are the most hazardous to the aquatic environment. In case of lower-tier organisms being affected by the substances under investigation a damage propagation among species-levels, which results in the weakening of higher-tier organisms, must be taken into account. Hereby, it must be considered that standard testing procedures cover the effect measuring using indicators like the uptake/elimination of test chemicals, growth rate or mobility which result in the classical toxicologic endpoints, e.g. EC_{10}. Hence, no conclusion can be drawn regarding the kind of effect that influences the respective organism.

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CHAPTER 5

3. COMPARTMENT: ENVIRONMENT

Environmental risk assessment of antibiotics including synergistic and antagonistic combination effects

Environmental risk assessment of antibiotics including synergistic and antagonistic combination effects

Marx, C., Mühlbauer, V., Krebs, P., Kuehn, V.

ABSTRACT

The interaction-based hazard index (HI_{int}) allows a prediction of mixture effects different from linear additivity by including information on binary mixtures between the chemicals. The aim of this study is to make a solid estimate on the possible synergistic potential of combined antibiotics and to quantify the subsequent effect for the case of the receiving river Elbe, Germany. Information on binary interactions between antibiotic groups were used from literature and from knowledge on human antibiotic combination therapy. Applying a moderate and a worst-case scenario, in terms of the interaction magnitude, resulted in 50 to 200 % higher environmental risks, compared to the classical assessment approach applying simple concentration addition. A subsequent sensitivity analysis revealed that the data strength for some binary antibiotic combinations is too low to be considered for a solid estimate of synergistic effects. This led to the definition of certain preconditions in order to decide whether or not to include certain interaction information (e.g. the necessary number of interaction studies). The exclusion of information with low data strength resulted in an attenuated risk increase of 20 to 50 %, based on the currently available scientific information on binary antibiotic mixtures. In order to include antibiotics with the highest share in the overall risk (macrolides, quinolones, and cephalosporins) as well as their corresponding metabolites, investigations should focus on binary interactions between them.

KEYWORDS

Antibiotics, risk assessment, synergism, antagonism, hazard index

INTRODUCTION

The assessment on whether or not a chemical substance causes a threat to the environment is usually carried out on the basis of toxicity tests using established standard organisms like fish, algae or other appropriate sensitive species. The results of these set-ups provide reliable information on the toxicity of single substances towards target and non-target organisms (Baguer et al., 2000). In order to understand the toxicity effects of multiple substances, each chemical has to be adequately described regarding its individual toxicity threshold and concentration within the mixture. Accordingly, this procedure of risk assessment is not applicable to undefined mixtures where most of the required information is not available. Urban wastewaters contain a vast number of chemical substances like cleaning agents, heavy metals, personal care products and pharmaceuticals (Trapido et al., 2014) and can have a significant influence on the receiving stream's water quality (Eggen et al., 2014). The wide variety of substances and concentration ranges in wastewaters make it difficult to satisfactorily determine their actual toxicity to the environment. One concept which intends to transform the toxicity information of several individual substances to an overall mixture toxicity is termed concentration addition. It is based on the thought of a similar mode of action of all substances present in the mixture, irrespective of the presence of other substances (Berenbaum, 1985). Even though this assumption is rather simplistic, it becomes practical if only limited information on the mode of action is available. Nevertheless, due to the origin of the toxicity information of individually tested substances, no conclusion on synergism and antagonism can be drawn from this approach.

Various studies have been performed to investigate mixture effects between surfactants (Rosal et al., 2010), organic fluorinated chemicals (Boltes et al., 2012; Rodea-Palomares et al., 2012), pesticides (Laetz et al., 2009), prescription drugs (Cleuvers, 2003) and antibiotics in particular (Eguchi et al., 2004; Gonzalez-Pleiter et al., 2013; Zou et al., 2013). According to these findings, toxicity increases by a factor of up to 6 for surfactants and up to 29 for organic fluorinated chemicals. Due to the obvious potential of synergistic effects, Laetz et al. (2009) proposed the introduction of safety factors to ensure the protection of aquatic organisms. Hereby, the adequate consideration of different groups of substances calls for a differentiated approach that addresses the synergistic potential of each one of them.

Pharmaceuticals are of special importance due to their vast application in urban areas and their subsequent release from wastewater treatment plants along the embankment line of natural water bodies (Michael et al., 2013). The high number of anthropogenic organic substances requires the knowledge development for a variety of new synergistic combinations. For instance, antibiotics as a sub-group of pharmaceuticals are known to cause toxicological impacts on the fauna of natural water bodies (Kümmerer, 2009). Especially primary producers like photosynthetic aquatic organisms (Gonzalez-Pleiter et al., 2013) and bacteria (Backhaus et al.,

2000) which inter alia constitute the basis for the aquatic biota were shown to be negatively affected by antibiotic mixtures. Similar effects have been observed for detrivorous macroinvertebrates (Maul et al., 2006) and plants (Brain et al., 2004). The studies demonstrate the importance of considering antibiotic mixtures as an integral component of an adequate risk assessment. Hence, the study focuses on the application of the interaction-based hazard-index (HI_{int}) (EPA, 2000) to make a solid estimate on the possible synergistic potential of antibiotic mixtures. The method will be used to quantify the resulting risk for the case of the receiving river Elbe, Germany.

MATERIALS AND METHODS

Predicted environmental concentration in the river Elbe

The calculation of the predicted environmental concentration (PEC_{AB}) is based on in- and outpatient prescription data covering the catchment area of the wastewater treatment plant (WWTP) Dresden-Kaditz, Germany. The use of information on nationwide productions, sales or prescriptions is an often used method to estimate the PEC_{AB} in WWTP effluents and receiving streams (Bendz et al., 2005; Choi et al., 2008; Gobel et al., 2005). Ambulant prescription data for the time period under investigation (2005 – 2011) were provided by the statutory health insurance company AOK PLUS. About 41 % of the people of Dresden are insured at the AOK PLUS. It is assumed that the prescription data of AOK-insured people can be extrapolated to the entire catchment area of the WWTP and to all respective inhabitants. Furthermore, it is accepted that insurants of private health companies, which hold a share of about 10 % of the total inhabitants, receive the same type and amount of antibiotics as those being statutorily insured. Antibiotic consumption data were also provided by the three major hospitals covering about 65 % of hospital beds available in the catchment area of the WWTP. The 15 most prescribed antibiotics in Dresden were chosen for this study and are listed in Table 1.

The substances ofloxacin and levofloxacin are racemats and were considered as one substance. The total class coverage in Table 1 refers to the ratio between included and total antibiotic loads within one therapeutic class. This means, for example, that all cephalosporins listed in Table 1 represent 91 % of all prescribed cephalosporins antibiotics, whereas the remaining cephalosporins (cefpodoxime, ceftibuten, loracarbef, cephalexin and ceftriaxone) hold a minor share of only 9 % of the total cephalosporin load.

Table 1: Prescription data of investigated antibiotics

Antibiotic	Therapeutic class		Ambulant prescription in WWTP catchment (2005 – 2011) [kg/a][1]	Share in group	Total class cover-age
	Name	Abbrev.			
Cefadroxil	Cephalosporin (1. generation)	CE	9.7 ± 1.6	2 %	91 %
Cefuroxime	Cephalosporin (2. generation)	CE	309.2 ± 63.9	78 %	
Cefaclor	Cephalosporin (2. generation)	CE	25.8 ± 3.7	7 %	
Cefixime	Cephalosporin (3. generation)	CE	14.1 ± 2.3	4 %	
Clarithromycin	Macrolide	MA	83.3 ± 6.0	22 %	82 %
Azithromycin	Macrolide	MA	24.3 ± 1.7	6 %	
Roxithromycin	Macrolide	MA	23.1 ± 4.0	6 %	
Clindamycin	Macrolide	MA	179.8 ± 40.6	48 %	
Amoxicillin	Penicillin	PE	251.6 ± 23.6	42 %	88 %
Penicillin V	Penicillin	PE	282.4 ± 23.3	46 %	
Ciprofloxacin	Quinolone	QU	141.7 ± 6.4	55 %	86 %
Levofloxacin	Quinolone	QU	81.2 ± 7.5	31 %	
Sulfamethoxazole	Sulfonamide	SU	202.7 ± 23.4	100 %	
Doxycycline	Tetracycline	TC	43.2 ± 5.7	82 %	
Trimethoprim	Diaminopy-rimidine	DI	41.2 ± 5.0	98 %	

Antibiotics are partly eliminated during their passage through the human body. The main consecutive processes are adsorption by the gastric mucosa/gut and metabolism. The content that is not prone to adsorption is assumed to be excreted unchanged. The excreted ratio of the parent compound (E_{AB}) can be calculated using equation 1,

[1] Kg/a – kilogram per year

$$E_{AB} = (1 - f_{A,AB}) + f_{A,AB} \cdot (1 - f_{M,AB}) \quad \text{(Equation 1)}$$

where $f_{A,AB}$ characterizes the antibiotic-dependent adsorption ratio in the human body. The parameter $f_{M,AB}$ describes the metabolized part of the adsorbed fraction. The necessary information is usually provided by the pharmaceutical industry and scientific literature (see supplementary material S1, annex 4).

This study focuses on the parent antibiotics without taking into account metabolites from human metabolism or environmental degradation processes. Recent investigations on sulfonamides (Gros et al., 2013), tetracyclines (Zhang et al., 2015), macrolides (Rossmann et al., 2014) and quinolones (Maia et al., 2014) included a variety of metabolites which reflects the current effort to consider those substances in mass flow analyses of sewer systems and WWTP. However, the scarcity of reliable information regarding the excretion, formation and ecotoxicity of most antibiotic metabolites hinders a comprehensive risk assessment including combination effects.

After human excretion, antibiotics are prone to adsorption and degradation processes in the sewer system (length approx. 1,700 km) and the WWTP. Those characteristics significantly differ among antibiotic groups. Penicillins are easily biodegradable due to cleavage of the beta-lactam ring and hence rarely found in the effluent of WWTP operating an activated sludge system or a fixed-bed filter (Watkinson et al., 2007). Cephalosporins as a sub-group of penicillins, exhibit a higher persistence in raw wastewater and during the treatment processes. The macrolides azithromycin, clarithromycin and roxithromycin show highly variable elimination rates from - 45 to 55 % in conventional activated sludge systems with biological nitrogen removal (Gao et al., 2012; Goebel et al., 2007; Yan et al., 2014). Considering the uncertainties regarding macrolide's elimination in WWTP, no degradation or accumulation processes will be considered in this study. The main removal route of tetracyclines and fluoroquinolones during the wastewater treatment is expected to occur via excess sludge withdrawal (Golet et al., 2003). There is no evidence that tetracyclines are prone to biodegradation (Kim et al., 2005). Sulfonamides are partly removed in activated sludge systems and a fixed-bed filters whereas sorption to sludge seems to be insignificant (Watkinson et al., 2007; Yang et al., 2005). A summary of the excretion and elimination rates applied in this study is provided as supplementary material S1 (annex 4).

The PEC_{AB} at the mixing point of WWTP discharge and river Elbe was calculated using equation 2:

$$PEC_{AB} = \frac{\left(\frac{P_{AOK,Dresden,AB}}{0.41} + \frac{P_{hospitals,Dresden,AB}}{0.65} \right) \cdot E_{AB} \cdot (1 - \eta_{AB,E\,\mathrm{lim}\,ination})}{(Q_{WWTP} + Q_{Elbe})} \qquad \text{(Equation 2)}$$

with the ambulant prescription data of Dresden ($P_{AOK,Dresden,AB}$), the available hospital prescription ($P_{hospitals,Dresden,AB}$), the excretion ratio (E_{AB}), the daily WWTP outflow of Dresden-Kaditz (Q_{WWTP}), the daily flow of the receiving stream Elbe (Q_{Elbe}), the elimination during the wastewater treatment ($\eta_{AB,Elimination}$), the share of insured inhabitants (41 %) and the share of hospital beds covered by the hospital prescription (65 %). In the region of Dresden, agriculture plays a minor role and antibiotic inputs originating from veterinary use are not expected. The WWTP has a design capacity of 740,000 population equivalents and treats wastewater of approximately 650,000 inhabitants plus industrial wastewater. The plant is operated as activated sludge process with biological nitrogen removal and chemical phosphorus precipitation. Due to missing data the antibiotic preload of the river Elbe prior to the WWTP discharge of the WWTP Dresden-Kaditz could not be considered in this study. The mean daily low flow of the river Elbe constitutes about 102 m³/s (DWSO, 2014).

Additivity-based and interaction-based Hazard Index (HI_{add} and HI_{int})

The risk assessment of single substances is carried out using the quotient of PEC_{AB} and predicted no effect concentration ($PNEC_{AB}$, see supplementary material S2, annex 4). The resulting Hazard Quotient (HQ) is based on the approach proposed by the European Chemicals Bureau (EU, 2003). Exceeding the threshold value of 1 states a risk to the environment through the substance of interest. In order to assess the risk of more than one substance the hazard index (HI_{add}) is calculated by summing up the respective HQ_{AB} of the mixture under investigation. For the calculation of the hazard index the method of concentration addition is applied (equation 3) (EPA, 2000) which assumes similar toxicological relevance and neglects synergistic or antagonistic effects.

$$HI_{add} = \sum HQ_{AB} \qquad \text{(Equation 3)}$$

We define "synergistic" as amplifying effect and "antagonistic" as diminishing effect, compared to the reference of linear additivity. The complex composition of non-synthetic waters, e.g. effluents of WWTP, makes the development of reliable interaction studies nearly impossible. As a result, most investigations focused on binary interactions to gain a rough insight into risk drivers, in terms of substances provoking effects greater than additive. The US EPA therefore developed a procedure that includes interaction data from binary mixture experiments to modify HI_{add} in a manner that considers synergistic and antagonistic effects. The approach was developed for human health risk assessment and is based on earlier

investigations by Mumtaz and Durkin (1992) reflecting the significance of available data as well as the composition of the mixture. The HI_{int} uses the HQ_{AB} as basis and considers the relative toxic hazard of each substance (Exposure Factor F, see equation 5), the magnitude of interaction (M), the weight-of-evidence (WOE) as well as the degree to which two chemicals are present in equitoxic amounts (Weighting factor for relative proportions Θ, see equation 6. The calculation of the HI_{int} is given in equation 4:

$$HI_{\text{int}} = \sum_{i=1}^{n}\left[HQ_i \cdot \sum_{j\neq i}^{n}\left(F_{ij} \cdot M_{ij}^{\,WOE_{ij}\cdot\Theta_{ij}}\right)\right] \qquad \text{(Equation 4)}$$

with the exposure factor F:

$$F_{ij} = \frac{HQ_j}{HI_{add} - HQ_i} \qquad \text{(Equation 5)}$$

and Θ_{ij} being defined as the ratio between the geometric and arithmetic mean:

$$\Theta_{ij} = \frac{\sqrt{(HQ_i \cdot HQ_j)}}{(HQ_i + HQ_j)/2} \qquad \text{(Equation 6)}$$

Interaction Magnitude

In order to carry out the interaction-based risk assessment the knowledge of the actual interaction magnitude is required. In this study a symmetric interaction magnitude is assumed, which means that the interaction effect of two substances is identical in either direction.

The US EPA suggests a default value of M = 5 if no information on the specific components is available (EPA, 2000). The factor of M = 5 arises from a study on the joint action of 27 chemicals and their equivolume and equitoxic combinations. The factor of predicted to measured effect was in the range of 0.2 to 5.0, which indicates a deviation factor of 5 in either direction of interaction. Due to the fact that even more distinct interaction effects could be observed for other chemicals (EPA, 2000), literature data of binary antibiotic mixtures was explored to determine a specific interaction magnitude for antibiotics. Investigated antibiotics from literature partially differ from the targeted substances in this study. Therefore, all antibiotics are referred to their corresponding superior class to achieve a general picture on interactions between antibiotic classes. A total of 159 binary antibiotic interactions were found in 21 peer-reviewed publications. The provided data of

82 experiments are considered sufficient to determine the magnitude of synergistic or antagonistic interaction. 22 tested binary mixtures did prove to act neither greater nor lesser than additivity which equals a magnitude of 1. In literature, three parameters were applied to decide whether additivity can be accepted or not:

1) Toxicity Units (TU): The concentrations of both substances conjointly causing 50 % inhibition are individually related to their corresponding median effective inhibition concentration ($EC_{50,AB}$). The sum of these two fractional terms is defined as TU (formula see supplementary material S3, annex 4). Synergy is expected for TU < 0.8 and antagonism for TU > 1.2. Within the range of 0.8 < TU < 1.2 simple dose additivity is assumed (Broderius et al., 1995).
2) Combination Index (CI): The CI is very similar to TU but is not solely defined for an inhibition of 50 %. Both substance concentrations which cause x % effect in combination are individually related to their corresponding effect concentration representing an individual effect of x % (formula see supplementary material S3, annex 4). The threshold value of 1 indicates an additive effect while < 1 and > 1 characterize synergism or antagonism, respectively. Notwithstanding the foregoing, for reasons of conformity the decision criteria on synergistic effects for CI will be adapted from the TU approach.
3) Fractional inhibitory concentration (FIC): The definition of the FIC equals the CI in terms of calculation and decision criteria (Berenbaum, 1978) using the minimum inhibitory concentration of single and mixed drugs (see supplementary material S3, annex 4). A synergy study between aminoglycosides and cephalosporins based on the decision criteria of Berenbaum (1978) investigated the reproducibility of the FIC (Hallander et al., 1982). The investigation states as a result that a FIC below 0.75 indicates synergism which limits the former criteria for synergism. Nevertheless, for reasons of comparability the decision criteria for additivity, synergism and antagonism will also be adopted from the TU.

According to (Zhao et al., 2010) and applying the adopted decision criteria presented above, the magnitude M will be interpreted as 1/TU for synergism (TU ≤ 0.8), TU for antagonism (TU ≥ 1.2) and a value of 1.0 for simple additivity .

Weight-of-evidence (WOE)

To decide whether synergistic or antagonistic effects are significant, the number of studies with similar results was evaluated. If the majority of results for one combination of antibiotic groups found in literature was synergistic or antagonistic, the drug combination was classified accordingly. Simple additivity was assumed for a specific antibiotic combination if

1) No information on binary interaction studies is available from literature
2) All available interaction studies collectively indicate simple additivity
3) There is an equal amount of investigations demonstrating synergism and antagonism

The weighting was chosen on the basis of the weight-of-evidence classification proposed by US EPA (see Table 2). The classification between -1 to 0 and 0 to 1 indicates effects lesser (antagonistic) and greater (synergism) than additivity, respectively. Each direction of the classification consists of four categories and reflects the strength of evidence that two chemicals influence the toxicity of each other. The description of each category is provided in Table 2. The WOE is not intended to give evidence on the magnitude of interaction nor the relative amounts of substances in the mixture. It just reflects the data quality regarding the interaction of two chemicals in mixtures. This study is based on a composition of literature data and assuming an unequivocal evidence of interaction (category I) is not appropriate. Hence, the use of the second WOE category is more suitable for this study. Accordingly, synergism, antagonism and additivity will be accounted for as 0.75, -0.50 and 0.00, respectively (see Table 2). The applied comparison of interaction studies can be adapted if more information on toxicological interactions become available (EPA, 2000).

Table 2: Weight-of-evidence categories (adopted for environmental risk assessment)

Category	Description	Direction	
		Greater than additive	Less than additive
I	The interaction has been shown to be relevant to the environment and the direction of the interaction is unequivocal	1.00	-1.00
II	The direction of the interaction has been demonstrated and its relevance to the environment is likely	0.75	-0.50
III	An interaction in a particular direction is plausible, but the evidence supporting the interaction is weak	0.50	0.00
IV	The assumption of additivity has been demonstrated or must be accepted	0.00	0.00

A second possibility of estimating the WOE between antibiotic classes is to use known interactions from human combination therapy (Karow and Lang-Roth, 2013). Antibiotics can be classified into bactericidal and bacteriostatic substances (see Figure 1): bactericidal antibiotics will kill bacteria, whereas bacteriostatic components will only inhibit bacterial growth. Bactericidal antibiotics can again be subdivided into components that will only kill bacteria during proliferation and degenerative bactericidal antibiotics that will destroy them under any condition. The combination of antibiotics with different modes of action can therefore have additive, synergistic and antagonistic effects. In case of combining two bactericidal components (groups I and II in Figure 1) the effect is additive or synergistic, since the degenerative substance will destroy non-proliferating bacterial forms while the other component will not. The combination of degenerative bactericidal antibiotics with bacteriostatic ones can be additive or antagonistic – an additive effect can be achieved by slowing down bacterial growth through the bacteriostatic component and destroying them through the bactericidal one. However, due to the competition between both substances, the combination is less effective than additive, i.e. the substances cannot develop their full individual potential. The combination of a component that destroys only proliferating bacteria with one that inhibits bacterial growth will definitely lead to an antagonistic effect and has to be avoided in human combination therapy.

