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Development and dissemination of antibiotic resistance in the environment under environmentally relevant concentrations of antibiotics and its risk assessment

Literature Study

For our Environment

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Development and dissemination of antibiotic resistance in the environment under environmentally relevant concentrations of antibiotics and its risk assessment Literature Study

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Abstract

This report addresses the role of antibiotic residues for the development or dissemination of antibiotic resistance in environmental compartments. A literature study summarizes data on fate and occurrence of antibiotics in the environment. Next, an overview of the existing information on effects of antibiotics at environmentally-relevant concentrations is provided. It is concluded that there is too limited information to judge whether antibiotic residues in the environment can exert effects on resistance, but that such effects cannot be ruled out, when extrapolating from collections of minimum inhibitory concentrations (MIC) and from data on minimum selective concentrations. A range of test methods for the detection of antibiotic resistance are described and evaluated with respect to their suitability as regulatory test systems. A combination of culture-based and molecular methods appears most promising, in combination with tests for resistance gene transfer. The methods generally lack standardisation. An inclusion of the effects of environmental antibiotic residues on resistance in the environmental risk assessment procedures of human and veterinary antibiotics is discussed. Given that manure and sewage effluent can be relevant hotspots and sources of resistant bacteria in the environment and that other chemicals such as heavy metals can also increase resistance, surveillance of environmental resistance is also recommended. Finally, a possible test system is described, research needs are identified, and the results of an international expert meeting are summarized.

Kurzbeschreibung

In diesem Bericht wird die Rolle von Antibiotikarückständen in der Umwelt für die Entwicklung und Verbreitung von Antibiotikaresistenzen in Umweltkompartimenten untersucht. Zunächst wird ein Überblick über das Verhalten und Auftreten von Antibiotika in der Umwelt gegeben. Darauffolgend werden Daten zu Effekten von Antibiotika auf Resistenz in Umweltkompartimenten zusammengetragen. Es liegen nicht genügend Daten vor, um Effekte von Rückständen auf das Auftreten von Resistenz bei umweltrelevanten Konzentrationen nachzuweisen. Basierend auf Sammlungen von minimalen Hemmkonzentrationen und Daten zu minimalen selektiven Konzentrationen können solche Effekte jedoch nicht ausgeschlossen werden. Testmethoden für das Auftreten und die Verbreitung von Antibiotikaresistenz in der Umwelt werden beschrieben und bewertet. Eine Kombination von Kultur- und PCR-Techniken, kombiniert mit Tests zur Genübertragung, bietet die meisten Vorteile. Die vorhandenen Testsysteme sind jedoch noch nicht standardisiert. Die Berücksichtigung von Effekten von Antibiotikarückständen auf die Resistenzbildung in der Umwelt innerhalb der Umweltrisikoabschätzung von Antibiotika wird diskutiert. Ein Resistenzmonitoring in der Umwelt wird empfohlen, um auch resistente Bakterien, die mit Gülle oder Kläranlagenabläufen in die Umwelt gelangen, und die Effekte von andere Stoffgruppen (z.B. Schwermetallen) auf die Resistenz abzudecken. Ein mögliches Testsystem wird vorgestellt, Forschungsbedarf wird identifiziert und die Ergebnisse eines internationalen Expertentreffens zum Thema werden beschrieben.

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 Objective and report outline

It is internationally accepted that the presence of antibiotic resistance in human and veterinary pathogens is a great problem (WHO 2001; FAO 2003). The relation between antibiotic use and development of antibiotic resistance has been established both in human and veterinary practice. Antibiotic residues can also reach the environment, for example through animal manure or through sewage sludge. These residues could maintain a selective pressure favouring the development and / or dissemination of resistance in the respective environmental compartments. In turn, this could contribute to adverse effects on human health, if resistance occurring in primarily environmental bacteria is transferred to species associated with human infection (Martinez 2008). However, there are currently no legal requirements to monitor or prevent possible effects of antibiotic residues on the development or dissemination of antibiotic resistance in the environment.

This report addresses the role of antibiotic residues for the development or dissemination of antibiotic resistance in the environment. The possibility of including the potential of antibiotics in the environment to increase resistance in the environmental risk assessment procedures of human and veterinary antibiotics is discussed.

Specifically, the following questions are addressed:

Chapter 2: What are the concentration levels of antibiotics resulting from current human and veterinary practices in environmental compartments that are relevant for the development and dissemination of antibiotic resistance (manure, soil, surface water and sediment, groundwater)?

Chapter 3: Can antibiotic residues contribute to or enhance the development or dissemination of antibiotic resistance in the environment? Can threshold concentrations for effects of antibiotic residues on resistance in the environment be derived from existing information?

Chapter 4: Which experimental test methods can be used to derive threshold concentrations of antibiotics for resistance development or resistance dissemination in the environment?

Chapter 5: How is antibiotic resistance addressed in the current risk assessment of human and veterinary antibiotics?

Chapter 6: Is there a need to include testing for possible effects of antibiotics on antibiotic resistance in the environmental risk assessment of human and veterinary pharmaceuticals? Which test methods and test designs might be applied in regulatory tests for resistance development and dissemination in the environment?

This report does not address the role of the environment as transmission route for antibiotic resistant bacteria from animal or human sources. It has convincingly been shown that antibiotic resistant bacteria and antibiotic resistance genes can be emitted

from sources such as wastewater treatment plant effluents and animal manure, and can reach environmental compartments such as receiving surface waters and agricultural soils. The presence of resistant bacteria in environmental media might also lead to human exposure, for example through recreation in surface waters under the influence of wastewater treatment plants, or through contact with manure. While aspects of a human health risk assessment of antibiotic resistance in the environment are discussed in chapter 6.2, it is not the goal of this report to fully discuss the risks of transmission of resistant bacteria emitted from animals or humans. For the role of the environment as possible transmission route, the reader is therefore referred to recent reviews (Chee-Sanford et al. 2009; Heuer et al. 2011a; Ashbolt et al. 2013; Finley et al. 2013; Gaze et al. 2013; Wellington et al. 2013, Pruden et al. 2013).

Throughout this report, antibiotics (or alternatively, antimicrobials) are defined as "any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets" (Davies and Davies 2010), and antibiotic resistance as "the ability of microorganisms of a certain species to survive or even to grow in the presence of a concentration of an antimicrobial that is usually sufficient to inhibit or kill bacteria of the same species" (European Commission 1999b).

2 Antibiotic residues - occurrence in environmental media (literature study)

This chapter is investigating the environmental concentration levels of antibiotics resulting from human and veterinary practices. To this end, information on antibiotic usage, on the physicochemical properties and environmental fate of various antibiotic classes, and on the current knowledge on environmentally measured concentrations was gathered from the literature. Due to the extent of the available literature, an overview on these issues is provided rather than a complete description of the state of knowledge.

2.1 Literature review – methods

The literature search was based on the following search terms:

1. Compound definition

antibiotic* OR antimicrobi* OR tetracycli* OR sulfonamid* OR aminoglycosi* OR quinolon* OR fluoroquinolon* OR macrolid* OR trimethoprim* OR beta-lacta* OR cephalosporin* OR penicill*

2. Matrix definition

manure OR slurry OR lagoon OR soil OR surface water OR groundwater OR ground water OR occurrence AND Environment* OR clay OR organic carbon

3. Process definition

Fate OR sorption OR degradat* OR remove* OR transform* OR biotransform* OR environmental behavior

Terms 1. and 2. were combined to search for occurrence data. In order to gain knowledge of processes and behavior of these chemicals in the environment, 1. and 3. were combined. Additionally all searches were combined with refining terms to reduce and focus results as the number of results was excessive.

The selection criteria 1., 2. and 3. were combined in different search engines (PubMed/Scopus/Web of Science/Non-peer reviewed literature). The most relevant records were selected and downloaded if available through Utrecht University. This resulted in a database of approximately 200 relevant records.

As searched databases only contain peer reviewed records, additional reports were monitored from personal literature records. Additionally some search terms were included in more generic 'google' searches to reveal generic non peer-reviewed literature as well. In the Netherlands, Germany as well as other countries various reports and books have been published on the occurrence and fate of veterinary antibiotics in the environment. These reports are often not accessible via scientific search engines. Therefore, we finally identified relevant information via our connections within the governmental and private research institutes.

2.2 Consumption of antibiotics

Pharmaceuticals have been intensively studied in environmental science over the last decade. Most studies focused on the occurrence, fate and risks of human pharmaceuticals (Kümmerer 2008). Veterinary pharmaceuticals have drawn somewhat less attention, even though the consumption volumes can be similar in European regions with intensive farming (Rohweder 2003; Mevius et al. 2007; Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2008). The type of pharmaceuticals used in human medicine clearly differs from the pharmaceuticals used in veterinary practice. While a major fraction of the human pharmaceuticals are analgesics, medication against high blood pressure, heart-diseases, high cholesterol, diabetes and neurological disorders, veterinary pharmaceuticals are mainly antibiotics, anti-parasitics, and some anesthetics and tranquilizers. Veterinary antibiotics cover 89% of the total consumption of veterinary pharmaceuticals (Kools et al. 2008). The total consumption of veterinary antibiotics in Europe is estimated to be 5400 tons per year of which 784 tons are used in Germany. This number exceeds the human consumption of antibiotics in Germany (Rohweder 2003; Bergmann 2011). Furthermore the consumption pattern of human antibiotics differs from veterinary pharmaceuticals. Tetracyclines are, for example, used in the highest volumes in veterinary practice, while their use in human medication is less pronounced (see Table 1). It must be noted, though, that Table 1 is based on different usage metrices which cannot directly be compared.

Table 1: Antibiotic consumption

Antibiotic class	Contribution of antibiotic class to total veterinary consumption of antibiotics in Germany in 2005 (% of tons)[d]	Veterinary consumption in 19 European countries in 2010 (% of mg/population corrected units)[e]	Contribution of antibiotic class to total human consumption of antibiotics in Germany in 2008 (% of Defined Daily Doses, DDD)[f]
Aminoglycosides	4.6	4.3	- [a]
(Am)phenicols	0.6 (Bergmann 2011)	0.6	- [a]
β-lactams (penicillins, cephalosporins, carbapenems, monobactams)	25.4	0.5 [c]	15 [c]
Penicillins	- [b]	23	28
(fluoro)quinolones	0.5	2.6	10
Macrolides	6.7	6	15
Sulfonamides and trimethoprim	12.4	12.9	6
Tetracyclines	43	39	22
Others	6.8	11.4	4
Total consumption	784 tons		402 tons 2001 (Rohweder 2003) 571 tons 2009 (Bergmann 2011)

a: Not given
b: Included in β-lactams
c: Excluding penicillins
d: From Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (2008)
e: Data are obtained from Austria, Belgium, Czech Republic, Denmark, Estonia, Finland, France, Hungary, Iceland, Ireland, Latvia, Lithuania, Netherlands, Norway, Portugal, Slovenia, Spain, Sweden, United Kingdom (European Medicines Agency 2012).
f: From Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (2010)

The consumption of antibiotics can lead to the development of resistance in microorganisms. Currently, there is a political debate on the development of antimicrobial resistance as a result of the large consumption of antimicrobial agents in both human and veterinary medicine (Sarmah et al. 2006).

Additionally, there are considerable variations in quantity and combinations of antibiotics used in different European countries as a result of different medical practices for human pharmaceuticals (European Surveillance of Antibiotic Use) and different live stock species and management of farms in veterinary practice (Kools et al. 2008; European Medicines Agency 2011; European Medicines Agency 2012).

2.3 Antibiotics in the environment

Figure 1 gives a generic overview of the fate of veterinary and human pharmaceuticals in the environment. The route of entry of veterinary pharmaceuticals into the environment differs from human pharmaceuticals. Veterinary manure/slurry is often collected and used as fertilizer on land and excrements of animals that are kept in open fields are directly spread over the land. Alternative routes for animal excrements consist of mesophilic or thermophilic anaerobic digestion (biogas production), after which remaining digestates are applied to agricultural land. Excreted residues of human pharmaceuticals are transported to sewage treatment plants and are discharged to surface waters after treatment (Kümmerer 2008). When unused drugs are disposed of into toilets, these also mainly enter sewage treatment plants and surface water. In countries where sewage sludge is used as agricultural fertilizer, human drugs can also be transported to soil. Additionally, antibiotics can enter the soil environment when solid waste, containing disposed antibiotics, ends up in land-fills. Accidental spills during production and transport can lead to contamination of surface waters and possibly soil. There is only limited recent data on the environmental risks of antibiotic consumption in aquaculture (Grave et al. 2008). When antibiotics are applied in open aquaculture systems, emissions to surface waters can be high (Cabello 2006). Nevertheless, the general impact of these routes is not included in the further discussion.

Figure 1 illustrates that it is relevant to obtain data on the occurrence of antibiotics in wastewater, manure, soil, surface water and groundwater. Furthermore, it is relevant to obtain data on excretion, transformation and sorption of these compounds to predict their behavior in the environment and the relation between the concentrations in the different (environmental) matrices.

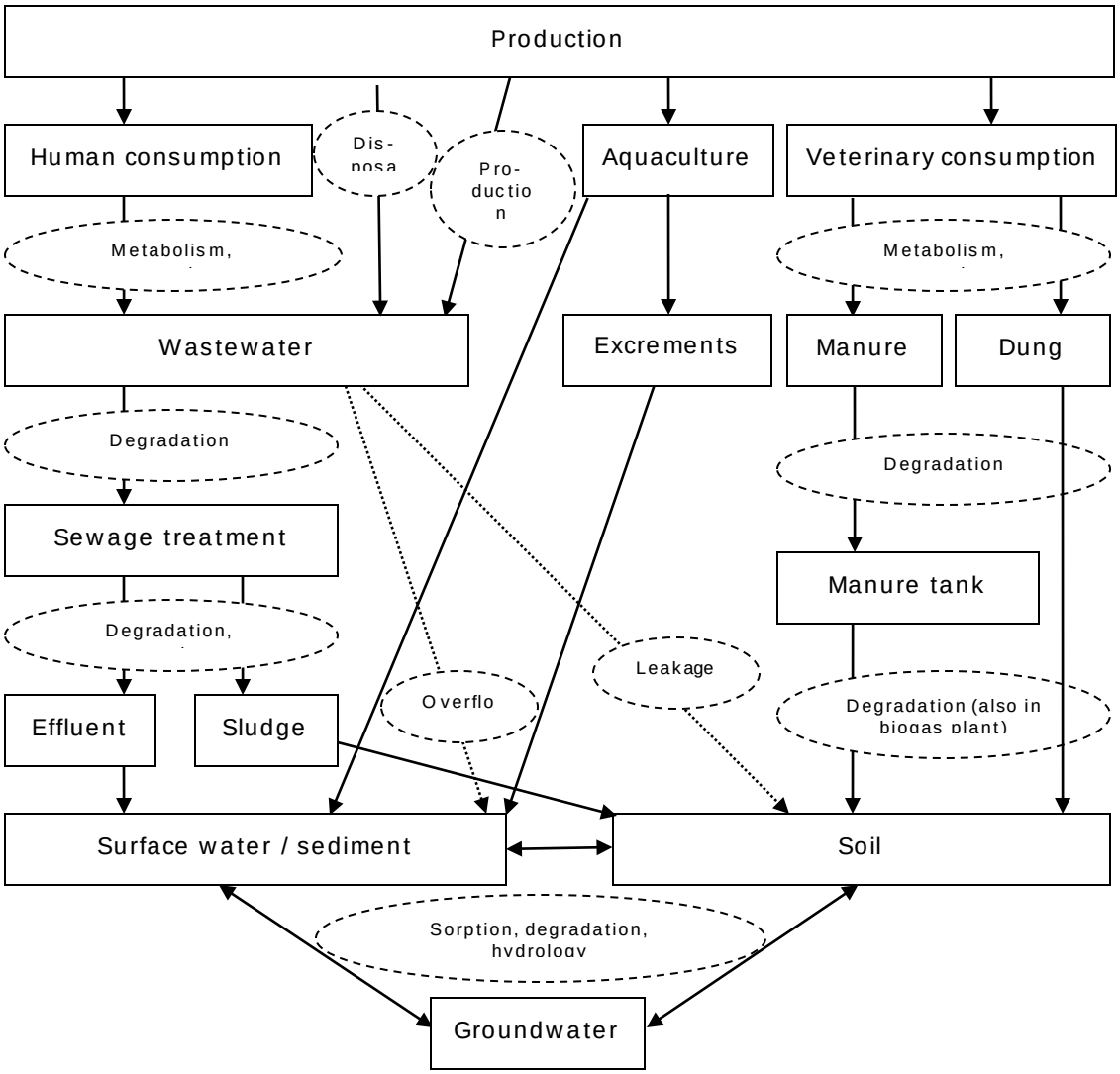

Fig. 1: Fate of antibiotics in the environment

2.3.1 Physicochemical characterization of antibiotics

Table 2 below gives some generic properties of the antibiotic classes. The indicated data ranges are based on a selection of compounds within these classes, and might therefore not cover the properties of the whole class. Especially the classes of β-lactams and macrolides include compounds with a very diverse array of chemical structures. The resulting diversity in properties within a class is not completely covered by the data in the table. Nevertheless, the table gives a generic impression of the properties of the antibiotics.

The physicochemical properties of the antibiotics affect their fate in the environment. Table 2 illustrates that properties of antibiotics vary to a large extent between antibiotic classes and even within antibiotic classes. Antibiotics are a rather diverse group of organic chemicals. However, some generalizations can be made. Practically all antibiotics consist of a non-polar core combined with polar and ionizable functional

groups (Thiele-Bruhn 2003). They often carry a negative, positive or both negative and positive charge at environmental pH. The polar and/or ionizable groups make the antibiotics rather soluble in water. Their solubilities range from the sub mg/L level of the large and somewhat hydrophobic macrolides towards several or even hundreds of grams per liter for the sugar-like aminoglycosides and the polar amphenicols. Their ionizable nature makes their aqueous solubility highly pH dependent. Additionally, large variations in properties can be observed within antibiotic classes (see Table 2). Solubilities of the ionized species are generally orders of magnitude higher than the solubility of their neutral counterparts (Schwarzenbach et al. 2003).

Table 2: Physicochemical characterization of various classes of antibiotics

Compound class	Molecular weight (Da)	Water solubility	pK_a, charge at pH 7	Soil Sorption [d]
Aminoglycosides	332.4 - 615.6 (Thiele-Bruhn 2003)	10000 - 500000 (Thiele-Bruhn 2003)	6.9-8.5 (+)(Thiele-Bruhn 2003)	-- [g]
(Am)phenicols	295.2 – 358.2 [b]	1320 – 640000 (U.S. Environmental Protection Agency 2000; Thiele-Bruhn 2003)	9 - 10 (+) [e]	Low (Subbiah et al. 2011) [f], (Boxall et al. 2006)
β-Lactams	334.4 – 470.3 (Thiele-Bruhn 2003)	22 - 10100 (Thiele-Bruhn 2003)	2.7 (-) (Thiele-Bruhn 2003)	Low [c, f] (Subbiah et al. 2011)(Boxall et al. 2006)
(Fluoro)quinolones	229.5 - 417.6 (Thiele-Bruhn 2003)	3.2 -17790 (Thiele-Bruhn 2003)	6.1/8.6 (+&-) (Thiele-Bruhn 2003)h	High (Boxall et al. 2006; Williams et al. 2009)
Macrolides	687.9 - 916.1 (Thiele-Bruhn 2003)	0.06 – 30 (Thiele-Bruhn 2003; Chee-Sanford et al. 2009[a]; Verlicchi et al. 2012)	7.7–9.5 (+)(Thiele-Bruhn 2003; Verlicchi et al. 2012)	Medium to High (Boxall et al. 2006; Verlicchi et al. 2012)
Sulfonamides	172.2 - 300.3 (Thiele-Bruhn 2003)	7.5-1500 (Thiele-Bruhn 2003)	2 - 3 / 4.5 – 10.6 (-) (Thiele-Bruhn 2003)	Low (Boxall et al. 2006; ter Laak et al. 2006a; ter Laak et al. 2006b)
Trimethoprim	290.3 [b]	0.4 [b]	7.3 (+/0) [b]	Low (Williams et al. 2009)
Tetracyclines	444.5 – 527.6 (Thiele-Bruhn 2003)	230 – 52000 (Thiele-Bruhn 2003)	3.3 / 7.7 / 9.3 (+&-) [a]	High (Boxall et al. 2006; ter Laak et al. 2006a; ter Laak et al. 2006b)

a Tetracyclins are zwitterions with positive and negative charged groups at neutral pH

b Data from http://www.chemspider.com/

c Limited data from http://www.msds-gsk.com/11060208.pdf

d Sorption is qualitatively defined as data for most substances are only available for a single soil or certain soil constituents (e.g. clay, organic matter); Low = log Ksoil < 1; Medium = log Ksoil 1-2, High = log Ksoil > 2

e Data from http://www.chem.agilent.com/Library/applications/5990-3615EN.pdf, the pKa and charge holds only for some (am)phenicols.

f No sorption coefficients determined but reference indicates high residues in soil supernatant

g No data available

h The quinolones are carboxylic acids and contain a quinolone ring. The fluoroquinolones also contain protonatable nitrogens and, therefore, can exist as zwitterions.

2.4 Fate of antibiotics in the environment

The potential occurrence and fate of antibiotics in the environment depends on the excretion (metabolism) of the compounds by humans or livestock, the interaction with various solid matrices in the environment (sorption) and the persistence of the compounds in various environmental matrices (transformation). The paragraphs below discuss these issues and how they affect the fate of antibiotics in the environment.

2.4.1 Excretion

The excretion of (unchanged) antibiotics varies per compound, application type (e.g. oral, intravenous or dermal) and organism (e.g. poultry, cattle, swine or human). However, in general large fractions are excreted as active compounds via urine and faeces (Van Loenen 2003; Chee-Sanford et al. 2009b; Du and Liu 2012) and in some cases relevant fractions can be excreted via sweat (Hoiby et al. 2000). The excretion of veterinary antibiotics by livestock is estimated to be 75% (Chee-Sanford et al. 2009a; Chee-Sanford et al. 2009b) or 30-90% (Du and Liu 2012) of the consumed amounts. However, human pharmacological data shows larger variation in excretion, ranging from 10 to >90% (Van Loenen 2003). Table 3 lists excretion rates of various antibiotics. It can be observed that excreted fractions of tetracyclines, (am)phenicols, (fluoro)quinolones, trimethoprim and penicillins (β-lactams) are generally around or above 50%, while sulfonamides show variable excretion, and most macrolides show lower excretion fractions ranging from 5 to 25%. Nevertheless, relevant fractions of the all consumed antibiotics are excreted unchanged.

2.4.2 Sorption

The sorption and desorption behaviour in soil, sediment, manure and sludge is an important aspect determining the fate of antibiotics in the environment and the bioavailability for microorganisms (Subbiah et al. 2011). Various studies have shown that the sorption of antibiotics to soil and manure does not follow a clear relation with the commonly applied octanol-water partition coefficient (Tolls 2001a; Loke et al. 2002a; Kreuzig et al. 2003; ter Laak et al. 2006b; Kümmerer 2008). Generally applied models underestimate sorption by orders of magnitude (Kümmerer 2008). Antibiotics can sorb to soil by electrostatic interactions or complexation to both organic (e.g. humic materials) and inorganic phases (minerals) in the soil or sediment (Porubcan et al. 1978; Sithole and Guy 1987; Tolls 2001b; Hamscher et al. 2005). This means that the sorption process can be affected by aqueous chemistry (pH, ionic strength, valence of (competing) ions in solution) as well as both organic and inorganic soil properties and structure (Tolls 2001b). Consequently, the sorption of polar ionic organic compounds can be highly variable and is difficult to predict. In manure, the fate of antibiotics can also vary substantially with different routes of administration and different manure parameters, and studies in spiked matrices might not always reflect processes in manure of treated animals. As an example, while a high proportion of non-extractable

residues was formed in sulfadiazine-spiked bovine manure (Kreuzig and Holtge 2005), the same compound showed negligible non-extractable residues when manure of sulfadiazine-treated pigs was studied (Lamshöft et al. 2010). With this in mind, some generic observations of the sorption behaviour of antibiotics are discussed below.

Most environmental sorbents carry a negative charge at environmental (near neutral) pH (Schwarzenbach et al. 2003). Consequently, weak organic acids are only weakly sorbed to these sorbents because their negative charge at neutral pH does not favor electrostatic interactions between compound and sorbent. Therefore, the sorption of highly water-soluble weak organic acids such as sulfonamides and β-lactams is low. Weak organic bases that carry a positive charge at neutral pH (e.g. macrolides) often sorb more strongly to environmental sorbents (Sposito 1989) due to favorable electrostatic interactions with the oppositely charged environmental sorbents. The zwitterionic tetracyclines and fluoroquinolones canhave both positive and negative charge at neutral pH and can form strong complexes with bivalent cations (Doluisio and Martin 1963). This enables them to form complexes in the aqueous phase and also with sorbent surfaces (Sithole and Guy 1987; Tolls 2001b). Consequently, their sorption coefficients are high and extraction of these compounds from sorbents such as soil can be very hard, despite their high aqueous solubility.

2.4.3 Transformation

Table 3 below lists half-lives of a broad array of antibiotics in manure, soil and water. Transformation rates or half-lives of antibiotics in wastewater treatment plants are generally not available. Therefore, removal rates of conventional activated sludge systems are listed. The half-lives range from hours to several months and removal efficiencies in wastewater treatment vary widely (Verlicchi et al. 2012). In some studies, the duration of the transformation experiments were too short to determine a robust degradation half-live, resulting in a ‘larger than’ value. These results suggest that degradation half-lives of some antibiotics can be months or even years.

Generally, reported half-lives in water are shorter than half-lives in manure or soil. This might be explained by reduced availability of the antibiotics for biological and chemical transformation due to sorption to solid matrices. However, for the macrolide antibiotic tylosin the half-live in water was much longer than in manure or soil. This is, likely due to the fact that this particular experiment was performed in ultra pure water that is not a suitable matrix to maintain a large and active bacterial community, thereby potentially hampering biological activity. Furthermore, the half-life of some antibiotics is considerably shortened in the presence of light due to photochemical transformation. Additionally, for some compounds that were tested frequently, large variations in half-lives can be observed between different studies. These differences might be attributed to the properties of the matrix (e.g. soil- or manure-type, water content, pH) and test conditions (e.g. temperature, light). Altogether, this illustrates that biological and chemical transformation processes of these compounds can be variable.

Removal efficiencies of a broad array of sewage treatment facilities have been recently collected by Verlicchi et al. (Verlicchi et al. 2012). A summary of this collection is given

in Table 3.The selected data for conventional activated sludge systems illustrate that there is a wide variety in removal efficiencies between antibiotics but also for single compounds. Only limited data are obtained for penicillins (β-lactams) as the removal efficiency is often high, and effluent concentrations are usually below limits of quantification (Monteiro and Boxall 2010). Removal efficiencies for tetracycline are generally high. This is probably due to sorption to the activated sludge, complexation with bivalent cations (Essington et al.) in solution and (photo)transformation (Thiele-Bruhn and Peters 2007; Jiao et al. 2008). Furthermore, the removal of amphenicols, and cephalosporins appears to be rather high as well, from the limited data available. Removal of sulfonamides, fluoroquinolones, and macrolides are highly variable, while the removal of trimethoprim is marginal.