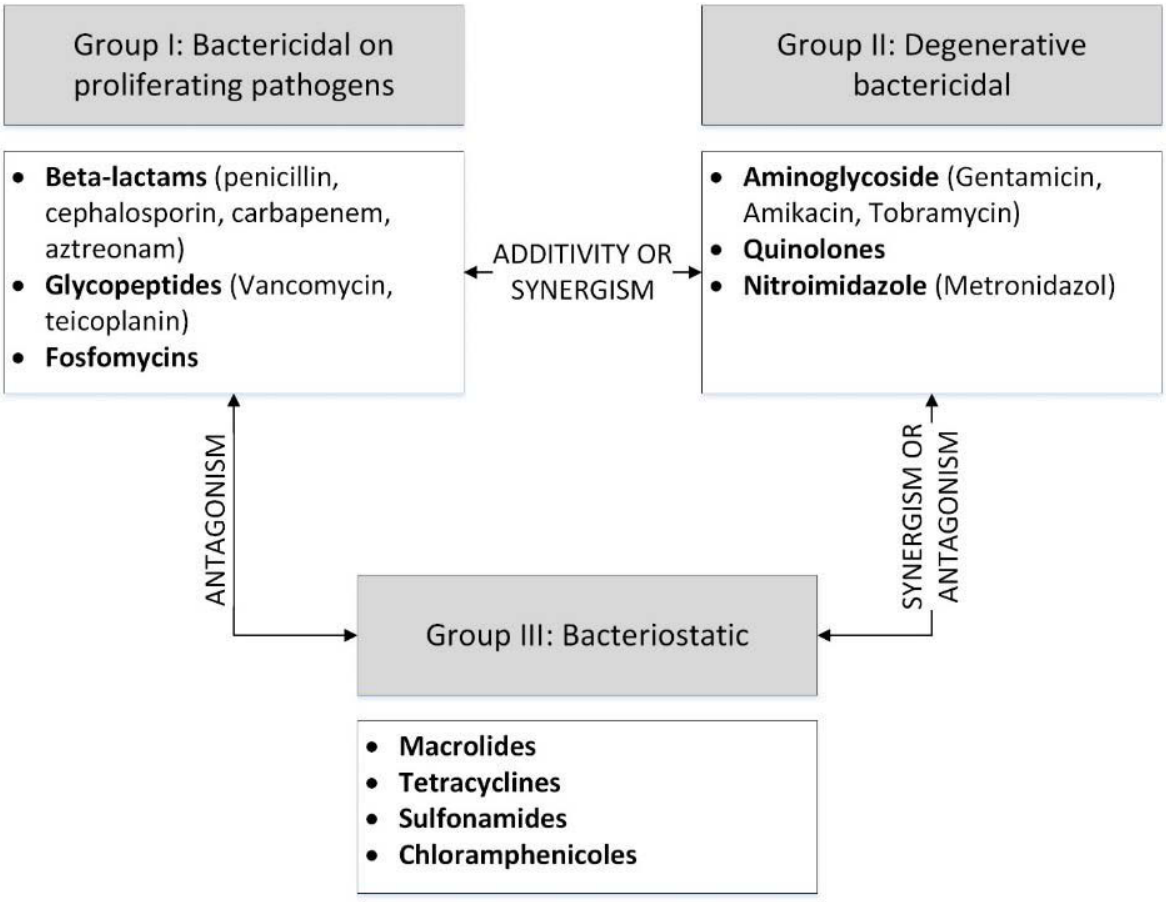

Figure 1: Interaction of chemotherapy agents in combination therapy (adopted from Karow and Lang-Roth, 2013)

The antibiotic concentrations applied for the treatment of bactericidal infections in humans is several orders of magnitude higher than those found in the environment. For example, clindamycin reaches a plasma concentration of several mg/l after its application in humans (Pharma, 2012) whereas environmental concentration can be found in the ng/l range (Gonzalez-Pleiter et al., 2013). This concentration difference can lead to an interaction effect that is deviating from what was shown for some binary mixtures of antibiotics at much higher concentrations (Gonzalez-Pleiter et al., 2013). Decrease of concentrations is assumed to cause the interaction effect to move either towards synergisms or towards antagonism, depending on the antibiotics in mixture. Ultimately, no conclusive estimation can be drawn on the consequences of adopting the information of antibiotic interactions in human therapy for environmental concentrations. Hence, for the present the information given by Karow and Lang-Roth (2013) will be evaluated with the same degree of evidence as those provided by the studies on binary mixtures of antibiotics (Karow and Lang-Roth, 2013). The influence of different concentration ranges on the interaction effect will be topic in the discussion section.

RESULTS

Additive Hazard Index HI_{add} in the receiving stream Elbe

The HQ of all investigated antibiotics were merged to their respective antibiotic classes and summed up to calculate HI_{add} in the river Elbe (see Figure 2). The mean HI_{add} resulted in 0.37 whereas 20 % of all weeks exceed a HI_{add} of 0.5. HI_{add} above 0.8 were detected during 11 weeks (3 %) of all weeks only and the threshold value of 1 was reached only during 1 week in the 7 years examination period. Quinolones (41 %), cephalosporins (31 %) and macrolides (21 %) contribute the highest proportion to the overall risk. Tetracyclines and penicillins are well eliminated during the wastewater treatment process and do not pose a hazard to the environment at the present concentrations. The hazard caused by sulfamethoxazole and trimethoprim is irrelevant due to comparatively high $PNEC_{AB}$ values and resulting low HQ. The corresponding PEC_{AB} of all antibiotics under investigation are provided as supplementary material S4.

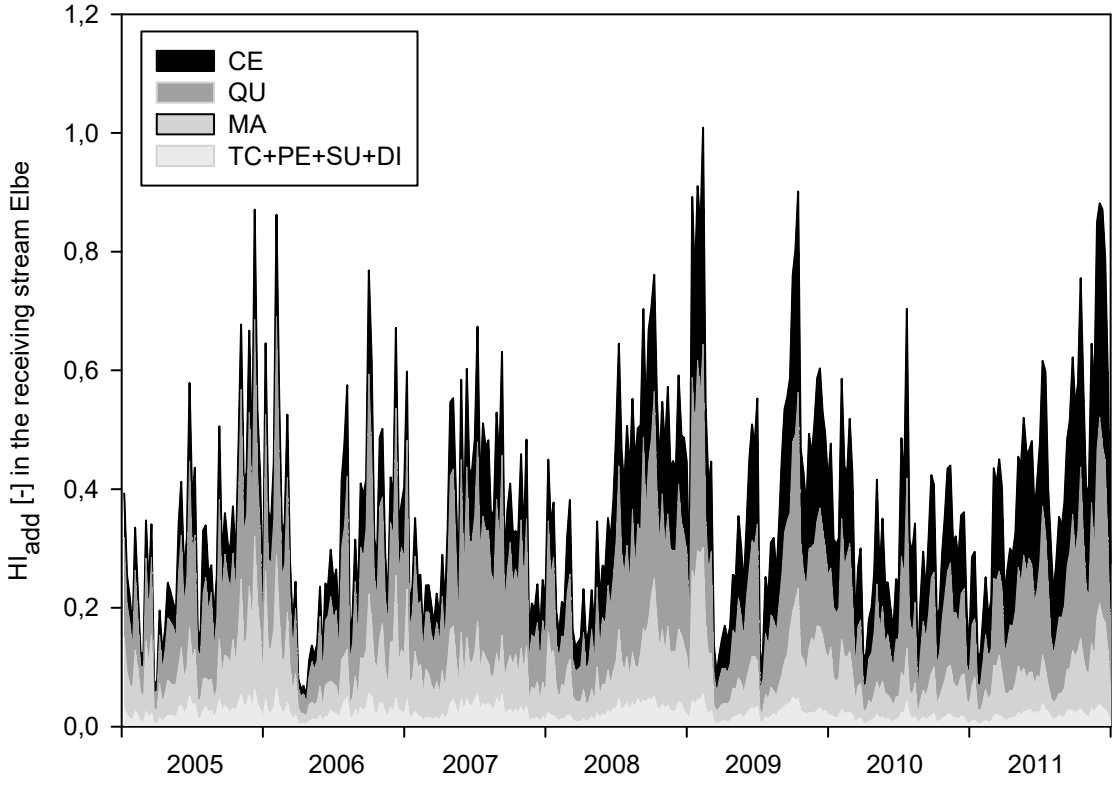

Figure 2: HI_{add} in the receiving stream Elbe (MA – Macrolides, QU – Quinolones, TC – Tetracyclines, PE – Penicillins, CE – Cephalosporins, SU – Sulfonamides, DI – Diaminopyrimidines)

Attention should be paid to the change of hazard distribution between antibiotic classes over time. From 2005 to 2011 the hazard share of the group of cephalosporins more than doubled from 20 to 45 %. Meanwhile macrolides and quinolones each lost about 10 % of their share in HI_{add} and hence became less important in

terms of hazard management. This instance highlights the fact that risk is constantly changing and acquires a continually recurring assessment. Despite the significant changes in risk distribution the HIadd did not noticeably change over time. A sensitivity analysis shows that the doubling of cefuroxime prescriptions was compensated by a close-to-equivalent decrease of roxithromycin. Both antibiotics have similar $PNEC_{AB}$ values ($PNEC_{CEF}$ = 0.15 µg/l, $PNEC_{ROX}$ = 0.10 µg/l) and hence mutually compensate (see supplementary material S2, annex 4).

Interaction Magnitude

Table 3 gives an overview on the magnitude M of all possible binary combinations of antibiotic classes. In this context, the direction of interaction is not considered in M and will be discussed in the next section regardind the weight-of-evidence. From Table 3 it can be seen that 15 out of 28 possible class interactions are covered by literature data of which two third are consolidated with more than 3 investigations. Quinolones (QU, 48 studies), tetracyclines (TC, 47 studies) and macrolides (MA, 41 studies) and penicillins (PE, 32 studies) are well investigated regarding binary interactions with at least 3 other antibiotic classes. The classes of diaminopyrimidines (DI, 18 studies), sulfonamides (SU, 14 studies) and cephalosporins (CE, 0 studies) are not well supported, in terms of synergy-studies covering a variety of other antibiotic classes. The scarcity of synergy data for cephalosporins is especially alarming since this antibiotic group is responsible for almost half of the entire risk in 2011 (45%, see 3.1) and synergistic effects will significantly increase the actual hazard to the environment.

Table 3: Magnitude of binary antibiotic interactions (mean/max values found in literature, number of studies is provided in brackets)

	MA[12]	QU[12]	TC[12]	PE[12]	CE[12]	SU[12]	DI[12]
MA	1.8/2.1 (2)	1.8/6.7 (17)	2.2/4.3 (10)	1.9/4.0 (11)	0.0/0.0 (0)	1.9/1.9 (1)	0.0/0.0 (0)
QU	1.8/6.7 (17)	2.2/4.2 (8)	2.4/4.8 (16)	1.5/2.0 (6)	0.0/0.0 (0)	2.2/2.2 (1)	0.0/0.0 (0)
TC	2.2/4.3 (10)	2.4/4.8 (16)	2.0/2.0 (1)	3.3/7.6 (11)	0.0/0.0 (0)	1.0/1.0 (1)	1.8/3.8 (8)
PE	1.9/4.0 (11)	1.5/2.0 (6)	3.3/7.6 (11)	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)
CE	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)	0.0/0.0 (0)
SU	1.9/1.9 (1)	2.2/2.2 (1)	1.0/1.0 (1)	0/0 (0)	0.0/0.0 (0)	1.0/1.0 (1)	4.1/6.3 (10)
DI	0.0/0.0 (0)	0.0/0.0 (0)	1.8/3.8 (8)	0.0/0.0 (0)	0.0/0.0 (0)	4.1/6.3 (10)	0.0/0.0 (0)

[12]MA – Macrolides, QU – Quinolones, TC – Tetracyclines, PE – Penicillins, CE – Cephalosporins, SU – Sulfonamides, DI – Diaminopyrimidines

A mean and a maximum (worst case) magnitude of interaction were determined to consider the range of available data (see Table 3). Tetracyclines exhibit the highest interaction magnitudes of all antibiotic classes. In combination with quinolones and penicillins a 4.8- to 7.6-fold increase in toxicity is observed. The interaction magnitudes between combinations of macrolides, quinolones and penicillins can be found predominantly in a moderate range between 1.5 and 1.9, while a maximum of 6.7 was identified. Various studies show that sulfonamides and diaminopyrimidine tend to significantly influence each other by an average factor of 4.1, exhibiting a maximum magnitude up to 6.3. Besides, information on interactions between sulfonamides and other antibiotic groups like macrolides, quinolones or tetracyclines are scarce und unreliable. For further calculations, the scenarios $M_1 = 2.7$ and $M_2 = 7.6$ will be examined. M_1 is the mean of the individual values of all interaction studies, excluding results indicating additivity. M_2 represents the maximum interaction value from the combination between tetracyclines and penicillins. The so-estimated magnitudes M_1 and M_2 agree well with results from the review regarding mixture toxicity studies between metals, pesticides and antifoulants (Cedergreen, 2014). The collected and evaluated magnitudes rarely exceed 10 and support the use of M_2 being above the suggestion of the US EPA.

Weight-of-evidence WOE of interaction scenarios

Table 4 summarizes the number of available literature results and corresponding WOE (in brackets). Regarding literature results on binary antibiotic mixtures, evidence is given for the combinations of tetracyclines – macrolides as well as cephalosporins – penicillins to exhibit a synergistic effect. In case of penicillins, two experiments (Ampicillin in combination with Cloaxillin and Penicillin) suggest an interaction greater than additive within this antibiotic group. Synergistic effects are also plausible for the combinations of macrolides – penicillins / cephalosporins, quinolones – tetracyclines / cephalosporins and sulfonamides – diaminopyrimidines. An antagonistic effect is evidently expected for tetracyclines in combination with diaminopyrimidines, as well as for the combinations macrolides – quinolones and tetracyclines – penicillins. A simple additive effect is assumed for the combination between quinolones – penicillins. The scarcity of synergy-studies covering mixtures with cephalosporins and sulfonamides (+ diaminopyrimidines) was already mentioned. There is a strong need to fill this particular knowledge gap to improve the toxicological assessment of these antibiotic classes.

Table 4: Weight-of-evidence from literature data – scenario 1 (number of investigations resulting in synergism/antagonism/additivity; resulting WOE is provided in brackets)

	MA[12]	QU[12]	TC[12]	PE[12]	CE[12]	SU[12]	DI[12]
MA	2/0/0 (0.75)	7/9/5 (-0.5)	11/0/0 (0.75)	5/2/6 (0.75)	1/0/1 (0.75)	0/1/0 (-0.5)	0/0/0 (0)
QU	7/9/5 (-0.5)	5/1/2 (0.75)	11/3/3 (0.75)	5/5/17 (0)	1/0/2 (0.75)	1/0/0 (0.75)	0/0/0 (0)
TC	11/0/0 (0.75)	11/3/3 (0.75)	1/0/0 (0.75)	10/9/2 (0.75)	0/0/0 (0)	0/0/1 (0)	0/6/2 (-0.5)
PE	5/2/6 (0.75)	5/5/17 (0)	10/9/2 (0.75)	2/0/0 (0.75)	2/0/0 (0.75)	0/0/0 (0)	0/0/0 (0)
CE	1/0/1 (0.75)	1/0/2 (0.75)	0/0/0 (0)	2/0/0 (0.75)	0/0/0 (0)	0/0/0 (0)	0/0/0 (0)
SU	0/1/0 (-0.5)	1/0/0 (0.75)	0/0/1 (0)	0/0/0 (0)	0/0/0 (0)	0/0/2 (0)	7/3/6 (0.75)
DI	0/0/0 (0)	0/0/0 (0)	0/6/2 (-0.5)	0/0/0 (0)	0/0/0 (0)	7/3/6 (0.75)	0/0/0 (0)

The instruction for the antibiotic combination therapy (Figure 1) is partially contrary, in terms of effect interaction between antibiotic classes. In order to cover the entire range of possible interactions (between groups I + II and II + III) a synergistic and an antagonistic scenario will be investigated. The synergistic scenario (scenario 2s) will consider the synergistic effects between groups I + II and between groups II + III. The antagonistic scenario (scenario 2a) considers an additive effect between groups I + II and an antagonistic effect between groups II + III. All antibiotic classes within one group are supposed to interact additively. As in the case of scenario 1 synergism, antagonism and additivity will be accounted for as 0.75, -0.50 and 0.00, respectively. A summary of the corresponding WOE is given in Table 5.

Table 5: Weight-of-evidence of scenarios 2s/2a

	MA^{12}	QU^{12}	TC^{12}	PE^{12}	CE^{12}	SU^{12}	DI^{12}
MA	0.00/ 0.00	0.75/ -0.50	0.00/ 0.00	-0.50/ -0.50	-0.50/ -0.50	0.00/ 0.00	0.00/ 0.00
QU	0.75/ -0.50	0.00/ 0.00	0.75/ -0.50	0.75/ 0.00	0.75/ 0.00	0.75/ -0.50	0.00/ 0.00
TC	0.00/ 0.00	0.75/ -0.50	0.00/ 0.00	-0.50/ -0.50	-0.50/ -0.50	0.00/ 0.00	0.00/ 0.00
PE	-0.50/ -0.50	0.75/ 0.00	-0.50/ -0.50	0.00/ 0.00	0.00/ 0.00	-0.50/ -0.50	0.00/ 0.00
CE	-0.50/ -0.50	0.75/ 0.00	-0.50/ -0.50	0.00/ 0.00	0.00/ 0.00	-0.50/ -0.50	0.00/ 0.00
SU	0.00/ 0.00	0.75/ -0.50	0.00/ 0.00	-0.50/ -0.50	-0.50/ -0.50	0.00/ 0.00	0.00/ 0.00
DI	0.00/ 0.00	0.00/ 0.00	0.00/ 0.00	0.00/ 0.00	0.00/ 0.00	0.00/ 0.00	0.00/ 0.00

Both scenarios 2s and 2a indicate antagonistic effects between penicillins – macrolides / tetracyclines, as well as between macrolides – cephalosporins which is contrary to scenario 1. Except for the latter combination all WOE of binary mixtures between macrolides, quinolones and cephalosporins in scenario 1 are covered by scenario 2s or 2a, respectively.

Distribution pattern and magnitude of HI_{int}

In the previous section, it becomes obvious that only a certain fraction of the considered mixture is affected by effects greater or lesser than additivity. Hence, the distribution of hazard-inducing antibiotic groups and subsequent risk management strategies may be subject to changes, depending on which interaction scenario
(1, 2a, 2s) is applied. In the following, the actual consequence of applying different WOE to the variety of binary antibiotic combinations will be determined to provide information on the robustness of the approach.

In Figure 3 the hazard distribution of antibiotic classes is depicted for the reference condition HI_{add} and the three investigated scenarios of HI_{int}. The distribution between antibiotic classes is also influenced by the interaction magnitude M. The influence is shown by plotting the range between 2.7 and 7.6 (M_1 and M_2) as error bars. The reference condition of HI_{add} was already described in 3.1. In scenario 1, the contribution of macrolide antibiotics to HI_{int} is lowered by 4 to 6 % for the share of cephalosporins (+ 4 to 5 %) which became nearly as important as quinolones. The HI of quinolones in turn remained nearly constant. Penicillins, sulfonamides and diaminopyrimidines did negligibly drop in terms of their involvement in the environmental hazard. The two scenarios 2s and 2a show to be

rather similar to scenario 1. The former differs slightly due to a lower share of cephalosporins and the highest share of quinolones. Overall, it can be concluded that no major changes in hazard distribution were induced by the HI_{int} approach, inasmuch as – despite the relative changes – the priority ranking remains the same. All changes of shares to HI were estimated to be well below 10 %.

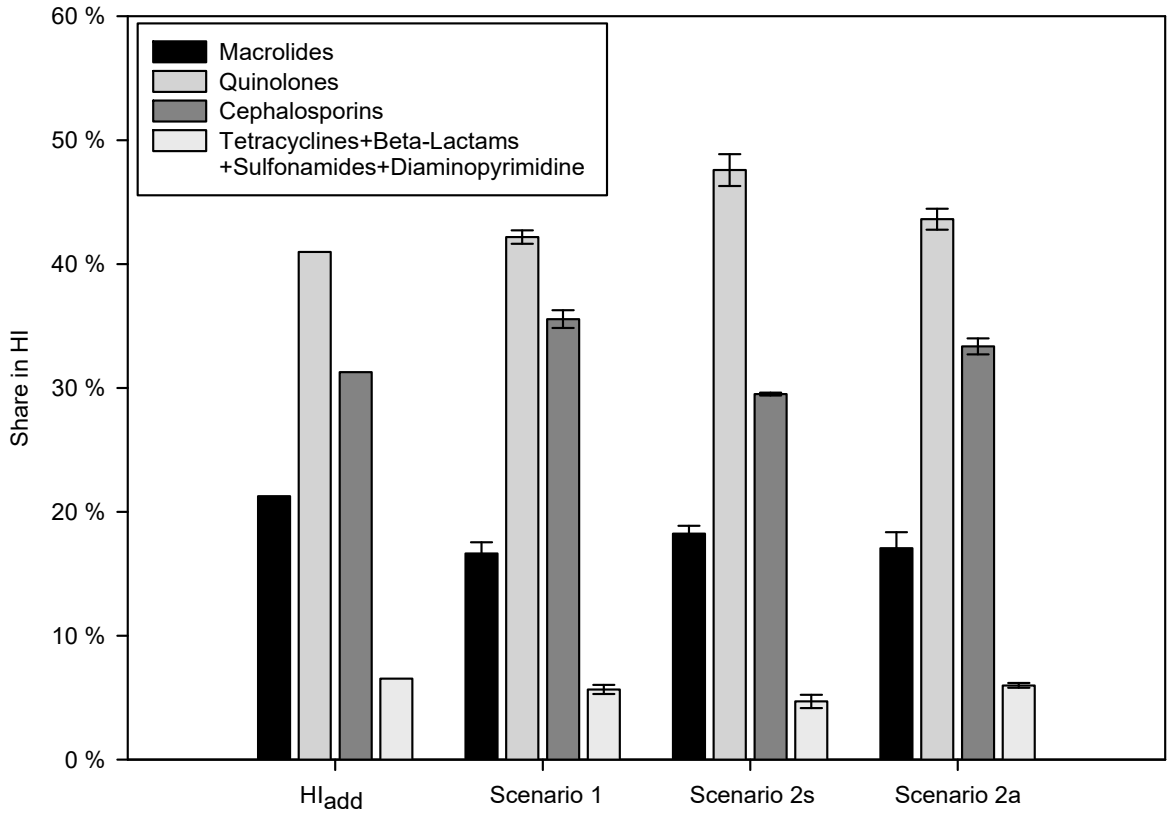

Figure 3: Hazard distribution of HI_{add} and HI_{int}-scenarios 1, 2s and 2a (error bars represent the variability due to applied interaction magnitudes M_1 and M_2)

Between the absolute values of HI_{add} and $HI_{int,n}$ (n=1,2s,2a), a quasi-linear relationship was observed (see Figure 4). Scenario 1 ($HI_{int,1}$) which is based on literature data was plotted against HI_{add} and results in a hazard increase of 50 % (R^2 = 0.9967) and 198 % (R^2 = 0.9886), respectively, depending on whether M_1 or M_2 is applied (see dots and triangles in Figure 4). The unevenly changing amounts of antibiotics (see Figure 2) do not essentially lead to a pronounced scatter in linearity. Hence, a linear approximation passing through the origin of the diagram seems feasible. The use of linear factors is practical to describe the order of magnitude HI_{int} differs from HI_{add} and will be applied for the evaluation of scenarios 2a and 2s.

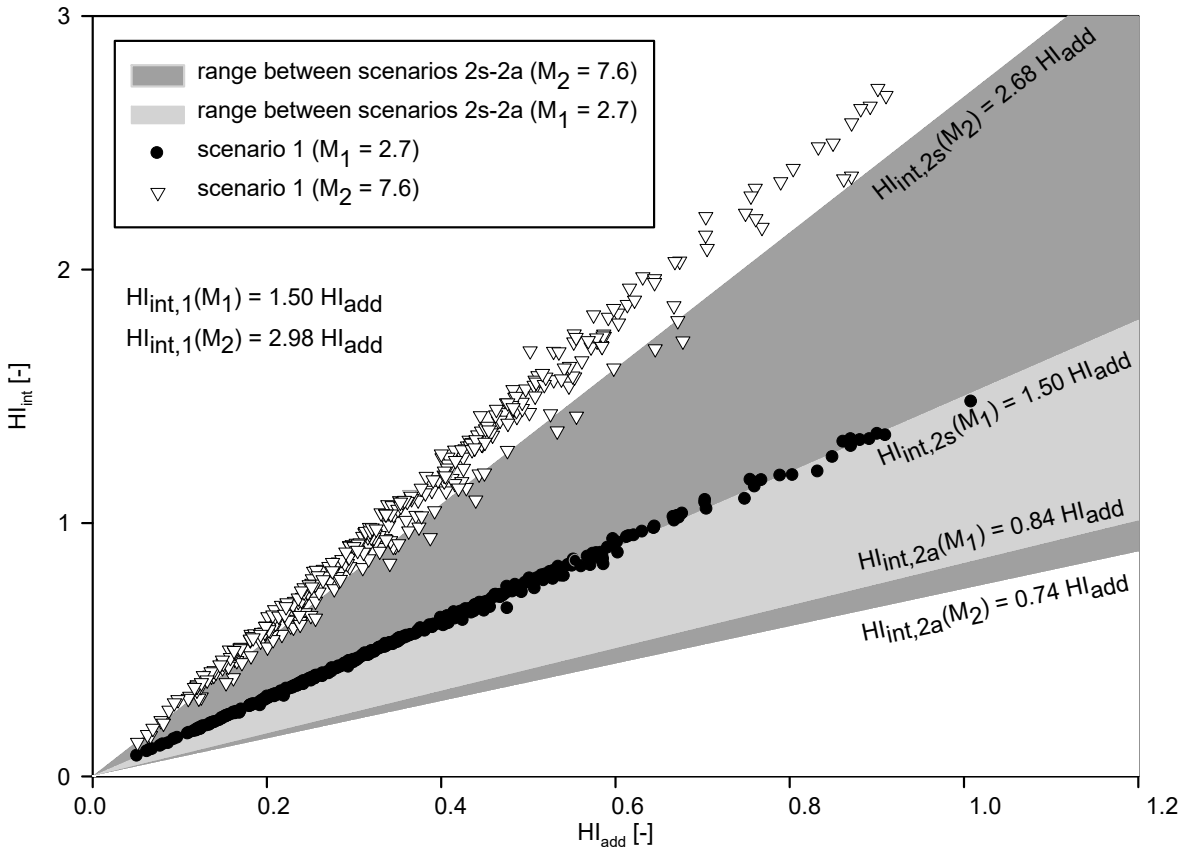

Figure 4: Relationship between HI_{add} and HI_{int} for the scenarios 1, 2s and 2a considering the selected interaction magnitudes M_1 and M_2

The approximated straight lines of scenarios 2s and 2a ($HI_{int,2s}$ and $HI_{int,2a}$) were connectedly plotted to depict the scenario-induced span of possibilities these approaches entail (see Figure 4). In case of M_1 (light grey), which states a solid estimate of the average interaction magnitude of antibiotics, the antagonistic scenario 2a falls close to the linear attenuation of the additivity-based approach HI_{add}. In case of a completely additive or antagonistic behavior between the antibiotic groups I + II and II + III, respectively (see Figure 3), the $HI_{int,2a}$(M1) diminishes the risk by 16 %, compared to HI_{add}. The maximum interaction magnitude M2 of 7.6 (dark grey) leads to a reduction of even 26 % ($HI_{int,2a}$(M2)). Considering the synergistic scenario 2s the worst case constitutes an additional increase in hazard of 168 %, referring to M_2. Applying M1, $HI_{int,2s}$(M1) raises the risk by about 50 %. From Figure 4 it can be seen that the synergistic scenario 2s arising from human combination therapy is well supported by scenario 1, both suggesting an increased environmental hazard between 50 and 200 % as compared to the additive approach.

DISCUSSION

Targeted organisms and concentration range

Two factors are considerably influencing the results of this approach dealing with the inclusion of synergistic effects in risk assessment: the diversity of tested organisms and the range of applied test concentrations. Literature on antibiotic mixtures mainly covers bacteria as test organism, due to the fact that antimicrobials specifically target this group of organisms. In contrast, the classical environmental risk assessment is based on the sensitivity of standard organisms like fish, daphnia and algae. Regarding the toxic effect of antibiotic mixtures, only the latter two were investigated so far. A total of 47 investigations were carried out with the non-target organisms algae (Pseudokirchneriella subcapitata, Selenastrum capricornutum) and crustacean (daphnia magna). On the other hand, investigations were carried out on a great variety of pathogenic bacteria (67 investigations) and two non-pathogenic bacteria (46 investigations), which also clearly demonstrates the division into clinical and environmental research purposes. With respect to the significance of this approach, the high share of environmentally relevant organisms (58 %) justifies this data set to be valid as a basis for a general statement on binary mixtures causing synergistic effects in the environment. An overview of tested organisms and antibiotic combinations can be found as supplementary material S5 (annex 4).

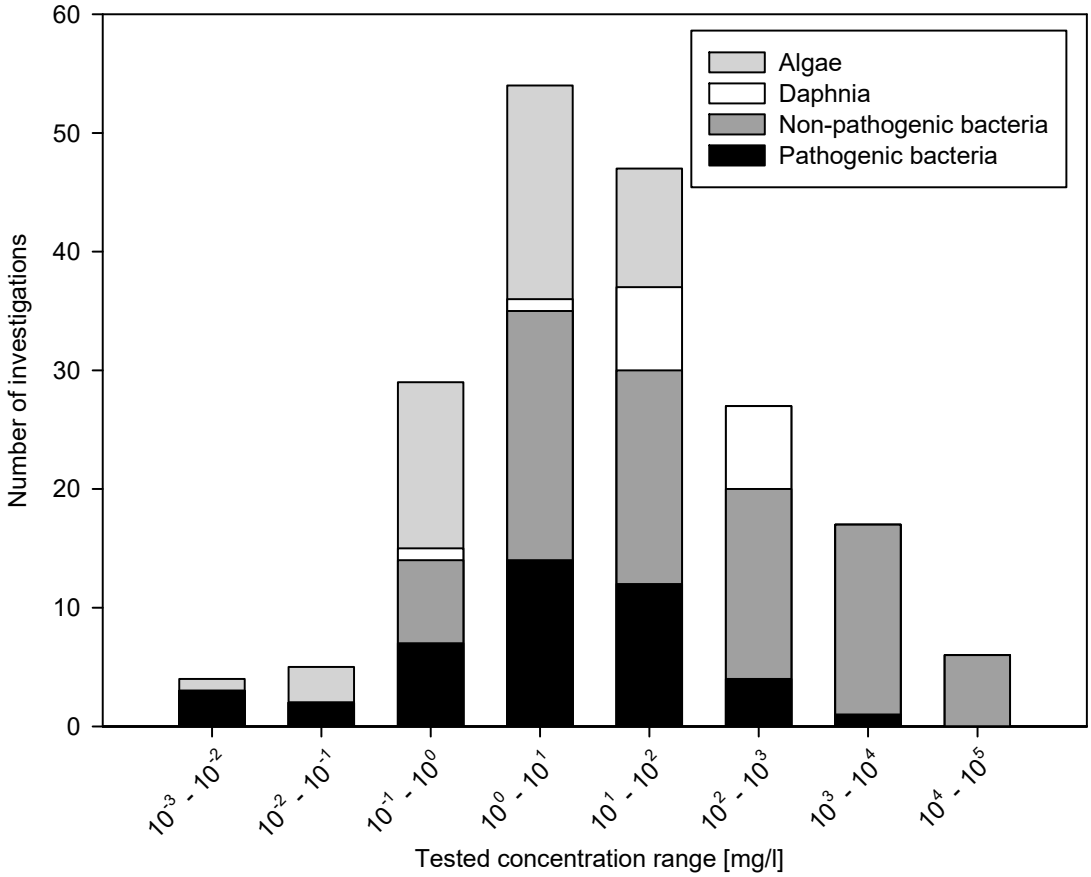

Figure 5: Concentration range of binary antibiotic interaction experiments among investigated groups of organisms

Based on the calculated PEC_{AB}, environmental concentrations in the Elbe can be found up to 100 ng/l (see supplementary material S4, annex 4), which is in the range of previously published data (Gonzalez-Pleiter et al., 2013). The majority of investigations regarding binary interactions of antibiotics were carried out at concentrations above 100 µg/l and up to 10 g/l (see Figure 5). Some results on algae (Chalkley and Koornhof, 1985; Gonzalez-Pleiter et al., 2013; Yang et al., 2008) and pathogenic bacteria (Gradelski et al., 2001; Olajuyigbe, 2012) are found below 100 µg/l and can be considered environmentally relevant. Nevertheless, it must be questioned if the number of investigations above 100 µg/l constitute a reliable data base.

Concerning a projection to environmentally relevant concentrations, the ratio of investigations which result in synergism related to the total number of investigations per concentration range shows a decreasing tendency with increasing concentration (see supplementary material S6, annex 4). In this context, it must be concluded that lower concentrations increase the probability of synergistic interactions between antibiotics. If this thesis is correct, adjustments of the present approach will be necessary to display environmentally relevant results. In this context, a few specific investigations cover the issue of the concentration dependency

of mixtures between heavy metals (Rodea-Palomares et al., 2009), pesticides (Laetz et al., 2009), antibiotics (Gonzalez-Pleiter et al., 2013) and surfactants (Rosal et al., 2010). The majority of the presented results are contrary to the thesis stated above and dependency is either directly proportional or non-existent. Nonetheless, in case of antibiotics and surfactants a few results support inverse proportionality showing an increase of synergism with decreasing concentration. Based on the Combination Index CI, the presence of both antibiotics tetracycline and erythromycin potentiated the effect on Pseudokirchneriella subcapitata and Anabaena CPB 4337 with decreasing concentration. The susceptibility of Anabena CPB 4337 also increased at lower concentrations of tetracycline-levofloxacin and 2,4,6-trichlorophenol-triclosan mixtures. The same behavior was observed for Pseudokirchneriella subcapitata being exposed to a mixture consisting of docusate and 2,4,6-trichlorophenol (Rosal et al., 2010), as well as a mixture consisting of 12 antimicrobial agents (Yang et al., 2008). In summary, Anabaena CPB 4337 and Pseudokirchneriella subcapitata showed increasing synergism inversely proportional to the applied concentration in 3 out of 15 and 2 out of 11 investigated mixtures, respectively. The results of investigations on vibrio fisheri do not give evidence on an indirect proportional relationship between decreasing concentration and a higher incident of synergism (Gonzalez-Pleiter et al., 2013; Rosal et al., 2010).

Due to the fact that the results regarding the concentration influence on synergism depend on the targeted organism as well as the combination of substances, no general statement can be made from the available information for a mixture consisting of multiple antibiotics. The results presented in 3.4 cannot be reasonably adapted to real environmental conditions. Hence, the findings of the literature research on binary mixtures and human combination therapy must be accepted to reflect the current state of knowledge.

Quantity of binary mixture data

The applied criteria to decide whether synergism or antagonism is predominating a certain mixture strongly depends on the total number of investigations (N) and the absolute difference between results indicating either one of the effect direction (D). If just a few studies support the occurrence of a certain effect, they only reflect a limited view on the concentration range and/or the variety of organisms. In consequence, only a minor statistical significance can be expected from these results (i.e. mixture between cephalosporins and macrolides). On the other hand, a high number of diverse investigations (i.e. mixture between penicillins and tetracyclines) also increases the uncertainty. In both cases the inclusion of a few new results can turn around the interaction direction and lead to results different from those presented in this study. In order to determine the robustness of the present data set on binary mixtures, a sensitivity analysis was performed. Therefore, the minimum number of investigations (N) and the required difference in number of investigations indicating synergism and antagonism (D) were independently varied from 1 to 5. In case of meeting both of the predetermined conditions, the WOE

was applied as presented in Table 4. If one of the conditions was not fulfilled, a WOE of 0 is applied due to ambiguity, indicating simple additivity.

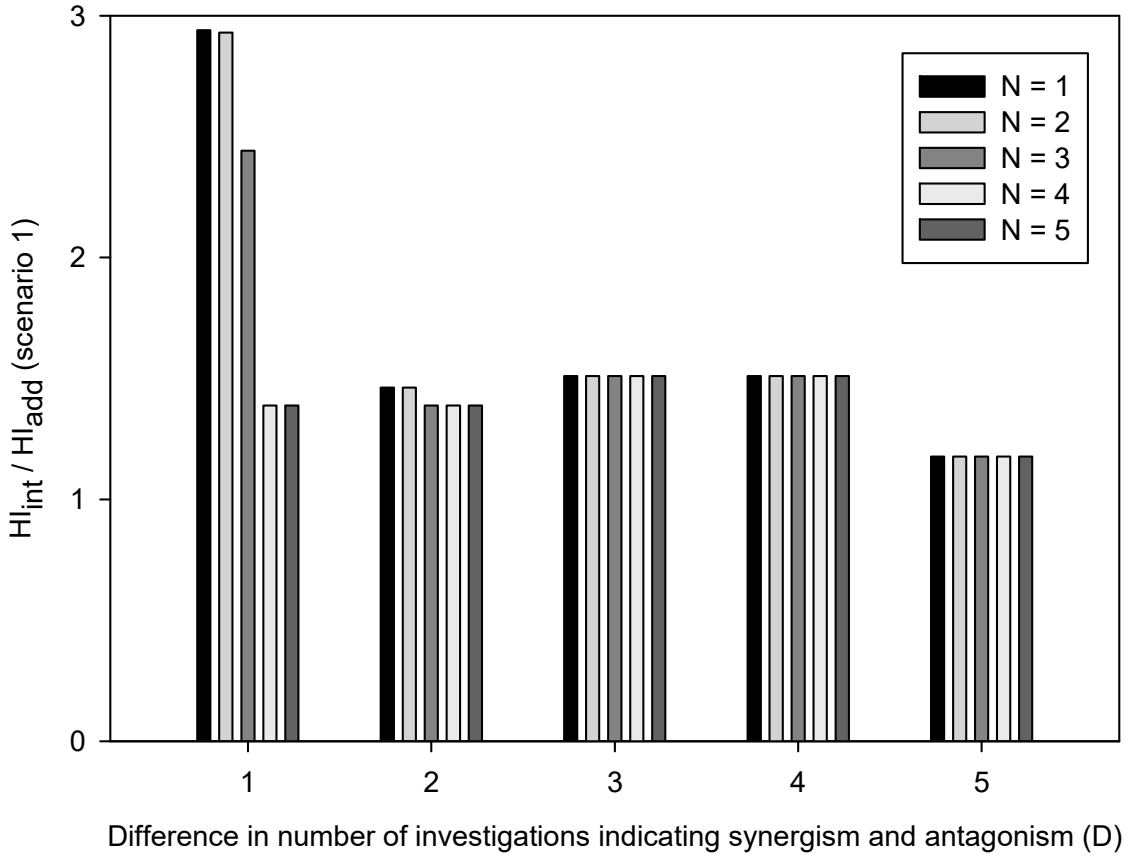

Figure 6: Sensitivity analysis on the HI_{int}/HI_{add} of scenario 1 (M_2 = 7.6) regarding the total number of available investigations (N) and the minimum difference in number of investigations indicating synergism and antagonism (D)

In Figure 6 the data set's sensitivity to preconditions (scenario 1) regarding a required minimum of data quantity N is depicted for M = 7.6. Assuming that $D \geq 1$ is sufficient to take an adequate decision, the HI_{int}/HI_{add}-ratio, as presented in 3.4, is significantly reduced demanding a minimum total data quantity of N = 3 and N = 4 interaction studies per mixture. At this preconditions high-impact synergy combinations like cephalosporins – macrolides and cephalosporins – quinolones do not longer have a share in determining HI_{int}, respectively, which leads to the significant decrease. Demanding a minimum D of 2, 3 or 4 results in an attenuated HI_{int}/HI_{add} ratio of around 1.5, which also leads to ignoring the above stated high-impact synergy combinations. Increasing D up to 5 leads to a further decrease down to about 1.2, due to the insignificance of the synergistic combinations among quinolones and between diaminopyrimidines and sulfonamides. Accordingly, applying M = 2.7 results in HI_{int}/HI_{add}-ratios between 1.1 ($N \geq 3$, $D \geq 5$) and 1.2 ($N \geq 3$, $2 \leq D \leq 4$). The sensitivity analysis clearly de-

monstrates the decision criteria's fragility on which the quantification of this approach is based. In the present study, accepting D equal to one leads to an exaggerated HI_{int}-value picturing an extreme scenario of synergistic interactions between antibiotics. To reduce the dependency on uncertain data, $D \geq 3$ should be applied for a more grounded estimation of HI_{int}. The number of interaction studies N does not significantly influence the results for $D \geq 3$ and hence is of minor importance. Nevertheless, for integrity reasons a minimum number of 5 investigations ($N \geq 5$) seems to be suitable for quantifying the change in hazard due to synergistic and antagonistic effects in antibiotic mixtures. This proposal is based on the data set for antibiotics and its validity needs to be confirmed for other chemical mixtures.

CONCLUSIONS AND SUMMARY

The interaction-based hazard index (HI_{int}) proposed by the US EPA was applied for antibiotics to evaluate and quantify possible interaction effects greater or lesser than additivity. Scenario 1, which is based on binary antibiotic mixture experiments using different organisms was confirmed by the synergistic scenario 2s which was derived from known synergistic and additive interactions of antibiotic groups from human combination therapy. Accordingly, it can be concluded that antibiotic mixtures tend to exhibit a synergistic overall effect. Compared to the classic HI_{add} approach an increased risk is expected. The sensitivity analysis on the available data quantity and its consistency provides the necessary evidence to reliably estimate the level of risk increase that is induced by synergism, based on the current state of available information. Applying a mean magnification factor of 2.7, which stands for a consolidated risk assessment, leads to a risk increase of about 20 %. An increase up to 50 % was determined if carrying out a worst-case risk assessment using the maximum determined magnitude of 7.6. Here it must be noted that high-impact synergy combinations like cephalosporins – macrolides and cephalosporins – quinolones were not included due to data scarcity. Furthermore, limited information on the amount and ecotoxicity of antibiotic metabolites resulted in the exclusion of this group of possible risk inducing substances. Providing information on high-impact synergy combinations and metabolites is essential to consolidate the findings of this study and to assess the overall risk induced by the application and release of antibiotics.