2.4.4 Relevance of sorption and transformation for the assessment of environmental concentrations

Consumption data, sorption coefficients (Table 2), excretion, removal in sewage treatment plants, and half-lives in various environmental matrices (Table 3) provide valuable input for the assessment of fate and concentrations of antibiotics in the environment. The total consumption and excretion of antibiotics can be estimated rather accurately from consumption/prescription data and pharmacological literature. The observed variation in wastewater treatment plant removal efficiencies are likely due to variable (1) physical conditions such as temperature and light, (2) wastewater quality (solid content and aqueous chemistry, that are related to the source characteristics such as communal wastewater, mixed communal and industrial wastewater and dilution due to storm water discharge), (3) operational conditions of the treatment plant (hydraulic and sludge retention times, design of the treatment plant). Furthermore, input concentrations of micro contaminants in sewage treatment plants can vary substantially over both short (i.e. day) and longer (e.g. year) periods of time (Goossens et al. 2005). Consequently, sampling procedures likely affect the quality and accuracy of determined removal efficiencies. However, with some additional work on data selection, as discussed in detail by Ort et al (Ort et al. 2010), the emissions of wastewater treatment plants might be described and predicted rather accurately (ter Laak et al. 2010).

The assessment of generic sorption affinity and transformation rates in environmental matrices remains cumbersome because data are limited for some (classes of) antibiotics. Additionally, available transformation and sorption parameters are often obtained from batch scale studies under standardised conditions. These studies do not always represent relevant matrices and processes in the environment and do not account for specific situations. These specific situations are for example heavy rain fall that enables preferential drain flow to groundwater (Kay et al. 2004), surface run-off to surface water, thereby bypassing sorption to top soils (Stoob et al. 2007; Kim et al. 2010) or specific effects of the presence of manure on the sorption affinity of antibiotics (Sukul et al. 2008). Additionally, even under rather standardised conditions, a large variation of half-lives and sorption coefficients can be observed within the literature data. The wide variety of conditions and matrices in the environment will probably result in even more variable half-lives and sorption coefficients under environmental conditions.

Furthermore, most lab scale transformation studies are performed at room temperature. The temperature in the environment of Western Europe is usually lower than 20°C to 25°C. This reduces biological transformation processes and chemical reactions compared to lab scale experiments. Besides that, the half-lives are generally determined with a one phase decay model and sorption studies are often determined in batch equilibrium studies with only one or several days of equilibration (OECD 2000). These phase decay models and batch sorption studies are not always applicable to transformation and sorption processes in the environment: concentration dependent biological transformation (Wang et al. 2006) and an increase in sorption with time (ageing) to environmental matrices (Alexander 2000; Gao and Pedersen 2010) can result in slowly or non-degradable residues and high sorption coefficients (Alexander 2000; Kuchta and Cessna 2009). For example, the sulfonamide sulfadiazine has been shown to increasingly form non-extractable residues in soil over 28 days, which include a fraction that is covalently bound to fulvic acids (Junge et al. 2011). An increase in the apparent sorption coefficients over time, possibly indicating formation of non-extractable residues, was also evident in field studies (Rosendahl et al. 2011). This means that half-lives obtained in the laboratory can differ from half-lives in the environment and sorption coefficients do not properly reflect the actual sorption for aged antibiotic residues in soil.

The variability of half-lives and removal rates are not suitable to derive general quantitative conclusions on the fate of antibiotics in various environmental matrices (Table 3). However, the observations can be used to propose some 'rules of thumb' for the most relevant antibiotic classes. Generally large fractions of consumed antibiotics are excreted via urine and faeces and substantial fractions likely end up in manure and sewage treatment plants. The half live of penicillins (β-lactams) in manure (storage tanks) is short (several days) and the removal in sewage treatment plants is high, so only a marginal fraction of these compounds are expected to reach or accumulate in the environment. Furthermore the removal of amphenicols and cephalosporins in sewage treatments is comparatively high, and the half live of cephalosporins in water is short (several days). These compounds are thus unlikely to accumulate in the environment. The (fluoro)quinolones and tetracyclines show longer half-lives in manure and soil (several months), but rather high removal in sewage treatment plants. This is probably related to their high sorption coefficients (Table 2). The high sorption hampers biological transformation in soil but increases removal in sewage treatment plants due to sorption to active sludge. Thereby, emissions in effluents of sewage treatment plants are largely reduced. Consequently it is likely that tetracyclines occur and accumulate in soils treated with manure, but it is unlikely that large fractions reach groundwater and surface water. Macrolides show rather low excretion rates (5-25%), variable removal in sewage treatment plants, variable half-lives in various matrices and moderate to high sorption coefficients. Consequently their fate is difficult to predict. Sulfonamides have moderate half-lives in various matrices and removal in sewage treatment plants and low sorption coefficients, so in contrast to tetracyclines, sulfonamides likely end up in groundwater and surface waters.

However, the rules of thumb listed above are rather unspecific and qualitative. A more accurate assessment of the environmental fate of antibiotics needs both knowledge on the behavior and properties of the compounds and monitoring data on the occurrence of these compounds in various environmental matrices for validation. Only the combined knowledge enables to improve the assessment of the environmental fate of these substances with complex environmental behavior, and the subsequent exposure assessment that is relevant for assessing risks of these compounds for microbial communities, resistance development and potential human health impacts. The following paragraph discusses the occurrence of antibiotics in manure, sewage, surface water, soil and groundwater.

In summary, rather large fractions of consumed antibiotics often ranging from 10 - 100% are excreted unchanged by their users (humans, and animals). The sorption to soil and sediment appears to be variable between different classes of antibiotics. Even within classes of antibiotics sorption can vary, depending on soil properties and pH dependant speciation of the molecules. Transformation also appears to be variable. Consequently, it is rather difficult to predict the fate of these molecules directly from their chemical structure. Further, environmental heterogeneity will lead to non-homogeneous distributions of antibiotics within matrices. Therefore, occurrence data is also necessary to assess the fate of these compounds in the environment.

Table 3: Excretion, removal and half-lives of antibiotics in environmental matrices

Antibiotic	Class	Human excretion rates (%)	Removal for conventional activated sludge systems (%) [a]	Manure half-live (d)	Soil half-live (d)	Water half-live (d)
Amoxicillin	penicillins	80-90 (Monteiro and Boxall 2010)	96	5 (Chee-Sanford et al. 2009b)[m]		
Ampicillin	penicillins	30-60 (Monteiro and Boxall 2010)		5 (Chee-Sanford et al. 2009b)[m]		
Azitromycin	macrolides	6 (Monteiro and Boxall 2010)	18-74	<2-20 (Chee-Sanford et al. 2009b)[n]		
Cefaclor	cefalosporins	60-85 (Van Loenen 2003)	98			2.7-18.5 [f] 2.2-5.0 (Jiang et al.)[g]
Cefalexin	cefalosporins	>90 (Van Loenen 2003)	53-100			
Cefotaxime	cefalosporins	40-60 (Van Loenen 2003)	43-83			
Chloramphenicol	amphenicols		92-97			
Chlorotetracycline	tetracyclines	>70 (Monteiro and Boxall 2010)	82-85	11-12 (Bao et al. 2009)[b] 87 (Bao et al. 2009)[c] 18 (Arikan 2008) 8.2 (Wu et al. 2011)[d] 100 (Chee-Sanford et al. 2009b)[q]	>2 (Allaire et al. 2006)	
Ciprofloxacin	fluoroquinolones	45-60 (Monteiro and Boxall 2010)	18-96	100 (Chee-Sanford et al. 2009b)[o]		0.0625 (Cardoza et al. 2005)[k]

Antibiotic	Class	Human excretion rates (%)	Removal for conventional activated sludge systems (%) [a]	Manure half-live (d)	Soil half-live (d)	Water half-live (d)
Clarithromycin	macrolides	~5 (urine), ~5 (faeces) (Van Loenen 2003)	0-83, 45 (Miege et al. 2009)	<2-20 (Chee-Sanford et al. 2009b)[n]		
Clindamycin	macrolides	10-20 (urine), 4 (faeces) (Van Loenen 2003)		<2-20 (Chee-Sanford et al. 2009b)[n]		
Cloxacillin	penicillins			5 (Chee-Sanford et al. 2009b)[m]		
Dimetridazole	nitroimidazoles					
Doxycycline	tetracyclines	41 ±19 (Monteiro and Boxall 2010)	14-100	52.5 & 25.7(Istvan et al. 2011), 100 (Chee-Sanford et al. 2009b)[q]		
Enoxacin	fluoroquinolones			100 (Chee-Sanford et al. 2009b)[o]		
Enrofloxacin	fluoroquinolones		38-70	113 (Wetzstein et al. 2009)		0.8 8 3.7 h 72 [f] (Knapp et al. 2005)
Erythromycin	macrolides	12-15 (Monteiro and Boxall 2010), 5-15 (Van Loenen 2003)	0-75, 67±16 (Miege et al. 2009)	41 (Schlüsener et al. 2006), <2-20 (Chee-Sanford et al. 2009b)[n]	20 (Schlüsener and Bester 2006)	
Flumequine	fluoroquinolones			100 (Chee-Sanford et al. 2009b)[o]	3.6-6.4 (Lai and Lin 2009)[e]	1.9-2.3 (Lai and Lin 2009)
lasalocid					<4 (Sassman and Lee 2007)	
Lincomycin	macrolides		0-57	<2-20 (Chee-Sanford et al. 2009b)[n]		

Antibiotic	Class	Human excretion rates (%)	Removal for conventional activated sludge systems (%) [a]	Manure half-live (d)	Soil half-live (d)	Water half-live (d)
Metronidazole	nitroimidazoles	34 (unchanged in urine), 14 (faeces) (Van Loenen 2003)	38-39			
Monesin					<4 (Sassman and Lee 2007)	
Norfloxacin	fluoroquinolones	30-50 (urine), 30 (faeces) (Van Loenen 2003)	18-96, 54.3 (Deblonde et al. 2011)[r]	100 (Chee-Sanford et al. 2009b)[o]		
Oleandomycin	macrolides			<2-20 (Chee-Sanford et al. 2009b)[n]	27 (Schlüsener and Bester 2006)	
Ofloxacin	fluoroquinolones	85-95 (Van Loenen 2003)	13-99, 64.5 (Deblonde et al. 2011)[r]			
Oxolinic acid	quinolones				9.5-15 (Lai and Lin 2009)[e]	2.3-4.8 (Lai and Lin 2009)
Oxytetracycline	tetracyclines	>80 (Monteiro and Boxall 2010)	44	>37 (Arikan et al. 2007), 56 (Arikan et al. 2006), 22 (Blackwell et al. 2007), 30 (De Liguoro et al. 2003), 8.1 (Wang and Yates 2008)[k], 1.1 (Wu et al. 2011)[d], 100 (Chee-Sanford et al. 2009b)[q]	33-56 (Wang and Yates 2008)	

Antibiotic	Class	Human excretion rates (%)	Removal for conventional activated sludge systems (%) [a]	Manure half-live (d)	Soil half-live (d)	Water half-live (d)
Penicillin V	penicillin	80-85 (Monteiro and Boxall 2010)	60	5 (Chee-Sanford et al. 2009b)m		
Roxithromycin	macrolides	>50 (Van Loenen 2003)	0-38, 0.37 ±9 (Miege et al. 2009) 39.5 (Deblonde et al. 2011)[r]	130 (Schlüsener et al. 2006), <2-20 (Chee-Sanford et al. 2009b)[n]	>120 (Schlüsener and Bester 2006)	
Salinomycin	macrolides			6 (Schlüsener et al. 2006), <2-20 (Chee-Sanford et al. 2009b)[n]	5 (Schlüsener and Bester 2006)	
Spiramycin	macrolides		0	<2-20 (Chee-Sanford et al. 2009b)[n]		
Sulfachloropyridazine (Sulfaclozine)	sulfonamides		26-82	<8-30 (Chee-Sanford et al. 2009b)[p]	21.3 (Accinelli et al. 2007)	
Sulfadiazine	sulfonamides	30-50 (Van Loenen 2003)	78-100	<8-30 (Chee-Sanford et al. 2009b)[p] Concentrations increasing due to deacetylation of metabolite (Lamshöft et al. 2010)	2-32 (easily extractable fraction) 23-330 (residual fraction) (Förster et al. 2009; Rosendahl et al. 2011)	
Sulfadimetoxine	sulfonamides		66-100	64 (De Liguoro et al. 2007), <8-30 (Chee-Sanford et al. 2009b)[p]	3-11 (Wang et al. 2006)	
Sulfadimidine (Sulfametazine)	sulfonamides		16-100	<8-30 (Chee-Sanford et al. 2009b)[p]	18.6 (Accinelli et al. 2007)[i]	