Applying the risk increase of 50 % to the situation of the Elbe river, it can be stated that the threshold value of $HI \geq 1$ is exceeded during 25 weeks (rather than 1 week applying HI_{add}) during the examined 7 years period. Due to this alarming influence more toxicologic investigations are necessary to further improve the robustness of the estimation on synergistic effects in the environment. In case of antibiotics, investigations should focus on binary combinations between macrolides, quinolones and cephalosporins in particular. The toxicologic set-up should definitely include varying concentrations down to the µg/l or even ng/l range, in order to additionally assess its influence on synergy-drifts.

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CHAPTER 6

Results and conclusions

Results and conclusions

INDIVIDUAL RESULTS

The first paper broaches the issue of adequacy applying a projection approach using prescription data to determine the influent loads of antibiotics to a WWTP (Marx et al. 2015d). The precise knowledge on in- and effluent loads of WWTP is the prerequisite to estimate predicted environmental concentration and hence to carry out a reliable risk assessment. The investigation focuses on uncertainties concerning a variety of necessary assumptions and the accuracy of expected input loads. Several antibiotics with and without seasonal influence were well recovered, i.e. the overall mass balance lies within an acceptable range, confirming the general suitability of the applied approach. As expected, antibiotics with low stability in raw wastewater (e.g. beta-lactams) exhibited a poor recovery at the WWTP inflow. The input variation of fluoroquinolones was unexpectedly high and an adequate seasonal prediction on the basis of the available prescription data is not possible. In this context, the assumption of an equal prescription practice in hospitals with different structures and core areas, the non-compliance in the use of antibiotics as well as delay and remobilization during the transport in the sewer system must be discussed in future research. Apart from the annual input variation, the mean recovery of fluoroquinolones was found to be significantly lower than expected. An insufficient analytical determination with an incomplete detection of the adsorbed fraction was suggested to be partly responsible for this unsatisfying recovery. In addition to the comparison between predicted and measured input loads, measuring data were also used to estimate the necessary sampling quantity in case no prescription or similar input data are available. As a result, it was proposed that a minimum of 20 to 40 samples is necessary to capture seasonal input dynamics of antibiotics and indicatively monitor influent (and effluent) loads of WWTPs. With respect to the verified model and the available amount of data, the recommended sample quantity can be considered generally valid for WWTP monitoring programs. In this context, it is advised to carry out additional experiments to prove the suitability of the applied analytical method and to determine correction factors, if necessary.

The second paper is based on long-term WWTP monitoring (12 months) and provides information on the removal efficiency of antibiotics and respective influencing factors regarding WWTP operations (Marx et al. 2015a). The applied evaluation of gapless time series generated robust elimination values, but no significant dependency between the removal of most antibiotics and operation parameters were identified. As an exception, clindamycin influent concentrations were inter alia correlated to the WWTP inflow, which led to the presumption of an existing but unknown transformation product in the raw wastewater. The subsequent re-transformation to the parent substance during the wastewater treatment equals the previously calculated "production" of clindamycin and supports the suggested transformation model. Concerning the MFA within the urban

wastewater system, no net removal of excreted clindamycin can be identified. Furthermore, the removal of clindamycin and ciprofloxacin seems to depend on the sludge retention time as well as the treatment temperature. A clear differentiation between both parameters was not feasible due to changing operation regimes of the WWTP. The subsequently performed MFA gives evidence on the fate of antibiotics during biological wastewater treatment, pointing out the partial fragility of available information. In this context, the fate of ciprofloxacin and levofloxacin in the solid phase cannot be reliably assessed and the influence of possible degradation processes during the wastewater and sludge treatment remains unclear. The majority of antibiotics could be well described according to their fate during the treatment process of the WWTP

How to prioritize antibiotics according to their environmental relevance is subject of the other two papers, which deal with risk assessment strategies at the examples of the WWTP effluent and the receiving stream, respectively. The third paper thereby addresses the species order according to their impact on the remaining food chain in combination with their specific susceptibility towards antibiotics (Marx et al. 2015c). Comparing toxicological investigations of antibiotics, differences according to the sensitivity ranking of standard species could be observed. The interference of lower-tier species causes damage propagation and leads in all probability to a weakening of higher-tier species due to the reduction of available food sources. Hereby, the classification of first-order priority substances was introduced to consider inconsistencies between the ranking of aquatic organisms in the food chain and their sensitivity towards antibiotics. The antibiotics ciprofloxacin, azithromycin and roxithromycin are the main disruptors for algae and considered to be first-order priority substances, while clarithromycin is mainly responsible for the risk towards crustaceans. In addition to investigating the risk distribution among species, available toxicological data were used to replace the simple linear dose-effect-relationship by a sigmoidal curve, which is more appropriate concerning this matter. The resulting attenuated risk reflects the range of tested concentrations and represents the variety of all investigated organisms.

The fourth paper deals with antibiotic mixtures and the consideration of synergistic and antagonistic effects (Marx et al. 2015b). The impossibility of practically testing the entire existing range of antibiotic combinations, including different concentration ranges, calls for a substantiated estimation of their effects. The interaction-based hazard index, which was proposed by the Environmental Protection Agency of the United States (EPA 2000), relies on binary interaction studies and considers several interaction characteristics like the magnitude of interaction or the weight-of-evidence. The results indicate an overall synergistic behavior of the considered mixture consisting of 15 antibiotics. Accepting a set of defined quality requirements regarding the available data, a risk increase of 20 to 50 % is expected, compared to the classical risk assessment applying simple concentration addition. Including the data of antibiotic combinations which do not yet meet the quality requirements (e.g. low data quantity) indicate environmental risks up to

300 % higher than the classical assessment approach. The results clearly highlight the necessity of including the synergistic potential of antibiotics to make solid estima-tions of the environmental risk. The increase of available binary interaction studies between antibiotics will be crucial to consolidate the determined results and pro-vide detailed information on antibiotic combinations causing the highest risk in-crease.

AGGREGATION OF RESULTS

The developed model for the comparison of predicted and measured input loads constitutes the basis of the environmental risk assessment based on secondary input data. The model verification for recalcitrant antibiotics was qualitatively carried out by means of laboratory experiments, considering probable uncertainties of the analytical approach. The high predictive quality of antibiotics with (azithromycin, clarithromycin, roxithromycin) and without (trimethoprim) seasonal variations justifies a general acceptance of the developed model. The verified model is hence suitable to integratively investigate the behavior of degradable and adsorbable antibiotics, which exhibit low recovery rates at the WWTP input, and draw conclusions on their fate within the urban wastewater system.

The sensitivity of antibiotics to abiotic and biotic degradation limits the accurate prediction of their input loads to the WWTP, but can provide first information on their possible fate in the environment. The good biological availability of some degradable antibiotics suggests a (nearly) complete disappearance after the biological treatment in the WWTP, resulting in further attenuated concentrations in the receiving water body. For example, the beta-lactam antibiotics penicillin V and cefotaxime exhibit a low recovery rate at the WWTP input and a predominantly complete elimination during the biological wastewater treatment, which complies well with the above interpretation. There are, however, other antibiotics with low recovery values at the WWTP input but significant concentrations in the WWTP effluent. Sulfamethoxazole, cefuroxime and piperacillin were shown to be degradable in raw wastewater, but it remains unclear why the elimination process remains incomplete during the biological wastewater treatment. The conducted mass flow analysis reveals, that only a minor fraction of the total input load is bound to sludge. This suggests, that mainly degradation processes are responsible for the partial removal during the wastewater treatment, rather than sorption processes. In addition to biodegradation, several studies also proved hydrolysis to be a relevant removal pathway for beta-lactams under typical pH and temperature conditions (Mitchell et al. 2014, Zhao et al. 2012), whereas the impact on sulfonamides was comparatively low (Loftin et al. 2008). These studies indicate, that hydrolysis as well as biodegradation must be considered as possible degradation processes. However, the discerning of abiotic and biotic degradation is rather speculative on the basis of monitoring data and hence were not carried out within the context of this work.

The mass flow analyses of ciprofloxacin and levofloxacin indicate, that about 200 % of the measured input load was found in the two output streams effluent plus digested sludge. Referring to the projected input load based on prescription data as well as considering a low to moderate elimination during the anaerobic sludge treatment, the estimated input load and the sum of both output streams are actually well comparable. On one hand, this finding supports the assumption of an inadequate analytical method regarding adsorbable compounds and/or an insufficient sampling strategy at the WWTP input, respectively. On the other hand, the constant removal rate during periods with high loads of ciprofloxacin and levofloxacin also indicate the simultaneous and proportionate increase of the effluent concentration, which is, compared to the influent, nearly free of particulate matter. Hence, an erroneous analytical method, as supposed in Marx et al. 2015a and Marx et al. 2015d, cannot be solely responsible for the input (and output) load fluctuations. It is rather likely that sampling and the projection model are the main sources of errors in this context, even though if it is not certain at which point.

Concerning the environmental risk, ciprofloxacin, levofloxacin and the group of cephalosporins significantly affect the aquatic environment. They either have the highest impact on (one of) the lowest trophic level(s) or disproportionately increase the ecotoxicological risk due to their synergistic characteristics. In this regard, the deficiencies regarding the input prediction of these antibiotics is of particular concern. Measurements suggest significantly higher fluctuations, along with higher load peaks, compared to the estimated input loads using the prediction model. The underestimation of such critical mass flow conditions weakens the approach of assessing environmental risks on the basis of secondary data like prescriptions. Hence, efforts must be made to further develop the projection model by improving the quality of secondary data, identifying additional emitters and understanding possible retention and degradation dynamics of antibiotics within the sewer system.

FINAL CONCLUSIONS

The prediction and projection model in combination with the conducted MFA are suited to estimate urban antibiotic emissions into the environment. However, the lack of critical information regarding the genesis of secondary data as well as the possibility of additional unidentified inputs partly limits their reliable estimation for short time periods, i.e. seasonal fluctuations Table V.1 presents a qualitative summary of the predictability of antibiotic emissions, covering the input recovery at the WWTP as well as the removal from the liquid phase during biological wastewater treatment. In combination with the prioritization of antibiotics according to their ecotoxicological relevance, it becomes obvious, that 4 out of 7 identified risk drivers cannot be adequately estimated on the basis of prescription data (see Table V.1, highlighted in dark grey). In any case, emission dynamics of unpredictable risk-drivers require measurements to capture load peaks and draw reliable conclusions on the resulting acute ecological impact. In contrast to the proposed sampling quantity regarding the indicative input measurements at WWTP,

about 30 to 80 samples per year are necessary to solidly estimate weekly fluctuations of the identified unpredictable risk-drivers. If verified in advance, antibiotic prescription data can be used to predict environmental loads of risk-drivers, as it is the case for investigated macrolides. Moreover, the identification of macrolides being risk-drivers is consistent with their inclusion in the European watch-list for substances that may pose a significant risk to or via the aquatic environment at Union level (EU 2015).

Table 1: Qualitative classification of investigated antibiotics regarding input predictability, WWTP removal and ecotoxicological relevance for the aquatic environment (without highlight – not identified as risk-driver; highlighted in light grey – predictable risk-driver; highlighted in dark grey – unpredictable risk-driver; n.a. – not assessed)

	Compartment			
	1		**2**	**3**
	Estimation of seasonal input recovery	**Estimation of annual input recovery[13]**	**Estimation of removal from liquid phase**	**Categorization as risk-driving antibiotic**
Amoxicillin	n.a.	possible	possible	no
Azithromycin	possible	possible	possible	yes
Cefotaxime	possible	possible	not possible	yes
Cefuroxime	not possible	possible	possible	yes
Ciprofloxacin	not possible	possible	possible	yes
Clarithromycin	possible	possible	possible	yes
Clindamycin	n.a.	possible	possible	no
Doxycyclin	not possible	not possible	not possible	no
Levofloxacin	not possible	possible	possible	yes
Penicillin V	n.a.	n.a.	possible	no
Piperacillin	n.a.	possible	possible	no
Roxithromycin	possible	possible	possible	yes
Sulfamethoxazole	possible	possible	possible	no
Trimethoprim	possible	possible	possible	no

In the context of fate characterization during biological wastewater treatment, a number of aspects remain to be dealt with. Until proven otherwise, the close-to-

[13] In combination with additional experiments regarding initial recovery and degradation of antibiotics in raw wastewater

constant removal rate along the year, which was determined for the majority of antibiotics, is still strictly limited to the investigated WWTP Dresden-Kaditz. The presented results must be confirmed by investigations similar in scale and process configuration (see Marx et al. 2015a). The integration of further plant configurations (probably) allows the distinction of state-of-the-art technologies according to their effectiveness in removing antibiotics. Such information can be valuable criteria for future plant planning. Furthermore, evaluating the sludge path of antibiotics is still very imprecise due to highly varying outcomes. It remains unclear to what extent load dynamics caused by sorption and desorption processes are responsible for the observed inconsistencies. With regard to the close-to-constant removal from the liquid phase in combination with the comparable insignificance of process conditions typically influencing sorption processes (pH, temperature), the adsorbed amount of antibiotics is expected to be mainly influenced by the influent load. The interpretation of results should be verified in future investigations focusing on long-term behavior of antibiotics in WWTP sludge.

Apart from antibiotics regularly entering the WWTP, emissions from combined sewer overflows must be part of future investigations regarding the assessment of environmental risks. About 10 % of the annual urban load of Dresden is intermittently released during only a limited number of combined wastewater discharges (Marx and Kuehn 2015). Low wastewater dilution at the beginning of such discharge events results in increased emission loads, which are expected to have the potential to induce acute ecotoxicological effects in the receiving water. Hence, future efforts should be made to develop appropriate sampling and evaluation techniques to quantify antibiotic loads from combined wastewater discharges and draw conclusions on the resulting acute impact on the aquatic environment.

The presented risk assessment approaches provide valuable additional information and alternative aspects to classical risk assessment methods, with the objective of prioritizing antibiotics according to their manifold impact on the environment. Both studies reveal a significant lack of toxicity data, in connection with only a few types of investigated bacteria, which impairs their comprehensive evaluation. In addition to the testing of the activated sludge biocenosis, a total of 2 - 5 different types of environmental bacteria were investigated, respectively, regarding mixture and single toxicity. The reason for the low number of tested bacteria can be found in the EMA-guideline, recommending standard organisms like algae, daphnia and fish for ecotoxicity studies of medical products for human use (EMA 2006). This recommendation originates from testing industrial chemicals and appears not fully applicable to substances with specific target organisms, like antibiotics (Agerstrand et al. 2015). Hence, the necessary consideration of a broader range of bacteria is obvious to reasonably generalize results from present approaches regarding the risk assessment of antibiotics. Prioritized antibiotics can subsequently serve as suitable indicator substances for future plant planning, with special regard to the advanced treatment using oxidation and adsorption processes. This procedure would ensure the removal of risk-driving antibiotics and help to maximally protect the abundance and diversity of organisms in the aquatic

environment. In this context, choosing the apparently most susceptible (or lowest) tier organism influences the definition of risk-driving antibiotics and should be evaluated in future research. Otherwise, the identified risk-drivers must be considered questionable.

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ANNEX 1

1. COMPARTMENT: CATCHMENT AND SEWER SYSTEM

Supplementary material Chapter 2

REPRESENTATIVE INPUT LOAD OF ANTIBIOTICS TO WWTPS: PREDICTIVE ACCURACY AND DETERMINATION OF A REQUIRED SAMPLING QUANTITY

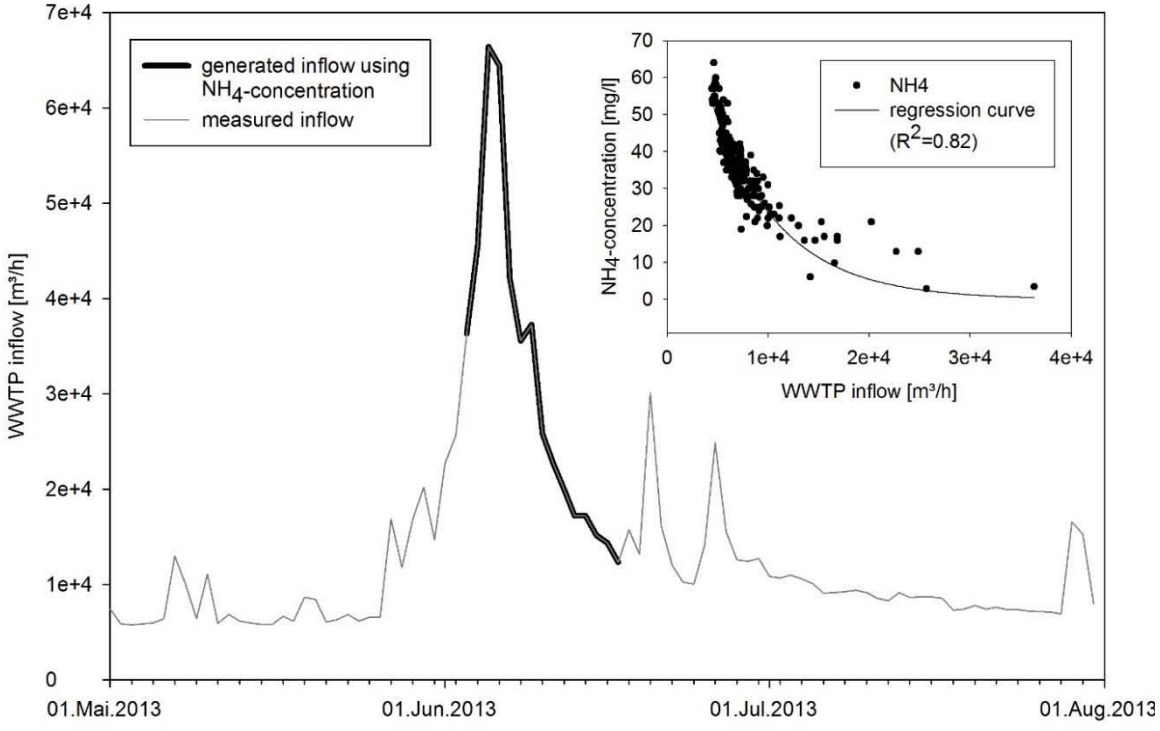

Figure S1: inflow generation using NH4-concentration

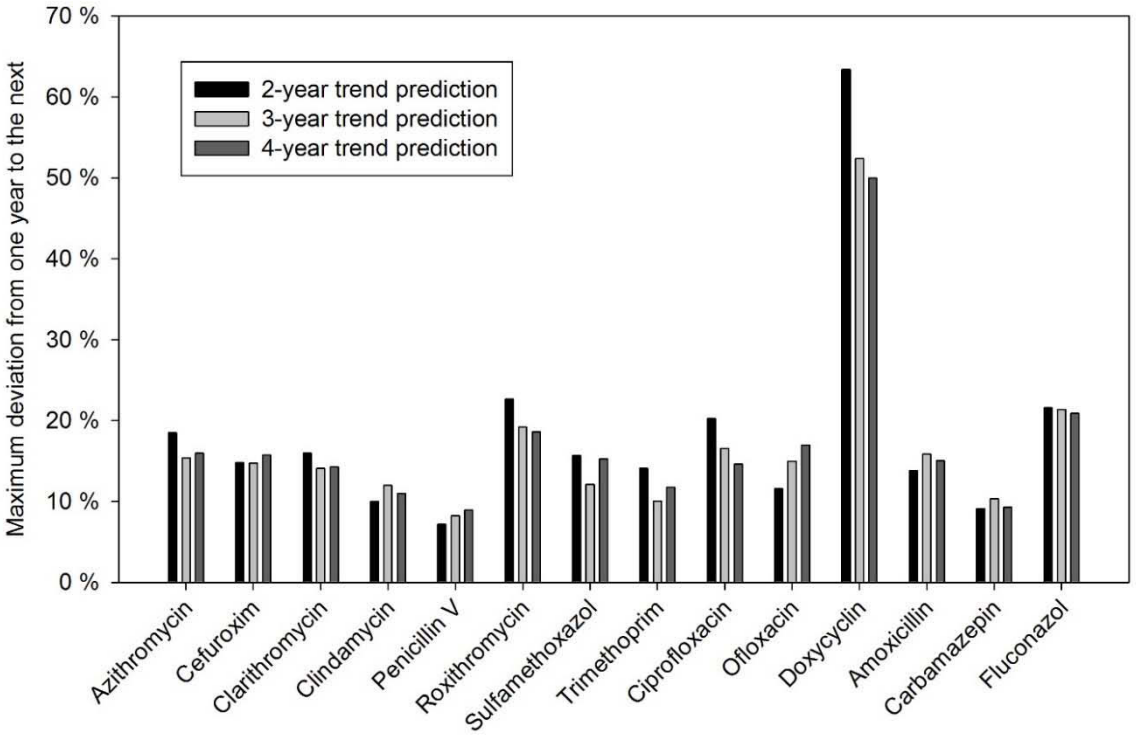

Figure S2: comparison of trend prediction approaches using 2, 3 and 4 previous years