Antibiotic	Class	Human excretion rates (%)	Removal for conventional activated sludge systems (%) [a]	Manure half-live (d)	Soil half-live (d)	Water half-live (d)
Sulfamethoxazole	sulfonamides	10-30 (Monteiro and Boxall 2010)	21-100, 73 ±35 (Vergouwen et al. 2011), 59 ±22 (Miege et al. 2009) 17.5 (Deblonde et al. 2011)[r]	<8-30 (Chee-Sanford et al. 2009b)[p]		
Sulfapyidine	sulfonamides		6-91 83.5 (Deblonde et al. 2011)[s]	<8-30 (Chee-Sanford et al. 2009b)[p]		
Sulfathiazole	sulfonamides		65-100	<8-30 (Chee-Sanford et al. 2009b)[p]		
Tetracycline	tetracyclines	80-90 (Monteiro and Boxall 2010)	24-80, 95.1 (Deblonde et al. 2011)[s]	10 (Wu et al. 2011)d, 100 (Chee-Sanford et al. 2009b)[q]	25 & 34 (Halling-Sorensen et al. 2005)	
Tiamulin	pleuromutilin			>180 (Schlüsener et al. 2006)	16 (Schlüsener and Bester 2006)	
Trimethoprim		50-60 (Van Loenen 2003)	5-85, 8 ±18 (Vergouwen et al. 2011), 16 ±20 (Miege et al. 2009) 1.4 (Deblonde et al. 2011)[r]			
Tylosin / Tilmicosin	macrolides			>45 (De Liguoro et al. 2003) <2-20 (Chee-Sanford et al. 2009b)[n]	>2 (Allaire et al. 2006), 67 & 49 (Halling-Sorensen et al. 2005), 8 (Schlüsener and Bester 2006)	200 (Hu et al. 2009)[j]

a Data range, obtained from Verlicchi et al. (2012) unless indicated otherwise, negative removal is not included

b Half-live in manure of hens

c Half-live in manure of hogs

d Half-live in compost

e Half-live in sediment slurry

f Half-live in the dark

g Half-live under light

h Half-live under low light conditions

i Still detectable after 1 year

j Tested in ultrapure water

k Wetted manure

l Half-live reduced by addition of DOC

m Estimation for β-lactams (penicillins) in general

n Range for macrolides in general

o Estimate for quinolones in general

p Range for sulfonamides in general

q Estimate for tetracyclines in general

r Average value

s Obtained from single observation

2.5 Occurrence of antibiotics in the environment

Table 4 lists observed concentrations of antibiotics in wastewater treatment plant influent and effluent, manure, soil, sediments, surface water and groundwater. As expected, concentrations of antibiotics are generally highest in manure and wastewater effluents, lower in soil and surface waters, and some residues are found in groundwater. Concentrations in soil are generally two orders of magnitude lower than in manure. This can be expected from common manure application loads and mixing with the top soil (Aust et al. 2008). Additionally, for human antibiotics, the lower concentrations in surface waters compared to effluents of wastewater treatment plants generally correspond to the dilution of the effluent after discharge (ter Laak et al. 2010; Ter Laak and Hofman in prep.). Moreover, due to difficulties in extracting the total amount of antibiotics from environmental matrices, and in estimating the bioavailable portion of the total detected amount, it is not trivial to translate observed concentrations to potentially effective concentrations.

2.5.1 Wastewater and large surface waters

Antibiotics in wastewater are practically all from human origin. However, new veterinary manure treatment techniques might lead to the occurrence of veterinary antibiotics in wastewater. These techniques include separation of concentrated aqueous phases with minerals from water (Velthof 2011) and discharge of the aqueous residues via the sewer or directly to surface waters. However, this technique is currently not used on a large scale, so this route is not (yet) relevant. Furthermore, antibiotics in regional surface waters can originate from veterinary use (Montforts et al. 2007), however it is likely that the vast majority of antibiotics in large river systems is from human origin. Additionally, open aquaculture systems can lead to emissions of antibiotics into surface waters. The consumption of antibiotics in aquaculture is limited in Germany but when antibiotics are applied in open aquaculture systems, emissions to surface waters are expected to be high (Cabello 2006). Although the risks of antibiotic consumption in aquaculture have been intensively discussed some decades ago, the limited recent data on the environmental risks of antibiotic consumption in open and closed aquaculture require attention as aquaculture is a growing industry worldwide (http://www.agroexpertise.de).

Concentrations of antibiotics in wastewater effluents can reach µg/L levels. Sulfamethoxazole, erythromycin and trimethoprim often show the highest concentrations in wastewater and surface waters. This corresponds to their high human consumption, high excretion rates and rather low removal during wastewater treatment (Table 3). Interestingly, β-lactams such as penicillins are only found at low concentrations in wastewater effluents, and there is hardly any data on their occurrence in surface waters (Monteiro and Boxall 2010) despite their rather high human consumption (Table 1). Apparently, they are rather easily degraded during sewage treatment (Table 3). It should, however, be noted that compounds that are rather degradable but are consumed in high quantities can remain present in waters at low concentrations due to the continuous emissions by wastewater treatment plants

(WWTPs; known as pseudo-persistence). Practically all occurrence data of tetracyclines in surface waters come from the United States, tetracyclines are usually not found in larger European surface waters such as the Rhine (RIWA Rijn 2010; RIWA Rijn 2011). The limited occurrence of these compounds in larger (European) surface waters is related to the relatively low consumption by humans (Table 1), their high removal during sewage treatment (Table 3) and the high sorption affinity for various environmental sorbents (Sassman and Laa 2005).

Effluents from drug manufacturing plants form a special case, as concentrations of antibiotics can be very high and even reach the mg/L range. So far, investigations have shown the occurrence of fluoroquinolones, sulfonamides, tetracyclines, and aminoglycosides in WWTP effluents treating manufacturing waste and the receiving rivers in countries including India (Larsson et al. 2007; Fick et al. 2009), China (Li et al. 2008a; Li et al. 2008b), Korea (Sim et al. 2011), and Kroatia (Babić et al. 2006).

2.5.2 Manure, local surface water, soil and groundwater

Concentrations in manure are often in the mg/kg range. Concentrations of the tetracyclines and sulfonamides (oxytetracycline, tetracycline and sulfadiazine) were often highest in manure. Hölzel et al. (Hölzel et al. 2010) observed median values of 0.73 mg/kg for four commonly applied tetracyclines and 0.15 mg/kg for fourteen commonly applied sulfonamides in pig slurry (n=305). However, concentrations did differ by orders of magnitude for individual samples, as total tetracycline and sulfonamide concentrations reached from <0.1 up to 52.6 mg/kg and <0.05 up to 38.4 mg/kg, respectively. Similar high concentrations were observed in dry manure (12-20% dry matter) (Bergmann 2011). Manure lagoons, a type of storage specific to North American agriculture, also contain high concentrations of these antibiotics. The high concentrations of tetracyclines correspond to the high dosage and consumption (Table 1) and the low gut wall absorption and metabolization that results in the almost complete excretion of the (oral) dose (Table 3). There is only limited knowledge on the occurrence of these antibiotics that originate from veterinary use in local surface waters in rural areas. A study of Watanabe (2010) et al. reports surface water concentrations of several sulfonamides and tetracyclines in the µg/L range (Watanabe et al. 2010). However these 'surface water' concentrations are from manure lagoons and can be considered a worst case situation. In Western Europe, manure is generally stored in manure tanks or directly disposed to soil by animals that are on pastures. Consequently these 'lagoon' concentrations are not applicable to the Western European situation. Excluding the data of Watanabe reveals surface water concentrations in the sub µg/L and ng/L range.

The concentrations of antibiotics in soil are generally below mg/kg level. Tetracyclines, fluoroquinolones and macrolides show the highest concentrations in soil reaching several hundreds µg/kg or several mg/kg. These values are likely a result of their high consumption in veterinary practice and rather high sorption coefficients to soil, which enables them to accumulate in the top soil. However, soil concentrations of sulfonamides are also rather high despite their rather low sorption coefficients towards soils (Loke et al. 2002b; ter Laak et al. 2006b).

There is only limited data on the occurrence of antibiotics in groundwater. As shown in Figure 1, antibiotics in soil can originate from manure application, leaching from manure lagoons (less relevant for Western European situation), leaching from contaminated surface water or direct leaching from sewers or solid waste disposal. Some sulfonamides, lincomycin and trimethoprim which is often administered together with sulfonamides (Mevius et al. 2007), have been observed in superficial groundwater (Hamscher et al. 2005). Their concentrations are generally in the sub µg/L to ng/L range. The sorption coefficients of these compounds to soil are low (Barber et al. 2009) which enables their transport through soil with water. Interestingly, tetracyclines and fluoroquinolones are not observed in groundwater despite their high consumption in veterinary practice. Probably, their high sorption to the soil generally prevents them from leaching to groundwater (Aust et al. 2008; Blackwell et al. 2009).

2.5.3 Contribution of veterinary and human antibiotics to the total emission

Some of the antibiotics listed in Table 1 are used in veterinary and human medicine. The origin of these antibiotics when detected in environmental matrices cannot be traced back to their use. However, residues in larger surface waters mainly originate from human consumption (Diaz-Cruz and Barcelo 2008; Walraven and Laane 2009; Monteiro and Boxall 2010; ter Laak et al. 2010). Their occurrence in manure lagoons is clearly linked to veterinary use and their occurrence in soil and groundwater also likely originates from veterinary practice (although this might be different in regions with limited sanitation). Table 1 shows that the consumption of antibiotics in veterinary practice and human practice is comparable (on a mass base) for Germany. The emission routes of veterinary antibiotics differ from human antibiotics. Human antibiotics are sorbed to activated sludge or end up in larger surface waters and eventually the sea. Contrastingly, veterinary pharmaceuticals likely end up in soil or possibly groundwater and local surface waters (ditches and small streams in agricultural areas). Figure 2 illustrates the fate of the most relevant classes of veterinary and human antibiotics. This figure is illustrative but not quantitative as metabolism, sorption and transformation of individual antibiotics vary.

2.5.4 Complexes, metabolites and transformation products

The dissipation of a parent compound does not necessarily imply that the compound is completely mineralized (Schmidt et al. 2008). Often, various transformation products or complexes are formed (Doluisio and Martin 1963; Halling-Sorensen et al. 2003; García-Galán et al. 2008, Li et al. 2008b; Kasteel et al. 2010; Wu et al. 2011). Transformation products can maintain their antimicrobial activity when the toxic moiety (the active region on the molecule) remains intact (Escher et al. 2008). Additionally, complexes can be transferred back to the parent species when the conditions in aqueous chemistry change. Therefore, the (apparent) dissipation of a certain compound does not necessarily mean that the antimicrobial activity is removed. Consequently, the effect can remain or return, when analytical tools show that the compound itself is no longer present in the environmental matrix. The transformation of compounds in the environment should therefore not be misinterpreted as a direct reduction of the environmental risk or effect (Kwon 2011). Consequently, the reduction of the risk of

antimicrobial compounds in environmental matrices due to transformation, complexation or sorption processes would ideally be tested by a combination of chemical analysis and effect based studies (Aga et al. 2003).

2.5.5 Summary

In conclusion, concentrations of antibiotics are highest in manure and municipal waste water. These concentrations are diluted by mixing manure with top soil and wastewater effluents with surface water. Soil mainly contains veterinary antibiotics with rather high sorption coefficients (e.g. tetracyclines, fluoroquinolones and macrolides), while more mobile veterinary antibiotics are more often found in groundwater (e.g. sulfonamide, lincomicides and trimethoprim). Surface waters mainly contain human antibiotics, such as sulfonamides, trimethoprim, and some more soluble macrolides. Interestingly, β-lactams such as penicillin are generally not found in surface waters soils and groundwaters while both veterinary and human consumption is significant. This is likely due to their low persistence in the environment. However, it should be noted that transformation of parent compounds can result in the formation of active transformation products. The dissipation of the compounds therefore does not equal the dissipation of the effect and subsequent risk.

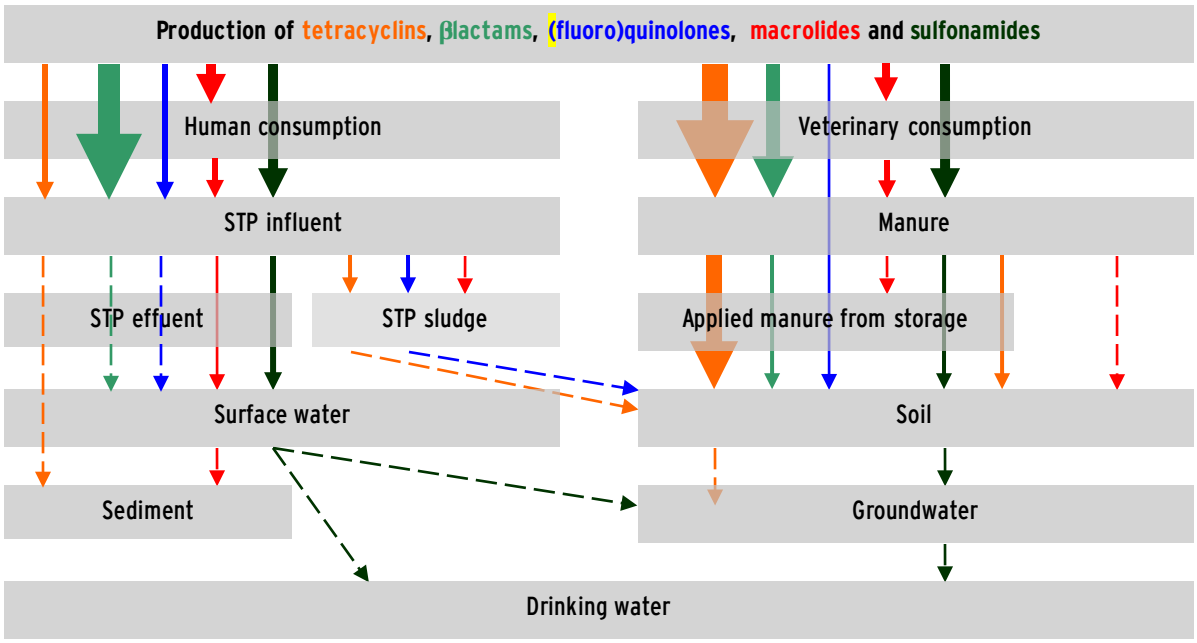

Fig. 2: Qualitative illustration of the fate of the most relevant classes of antibiotic classes in the environment

Table 4: Occurrence of antibiotics in the environment

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Chloramphenicol	amphenicols	0.56 (Monteiro and Boxall 2010)[a] <0.006-0.069 (Verlicchi et al. 2012)[a]			Nd[c]-0.06 (Monteiro and Boxall 2010)[r]	
Cefaclor	cephalosporins	0.009 (Verlicchi et al. 2012)[a]				
Cefalexin	cephalosporins	Nd-0.33 (Verlicchi et al. 2012)[a]				
Cefotaxime	cephalosporins	Nd-0.034 (Verlicchi et al. 2012)[a]				
Ciprofloxacin	fluoroquinolones	<0.020-0.251 (Monteiro and Boxall 2010)[a] 0.007-2.37 (Verlicchi et al. 2012)[a] 0.43 influent-0.072 effluent (Golet et al. 2002)[m] 31 000 (Larsson et al. 2007)[s] 0.28-0.45 effluent (Luo et al. 2011)	0.028 (Bergmann 2011)[m]	0.0269 -0.1198 (Li et al. 2011)[r]	Nd[c] – 0.03 (Monteiro and Boxall 2010)[r] Nd-1.7 (Luo et al. 2011)[e]	
Enoxacin	fluoroquinolones	0.01-0.03 (Monteiro and Boxall 2010)[a]			0.06 (Bergmann 2011)[m]	
Enrofloxacin	fluoroquinolones	0.01 (Verlicchi et al. 2012)[a] >9.4 (Babić et al. 2006)[s] 210 (Fick et al. 2009) [s]	8.3 (Bergmann 2011)m	0.0994-1.3476 (Li et al. 2011)[r] 3.8 (Bergmann 2011)[m]		

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Flumequine	fluoroquinolones			0.0011-0.0025 (Montforts et al. 2007) 0.0069 (Tamtam et al.)[m]	Nd (Monteiro and Boxall 2010)	
Lomefloxacin	fluoroquinolones	0.13-0.32 (Monteiro and Boxall 2010)[a] 0.22-0.32 (Verlicchi et al. 2012)[a]		0.0074-0.00137 (Li et al. 2011)[r]		
Norfloxacin	fluoroquinolones	0.03-0.112 (Monteiro and Boxall 2010)[a] 0.007-0.21 (Verlicchi et al. 2012)[a] 25 (Fick et al. 2009)[s]		0.0619-0.1502 (Li et al. 2011)[r]	0.12 (Monteiro and Boxall 2010)[c]	
Olfloxacin	fluoroquinolones	0.045-0.600 (Monteiro and Boxall 2010)[a] 0.019-0.86 (Verlicchi et al. 2012)[a]			0.033-0.306 (Monteiro and Boxall 2010)[r]	
Clindamycin	lincosamides	0.005 (Verlicchi et al. 2012)[a]			2.0 (Bergmann 2011)[m]	
Lincomycin	lincosamides	0.0305 (Monteiro and Boxall 2010)[a,c] 0.05-0.06 (Verlicchi et al. 2012)[a] 568 (Sim et al. 2011)[s]		0.046–0.117 (Kuchta et al. 2009)[r]	0.008-0.84 (Kuchta and Cessna 2009)[a, f] 0.054 (Watanabe et al. 2010)[d, k] 0.001-0.73 (Monteiro and Boxall 2010)[r]	Nd-1.9 (Watanabe et al. 2010)[r]

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Azithromycin	macrolides	0.085d-0.255 (Monteiro and Boxall 2010)[a] 0.04-0.38 (Verlicchi et al. 2012)[a]			0.58 (Bergmann 2011)[m]	
Clarithromycin	macrolides	<0.050-0.536 (Monteiro and Boxall 2010)[a] 0.15-0.46 (Verlicchi et al. 2012)[a]			Nd[c] – 0.26 (Monteiro and Boxall 2010)[r]	<0.02 (Monteiro and Boxall 2010)
Erythromycin (hydroxy)	macrolides	<0.010-6.00 (Monteiro and Boxall 2010)[a] 0.009-2.77 (Verlicchi et al. 2012)[a]			0.0032[c] – 1.70 (Monteiro and Boxall 2010)[r]	0.049 (Monteiro and Boxall 2010)[d]
Roxithromycin	macrolides	Nd-1.0 (Monteiro and Boxall 2010)[a] 0.01-5.0 (Verlicchi et al. 2012)[a]			Nd[c] – 0.56 (Monteiro and Boxall 2010)[r]	<0.02 (Monteiro and Boxall 2010)
Tylosin / Tilmicosin	macrolides	Nd (Verlicchi et al. 2012)[a]			0.0025 (Watanabe et al. 2010)[d, k]	
Spyramicin	micromonospora			0.19 (Bergmann 2011)[m]	0.0098[c] – 0.0742 (Monteiro and Boxall 2010)[r]	
Metronidazole	nitroimidazoles	0.055-0.56 (Verlicchi et al. 2012)[a]				
Amoxicillin	penicillins	0.0047 (Monteiro and Boxall 2010)[c] 0.007 (Verlicchi et al. 2012)[a]			<0.002-0.061 (Montforts et al. 2007)[r] 0.1 (Bergmann 2011)[m]	
Cloxaxillin	penicillins	0.001 (Verlicchi et al. 2012)[a]				

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Penicillin G	Penicillins	1.68 (Li et al. 2008b)[s]			0.31(Li et al. 2008b)[s]	
Nalidixic acid	quinolones			0.0221 (Tamtam et al.)[m]		
Oxolinic acid	quinolones			0.0059 (Tamtam et al.)[m]		
Sulfacetamide	sulfonamides	0.064c-0.151 (Monteiro and Boxall 2010)[a]				
Sulfachloropyridazine (Sulfaclozine)	sulfonamides	<0.03-0.14 (Verlicchi et al. 2012)[a]			0.005 (Arikan et al. 2008)[m] Nd (Bergmann 2011)	
Sulfadiazine	sulfonamides	Nd-0.019[c] (Monteiro and Boxall 2010)[a] 0.07 (Verlicchi et al. 2012)[a]	91 (Martinez-Carballo et al. 2007)& (Bergmann 2011)[m]	0.0134-0.0855 (Li et al. 2011)[r] 0.06 (Bergmann 2011)[m]	0.23 (Hamscher et al. 2006)[m] <0.0005-0.0028 (Montforts et al. 2007)[r]	<20-1160 (Monteiro and Boxall 2010)[r]
Sulfadimidine (Sulfamethazine)	sulfonamides	Nd-0.363 (Monteiro and Boxall 2010)[a] >400 (Babi et al. 2006)[s] 9.03 (Sim et al. 2011)[s]		0.0055-0.074 (Li et al. 2011)[r] 0.036 (Watanabe et al. 2010)[l, m] 0.06 (Bergmann 2011)[m]	8.6 (Watanabe et al. 2010)[k, m] <0.001-0.22 (Monteiro and Boxall 2010)[r] 0.006 (Arikan et al. 2008)[m]	0.11-3.6 (Watanabe et al. 2010)[r] <20 – 900 (Monteiro and Boxall 2010)[r]
Sulfadimetoxine	sulfonamides	<0.01-0.7 (Verlicchi et al. 2012)[a]	10 (Aust et al. 2008)[g, m] 20 (Martinez-Carballo et al. 2007)[m]	0.0077 (Hamscher et al. 2006)[h, d] 4.9-40.4 (Li et al. 2011)[r]	0.14-0.88 (Hamscher et al. 2006)[r] 0.9 (Watanabe et al. 2010)[d, k] <0.001-0.009 (Arikan et al. 2008)[m]	0.01-0.13 (Watanabe et al. 2010)[r]
Sulfaguanidine	sulfonamides					<20-1600 (Monteiro and Boxall 2010)[r]

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Sulfamerazine	sulfonamides			0.016-0.0935 (Li et al. 2011)[r]	Nd – 0.19 (Monteiro and Boxall 2010)[r] 0.005-0.694 (Arikan et al. 2008)[r] 0.01 (Bergmann 2011)m	
Sulfameter	sulfonamides			0.0514-0.1204 (Li et al. 2011)[r]		
Sulfamethizole	sulfonamides				0.005 (Arikan et al. 2008)[m]	<20 – 330 (Monteiro and Boxall 2010)[r]
Sulfamethoxazole	sulfonamides	Nd-2.140 (Monteiro and Boxall 2010)[a] Nd-0.84 (Verlicchi et al. 2012)[a] 13.7 (Sim et al. 2011)[s]		0.0235-0.0545 (Li et al. 2011)[r] 0.0062 (Watanabe et al. 2010)[l, m] 0.0025 (Tamtam et al.)[m] Nd-1.15 (Bergmann 2011)[p]	0.002 (Arikan et al. 2008)[m] <0.001-0.056 (Montforts et al. 2007)[r] 4.9 (Watanabe et al. 2010)[k, m] Nd-1.9 (Monteiro and Boxall 2010)[r] <0.001-0.007 (Arikan et al. 2008)	0.037 (Avisar et al. 2009)[d] 0.17 (Fram and Belitz 2011)[d] Nd-0.5 (Bergmann 2011)[p]
Sulfapridine	sulfonamides	0.081c –0.228 (Monteiro and Boxall 2010)[a] 0.02-1.11 (Verlicchi et al. 2012)[a]				
Sulfathiazole	sulfonamides	0.005 (Verlicchi et al. 2012)[a] 24.6 (Sim et al. 2011)[c]			<0.001 (Monteiro and Boxall 2010) 0.004 (Arikan et al. 2008)[m] 0.01 (Bergmann 2011)[m]	

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Trimethoprim	dihydropyrimidine	0.009-1.760 (Monteiro and Boxall 2010)[a] <0.01-6.7	17 (Bergmann 2011)[m]	0.1 (Bergmann 2011)[m]	<0.001-0.003 (Montforts et al. 2007)[r] Nd – 0.71 (Monteiro and Boxall 2010)[j, r]	0.018 (Fram and Belitz 2011)[d]
Chlorotetracycline	tetracyclines	Nd (Monteiro and Boxall 2010) (Verlicchi et al. 2012)[a]	0.410 (Aust et al. 2008)[m] 0.1 (Hamscher et al. 2002)[m] 46 (Martinez-Carballo et al. 2007)[m] 0.33 (0.1-50.8) (Hölzel et al. 2010) 7.6 & 203 (Bergmann 2011)[m]	0.005 (Andreu et al. 2009)[m] 0.0046-0.0073 (Hamscher et al. 2002)[o] 0.0322-0.1046 (Li et al. 2011)[r] 0.013 (Hamscher et al. 2006)[e] 0.176 (Watanabe et al. 2010)[l, m] 0.82 (Bergmann 2011)[m]	1.5 (Watanabe et al. 2010)[k, m] Nd-0.69 (Monteiro and Boxall 2010)[r] <0.001-0.180(Arikan et al. 2008)[r]	
Democycline	tetracyclines	0.09 (Monteiro and Boxall 2010)[a]			Nd – 0.44 (Monteiro and Boxall 2010)[r]	
Doxycycline	tetracyclines	0.038[c] – 0.09(Monteiro and Boxall 2010)[a] Nd- 0.064 (Verlicchi et al. 2012)[a]	0.29 (0.1-0.7) (Hölzel et al. 2010)[n]	0.012 (Hamscher et al. 2002)[m]	Nd – 0.08 (Monteiro and Boxall 2010)[r] <0.001-0.143(Arikan et al. 2008)[r]	

Antibiotic	Class	Wastewater influent and effluent (µg/L)[b]	Manure (mg/kg dry weight)	Soil (mg/kg dry weight)	Surface water (µg/L)	Ground water (µg/L)
Oxytetracycline	tetracyclines	0.038[c] – 0.09(Monteiro and Boxall 2010) [a] Nd- 0.064 (Verlicchi et al. 2012)[a]Nd (Monteiro and Boxall 2010)[a] Nd-0.02 (Verlicchi et al. 2012)[a] 19 500 (Li et al. 2008a)[s]	29 (Martinez-Carballo et al. 2007) & (Bergmann 2011)[m] 0.14 (0.1-0.9) (Hölzel et al. 2010)[n]	0.015 (Andreu et al. 2009)[m] 0.2 (Karci and Balcioglu 2009)[m] 0.0096-0.0797 (Li et al. 2011) [r] 0.109 (Watanabe et al. 2010)[l, m] 0.322 (Bergmann 2011)[m]	<0.002-0.014 (Montforts et al. 2007)[r] 0.66 (Watanabe et al. 2010)[k, m] Nd-0.34 (Monteiro and Boxall 2010)[r] <0.001-0.388 (Arikan et al. 2008)[r] Nd-1.34 (Lindsey et al. 2001)[r] Nd-4.2, average 0.004 (Luo et al. 2011)[r]	
Tetracycline	tetracyclines	Nd-1.00 (Monteiro and Boxall 2010)[a]	4.0 (Hamscher et al. 2002)[m] 23 (Martinez-Carballo et al. 2007)[m] 0.71 (0.1-46.0) (Hölzel et al. 2010)[n] 0.132, 66.0 (Bergmann 2011)[m]	0.018 (Andreu et al. 2009)[m] 0.15 (Hamscher et al. 2005)[d] 0.086-0.199 (Hamscher et al. 2002)[o] 0.0441-0.0744 (Li et al. 2011) [r] 0.105 (Watanabe et al. 2010)[l, m] 0.395 (Bergmann 2011)[m]	0.11 (Watanabe et al. 2010)[k] Nd – 0.11 (Kolpin et al. 2002, Monteiro and Boxall 2010) [r,t] <0.001-0.005 (Arikan et al. 2008)[r] Nd-1.9, average 0.003 (Luo et al. 2011)[r]	

a Effluent data, presented in minimum to maximum values (ranges).

b Two references, Verlicchi et al. (2012) and Monteiro and Boxall (2010), are both extensive reviews, so the source data within these reviews partially overlap

c Median value

d Mean value

e Observed in sediment

f Observed in snowmelt (surface run off water)

g Concentration on dry weight basis

h Observed in sediment

i Groundwater under manure lagoons

j Groundwater well

k Observed in manure lagoon water

l Observed in manure lagoon sediment

m Maximum values

n Median of positive detections (minimum and maximum value)

o Maximum values for different sub-layers of soil

p Large aggregation of data from Germany also includes dry manure (12-20% dry matter) (Bergmann 2011)

q Mean of positive detections

r minimum to maximum values

s in wastewater or downstream of antibiotic production site

t wrongly cited in Monteiro and Boxall (2010) (0.14)

Nd = not detected

3 The role of antibiotic residues for the development and dissemination of antibiotic resistance in environmental media

3.1 Introduction and scope

Antibiotic resistant bacteria are present in environmental media, most importantly soil, without human influence. It is generally thought that the synthesis of antibiotics by soil microorganisms provides them with an advantage over their competitors. Recently, though, antibiotics have also been discussed to have additional functions as cellular communication means (Martinez 2008).