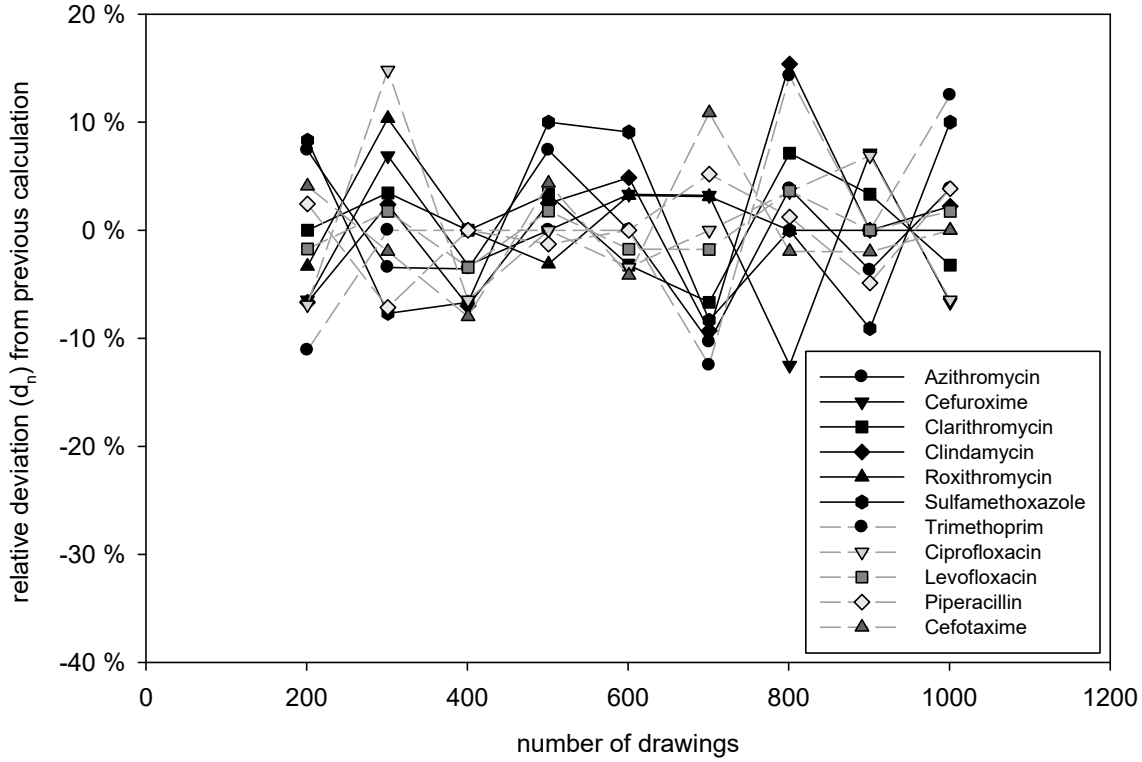

Figure S3: Influence of the number of repetitions on the deviation of the necessary sampling quantity

Calculations were carried out for 100, 200, … and 1000 repetitions. The illustrated deviation dn (y-axis) was calculated using the following formula:

$$d_n = \frac{N_n - N_{n-100}}{N_{n-1}} \quad \text{for n=200, 300, …, 1000}$$

N_n … necessary number of samples using n repetitions

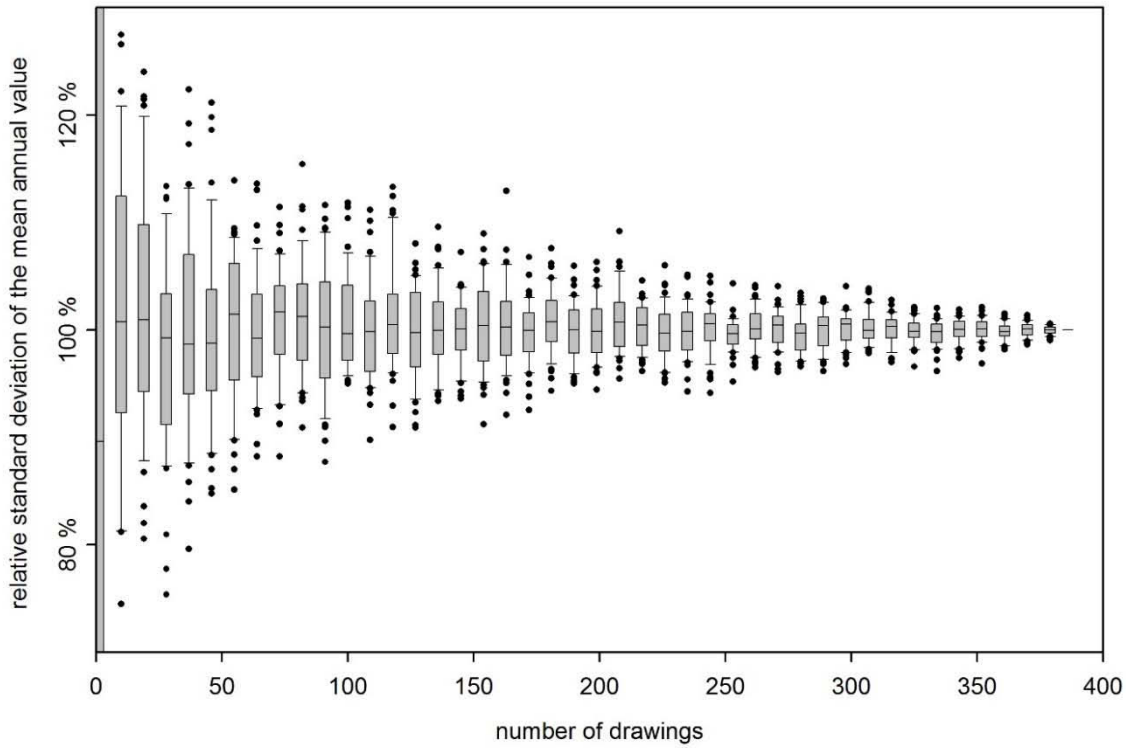

Figure S4: Relative standard deviation of different drawing ranges applying 500 repetitions by the example of roxithromycin

Table S1 Results derived from the monitoring program classification concerning the predicted input loads

	Number of values	within the predicted range (grey area)		mean recovery
Azithromycin	303	95	32%	110%
Cefuroxime	379	70	18%	53%
Clarithromycin	392	172	43%	58%
Clindamycin	395	62	16%	7%
Penicillin V	84	0	0%	3%
Roxithromycin	386	207	53%	76%
Sulfamethoxazole	387	281	85%	85%
Trimethoprim	395	268	67%	76%
Ciprofloxacin	381	67	18%	41%
Levofloxacin	381	61	15%	49%
Doxycycline	342	294	86%	155%
Amoxicillin	158	0	0%	8%
Piperacillin	377	0	0%	9%
Cefotaxime	292	3	1%	22%

Equation S1: Determination of the excretion ratio using detailed information on adsorption and metabolism

$$E_{AB} = (1 - f_{A,AB}) + f_{A,AB} \cdot (1 - f_{M,AB})$$

Antibiotics are partially eliminated during their passage through the human body. They are partly adsorbed by the gut and/or gastric mucosa and may be prone to metabolizing processes. If no data on faecal excretion is available, it is assumed that the non-adsorbed content will be excreted unchanged. Equation 3 is hence valid to calculate the excreted ratio of the parent compound (E_{AB}). $f_{A,AB}$ characterizes the adsorption ratio in the human body. The share in the adsorbed fraction that is prone to metabolism is determined by the parameter $f_{M,AB,}$ which in turn decreases the amount of the parent substance in the faeces. The information on adsorption and metabolism of the parent compound are usually provided by the pharmaceutical industry and research papers.

ANNEX 2

2. COMPARTMENT: WASTEWATER TREATMENT PLANT

Supplementary material Chapter 3

MASS FLOW OF ANTIBIOTICS IN A WASTEWATER TREATMENT PLANT FOCUSING ON REMOVAL VARIATIONS DUE TO OPERATIONAL PARAMETERS

Table S1: Routine analysis parameters of each sampling point and applied analytical regulations

Parameter	Influent	Effluent	Aeration tank	Sludge	Regulation / device
Total Kjeldahl nitrogen	X				DIN EN 25663 H11
Ammonia nitrogen	X	X			DIN EN ISO 11732E 23
Nitrite nitrogen	X	X			EN ISO 10304-1 D 20
Nitrate nitrogen	X	X			EN ISO 10304-1 D 20
Total phosphorus	X	X		X	DIN EN ISO 6878 D11 DIN EN ISO 11885 E22
Total suspended solids	X	X	X	X	DIN 38414 S2 DIN 38409 H2-2
divalent iron	X	X		X	DIN EN ISO 11885 E22
Temperature	X		X		WTW pH 3210
pH	X		X		WTW pH 3210
Flow		X		X	Electromagnetic flow-meter ABB DM 43 F
Automatic sampler	X	X			Endress + Hauser ASP Station 2000

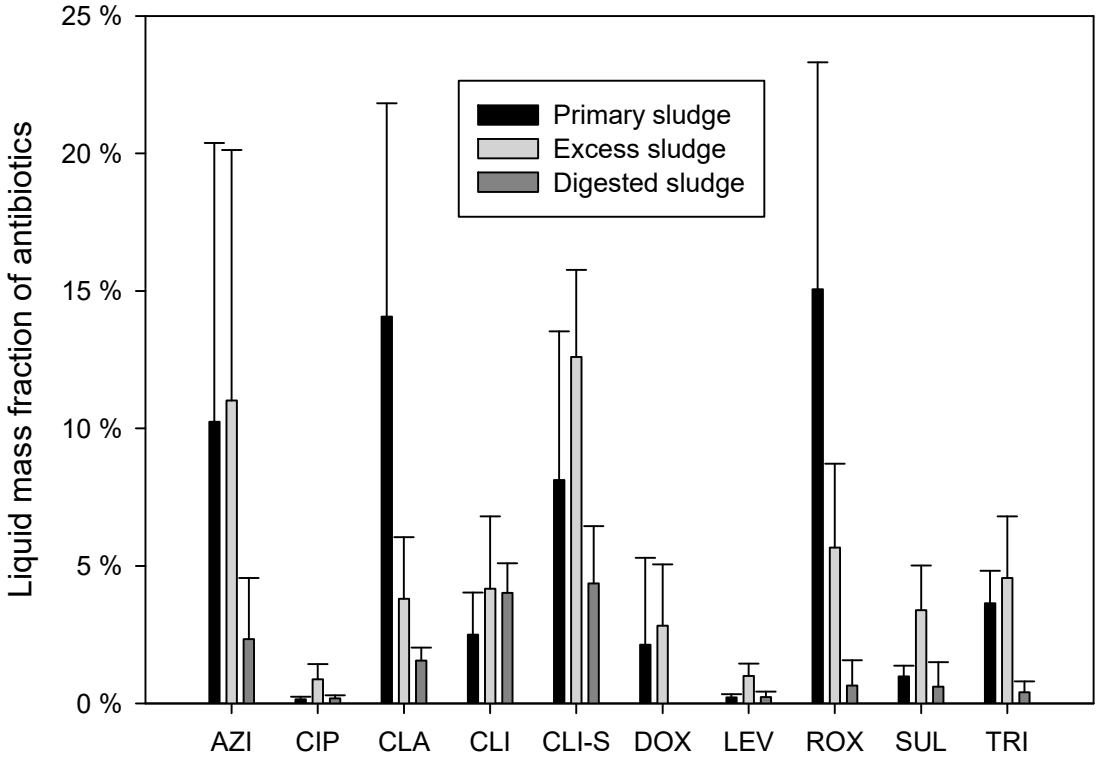

Figure S2: Mass fraction of dissolved antibiotics after centrifugation at 6000 rpm for 6 min, related to the total mass of extracted antibiotics

Table S3: Identified time series of gapless in- and effluent loads

Time series	Number of values	Removal efficiency	Coefficient of determination	Time period	
				from	to
Azithromycin					
1	14	-17%	0.9969	30.09.2013	13.10.2013
2	11	10%	0.9988	15.10.2013	25.10.2013
3	10	2%	0.9895	20.05.2013	29.05.2013
4	8	-5%	0.9893	17.09.2013	24.09.2013
Cefotaxime					
1	15	100%	1.0000	04.09.2013	18.09.2013
2	14	42%	0.9928	12.02.2013	25.02.2013
3	13	100%	1.0000	01.05.2013	13.05.2013
4	13	100%	1.0000	02.10.2013	14.10.2013
Cefuroxime					
1	44	61%	0.9982	17.01.2013	01.03.2013
2	34	69%	0.9922	22.08.2013	24.09.2013
3	30	73%	0.9963	01.05.2013	30.05.2013
4	29	61%	0.9938	23.07.2013	20.08.2013
5	26	63%	0.9986	30.09.2013	25.10.2013
6	16	65%	0.9970	03.03.2013	18.03.2013
7	8	66%	0.9830	08.01.2013	15.01.2013
Clarithromycin					
1	34	7%	0.9990	22.08.2013	24.09.2013
2	29	10%	0.9975	23.07.2013	20.08.2013
3	27	12%	0.9970	03.02.2013	01.03.2013
4	26	14%	0.9972	30.09.2013	25.10.2013
5	25	6%	0.9915	08.01.2013	01.02.2013
6	23	21%	0.9864	08.05.2013	30.05.2013
7	16	18%	0.9886	03.03.2013	18.03.2013
8	8	-3%	0.9988	20.03.2013	27.03.2013
9	8	16%	0.9918	03.07.2013	10.07.2013
Clindamycin					
1	53	-155%	0.9954	08.01.2013	01.03.2013
2	34	-288%	0.9993	22.08.2013	24.09.2013
3	30	-241%	0.9963	01.05.2013	30.05.2013
4	29	-227%	0.9955	23.07.2013	20.08.2013
5	26	-304%	0.9922	30.09.2013	25.10.2013
6	16	-183%	0.9963	03.03.2013	18.03.2013
7	8	-174%	0.9946	20.03.2013	27.03.2013

Clindamycin (corrected using the re-transformation model)					
1	38	7,66%	0.9969	20.06.2013	27.07.2013
2	34	20,70%	0.9998	22.08.2013	24.09.2013
3	30	-3,38%	0.9994	01.05.2013	30.05.2013
4	26	10,64%	0.9997	30.09.2013	25.10.2013
5	13	24,77%	0.9991	08.08.2013	20.08.2013
6	10	-16,14%	0.9969	06.03.2013	15.03.2013
7	8	-26,08%	0.9988	20.03.2013	27.03.2013
Clindamycin-Sulfoxid					
1	62	0,76%	0.9998	20.06.2013	20.08.2013
2	34	-9,70%	0.9996	22.08.2013	24.09.2013
3	30	-8,62%	0.9997	01.05.2013	30.05.2013
4	26	-6,42%	0.9988	30.09.2013	25.10.2013
5	10	-9,64%	0.9859	06.03.2013	15.03.2013
6	8	7,43%	0.9988	20.03.2013	27.03.2013
Ciprofloxacin					
1	34	64%	0.9998	22.08.2013	24.09.2013
2	31	54%	0.9972	30.01.2013	01.03.2013
3	30	68%	0.9938	01.05.2013	30.05.2013
4	29	67%	0.9979	23.07.2013	20.08.2013
5	26	65%	0.9972	30.09.2013	25.10.2013
6	21	61%	0.9984	08.01.2013	28.01.2013
7	12	62%	0.9958	20.06.2013	01.07.2013
8	8	55%	0.9896	20.03.2013	27.03.2013
Levofloxacin					
1	34	56%	0.9993	22.08.2013	24.09.2013
2	31	47%	0.9956	30.01.2013	01.03.2013
3	30	63%	0.9965	01.05.2013	30.05.2013
4	29	53%	0.9966	23.07.2013	20.08.2013
5	26	56%	0.9991	30.09.2013	25.10.2013
6	21	56%	0.9976	08.01.2013	28.01.2013
7	16	64%	0.9964	03.03.2013	18.03.2013
8	12	56%	0.9964	20.06.2013	01.07.2013
9	8	57%	0.9924	20.03.2013	27.03.2013
10	8	46%	0.9913	03.07.2013	10.07.2013
Piperacillin					
1	42	28%	0.9985	17.01.2013	27.02.2013
2	34	37%	0.9939	22.08.2013	24.09.2013
3	30	54%	0.9903	01.05.2013	30.05.2013
4	29	57%	0.9980	23.07.2013	20.08.2013
5	26	39%	0.9656	30.09.2013	25.10.2013
6	16	32%	0.9784	03.03.2013	18.03.2013
7	8	48%	0.9626	08.01.2013	15.01.2013

Roxithromycin					
1	37	-6%	0.9970	16.01.2013	21.02.2013
2	34	-10%	0.9989	22.08.2013	24.09.2013
3	29	4%	0.9988	23.07.2013	20.08.2013
4	26	6%	0.9977	30.09.2013	25.10.2013
5	13	15%	0.9924	18.05.2013	30.05.2013
6	12	-3%	0.9984	01.05.2013	12.05.2013
Sulfamethoxazole					
1	34	70%	0.9978	22.08.2013	24.09.2013
2	30	52%	0.9945	17.01.2013	15.02.2013
3	29	58%	0.9966	23.07.2013	20.08.2013
4	26	65%	0.9989	30.09.2013	25.10.2013
5	16	60%	0.9933	03.03.2013	18.03.2013
6	16	78%	0.9822	01.05.2013	16.05.2013
7	13	73%	0.9923	17.02.2013	01.03.2013
8	8	59%	0.9969	08.01.2013	15.01.2013
9	8	55%	0.9862	20.06.2013	27.06.2013
10	8	59%	0.9969	08.01.2013	15.01.2013
Trimethoprim					
1	43	-7%	0.9990	18.01.2013	01.03.2013
2	34	-12%	0.9993	22.08.2013	24.09.2013
3	30	-11%	0.9995	01.05.2013	30.05.2013
4	29	-10%	0.9991	23.07.2013	20.08.2013
5	26	-7%	0.9995	30.09.2013	25.10.2013
6	15	-8%	0.9988	04.03.2013	18.03.2013
7	12	1%	0.9964	20.06.2013	01.07.2013
8	8	1%	0.9979	20.03.2013	27.03.2013
9	8	5%	0.9982	03.07.2013	10.07.2013

S4: Calculation of normalized mass flows

The WWTP has the wastewater influent as input stream and the effluent as well as the digested sludge as output streams. The overall removal from the liquid phase will be calculated as the mean of all removal efficiencies ($R_{AB,liquid,i}$) determined according to the procedure presented in paragraph 2.3 in the manuscript. The proportion of antibiotics emitted with the effluent stream ($M_{AB,effluent}$) is subsequently determined according to equation SE1. Within this study the concentration of antibiotics in primary sludge ($c_{AB,PS}$), excess sludge ($c_{AB,ES}$) and digested sludge ($c_{AB,DS}$) were determined as antibiotics mass per dried sludge mass. The removal rate of antibiotics during the anaerobic treatment ($R_{AB,digester}$) was determined according to equation SE2 using the associated daily loads of total suspended solids (TSS) L_{PS}, L_{ES} and L_{DS}. A value for $R_{AB,digester}$ above 0 % indicates a removal during the anaerobic treatment while values below 0 % theoretically account for a production/re-transformation/desorption. The antibiotic load emitted from the WWTP via sludge ($M_{AB,DS}$) was calculated on the basis of the digested sludge (see equation SE3) which was associated with the diurnal antibiotic input load to the WWTP ($L_{AB,input}$). If the cumulative value of $M_{AB,effluent}$ and $M_{AB,DS}$ equals 100 %, the entire influent mass flow is described by these two process streams, i.e. there is neither an overall degradation nor a production of the substance. In case the value falls below or exceeds the value of 100 % the difference indicates the degradation or production of the respective antibiotic, respectively (equation SE4).

$$M_{AB,effluent} = 1 - R_{AB,liquid} = 1 - \frac{\sum_{i=1}^{n_{AB}} R_{AB,liquid,i}}{n_{AB}} \quad [\%] \quad \text{(Equation SE1)}$$

$$R_{AB,digester} = 1 - \frac{C_{AB,DS} \times L_{DS}}{C_{AB,PS} \times L_{PS} + C_{AB,ES} \times L_{ES}} \quad [\%] \quad \text{(Equation SE2)}$$

$$M_{AB,DS} = \frac{C_{AB,DS} \times L_{DS}}{L_{AB,input}} \quad [\%] \quad \text{(Equation SE3)}$$

$$M_{AB,degradation} = -M_{AB,production} = 100\% - M_{AB,effluent} - M_{AB,DS} \quad [\%] \quad \text{(Equation SE4)}$$

The verification of sludge streams and the determination of the SRT was carried out on the basis of an iron balance. The SRT based on the sludge withdrawal showed inconsistencies during the flood event in 2013 (see supplementary material S6). Accordingly, for integrity reasons the SRT based on the iron balance was

used for the identification of relationships between SRT and the calculated removal rates of antibiotics. The chemical phosphorus precipitation using iron was realized with a combination of iron sulphate ($FeSO_4$) and ferrous chloride sulphate ($FeClSO_4$) of which both were considered in the balance approach. N-removal was calculated based on the following equation:

$$N - removal = 1 - \frac{C_{NH4-N,out} + C_{NO2-N,out} + C_{NO3-N,out} + C_{org,out}}{C_{TKN,in}} \quad [\%] \quad \text{(Equation SE5)}$$

with concentrations of ammonia nitrogen ($c_{NH4\text{-}N,out}$), nitrite nitrogen ($c_{NO2\text{-}N,out}$), nitrate nitrogen ($c_{NO3\text{-}N,out}$) and dissolved organic nitrogen compounds ($c_{Norg,out}$) in the effluent as well as the TKN concentration in the influent ($c_{TKN,in}$). The dissolved organic nitrogen was not measured during the monitoring and estimated to 4 mg/L.