Still, human activities can give rise to an increase in the presence of antibiotic resistance in the environment (e.g. Finley 2013). Antibiotic usage in human and veterinary pharmaceutical practice is known to increase antibiotic resistance, as reflected in the resistance development of animal and human pathogens. Both human and veterinary use can affect the environment, with water as most important environmental reservoir receiving human antibiotics (through human excretions entering the wastewater system) and the terrestrial environment receiving veterinary antibiotics (through the application of manure of antibiotic-treated farm animals, or the presence of animals on pasture). The presence of antibiotic resistance in the environment has therefore been investigated widely during the last years.

It has often been assumed that the antibiotic residues present in the environment can increase the environmental level of resistance. However, the input of resistant (intestinal) bacteria with manure or wastewater is a parallel process which can increase the amount of resistance in the environment. As human and animal excrements simultaneously contain both, antibiotic residues and resistant bacteria, it is generally difficult to study the relative effect of either the presence of resistant bacteria or antibiotic residues. Generally speaking, antibiotic residues might have several effects (see also Figure 3):

- The selective pressure of antibiotic residues leads to an increase in antibiotic resistance in the environment, possibly including selection of resistant species originating from gene transfer from naturally resistant bacteria or after mutations
- Animal manure and human wastewater contain resistant bacteria (contributed to by the selective pressure of antibiotics during animal and human pharmacotherapy), which can enter the environment. The presence of antibiotic residues maintains a selective pressure and therefore increases survival of these resistance carriers.
- As resistance is often located on mobile genetic elements, the selective pressure exerted by antibiotic residues in the environment confers a selective advantage on organisms that acquire these genetic elements. Transfer of mobile genetic elements might occur from and to resistant enteric bacteria introduced with human / animal waste. It has also been shown that the mutation rates or horizontal transfer rates can be increased in the presence of specific antibiotics,

thus antibiotics do not only select for existing resistant subtypes, but also increase the rate of their formation (Andersson and Hughes, 2012).

- It has to be noted that the 'costs' of resistance in terms of fitness can be so low that survival of resistance genes is possible without selective pressure (Dahlberg and Chao 2003; Moritz and Hergenrother 2007).

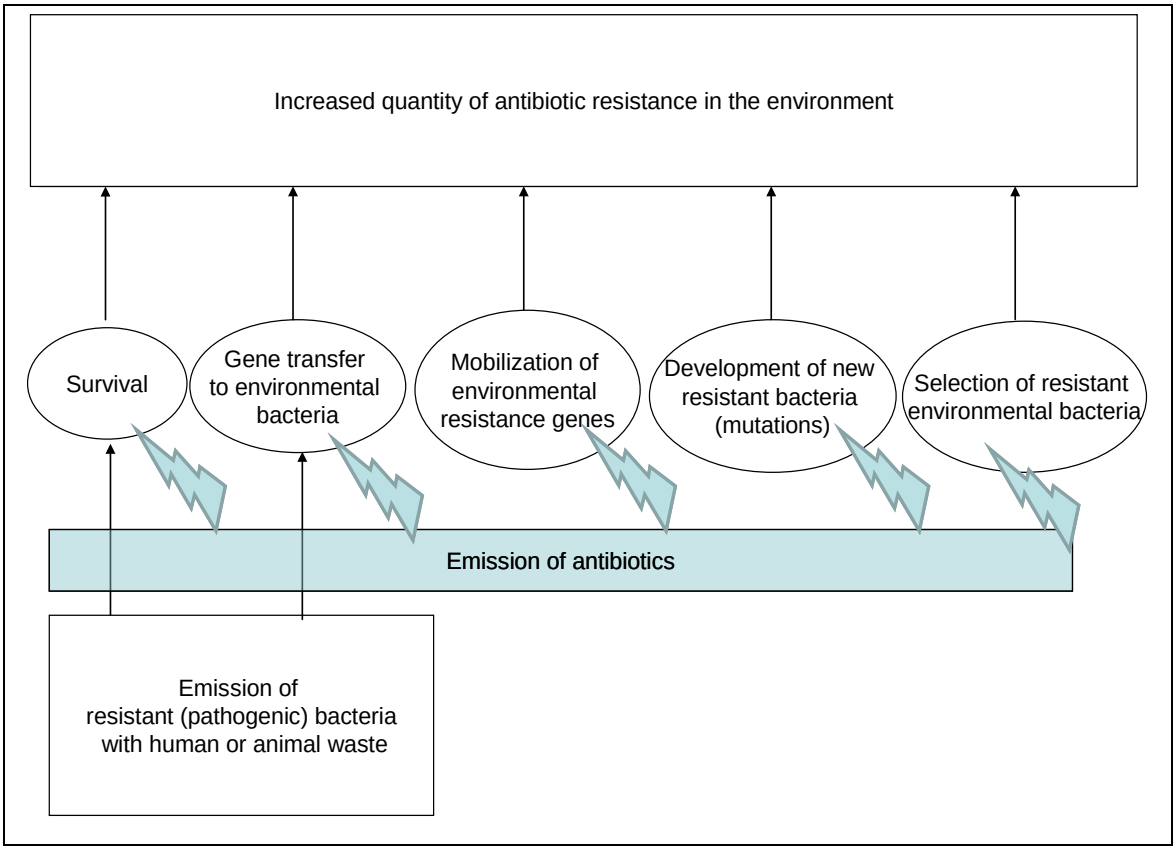

Fig. 3: Possible effects of antibiotics on resistance in the environment

In this section, studies on the role of antibiotic residues for resistance development or dissemination have been identified by means of a literature study which is described in the section 3.1 In addition, indirect evidence for the effects of antibiotic residues on resistance development is described and the natural background of resistance is considered. The following subsections contain a discussion of the available literature including the following questions:

1. Can antibiotic residues affect the development and dissemination of antibiotic resistance?
2. Can the role of antibiotics be distinguished from the input of resistant bacteria with e.g. manure or sewage?
3. Are sufficient data available to derive threshold values for an increase in resistance for selected antibiotics?
4. Can effects on resistance in environmental compartments be expected at environmentally realistic concentrations?

5. Can different effects of antibiotics (such as effects on mutation or gene transfer in environmental bacteria or survival of fecal bacteria, see Figure 3) be distinguished?

3.2 Role of antibiotics for resistance development in the environment (literature study)

A literature search was set up through a combination of strings for antibiotic resistance, environmental compartments (such as manure, slurry or surface water), and antibiotic compounds. Literature data bases searched included scopus, Web of Science and PubMed. This initial search strategy yielded a high number of publications (1500) in which both "resistance" and the antimicrobial compounds occurred. From those, publications were selected manually that not only investigated "mixed" samples (thus, manure which might contain both resistant bacteria and antibiotic residues), but also explicitly studied the effect of antibiotic residues. Overall, about 20 studies were found that fulfilled this criterion.

The number of studies directly investigating the role of antibiotic residues is very limited. Most publications have rather investigated whether the application of manure increases the likelihood of encountering resistance in groundwater or in manure-fertilized soils (Koike et al. 2007; Zhou et al. 2009), or whether the presence of resistance in surface water is predominantly the result of animal or human sources, i.e. animal lagoons or sewage effluent (Storteboom et al. 2010a). However, in these studies, the role of antibiotic residues present in manure or sewage cannot be distinguished from the impact of resistant bacteria also present in the manure or sewage.

A handful of studies make use of study designs with antibiotics spiked to samples, thus enabling a comparison of the development amount of resistance with or without additional selective pressure. These studies have been analysed in detail with respect to the environmental matrices used, their methodology and investigated endpoints, with respect to the antibiotic classes and concentrations applied, and with respect to their outcome. An overview of all identified studies is given in Table 5. Annex I includes more information on the test methods for each study. Additional studies in which an increase in antibiotic resistance has been found exist. These have been performed in e.g. effluents of antibiotic production plants or aquaculture ponds and are thus likely to contain antibiotic residues, however the concentrations of antibiotics were not monitored in these studies (e.g. (Guardabassi et al. 1998; Tendencia and De La Peña 2001)).

A typical study setup consists of an environmental matrix which is amended with possible sources of resistant bacteria, such as manure, animal lagoon wastewater, or human wastewater. A part of the microcosms is then additionally spiked with antibiotics. Resistance is monitored after set timepoints. To illustrate such 'typical' designs, one aquatic and one terrestrial study are described below in more detail.

In (Engemann et al. 2008), the effect of two concentrations of oxytetracycline (25 and 250 µg/L) on the decay rate of tetracycline resistance genes over time was studied in aquatic microcosms with river water receiving wastewater from a beef feedlot lagoon. The aim was to elucidate whether bacterial hosts carrying resistance genes might die in

the water column, or whether resistance gene transfer might increase the amount of genes. Samples were taken both from the water column and from biofilms. The measured parameters were concentrations of 6 tetracycline resistance genes, measured by real-time PCR. The concentrations of oxytetracycline were verified by ELISA. It was found that the concentrations of all genes declined according to a first order process, while absolute concentrations of bacteria (measured by qPCR of the 16S rRNA gene) remained nearly stable. Within samples with the same abiotic regime (light or dark treatment), there was no significant difference between the first order decay rates of the gene abundance of oxytetracycline treated- and untreated samples. Thus, additional spiking of the samples with oxytetracycline did not favour the survival of resistant bacteria or gene transfer to other bacteria. Different decay rates were found for light and dark treatments.

In Heuer and Smalla (2007), soil microcosms were established from control soil, soil amended with manure only, and soil amended with manure and antibiotic (sulfadiazine at 10 and 100 mg/kg dry weight). The aim was to elucidate whether antibiotic residues influence the abundance, diversity and transfer potential of sulfonamide resistance genes. Samples were taken over a time course of 61 days. No chemical analysis of sulfadiazine was performed in this study. The measured parameters were the abundance of sulfadiazine-resistant bacteria, the abundance of the sulfonamide resistance gene sul1 (real-time PCR), and the frequency of sulfonamide resistance transfer. It was found that both concentrations of sulfadiazine increased all, the culturable resistant bacteria, the abundance of the resistance gene, and the transfer frequency (detected through mating experiments). However, manure addition alone (without added sulfadiazine) also had a significant effect on all three parameters.

When comparing all identified studies with respect to the environmental matrices studied, the majority of these focused on soil. Most often, samples are amended with animal manure or also septic tank effluents as a possible source of resistant bacteria. Studies without manure or sludge are rare (Čermák et al. 2008). Studies on the aquatic environment also exist, as well as a limited number of studies with wastewater communities or lagoons of animal waste.

While nearly all of the studies deliberately added antibiotics to the test matrix, about half of the studies used an antibiotic at one single concentration. The other studies applied a dose-response design including a range of increasing concentrations (see section 4.2).

In ecotoxicology, an analytical verification of toxicant concentrations is commonly required for studies of environmental effects of a given substance. In only a part of the studies identified, and more often in aquatic studies, were the spiked concentrations of antibiotics verified through analytical chemistry (e.g. (Engemann et al. 2006; Engemann et al. 2008; Knapp et al. 2008)) Also, data on the possible bioavailability of the antibiotics in these matrices is generally lacking.

The concentrations applied are sometimes beyond environmentally realistic concentrations (e.g. (Rysz and Alvarez 2004; Atoyan et al. 2007)), and most often around worst case concentrations (Heuer and Smalla 2007; Heuer et al. 2009; Heuer et

al. 2011b). Only few studies apply a sufficiently wide concentration range of the spiked antibiotics to distinguish between effective and non-effective concentrations (Schmitt et al. 2006; Stepanauskas et al. 2006).

The time frame of the studies varies between 7 and 180 days for both terrestrial and aquatic studies, with one study extending over 300 days (Rysz and Alvarez 2004). Median study length is 51 (terrestrial) and 40 days (aquatic studies).

With respect to methodology, most of the studies used either real-time quantitative PCR of resistance genes or culturing of resistant bacteria (either specific bacteria or media that do not select for specific bacterial species). Other methods include testing the presence of resistance genes by PCR and analysing the resistance pattern of retrieved bacterial isolates.

In more than half of the studies, an effect of antibiotics was established. In several studies, no statistically significant effect was found, and in some studies, some effect of antibiotics was seen, but not on all studied parameters (i.e. the effect was either limited in time or in size).

In summary, there is only a limited number of studies in which the effect of antibiotic residues can be distinguished from the effect of spiking with manure or sewage, which might potentially contain both resistant bacteria and antibiotic residues. These studies have most often spiked additional antibiotics to microcosms also amended with manure or sewage. Often, the range of antibiotic concentrations was not wide enough to also include environmentally relevant concentrations of antibiotics or concentrations without antibiotic effects. (Quantitative) PCR and cultivation of resistant bacteria have most often been used to establish effects on resistance. Effects of antibiotics on resistance development or dissemination could be established in more than half of the studies.

Table 5: Experimental studies on effects of antibiotics on resistance in the environment

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Binh 2007	Amoxicillin	soil amended with manure	8-18 days	10 and 100 mg/kg	exogenous isolation of resistance plasmids / gene abundance / resistance on R2A agar	log resistance quotient / intensity of resistance gene dot-blots / numbers of transconjugants	1 - SOME) short term effect of amoxicillin on ampR bacteria in 1/2 soils significant 2-NO) amoxicillin does not increase the intensity of bla-TEM blots above the intensity seen in manure-treated soils only 3-NO) no clear effect of amoxicillin on number of transconjugants	n.d.
Stepanauskas 2006	Ampicillin	surface water microcosms	7 days	0.1, 1, 10 mg/L	resistance in cultivable bacteria	resistance of isolated strains	YES: ampicillin statistically significantly increases ampicillin resistance at 10 mg/L (and gentamycin resistance at 10 mg/L)	n.d.
Yu 2009	ciprofloxacin	water / sediment mesocosms, some also containing sterile pig faeces or feed	120 d (sampling on 7 timepoints)	2 mg/L	resistance in isolates	% resistance in isolates	YES: increase in *E. faecalis* resistance from 0% to 65-90% after 3 days, then zero-order decline until 0-40% over 120 days	0.00003
Subbiah 2012	ceftiofur	soil microcosms saturated with calf urine	100 d	Equivalent to 13 mg/kg	selective effects	Survival kinetics of spiked resistant strain of *E. coli*	YES: concentration of ceftiofur-resistant *E. coli* spiked to the microcosms declines slowlier than of non-resistant *E. coli*	n.d.
Munoz-Aguayo 2007	Chlortetra-cycline	chemostats with river water samples, fed 1/10 LB broth	10 days (sampling on several timepoints)	8, 800, 32000 µg/L	resistance in cultivable bacteria / resistance genes present in chemostat water	total counts of resistant bacteria and resistance percentages among treatments	YES: more resistant bacteria and higher resistance% in the 800 µg/L treatment, but not in the 8 µg/L	0.0009

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Engemann 2006	Oxytetra-cycline	river water microcosms receiving feedlot (beef) lagoon wastewater	29 days	0, 25, 250 µg/L oxytetracycline	resistance genes / cultivable bacteria	gene abundance / 16S normalized gene abundance / percent resistant isolates	NO: no effect of both OTC concentrations on decay rates of genes / not stated or similar for resistant isolates	0.017
Engemann 2008	Oxytetra-cycline	aquatic mesocosms of river water receiving feedlot waste	14 days	250 µg /L oxytetracycline	resistance genes	gene abundance	NO: no effect of OTC on decay rates of genes	0.017
Knapp 2008	Oxytetra-cycline	mesocosms fed with lake water	around 60 days (sampling on several timepoints)	0, 5, 20, 50, 250 µg /L	resistance genes	normalized and total resistance genes, increase in normalized resistance genes over time	YES: abundances over time for sum of resistance genes increase (although only convincingly for highest treatment) / "selection rate", the first order rate constant for the increase of sum of resistance genes in time is also correlated with OTC levels	0.017
Yu 2009	Oxytetra-cycline	water / sediment mesocosms, some also containing sterile pig faeces or feed	120 d (sampling on 7 timepoints)	5 mg/L	resistance in isolates	% resistance in isolates	YES: increase in *E. faecalis* resistance from 10% to 100% after 3 days, then zero-order decline until 0-40% over 120 days	0.0008

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Li 2010	Oxytetra-cycline	WWTP treating effluent from OTC production, and its receiving river up- and downstream		19.5 mg/L in WWTP effluent, 377-641 µg /L in receiving river sites	resistance in cultivable bacteria / resistance genes present in isolates	percentage of resistance and multidrug resistance among isolates / MIC50 and MIC90 of isolates / carriage of resistance genes among isolates	YES: higher resistance percentage in isolates from WWTP and downstream river / higher MIC50 adn MIC90 / more multidrug resistance (resistance genes not compared with upstream river samples)	field study
Rodríguez-Sánchez 2008	Oxytetra-cycline and gentamycin	field plots of coriander	16 months (antibiotic application on four occasions)	1.6 kg/ha as spray	resistance in cultivable bacteria / resistance genes present in soil / exogenous plasmid isolation from soil	total counts of resistant bacteria and %resistance /	NO: no difference in resistant cultivable bacteria between treated and control plots / no difference in gene carriage	1
Kim 2007	tetracycline	sequencing batch reactors fed wastewater	51 days	250 µg /L tetraycline	resistance in cultivable bacteria	concentrations of tetR bacteria / tetR bacteria growth rates and production / percentage of tet resistance	YES / SOME: all 4 studied resistance parameters give significantly higher values under at least some SBR conditions in tet treated SBRs and for some bacteria	0.008
Hund-Rinke 2004	tetracycline	soil amended with and without pig slurry	26 weeks	5, 50, 500 mg/kg (dw)	resistance genes	presence / absence of resistance genes	NO: no difference in the amount of detected resistance genes between control treatments and treatments with tetracycline. In samples with additional manure spiking, more resistance genes were observed until 16 weeks.	0.0008

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Rysz 2004	tetracycline	soil flow-through columns	300 days	50 mg/L in column influent	resistance in cultivable isolates	total counts of resistant bacteria	YES: absolute concentration of resistant bacteria in effluent almost doubled, total counts reduced by almost 10 times	n.d.: influent concentration cannot be recalculated to soil concentration
Atoyan 2007	tetracycline	soil lysimeters receiving septic tank effluent	51 days (of which antibiotic spiking during the first 10)	5 mg/L	resistance in *E. coli* isolated from leachate / influent	Theta=fraction of resistant bacteria in lysimeter drainage / fraction of resistant bacteria in influent (which is septic tank effluent)	NO: in 1/2 treatmetns, nonsignificant increase in resistant E.coli during lysimeter passage seen, but overall, significant reductions during several dates during and after tetracycline administration to lysimeter influent	n.d.: influent concentration cannot be recalculated to soil concentration
Atoyan 2007	tetracycline	soil lysimeters receiving septic tank effluent	51 days (of which antibiotic spiking during the first 10)	5 mg/L	resistance in E.coli isolated from lysimeter soil	% resistant	SOME: % resistance of both *E. coli* and Streptococci declines over the time course, including during tetracycline treatment of influent, but fecal streptococci in aerobic lysimeter increases insignificantly from 9% to about 155	n.d.: influent concentration cannot be recalculated to soil concentration
Stepanauskas 2006	tetracycline	surface water microcosms	7 days	0.03, 0.3, 3, 30 mg/L	resistance in cultivable bacteria	resistance of isolated strains	YES: tetracycline statistically increases tetracycline resistance at 30 mg/L and ampicillin and gentamycin resistance at 3 mg/L	0.0006

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Schmitt 2006	tetracycline, oxytetra-cycline	soil microcosms amended wih manure	8 and 14 days	0, 0.5, 5, 15, 50, 150, 500, 1500 mg/kg oxytetracycline // 0, 0.1, 1, 10, 100 oxytetra-cycline // 0, 0.1, 1, 10, 100, 1000 mg/kg tetracycline	resistance genes by PCR	presence of resistance genes	SOME: some genes less often present in oxytetracycline unspiked soils compared to manure -only soils // NO: no further effect of oxytetracycline or tetracyclin on top of the effect of manure spiking (but difficult to detect, as manure effect was big)	
Quinlan 2011	tetracycline	Aquatic mesocosms	28 days exposure _+ 28 days recovery	0, 0.5, 1,10, 100 µg /L	resistance in cultivable bacteria	% resistant	YES: during the exposure period, all concentrations of tetracycline led to an increase in the % resistance (significant increases only observed at 0.5 µg/L., but not at higher concentrations). During the recovery period, 100 µg/L significantly increased the % resistance.	0.019-3.8
Cermak 2008	lincomycin	soil (forest soil)	40 days with 4 sampling events	0.050, 5, 500 mg/kg	resistance in cultivated bacteria /	unclear, no data given	NO: according to the discussion, no effect of lincomycin on % resistance in total bacteria, although data not shown	0.0002
Duffy 2011	streptomycin	soil	not stated - 3x repeated application of streptomycin	3 x commercial dose	presence of resistant bacteria after selective enrichment	presence of resistant bacteria in soil samples	NO - resistant bacteria present in control and streptomycin treated soil	n.d.: no MEC values

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Heuer 2007	sulfadiazine	soil microcosms amended with manure	61 days (sampling on two timepoints)	10, 100 mg/kg	resistance genes / cultivable bacteria / class1 integrons / transfer frequencies of sulfonamide resistance	total abundance resistant isolates / normalized gene abundance / presence of class1 integrons by PCR	YES 1): more resistant isolates, YES 2) 10-100 times more sul1 in SDZ+ manure treated soils as compared to manure-treated soils at day 31 and 62 - 10 mg/kg significant, YES 3) higher transfer frequencies in SDZ+ manure soils as compared to manure soils, 10 mg/kg significant	0.009
Heuer 2009	sulfadiazine	soil microcosms amended with manure	61 days (sampling on two timepoints)	10, 100 mg/kg	resistance genes / plasmid genes / exogeneous plasmid isolation and sequencing	normalized resistance genes / plasmid characterization	YES, 10-100 times more sul2 in SDZ+manure treated soil as compared to manure-treated soils at day 31 and 62 - 10 mg/kg significant. Less clear for traN	0.009
Heuer 2011	sulfadiazine	soil microcosms amended with manure	180 days (sampling on three timepoints)	10, 100 mg/kg	resistance genes	normalized resistance genes	YES, 10-100 times more sul1 and sul2 in SDZ+manure treated soil as compared to manure-treated soils 60 days after each repeated manure application	0.009
Norpoth 1989	sulfadimidine	Stable manure	1 week	4 mg/m2 stable surface	resistance in intestinal cultivable bacteria in sows held on a spiked stable surface	% resistance of total E.coli and % of multiresistant *E. coli*	YES: increase in the percentage of multiresistant E.coli after 1 day	n.d.
Westergaard 2001	tylosin	soil microcosms	60 days	2 mg/kg (dw)	resistance in cultivable bacteria	% resistance in isolates	YES: increase in resistance up to 60% (control: 0.8%), maintained at >30% for 20 days	n.d.

Source	Compound	Environmental matrix	Exposure duration	Compound concentrations	Method of resistance measurement	Parameter measured	Effects of antibiotics established?	MEC /effect concentration[a]
Pei 2007	mix of oxytetracycline, sulfamethoxazole, tylosin and monensin	microcosms of dairy lagoon water (other parameters tested: oxygen state and temperature)	140 days	each at 20 mg/L	resistance genes present in lagoon water	normalized resistance genes over time	YES: increase in sul resistance genes in antibiotic treatments vs background SOME: temporary increase in tet resistance in antibiotic treatments, however greatest increase in killed treatments NO: no influence on macrolide resistance genes	0.0002
Xiong 2015	mix of enrofloxacin, norfloxacin and ciprofloxacin	soil microcosms amended with manure	60 days	each at 5 mg/kg	resistance genes	normalized resistance genes (over time)	YES: quinolone resistance genes decline more slowly in the treatment with manure and quinolones as compared to the manure only treatment	n.d.: mixture of antibiotics
Xiong 2015b	mix ofa) 3 tetracyclines, b) 3 sulfonamides, c) 3 quinolones	surface water / sediment microcosms amended with manure	14 days	Each antibiotic at 1 mg/L	resistance genesin water and sediment	normalized resistance genes	YES: in creased relative abundance of several resistance genes as compared to the control (the control also received manure), but statistical significance not tested	n.d.: mixture of antibiotics
Berglund 2014	12 antibiotics	Constructed wetlands	460 days	10x wastewater concentrations	resistance genes present in sediment	Normalized resistance genes	NO: abundance of resistance genes did not differ between antibiotic-treated and control wetlands	n.d.

a ratio of MEC to lowest effect or highest no effect concentration

3.2.1 Indirect evidence for the role of antibiotics for resistance in the environment

Next to publications on the direct effect of antibiotics, information on the role of antibiotics for the development and dissemination of resistance in the environment can also be gained from studies on the transmission of resistant bacteria or resistance genes from sources like manure or sewage to the environment.

An investigation of the levels of cultivable resistant bacteria in farmland after manuring (Sengeløv et al. 2003) concludes that the selective pressure of manure-borne tetracyclines in the soil might be low, as there was no correlation between the predicted tetracycline and macrolide soil concentrations and the resistance level among 4 farms. In faeces droppings from antibiotic-treated steers monitored over several months, resistance genes showed similar survival than genes from untreated steers (Alexander et al. 2011), again pointing to a limited role of residues for resistance selection. In a study on the occurrence of resistance genes in a watershed impacted by both sewage effluent and agricultural effluent, Storteboom et al. (2010b) argue that the difference in resistance gene patterns between pristine sites and impacted sites points to a limited selection caused by antibiotic residues in the water, as otherwise, the resistance genes in the pristine sites would be more abundant. Last, in groundwater impacted by slurry lagoons, Aminov (2002) noted that the similarity of the resistance patterns in the faeces and in the impacted sites suggests little selective pressure, as otherwise, additional resistance genes might have been selected for in the impacted sites.