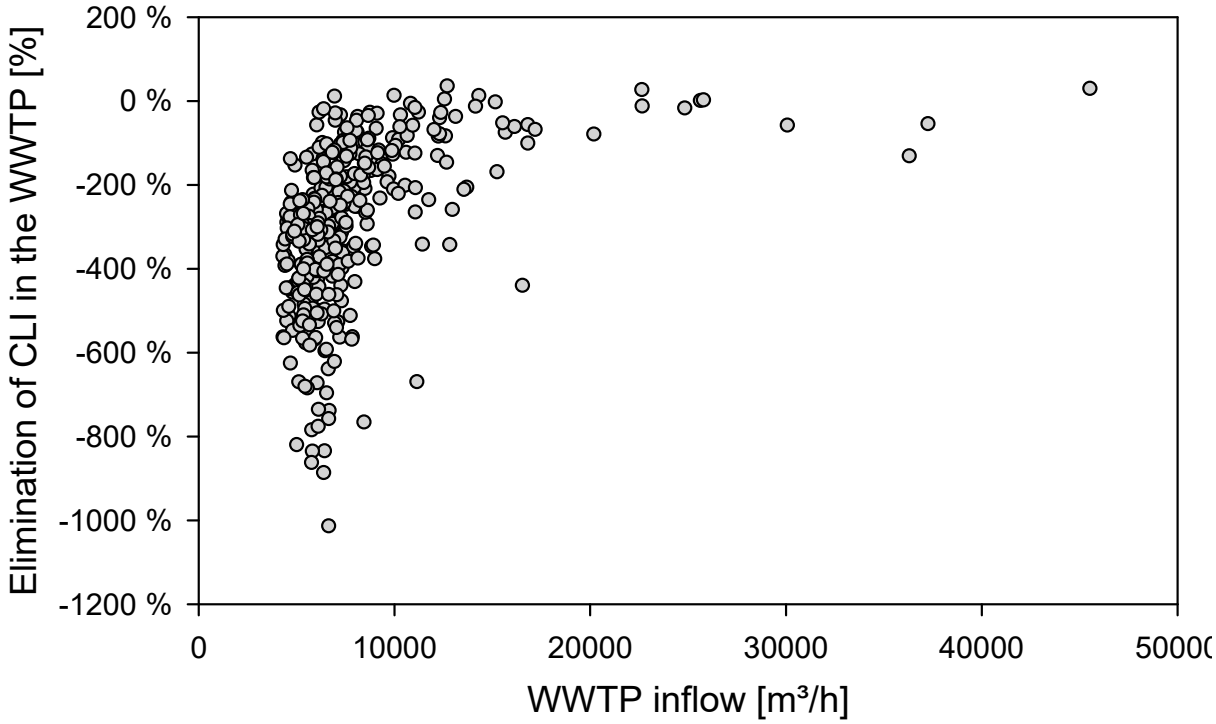

Figure S5: Dependency between measured elimination of CLI and WWTP inflow

Table S6: Kd-values of antibiotics in primary (PS), excess (ES) and digested sludge (DS) in l/kg and the corresponding removal efficiency during the anaerobic treatment (mean ± SD; number of values in brackets, n.d. – not detected)

Antibiotic	Primary sludge	Excess sludge	Digested sludge	$R_{AB,digester}$
	[l/kg]	[l/kg]	[l/kg]	
AMO	n.d.	n.d.	n.d.	n.d.
AZI	109 ± 63 (8)	211 ± 127 (8)	473 ± 287 (10)	30% ± 65% (9)
CEFO	n.d.	n.d.	n.d.	n.d.
CEF	n.d.	n.d.	n.d.	n.d.
CIP	4768 ± 1884 (8)	1414 ± 539 (6)	5044 ± 3246 (10)	62% ± 29% (8)
CLA	54 ± 20 (8)	428 ± 147 (8)	437 ± 133 (3)	81% ± 16% (5)
CLI	294 ± 111 (8)	405 ± 159 (8)	166 ± 35 (10)	22% ± 75% (9)
CLI-S	89 ± 57 (7)	93 ± 31 (7)	183 ± 80 (9)	56% ± 21% (5)
DOX	236 ± 102 (4)	430 ± 285 (7)	n.d.	5% ± 42% (8)
LEV	3089 ± 989 (8)	1600 ± 777 (8)	4074 ± 2025 (10)	-21% ± 98% (9)
PEN	n.d.	n.d.	n.d.	n.d.
PIP	n.d.	n.d.	n.d.	n.d.
ROX	40 ± 20 (8)	289 ± 141 (5)	390 (1)	70% ± 13% (5)
SUL	526 ± 136 (6)	403 ± 173 (6)	1315 ± 1286 (5)	48% ± 11% (4)
TRI	176 ± 44 (8)	346 ± 145 (8)	1063 ± 346 (6)	23% ± 72% (7)

The calculation was carried out according to the following formula:

$$K_d = \frac{c_{AB,solid}}{c_{AB,liquid}} \times 1000 \quad \text{[l/kg]} \quad \text{(Equation SE6)}$$

with the sludge concentration $c_{AB,sludge}$ [ng/g] and the liquid concentration $c_{AB,liquid}$ [ng/l], assuming an equilibrium state.

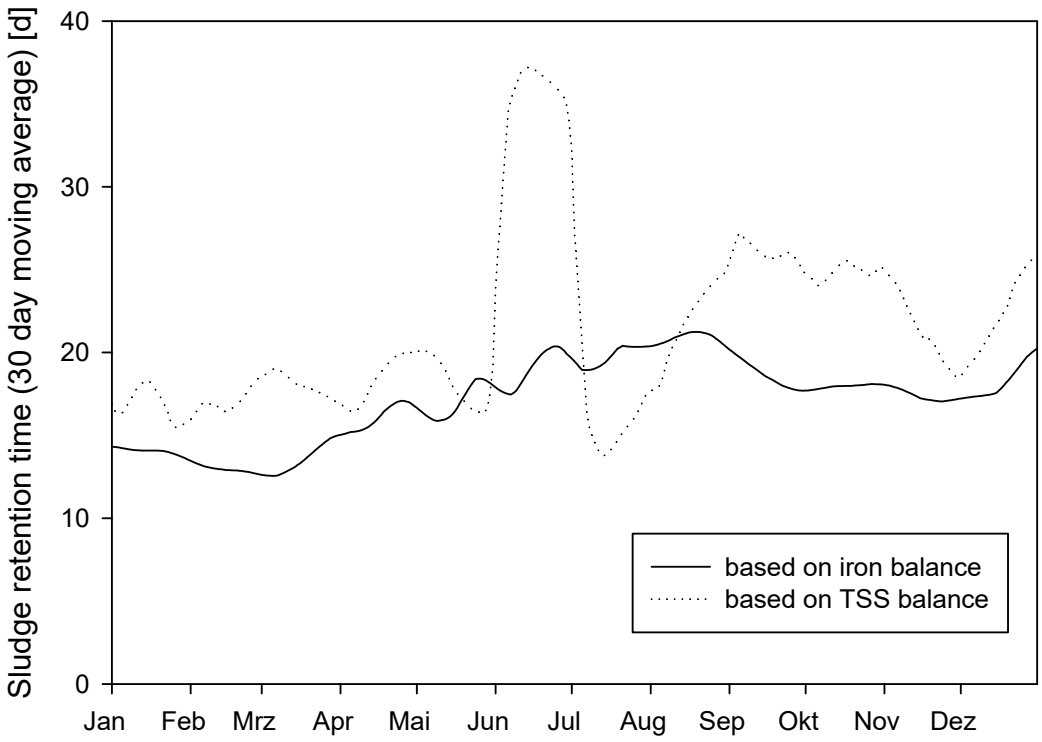

Figure S7: Calculated sludge retention time based on an iron and TSS balance for the year 2013

ANNEX 3

3. COMPARTMENT: ENVIRONMENT

Supplementary material Chapter 4

SPECIES-RELATED RISK ASSESSMENT OF ANTIBIOTICS USING THE PROBABILITY DISTRIBUTION OF LONG-TERM TOXICITY DATA AS WEIGHTING FUNCTION – A CASE STUDY

Table S1: Toxicity values

	Species	Group of species	Number of investiga-tions	Geometric Mean [µg/l]	Standard deviation [µg/l]	Literature
Amoxicillin						
1	Synechococcus leopo-liensis	Algae, Moss, Fungi	2	1.17	0.39	(Andreozzi et al. 2004)
2	Selenastrum capricor-nutum	Algae, Moss, Fungi	1	530000.00		(GlaxoSmithKline 2006)
3	primary rainbow trout hepatocytes (PRTH) cell line	Fish	1	182700.00		(Laville et al. 2004)
4	PLHC-1 cell line	Fish	1	182700.00		(Laville et al. 2004)
5	Oncorhynchus mykiss	Fish	1	1000000.00		(GlaxoSmithKline 2006)
6	leopornis macrochirus	Fish; U.S. Exotic/Nui-sance Species	1	930000.00		(GlaxoSmithKline 2006)
7	Lemna gibba	Flowers, Trees, Shrubs, Ferns	5	1000.00	0.00	(Brain et al. 2004)
8	Daphnia magna	Crustaceans	1	2300000.00		(GlaxoSmithKline 2006)
9	bacterial short-term genotoxicity assay	Bacteria	1	20000.00		(Hartmann et al. 1998)

Azithromycin						
1	salmo trutta	Fish	1	84000.00		(fass.se 2014)
2	Ceriodaphnia dubia	Crustaceans	1	4.40		(fass.se 2014)
Cefuroxime						
1	Selenastrum caprocornutum	Algae, Moss, Fungi	1	76000.00		(fass.se 2014)
2	Oncorhynchus mykiss	Fish	1	100000.00		(fass.se 2014)
3	Daphnia magna	Crustaceans	1	831000.00		(fass.se 2014)
Ciprofloxacin						
1	Selenastrum capricor-nutum	Algae, Moss, Fungi	1	3.00		(Kummerer et al. 2000)
2	Pseudomonas putida	Bacteria	2	4.90	4.38	(Golet et al. 2002, LUA 2002)
3	Lemna gibba	Flowers, Trees, Shrubs, Ferns	15	402.87	326.41	(Brain et al. 2004)
4	Anabaena flos-aquae	Algae, Moss, Fungi	2	3.43	2.23	(Ebert et al. 2011)

Clarithromycin						
1	Pseudomonas putida	Bacteria	1	12692.00		(Alexy 2003)
2	Pseudokirchneriella subcapitata	Algae, Moss, Fungi	5	11.54	16.00	(Harada et al. 2008, Suzuki et al. 2007, Yamashita et al. 2006, Yang et al. 2008)
3	Enterococcus faecalis	Bacteria	1	42.00		(Alexy 2003)
4	Daphnia magna	Crustaceans	2	4.70	2.26	(Yamashita et al. 2006)
5	Danio rerio	Fish	1	1000000		(Isidori et al. 2005)
6	Activated sludge	Bacteria	1	50.00		(Ghosh et al. 2009)
7	bacterial short-term genotoxicity assay	Bacteria	1	5.00		(Hartmann et al. 1998)
Doxycyclin						
1	Lemna gibba	Flowers, Trees, Shrubs, Ferns	13	310.00	321.29	(Brain et al. 2004)

Levofloxacin						
1	Xenopus laevis	Amphibians	2	100000.00	0.00	(Richards and Cole 2006)
2	Vibrio fischeri	Bacteria	3	1.99	1.35	(Backhaus and Grimme 1999, Backhaus et al. 2000)
3	Lemna gibba	Flowers, Trees, Shrubs, Ferns	30	294.30	284.30	(Brain et al. 2004)
Roxithromycin						
1	Pseudokirchneriella subcapitata	Algae, Moss, Fungi	2	25	15.00	(Yang et al. 2008)
2	Lemna gibba	Flowers, Trees, Shrubs, Ferns	5	1000	0.00	(Brain et al. 2004)
Sulfamethoxazole						
1	Xenopus laevis	Amphibians	2	100000.00	0.00	(Richards and Cole 2006)
2	stauropigiana Leopolensis	Algae, Moss, Fungi	1	5.90		(Ferrari et al. 2004)
3	Selenastrum capricornutum	Algae, Moss, Fungi	1	614.00		(Eguchi et al. 2004)
4	Pseudomonas putida	Bacteria	1	17393.00		(Alexy 2003)

5	Pseudokirchneriella subcapitata	Algae, Moss, Fungi	4	501.00	300.61	(Eguchi et al. 2004, Ferrari et al. 2004, Liu et al. 2001, Yang et al. 2008)
6	Lemna gibba	Flowers, Trees, Shrubs, Ferns	19	74.23	68.79	(Brain et al. 2004, (Brain et al. 2008)
7	Hydra attenuata	Invertebrates	5	63000.00	50695.17	(Quinn et al. 2008)
8	Escherichia faecalis	bacteria	1	153592.00		(Alexy 2003)
9	Danio rerio	Fish	30	33848.57	182476.87	(Isidori et al. 2005, Madureira et al. 2011)
10	Cyclotella meneghiniana	Algae, Moss, Fungi	1	1250.00		(Ferrari et al. 2004)
11	Ceriodaphnia dubia	Crustaceans	1	250.00		(Ferrari et al. 2004)
12	Caenorhabditis elegans	Worms	14	1459.31	3703.08	(Yu et al. 2011)
13	Brachionus calyciflorus	Invertebrates	1	25000.00		(Ferrari et al. 2004)
14	Activated sludge	Bacteria	1	50.00		(Gosh et al. 2009)

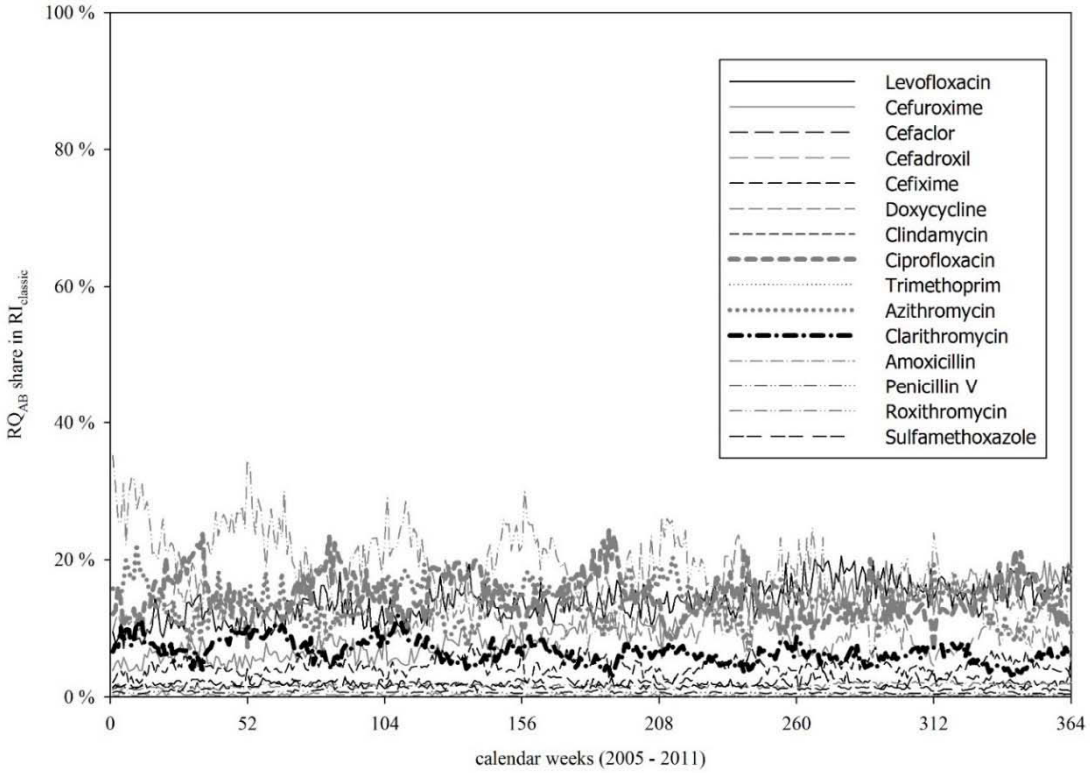

Figure S2: Share of antibiotics in RI using the classical PNEC-approach

S3: Conversion of formulas

Hill equation

$$E = \frac{1}{\left(\frac{Dm}{PNEC}\right)^{m} + 1}$$

$$\left(\frac{Dm}{PNEC}\right)^{m} = \frac{1-E}{E}$$

$$m \log\left(\frac{Dm}{PNEC}\right) = \log\left(\frac{1-E}{E}\right)$$

$$m = \frac{\log\left(\frac{1-E}{E}\right)}{\log\left(\frac{Dm}{PNEC}\right)}$$

equation from [Chèvre et al. 2006]

$$\log HC5 = \log HC50 - \left(\frac{1}{slope}\right) \bullet \log\left(\frac{95}{5}\right)$$

$$\frac{\log HC50 - \log HC5}{\log\left(\frac{95}{5}\right)} = \left(\frac{1}{slope}\right)$$

$$\frac{\log\left(\frac{95}{5}\right)}{\log\left(\frac{HC50}{HC5}\right)} = slope$$

for E = 0.05; Dm = HC50; PNEC = HC5; slope = m

Table S4: PNEC values (applied values are printed in bold-face type)

	Own data (assessment factor in brackets) [µg/l]	Literature values (assessment factor in brackets) [µg/l]	Literature
Amoxicillin			
	0.078 (10)	-	Own data
		0.1 (100)	(Kummerer and Henninger 2003)
		10 (n/a)	(Turkdogan and Yetilmezsoy 2009)
Azithromycin			
	0.088 (50)	-	Own data
	-	0.09 (n/a)	(EcotoxCentre 2013)
	-	0.15 (100)	(Kummerer and Henninger 2003)
Cefaclor			
		0.6 (100)	(Kummerer and Henninger 2003)
Cefadroxil			
		1.1 (100)	(Kummerer and Henninger 2003)
Cefixime			
		0.04 (100)	(Kummerer and Henninger 2003)
Cefuroxime			
		0.15 (100)	(Kummerer and Henninger 2003)
		76 (1000)	(fass.se 2014)
Ciprofloxacin			
	0.12 (10)		Own data
		3 (100)	(Turkdogan and Yetilmezsoy 2009)
		0.02 (100)	(Kummerer and Henninger 2003)

		0.5 (100)	(Halling-Sorensen et al. 2000)
Clarithromycin			
	0.94 (5)	-	Own data
		0.04 (100)	(Kummerer and Henninger 2003)
		0.002 (1000)	(Zhang et al. 2012)
Clindamycin			
		0.5 (100)	(Kummerer and Henninger 2003)
Doxycyclin			
	0.054 (100)		Own data
		0.3 (100)	(Kummerer and Henninger 2003)
Levofloxacin			
	0.1 (100)		Own data
		0.04 (100)	(Kummerer and Henninger 2003)
		4.74 (1000)	(Turkdogan and Yetilmezsoy 2009)
Penicillin V			
		0.1 (100)	(Kummerer and Henninger 2003)
		177 (n/a)	(Jones et al. 2002)
Roxithromycin			
	0.1 (100)	-	Own data
		0.15(100)	(Kummerer and Henninger 2003)
Sulfamethoxazole			
	0.59 (10)	-	Own data
		0.027 (1000)	(Zhang et al. 2012)
		0.6 (n/a)	(EcotoxCentre 2013)

Trimethoprim			
	15.7 (10)		Own data
		16 (1000)	(Turkdogan and Yetilmezsoy 2009)
		60 (n/a)	(EcotoxCentre 2013)

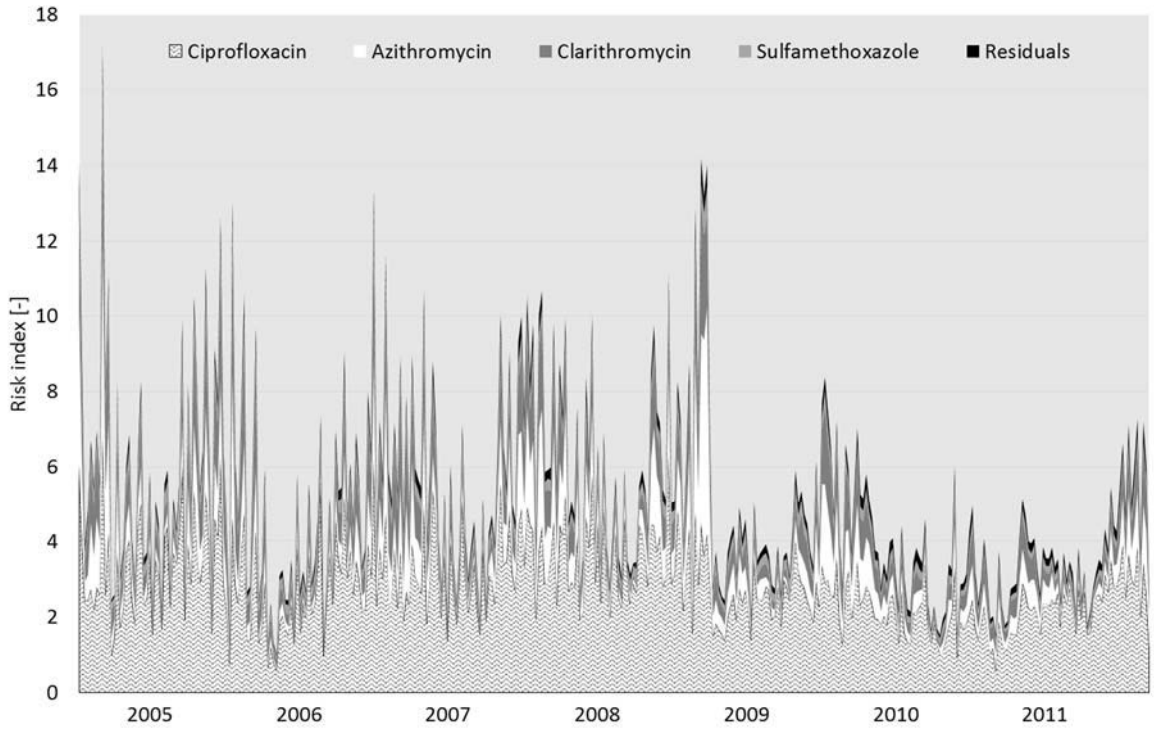

Figure S5: Risk index of the species algae (RI_{algae}) – remaining RQ_{AB} are summed up and displayed as residuals

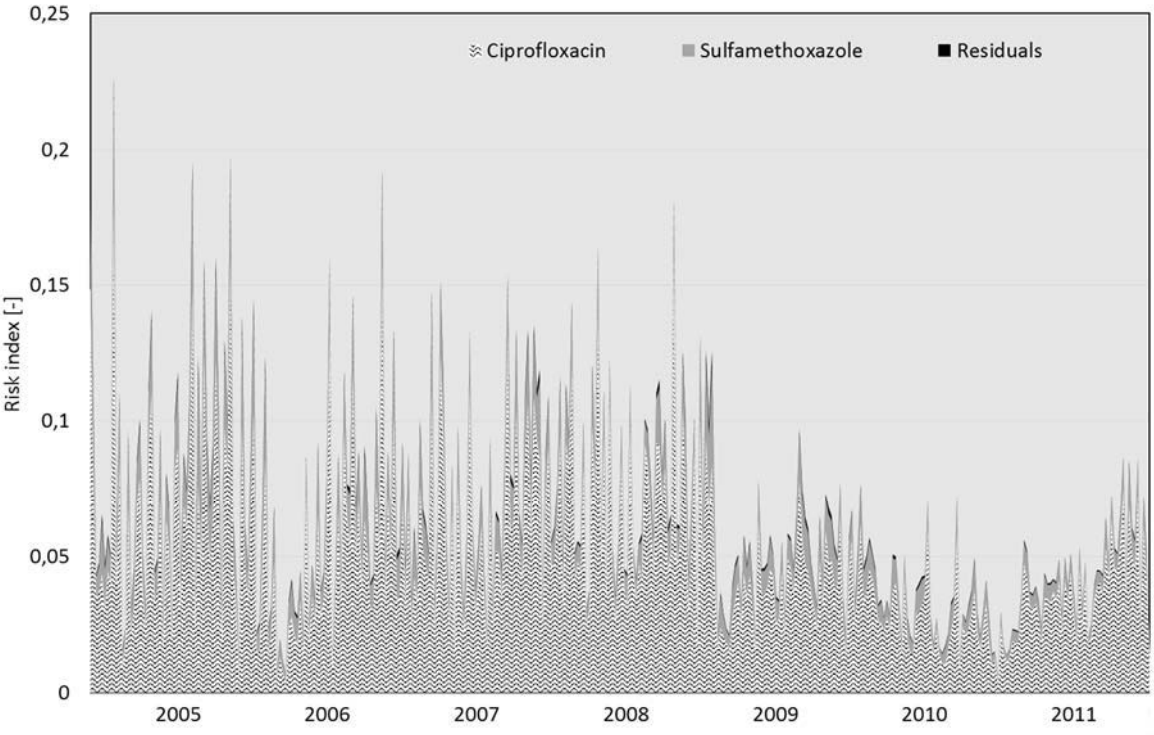

Figure S6: Risk index of the species fish (RI_{fish}) – remaining RQ_{AB} are summed up and displayed as residuals

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ANNEX 4

3. COMPARTMENT: ENVIRONMENT

Supplementary material Chapter 5

ENVIRONMENTAL RISK ASSESSMENT OF ANTIBIOTICS INCLUDING SYNERGISTIC AND ANTAGONISTIC COMBINATION EFFECTS

Table S1: Excretion and elimination rate of antibiotics under investigation

Antibiotic	Excretion rate	Elimination during wastewater treatment
Amoxicillin	60 – 85 % (mean: 72.5 %) (Aktories et al. 2009, Martindale 1993)	60 – 85 % (mean: 72.5 %) (Aktories et al. 2009, Martindale 1993)
Azithromycin	99 %[14] (Watkinson et al. 2009)	99 % (Watkinson et al. 2009)
Cefaclor	67.4 % (Sandoz 2009)	67.4 % (Sandoz 2009)
Cefadroxil	0 % (Goebel et al. 2007)	0 % (Goebel et al. 2007)
Cefixime	53 % (Lode et al. 1979)	53 % (Lode et al. 1979)
Cefuroxime	100 % (Watkinson et al. 2007) (used in study: 99 %)	100 % (Watkinson et al. 2007) (used in study: 99 %)
Ciprofloxacin	88 % (Pfeffer et al. 1977)	88 % (Pfeffer et al. 1977)
Clarithromycin	50 % (assumption, no data available)	50 % (assumption, no data available)
Clindamycin	18 % (Brittain et al. 1985)	18 % (Brittain et al. 1985)
Doxycyclin	50 % (assumption, no data available)	50 % (assumption, no data available)
Levofloxacin	50 % (ODDB 2014)	50 % (ODDB 2014)
Penicillin V	60 % (unpublished data)	60 % (unpublished data)
Roxithromycin	40 % (urine), 15 % (faeces) (sum: 55 %) (Aktories et al. 2009, Vancebryan et al. 1990)	40 % (urine), 15 % (faeces) (sum: 55 %) (Aktories et al. 2009, Vancebryan et al. 1990)
Sulfamethoxazole	66 % (Li &Zhang 2011)	66 % (Li &Zhang 2011)
Trimethoprim	60 % (Hirsch et al. 1999)	60 % (Hirsch et al. 1999)

[14] Using equation 5 with $f_{A,AB}$ = 37 % and $f_{M,AB}$ = 88 %

Table S2: PNEC values (applied values are printed in bold-face type)

	Own data (assessment factor in brackets) [µg/l]	Literature values (assessment factor in brackets) [µg/l]	Literature
Amoxicillin			
	0.078 (10)	-	Own data
		0.1 (100)	(Kummerer and Henninger 2003)
		10 (n/a)	(Turkdogan and Yetilmezsoy 2009)
Azithromycin			
	0.088 (50)	-	Own data
	-	0.09 (n/a)	(EcotoxCentre 2013)
	-	0.15 (100)	(Kummerer and Henninger 2003)
Cefaclor			
		0.6 (100)	(Kummerer and Henninger 2003)
Cefadroxil			
		1.1 (100)	(Kummerer and Henninger 2003)
Cefixime			
		0.04 (100)	(Kummerer and Henninger 2003)
Cefuroxime			
		0.15 (100)	(Kummerer and Henninger 2003)
		76 (1000)	(fass.se 2014)
Ciprofloxacin			
	0.12 (10)		Own data
		3 (100)	(Turkdogan and Yetilmezsoy 2009)
		0.02 (100)	(Kummerer and Henninger 2003)

		0.5 (100)	(Halling-Sorensen et al. 2000)
Clarithromycin			
	0.94 (5)	-	Own data
		0.04 (100)	(Kummerer and Henninger 2003)
		0.002 (1000)	(Zhang et al. 2012)
Clindamycin			
		0.5 (100)	(Kummerer and Henninger 2003)
Doxycyclin			
	0.054 (100)		Own data
		0.3 (100)	(Kummerer and Henninger 2003)
Levofloxacin			
	0.1 (100)		Own data
		0.04 (100)	(Kummerer and Henninger 2003)
		4.74 (1000)	(Turkdogan and Yetilmezsoy 2009)
Penicillin V			
		0.1 (100)	(Kummerer and Henninger 2003)
		177 (n/a)	(Jones et al. 2002)
Roxithromycin			
	0.1 (100)	-	Own data
		0.15(100)	(Kummerer and Henninger 2003)
Sulfamethoxazole			
	0.59 (10)	-	Own data
		0.027 (1000)	(Zhang et al. 2012)
		0.6 (n/a)	(EcotoxCentre 2013)

Trimethoprim			
	15.7 (10)		Own data
		16 (1000)	(Turkdogan and Yetilmezsoy 2009)
		60 (n/a)	(EcotoxCentre 2013)

S3: Synergy parameters

Toxic Units

$$TU = \sum_{AB=1}^{n} \left(\frac{C_{AB,50}}{IC_{50,AB}} \right)$$ (Yang et al., 2008)

With $IC_{x,A}$ and $IC_{x,B}$ being the concentration of subtances A and B to produce 50 % effect and $C_{A,x}$ and $C_{B,x}$ in combination also causing 50 % effect

Combination index

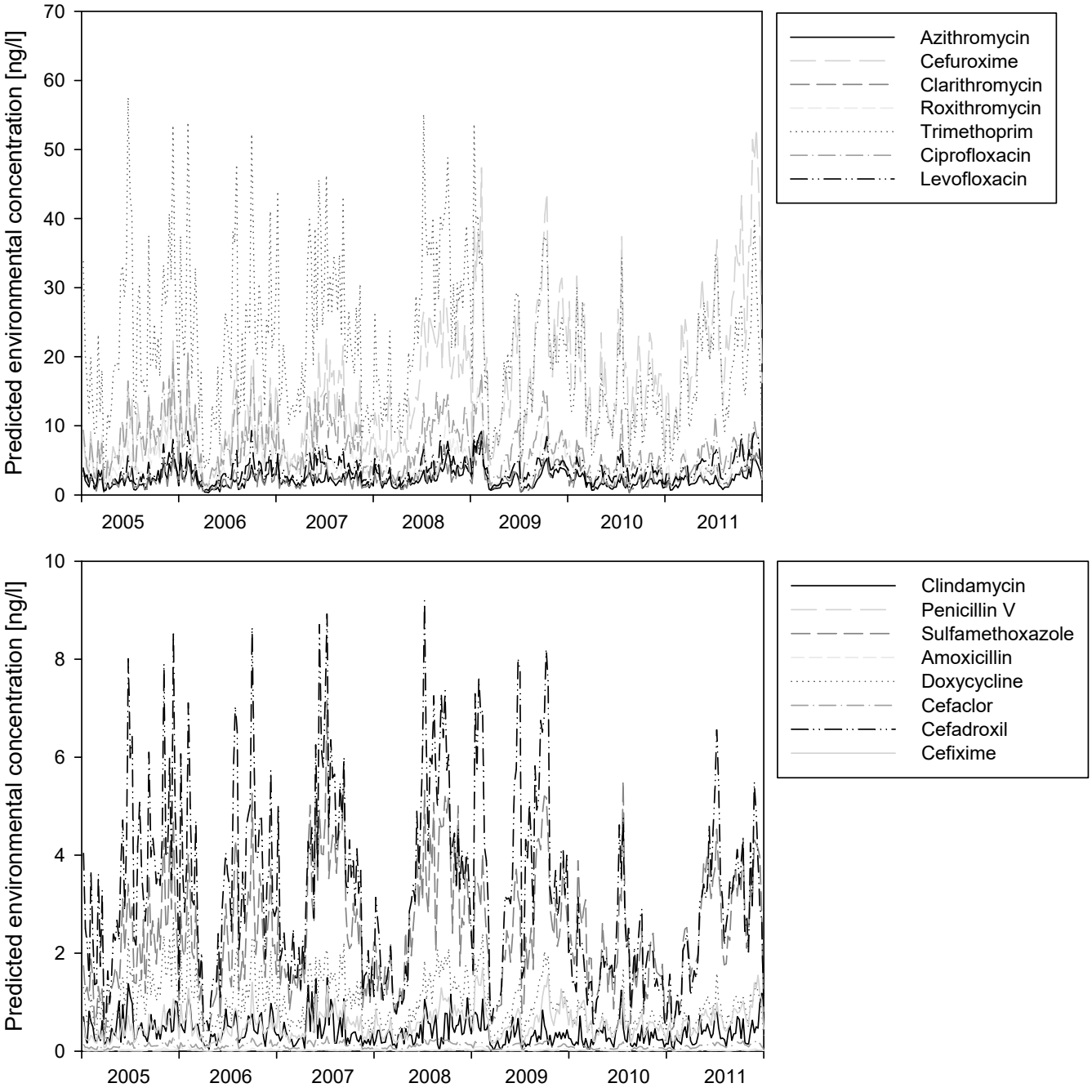
$$CI = \sum_{AB=1}^{n} \left(\frac{C_{AB,x}}{IC_{x,AB}} \right)$$ (Zhao et al., 2010)

With $IC_{x,A}$ and $IC_{x,B}$ being the concentration of subtances A and B to produce a given effect x (e.g. IC_{50}) and $C_{A,x}$ and $C_{B,x}$ in combination causing the same effect x

Fractional inhibitory concentration

$$FIC = \sum_{AB=1}^{n} \left(\frac{MIC_{combinatio\ n,AB}}{MIC_{alone,AB}} \right)$$ (Hallander et al., 1982)

With $MIC_{combination,AB}$ being the lowest concentration of antibiotic combination permitting no visible growth and $MIC_{alone,AB}$ being the lowest concentration of the single antibiotic permitting no visible growth



Figures S4: Predicted environmental concentration of antibiotics under investigation

Table S5: Binary interaction studies on antibiotics

Substance A	Class A	Substance B	Class B	organism	Parame-ter	Synergism/ Antagonism	Concentra-tion range A [mg/l]	Concentra-tion range B [mg/l]	Literature
Trime-thoprim	DI	Sulfameth-oxazole	SU	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	176.79 - 17679.2	(Zou et al., 2013)
Trime-thoprim	DI	Sulfadox-ine	SU	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	273.67 - 27367.2	(Zou et al., 2013)
Trime-thoprim	DI	Sulfachlo-ro-pyridazine	SU	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	134.01 - 13400.8	(Zou et al., 2013)
Trime-thoprim	DI	Tetracyclin	TC	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	15.34 - 1383.2	(Zou et al., 2013)
Trime-thoprim	DI	Oxytetra-cycline	TC	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	16.58 - 1657.6	(Zou et al., 2013)
Trime-thoprim	DI	Doxycy-clin	TC	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	16.41 - 1640.8	(Zou et al., 2013)
Trime-thoprim	DI	Chlortetra-cyclin	TC	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	17.08 - 1708	(Zou et al., 2013)
Trime-thoprim	DI	Tetracyclin	TC	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	15.34 - 1534.4	(Zou et al., 2013)
Trime-thoprim	DI	Oxytetra-cycline	TC	*Vibrio Fisheri*	TU	antagonistic	75.6 - 7560	16.58 - 1657.6	(Zou et al., 2013)
Trime-thoprim	DI	Chloram-phenicol	Feni-cole	*Vibrio Fisheri*	TU	none	75.6 - 7560	22.96 - 2296	(Zou et al., 2013)

Trime-thoprim	DI	Chloram-phenicol	Feni-cole	*Vibrio Fisheri*	TU	none	75.6 - 7560	22.96 - 2296	(Zou et al., 2013)
Trime-thoprim	DI	Doxycy-clin	TC	*Vibrio Fisheri*	TU	none	75.6 - 7560	22.01 - 2200.8	(Zou et al., 2013)
Trime-thoprim	DI	Chlortetra-cyclin	TC	*Vibrio Fisheri*	TU	none	75.6 - 7560	17.08 - 1708	(Zou et al., 2013)
Trime-thoprim	DI	Sulfameth-oxazole	SU	*Vibrio Fisheri*	TU	synergistic	75.6 - 7560	176.79 - 17679.2	(Zou et al., 2013)
Trime-thoprim	DI	Sulfadox-ine	SU	*Vibrio Fisheri*	TU	synergistic	75.6 - 7560	273.67 - 27367.2	(Zou et al., 2013)
Trime-thoprim	DI	Sulfachlo-ro-pyridazine	SU	*Vibrio Fisheri*	TU	synergistic	75.6 - 7560	134.01 - 13400.8	(Zou et al., 2013)
Amoxicil-lin	BL	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	5.9 - 13.9	4.2 - 13.6	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Levofloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	5.4 - 10.0	5.4 - 10.0	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Erythro-mycin	MA	*Anabaena sp. CPB4337*	CI	antagonistic	4.2 - 13.6	4.2 - 13.6	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	0.8 - 1.8	0.8 - 1.8	(Gonzalez-Pleiter et al., 2013)

Norfloxacin	QU	Tetracyclin	TC	*Pseudokirchneriella subcapitata*	CI	antagonistic	1.4 - 2.4	1.4 - 2.4	(Gonzalez-Pleiter et al., 2013)
Norfloxacin	QU	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	antagonistic	1.5 - 2.3	1.5 - 2.3	(Gonzalez-Pleiter et al., 2013)
Erythromycin	MA	Levofloxacin	QU	*Pseudokirchneriella subcapitata*	CI	antagonistic	1.0 - 1.6	1.0 - 1.6	(Gonzalez-Pleiter et al., 2013)
Erythromycin	MA	Levofloxacin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	0.57 - 1.17	0.57 - 1.17	(Gonzalez-Pleiter et al., 2013)
Erythromycin	MA	Norfloxacin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	0.46 - 1.46	0.46 - 1.46	(Gonzalez-Pleiter et al., 2013)
Amoxicillin	BL	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	1.2 - 2.4	1.2 - 2.4	(Gonzalez-Pleiter et al., 2013)
Levofloxacin	QU	Norfloxacin	QU	*Pseudokirchneriella subcapitata*	CI	synergistic	0.7 - 1.5	0.7 - 1.5	(Gonzalez-Pleiter et al., 2013)
Levofloxacin	QU	Tetracyclin	TC	*Pseudokirchneriella subcapitata*	CI	synergistic	0.24 - 0.44	0.24 - 0.44	(Gonzalez-Pleiter et al., 2013)
Levofloxacin	QU	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	0.25 - 0.49	0.25 - 0.49	(Gonzalez-Pleiter et al., 2013)

Erythro-mycin	MA	Norfloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	synergistic	2.4 - 3.6	2.4 - 3.6	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	0.0022 - 0.007	0.0022 - 0.007	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	0.17 - 0.39	0.17 - 0.39	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	11.4 - 18.6	11.4 - 18.6	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Levofloxa-cin	QU	*Anabaena sp. CPB4337*	CI	none	9.3 - 13.5	9.3 - 13.5	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Erythro-mycin	MA	*Anabaena sp. CPB4337*	CI	antagonistic	10.7 - 21.7	10.7 - 21.7	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	none	1.5 - 2.5	1.5 - 2.5	(Gonzalez-Pleiter et al., 2013)
Norfloxa-cin	QU	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	none	2.3 - 3.1	2.3 - 3.1	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Levofloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	1 - 1.6	1 - 1.6	(Gonzalez-Pleiter et al., 2013)

Erythro-mycin	MA	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	1.1 - 2.3	1.1 - 2.3	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	2.4 - 3.4	2.4 - 3.4	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	0.39 - 0.87	0.39 - 0.87	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	0.54 - 0.8	0.54 - 0.8	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	none	29.8 - 36.4	29.8 - 36.4	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Levofloxa-cin	QU	*Anabaena sp. CPB4337*	CI	synergistic	20.9 - 23.9	20.9 - 23.9	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Erythro-mycin	MA	*Anabaena sp. CPB4337*	CI	none	27.5 - 44.5	27.5 - 44.5	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	synergistic	3.9 - 4.7	3.9 - 4.7	(Gonzalez-Pleiter et al., 2013)
Norfloxa-cin	QU	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	none	4.6 - 5.6	4.6 - 5.6	(Gonzalez-Pleiter et al., 2013)

Erythro-mycin	MA	Levofloxa-cin	QU	*Anabaena sp. CPB4337*	CI	none	2.3 - 2.7	2.3 - 2.7	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Norfloxa-cin	QU	*Anabaena sp. CPB4337*	CI	antagonistic	3.8 - 5.2	3.8 - 5.2	(Gonzalez-Pleiter et al., 2013)
Amoxicil-lin	BL	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	6 - 7	6 - 7	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	1.3 - 1.9	1.3 - 1.9	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Tetracyclin	TC	*Anabaena sp. CPB4337*	CI	synergistic	2.7 - 3.3	2.7 - 3.3	(Gonzalez-Pleiter et al., 2013)
Norfloxa-cin	QU	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	3.2 - 3.8	3.2 - 3.8	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Levofloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	antagonistic	22.7 - 23.3	22.7 - 23.3	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Norfloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	synergistic	2.2 - 3.6	2.2 - 3.6	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	0.75 - 1.03	0.75 - 1.03	(Gonzalez-Pleiter et al., 2013)

Erythro-mycin	MA	Norfloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	synergistic	5.1- 6.5	5.1- 6.5	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	0.014 - 0.028	0.014 - 0.028	(Gonzalez-Pleiter et al., 2013)
Norfloxa-cin	QU	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	8.3 - 10.1	8.3 - 10.1	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Levofloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	synergistic	2.4	2.4	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Norfloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	synergistic	13.7 - 16.3	13.7 - 16.3	(Gonzalez-Pleiter et al., 2013)
Levofloxa-cin	QU	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	4.1 - 5.1	4.1 - 5.1	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Norfloxa-cin	QU	*Pseudokirchneri-ella subcapitata*	CI	synergistic	16.7 - 19.7	16.7 - 19.7	(Gonzalez-Pleiter et al., 2013)
Erythro-mycin	MA	Tetracyclin	TC	*Pseudokirchneri-ella subcapitata*	CI	synergistic	0.23 - 0.31	0.23 - 0.31	(Gonzalez-Pleiter et al., 2013)
Sulfadi-methoxine	SU	Py-rimetham-ine	xx	*Selenastrum capricornutum*	compari-son EC50	none	2.17 - 2.57	2.17 - 2.57	(Eguchi et al., 2004)

Sulfameth-oxazole	SU	Trime-thoprim	DI	*Selenastrum capricornutum*	compari-son EC50	synergistic	0.239 - 0.309	0.239 - 0.309	(Eguchi et al., 2004)
Sulfadia-zine	SU	Trime-thoprim	DI	*Selenastrum capricornutum*	compari-son EC50	synergistic	0.419 - 0.517	0.419 - 0.517	(Eguchi et al., 2004)
mixture	SU	mixture	SU	*Daphnia Magna*	compari-son EC50	none	0.28 - 657.2	0.28 - 657.2	(De Liguoro et al., 2009)
sulfadia-zine	SU	Trime-thoprim	DI	*Daphnia Magna*	compari-son EC50	none	17.6 - 515.9	10.7 - 345.9	(De Liguoro et al., 2009)
sulfaguani-dine	SU	Trime-thoprim	DI	*Daphnia Magna*	compari-son EC50	none	0.28 - 8.97	10.7 - 345.9	(De Liguoro et al., 2009)
sulfamera-zine	SU	Trime-thoprim	DI	*Daphnia Magna*	compari-son EC50	none	23.1 - 675.8	10.7 - 345.9	(De Liguoro et al., 2009)
sulfadi-methoxine	SU	Trime-thoprim	DI	*Daphnia Magna*	compari-son EC50	none	22.4 - 657.2	10.7 - 345.9	(De Liguoro et al., 2009)
sulfame-thazine	SU	Trime-thoprim	DI	*Daphnia Magna*	compari-son EC50	none	16.2 - 486	10.7 - 345.9	(De Liguoro et al., 2009)
sulfa-quinoxa-line	SU	Trime-thoprim	DI	*Daphnia Magna*	compari-son EC50	none	9.4 - 303.4	10.7 - 345.9	(De Liguoro et al., 2009)
Erythro-mycin	MA	Oxolinic acid	QU	*Pseudokirchneri-ella subcapitata*	λ	none	0.15	0.7 - 1.1	(Munch Christensen et al., 2006)
Oxytetra-cyclin	TC	Flumequine	QU	*Pseudokirchneri-ella subcapitata*	λ	antagonistic	0.46 - 0.48	7.2 - 11	(Munch Christensen et al., 2006)

Erythro-mycin	MA	Flumequine	QU	*Pseudokirchneri-ella subcapitata*	λ	antagonistic	0.23 - 0.24	8.0 - 8.1	(Munch Christensen et al., 2006)
Oxytetra-cyclin	TC	Oxolinic acid	QU	*Pseudokirchneri-ella subcapitata*	λ	none	1.8 - 2.2	9.9 - 31	(Munch Christensen et al., 2006)
Erythro-mycin	MA	Oxolinic acid	QU	*Belebtschlamm*	λ	synergistic	33 - 44	1.1 - 2.3	(Munch Christensen et al., 2006)
Flumequine	QU	Oxolinic acid	QU	*Belebtschlamm*	λ	none	0.60 - 0.67	0.73 - 0.91	(Munch Christensen et al., 2006)
Oxytetra-cyclin	TC	Flumequine	QU	*Belebtschlamm*	λ	synergistic	2.4 - 3.6	1.1 - 1.3	(Munch Christensen et al., 2006)
Erythro-mycin	MA	Flumequine	QU	*Belebtschlamm*	λ	synergistic	30 - 51	0.65 - 1.80	(Munch Christensen et al., 2006)
Erythro-mycin	MA	Oxytetra-cyclin	TC	*Pseudokirchneri-ella subcapitata*	λ	synergistic	0.13	1.4	(Munch Christensen et al., 2006)
Erythro-mycin	MA	Oxytetra-cyclin	TC	*Belebtschlamm*	λ	synergistic	33 - 49	3.1 - 4.2	(Munch Christensen et al., 2006)
Oxytetra-cyclin	TC	Oxolinic acid	QU	*Belebtschlamm*	λ	synergistic	2.7 - 4.9	0.75 - 0.86	(Munch Christensen et al., 2006)

Ceftazidim	CE	Clarithro-mycin	MA	*Pseudomonas aeruginosa*	Survival rate	none	0.5 - 32	16 - 1024	(Bui et al., 2000)
Ceftazidim	CE	Clarithro-mycin	MA	*Pseudomonas aeruginosa*	Survival rate	synergistic	1	16 - 128	(Bui et al., 2000)
Levofloxa-cin	QU	Clarithro-mycin	MA	*Pseudomonas aeruginosa*	No. Of vi-able bacte-ria	synergistic	-	-	(Yanagihara et al., 2000)
Ciproflox-acin	QU	Mero-penem	BL	*Staphylococcus aureus*	FIC	none	-	-	(Sueke et al., 2010)
Carbenicil-lin	BL	cepha-lothin	CE	*Escherichia coli*	MIC	synergistic	-	-	(Klasters.J et al., 1972)
Carbenicil-lin	BL	cepha-lothin	CE	*Klebsiella-En-terobacter*	MIC	synergistic	-	-	(Klasters.J et al., 1972)
Ciproflox-acin	QU	Ampicillin	BL	*Staphylococcus aureus*	FIC	none	2	0.5	(Gradelski et al., 2001)
Ciproflox-acin	QU	Ceftriax-one	BL	*Staphylococcus aureus*	FIC	none	2	2	(Gradelski et al., 2001)
Ciproflox-acin	QU	Cefepim	BL	*Staphylococcus aureus*	FIC	none	2	2	(Gradelski et al., 2001)
Ciproflox-acin	QU	Ceftriax-one	BL	*Escherichia coli*	FIC	none	0.032	0.015	(Gradelski et al., 2001)
Ciproflox-acin	QU	Ampicillin	BL	*Escherichia coli*	FIC	none	0.032	2	(Gradelski et al., 2001)
Ciproflox-acin	QU	Cefepim	BL	*Escherichia coli*	FIC	none	0.032	0.015	(Gradelski et al., 2001)
Ciproflox-acin	QU	Cefepim	BL	*Pseudomonas aeruginosa*	FIC	none	2	2	(Gradelski et al., 2001)

Ciproflox-acin	QU	Ampicillin	BL	*Enterococcus faecalis*	FIC	none	8	0.25	(Gradelski et al., 2001)
Ciproflox-acin	QU	Clarithro-mycin	MA	*Staphylococcus aureus*	FIC	none	2	0.125	(Gradelski et al., 2001)
Ciproflox-acin	QU	Clarithro-mycin	MA	*Enterococcus faecalis*	FIC	none	8	0.5	(Gradelski et al., 2001)
Penicillin	BL	Chlortetra-cyclin	TC	*Klebsiella pneu-moniae*	No. Of vi-able bacte-ria	antagonistic	12	1	(Jawetz et al., 1954)
Aureomy-cin	TC	Penicillin	BL	*Streptococcus Faecalis*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)
Oxytetra-cyclin	TC	Penicillin	BL	*Streptococcus Faecalis*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)
Aureomy-cin	TC	Penicillin	BL	*Streptococcus Pyogenes*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)
Oxytetra-cyclin	TC	Penicillin	BL	*Streptococcus Pyogenes*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)
Oxytetra-cyclin	TC	Penicillin	BL	*Micrococcus Py-ogenes var. au-reus*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)
Aureomy-cin	TC	Penicillin	BL	*Klebsiella pneu-moniae*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)

Oxytetra-cyclin	TC	Penicillin	BL	*Klebsiella pneu-moniae*	No. Of vi-able bacte-ria	antagonistic	-	-	(Jewetz et al., 1952)
Erythro-mycin	MA	Amoxicil-lin	BL	*Escherichia coli*	FIC	none	3.125	7.813	(Olajuyigbe and Ani-mashaun, 2012)
Erythro-mycin	MA	Amoxicil-lin	BL	*Acinetobacter calcoaceticus*	FIC	none	0.195	250	(Olajuyigbe and Ani-mashaun, 2012)
Erythro-mycin	MA	Amoxicil-lin	BL	*shigella flexneri*	FIC	none	0.195	31.25	(Olajuyigbe and Ani-mashaun, 2012)
Erythro-mycin	MA	Amoxicil-lin	BL	*Staphylococcus aureus*	FIC	none	0.195	125	(Olajuyigbe and Ani-mashaun, 2012)
Erythro-mycin	MA	Amoxicil-lin	BL	*Proteus vulgaris ATCC 6830*	FIC	none	3.125	62.5	(Olajuyigbe and Ani-mashaun, 2012)
Erythro-mycin	MA	Amoxicil-lin	BL	*Proteus vulgaris CSIR 0030*	FIC	synergistic	6.25	0.12	(Olajuyigbe and Ani-mashaun, 2012)

Erythro-mycin	MA	Amoxicil-lin	BL	*Salmonella Typhi*	FIC	synergistic	3.906	7.813	(Olajuyigbe and Ani-mashaun, 2012)
Erythro-mycin	MA	Amoxicil-lin	BL	*Streptococcus Pyogenes*	FIC	synergistic	0.781	1.953	(Olajuyigbe and Ani-mashaun, 2012)
Ampicillin	BL	Ciproflox-acin	QU	*Staphylococcus aureus*	FIC	antagonistic	10.4	26.1	(Singh and Mishra, 2012)
Penicillin	BL	Ciproflox-acin	QU	*Staphylococcus aureus*	FIC	antagonistic	26.8	26.1	(Singh and Mishra, 2012)
Azithro-mycin	MA	Ofloxacin	QU	*Staphylococcus aureus*	FIC	antagonistic	27.4	20.7	(Singh and Mishra, 2012)
Ampicillin	BL	Ofloxacin	QU	*Staphylococcus aureus*	FIC	none	10.4	20.7	(Singh and Mishra, 2012)
Ampicillin	BL	Cloaxillin	BL	*Staphylococcus aureus*	FIC	synergistic	10.4	-	(Singh and Mishra, 2012)
Ampicillin	BL	Penicillin	BL	*Staphylococcus aureus*	FIC	synergistic	10.4	26.8	(Singh and Mishra, 2012)

Ampicillin	BL	Azithro-mycin	MA	*Staphylococcus aureus*	FIC	synergistic	10.4	27.4	(Singh and Mishra, 2012)
Penicillin	BL	Azithro-mycin	MA	*Staphylococcus aureus*	FIC	synergistic	26.8	27.4	(Singh and Mishra, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Proteus vulgaris*	FIC	antagonistic	1.953	1.953	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Bacillus cereus*	FIC	synergistic	0.007625	0.007625	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Staphylococcus aureus*	FIC	synergistic	1.953	1.953	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Salmonella Typhi*	FIC	synergistic	0.0038	0.0038	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Acinetobacter calcoaceticus*	FIC	synergistic	62.5	62.5	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Klebsiella pneu-moniae*	FIC	synergistic	3.9063	3.9063	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Enterococcus faecalis*	FIC	synergistic	0.488	0.488	(Olajuyigbe, 2012)
Amoxicil-lin	BL	Tetracy-cline	TC	*Staphylococcus aureus*	FIC	synergistic	0.488 - 1.953	0.488 - 1.953	(Olajuyigbe, 2012)
Azlocillin	BL	Ciproflox-acin	QU	*Klebsiella pneu-moniae*	FIC	none	-	-	(Chin et al., 1986)
Azlocillin	BL	Ciproflox-acin	QU	*Enterobacter spp*	FIC	none	-	-	(Chin et al., 1986)

Azlocillin	BL	Ciproflox-acin	QU	*Escherichia coli*	FIC	none	-	-	(Chin et al., 1986)
Azlocillin	BL	Ciproflox-acin	QU	*Branhamella catarrhalis*	FIC	none	-	-	(Chin et al., 1986)
Azlocillin	BL	Ciproflox-acin	QU	*Pseudomonas aeruginosa*	FIC	synergistic	-	-	(Chin et al., 1986)
Azlocillin	BL	Ciproflox-acin	QU	*Pseudomonas aeruginosa*	FIC	none	-	-	(Bamberger et al., 1986)
Ciproflox-acin	QU	Ceftizox-ime	CE	*Serratia marcescens*	FIC	none	-	-	(Bamberger et al., 1986)
Ciproflox-acin	QU	Ceftizox-ime	CE	*Pseudomonas aeruginosa*	FIC	none	-	-	(Bamberger et al., 1986)
Azlocillin	BL	Ciproflox-acin	QU	*Serratia marcescens*	FIC	synergistic	-	-	(Bamberger et al., 1986)
Ciproflox-acin	QU	Azlocillin	BL	*Pseudomonas aeruginosa*	No. Of viable bacteria	synergistic	0.000025	0.0025	(Chalkley and Koornhof, 1985)
Ciproflox-acin	QU	cefotaxime	CE	*Escherichia coli*	No. Of viable bacteria	synergistic	0.00001	0.00005	(Chalkley and Koornhof, 1985)
Ciproflox-acin	QU	Azlocillin	BL	*Pseudomonas aeruginosa*	No. Of viable bacteria	synergistic	0.00001	0.01	(Chalkley and Koornhof, 1985)
Clindamycin	MA	Tetracyclin	TC	*Staphylococcus epidermidis*	No. Of viable bacteria	synergistic	0.25 - 2	1 - 8	(Monzon et al., 2001)

Penicillin	BL	Oxytetra-cyclin	TC	*Streptococcus faecalis*	No. of viable bacteria	none	-	-	(Gunnison et al., 1955)
Penicillin	BL	Oxytetra-cyclin	TC	*Staphylococcus aureus*	No. of viable bacteria	none	-	-	(Gunnison et al., 1955)
Tylosin	MA	Roxithro-mycin	MA	*Pseudokirchneriella subcapitata*	TU	synergistic	0.105	0.0235	(Yang et al., 2008)
Clarithro-mycin	MA	Roxithro-mycin	MA	*Pseudokirchneriella subcapitata*	TU	synergistic	0.023	0.0235	(Yang et al., 2008)
Sulfame-thazine	SU	Sulfameth-oxazolee	SU	*Pseudokirchneriella subcapitata*	TU	none	4.35	0.95	(Yang et al., 2008)
Chlortetra-cycline	TC	Tetracy-cline	TC	*Pseudokirchneriella subcapitata*	TU	synergistic	0.9	0.5	(Yang et al., 2008)
Ciproflox-acin	QU	Norfloxa-cin	QU	*Pseudokirchneriella subcapitata*	TU	synergistic	3.35	90	(Yang et al., 2008)
Tylosin	MA	Chlortetra-cycline	TC	*Pseudokirchneriella subcapitata*	TU	synergistic	0.105	0.9	(Yang et al., 2008)
Tylosin	MA	Tetracy-cline	TC	*Pseudokirchneriella subcapitata*	TU	synergistic	0.105	0.5	(Yang et al., 2008)
Trime-thoprim	DI	Sulfame-thazine	SU	*Pseudokirchneriella subcapitata*	TU	synergistic	20	4.35	(Yang et al., 2008)
Trime-thoprim	DI	Sulfameth-oxazolee	SU	*Pseudokirchneriella subcapitata*	TU	synergistic	20	0.95	(Yang et al., 2008)
Tylosin	MA	Sulfame-thazine	SU	*Pseudokirchneriella subcapitata*	TU	antagonistic	0.105	0.95	(Yang et al., 2008)

Tylosin	MA	Norfloxacin	QU	*Pseudokirchneriella subcapitata*	TU	none	0.105	90	(Yang et al., 2008)
Sulfamethazine	SU	Chlortetracycline	TC	*Pseudokirchneriella subcapitata*	TU	none	4.35	0.9	(Yang et al., 2008)
Sulfamethazine	SU	Norfloxacin	QU	*Pseudokirchneriella subcapitata*	TU	synergistic	4.35	90	(Yang et al., 2008)
Chlortetracycline	TC	Norfloxacin	QU	*Pseudokirchneriella subcapitata*	TU	synergistic	0.9	90	(Yang et al., 2008)

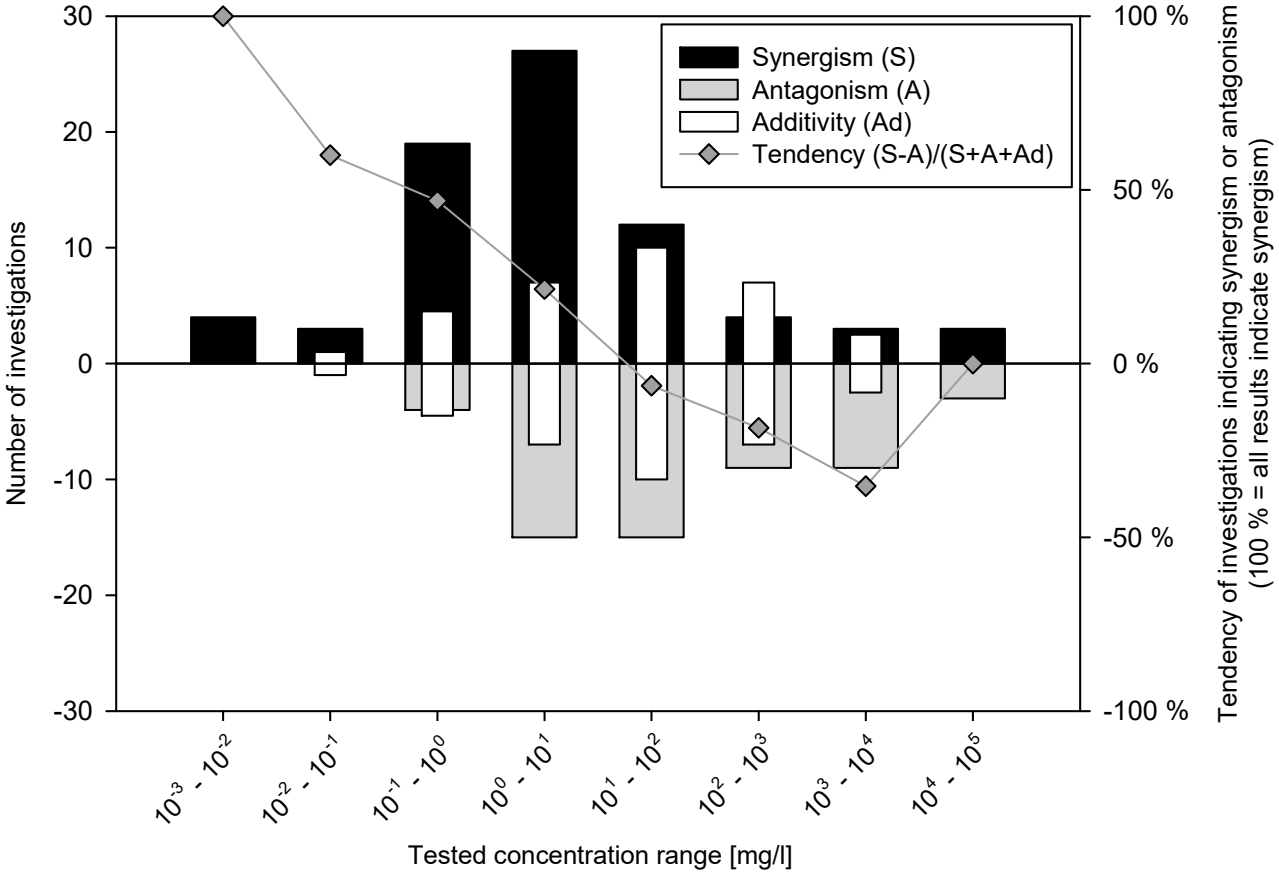


Figure S6: Tendency of synergism according to the tested concentration range of investigations

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