In summary, indirectly, limited effects of antibiotic residues at environmentally-relevant concentrations were assumed to occur in a handful of studies on levels of resistance in environmental matrices that received manure or sewage.

3.2.2 Natural background of resistance in the environment

Often, a certain degree of antibiotic resistance has also been found in the investigated studies in samples that had neither been treated with antibiotics nor with possible sources of resistant bacteria. The occurrence of resistance genes in environmental samples without influence of human activities or exposure to antibiotics has also been convincingly demonstrated in many other studies. One such example is the finding of a sulfonamide and streptomycin resistance gene in a *Pseudomonas* strain isolated from permafrost (Petrova et al. 2011). In this strain, the resistance gene was part of a mobile genetic element, namely a class 1 integron located on a transposon – in contrast to the idea that not the diversity of resistance genes, but their linkage with mobile genetic elements might have been increasing throughout the last decades. Moreover, an investigation into resistance genes coding for beta-lactamases in a metagenomic library obtained from Alaskan soils unlikely to be exposed to antibiotics also identified resistance genes, in this case genes that were only distantly related to known clinical genes (Allen et al. 2008).

To start with, bacterial species can be "intrinsically resistant" to an antibiotic, i.e. all (or almost all) strains of this particular species are normally not susceptible to an antibiotic. This is caused by structural or functional characteristics, leading e.g. to the inability of the drug to enter the bacterial cell or to the absence of the drug target. In contrast, "acquired" resistance can be defined as the emergence of resistance in a particular strain, which has previously been sensitive to the activity of this particular antibiotic (European Commission 1999b).

Resistance has been encountered in bacterial species that are natural producers of antibiotics. Initially, it has been hypothesized that the production of antibiotics might be part of a "chemical war" in environmental compartments, and specifically in soil (e.g. Waksman and Woodruff 1940; Martinez 2008). Soil microorganisms able to synthesize antibiotics might therewith gain an advantage against competitors. Resistance genes are thought to further improve fitness of the producers due to protection against their own products, which is supported by the finding that, in antibiotic producing strains, resistance genes often reside on gene clusters which also harbour antibiotic synthesis genes (Allen et al. 2010). Recently, though, different "natural" roles of antibiotics at lower, subinhibitory concentrations have been highlighted, such as involvement in cell-to-cell signalling (Martinez 2008, Fajardo 2008). Last, environmental bacteria contain genes that have a wide range of possible functions, of which antibiotic resistance is only one. For example, the gene family of multidrug efflux pumps can also detoxify metabolic intermediates and increase bacterial virulence (Fajardo and Martínez 2008; Martinez 2008). The diversity of resistance determinants in environmental compartments such as soil can thus be high.

It has been hypothesized, though, that the genetic organisation of resistance genes in 'natural' hosts and in pathogenic bacteria might be different. Exposure to higher concentrations of antibiotics as well as to other stressors can increase the frequency of mutations, gene transfer and recombination. By these processes, resistance genes can be transferred to mobile genetic elements such as integrons which lack the tight regulatory control that resistance gene promotors exert in the host organisms (Martinez 2008). For example, while genes encoding for extended-spectrum beta-lactamase enzymes (conferring resistance to cephalosporins) that are found in pathogenic bacteria isolated in clinical environments are mostly located on plasmids, environmental strains that potentially served as the first sources of these genes contain CTX-M genes on their chromosomes, but not on mobile genetic elements (Poirel et al. 2002).

In conclusion, there is a natural level of resistance in environmental compartments, on top of which effects of antibiotics might occur.

3.3 Discussion of the role of antibiotics for antibiotic resistance in the environment

There is clear and sufficient evidence that elevated concentrations of antibiotics in the environment can increase the level of antibiotic resistance, as demonstrated by the studies summarized in 3.2. Endpoints affected by antibiotics were

- an increase in the frequency of resistance or multidrug resistance in isolated bacteria (Stepanauskas et al. 2006; Kim et al. 2007; Yu et al. 2009; Li et al. 2010), or increased MIC_{50} values in isolated bacteria (Li et al. 2010),
- an increase in the absolute counts or the percentage of cultivable bacteria that are resistant (Rysz and Alvarez 2004; Heuer and Smalla 2007; Munoz-Aguayo et al. 2007),
- an increase in the absolute amount of resistance genes or the relative amount of resistance genes per total DNA (Heuer and Smalla 2007; Pei et al. 2007; Knapp et al. 2008; Heuer et al. 2009; Heuer et al. 2011b), or an increase in the rate by which the amount of resistance genes increases ((Pei et al. 2007; Knapp et al. 2008)), and
- an increase in the rate of horizontal transfer of resistance (Heuer and Smalla 2007).

Thus, antibiotics can affect both the development of resistance (Knapp et al. 2008) and the dissemination of resistance (Heuer and Smalla 2007). Mechanistically, the changes observed in the other studies can be caused by a prolonged survival or growth of resistant bacteria introduced with e.g. manure or wastewater, transmission of resistance from introduced bacteria to environmental bacteria, or resistance development in situ. A distinction of these processes on the basis of the given data is impossible.

Effects were observed both in aquatic and terrestrial test systems, and for the antibiotic classes of tetracyclines, sulfonamides and beta-lactams. Tetracycline effects were most often found in aquatic studies, while sulfonamide studies were most often conducted in soil.

As antibiotic resistance also naturally occurs in the environment, the question remains whether a possible increase of resistance induced by antibiotic residues is relevant when compared with the natural background. Here, the studies in which an antibiotic effect was established suggest that while the diversity of resistance in the environment might be too high to be changed by antibiotic residues, the quantity of resistance (abundance of resistance genes in the total population, or abundance of certain resistant species) might indeed change.

In summary, there is clear evidence that antibiotics can increase the amount or dissemination of antibiotic resistance in the environment at sufficient concentrations.

3.3.1 Can a main effect of antibiotics be identified?

The following effects that antibiotic residues might have in the environment cannot be distinguished in nearly all of the available studies:

- selection and/or induction of resistance in environmental bacteria (through mutations or gene transfer)

- providing a selective advantage for resistant bacteria / genes stemming from manure or WWTP sludge (survival of clones)
- providing a selective advantage for gene transfer between resistant bacteria and environmental bacteria (here, selected publications are available (Heuer and Smalla 2007; Rodríguez-Sánchez et al. 2007)

Thus, there is yet insufficient evidence to estimate the degree of resistance occurring/ building up in environmental bacteria as compared to the survival of intestinal bacteria. Many analyses focused on genes, of which the host is not known (and could be an environmentally-adapted bacterium or an enteric bacterium). Other publications only focused on intestinal bacteria (e.g. *E. coli* (Atoyan et al. 2007)), or used nonselective media allowing for the growth of both enteric and environmental bacteria. Thus, the amount of resistance located on enteric or environmental bacteria cannot be distinguished in the available studies.

These different effects of residues might not lead to different regulatory decisions. However, resistance genes on mobile genetic elements which can be transferred between different species of bacteria (such as plasmids or transposons) might represent the highest human risks as they might be transferred to human pathoges most easily. Such resistance genes on mobile genetic elements might be selected for through each of the three before mentioned effects.

3.3.2 Antibiotic residues versus input of resistant bacteria

The studies evaluated in sections 3.2-3.4 were successful in establishing the specific effect of the presence of antibiotic residues in the environment, on top of the effect of transmission of resistant bacteria with animal- or human-borne matrices like manure or wastewater. Thus, a test setup of spiking environmental matrices with both, sources of resistant bacteria and antibiotics themselves, is successful in 'isolating' the role of antibiotics. However, manure or wastewater used as source of resistant bacteria can also contain additional antibiotic residues, the effect of which cannot be separated from the effect of input of resistant bacteria.

However, the input of resistant bacteria and / or resistance genes with such matrices also significantly influenced the amount of resistance found in the test systems, as e.g. shown in studies by Heuer (Heuer and Smalla 2007; Heuer et al. 2009), (Hund-Rinke et al. 2004) and (Schmitt et al. 2006). There, resistance in soils was compared with resistance in soils spiked with manure (additional treatments consisted of manure-spiked soils additionally spiked with antibiotics). In these studies, a significant effect of manure alone was noted on the amount of sulfonamide resistance genes (Heuer and Smalla 2007; Heuer et al. 2009; Heuer et al. 2011b), or on the diversity of tetracycline resistance genes (Hund-Rinke et al. 2004; Schmitt et al. 2006).

Note that there are plenty more investigations analysing the 'gross' effect of input of resistant bacteria and antibiotic residues, without enabling to distinguish the effect of

both. Relevant reviews on this topic include (Baquero et al. 2008; Gaze et al. 2008; Chee-Sanford et al. 2009a; Kümmerer 2009; Heuer et al. 2011a).

In summary, while it is possible to use test designs exclusively analysing the role of antibiotic residues for environmental resistance, it is also important to include the possible increase of resistance in the environment through the introduction of resistant bacteria with manure or sewage, as these can increase the level of resistance in the environment significantly.

3.3.3 Threshold values for effects of antibiotics on resistance in the environment

Despite the existence of studies on the role of antibiotics for resistance in the environment, only four publications are suitable for the establishment of threshold values of antibiotics (Stepanauskas et al. 2006; Munoz-Aguayo et al. 2007; Knapp et al. 2008, Quinlan et al. 2011) – see section 3.2 for a summary of those studies). The main reason is that the study design of the majority of the studies does not allow for the identification of concentrations with and without statistically significant effects. In other words, while effects were found in a considerable number of studies, these studies were conducted with a too limited number of antibiotic concentrations to establish a threshold concentration (i.e. a LOEC). Although studies applying a wide range of concentrations do exist in additional studies, no or limited effects of antibiotic residues were found in these studies (Schmitt et al. 2006; Engemann et al. 2008).

A summary of the findings of the four abovementioned studies is shown in Table 6. All studies target aquatic environments, and focus on the group of tetracyclines (with one study also including ampicillin). NOECs are in the range of 5 µg/L – 1 mg/L.

Table 6: Experimental studies on effects of antibiotics on environmental resistance allowing for derivation of threshold concentrations.

Source	Com-pound	Test system	Endpoint	Statisti-cally significant effect on resistance at	Effect size at the concen-tration with significant effects	NOEC for increase in resistance [µg/L]	EC_{50} for increase in resistance	MEC surface water [µg/L]	Max. ratio of MEC to NOEC for resistance development[a]	Effect concentration in ecotoxicity tests with bacteria / cyanobacteria [µg/L][b]
Stepanaus-kas 2006	Tetra-cycline	surface water micro-cosms	% ampicillin resistance of isolated strains (an increase in tetracycline resistant bacteria was also found, but at higher concentra-tions)	3 mg/L	increase from 0% resistance to approx. 42% resistance	300	between 3 and 30 mg/L	Nd – 0.11 (US rivers, Kolpin et al. 2002, Arikan et al. 2008) [r] Nd-1.9, average 0.003 (Chinese rivers, Luo et al. 2011) 0.11 (US manure lagoon, Watanabe et al. 2010)	0.0004 (max. US surface water) 0.006 (max. Chinese river tributary)	24.1 (EC_{50}, *Vibrio fisheri*, Backhaus et al. 1997) 90 (EC_{50}, *Microcystis aeruginosa*, Halling-Sørensen 2000) 10 (NOEC, *Synechocystis* sp., Pomati et al. 2004)
Quinlan 2011	Tetra-cycline	surface water meso-cosms	% tetracycline resistance of isolated strains	0.5 µg/L (during exposure to tetra-cycline), 100 µg/L (during recovery)	increase in % resistant bacteria from 2% to 6% (4% to 12% during recovery)	no NOEC during exposure (lowest concentra-tion tested), 10 µg/L during recovery	n.d.	see above	0.0011-0.22 (max. US surface water) 0.02-3.8 (max. Chinese river tributary)	see above

Source	Com-pound	Test system	Endpoint	Statisti-cally significant effect on resistance at	Effect size at the concen-tration with significant effects	NOEC for increase in resistance [µg/L]	EC_{50} for increase in resistance	MEC surface water [µg/L]	Max. ratio of MEC to NOEC for resistance development[a]	Effect concentration in ecotoxicity tests with bacteria / cyanobacteria [µg/L][b]
Stepanaus-kas 2006	Ampicillin	surface water micro-cosms	% ampicillin resistance of isolated strains	10 mg/L	increase from 3-20% resistance to approx. 63% resistance	1 000	between 1 and 10 mg/L			0.2->200 000 (EC_{50}) / 0.31-100 000 (NOEC), 9 species of cyanobcteria, Ando et al. 2007)
Munoz-Aguayo 2007	Chlortetra-cycline	Chemo-stats with Missi-ssippi river water samples, fed 1/10 LB broth	total counts of resistant bacteria and resistance percentages	800 µg/L	increase in total counts of resistant bacteria by a factor of 100	8	n.d.	Nd-0.69 (US rivers, Monteiro and Boxall 2010, Arikan et al. 2008) Nd-1.5 (US manure lagoon, Watanabe et al. 2010)	0.09 (US surface water) 0.2 (US manure lagoon)	30 (EC_{50} aerobic sludge bacteria, Halling-Sørensen et al. 2002) 50 (EC_{50}, *Microcystis aeruginosa*, Halling-Sørensen 2000) 138 / 496 (EC_{50} limnic bacterial communities, Brosche et al. 2010)

Source	Com-pound	Test system	Endpoint	Statisti-cally significant effect on resistance at	Effect size at the concen-tration with significant effects	NOEC for increase in resistance [µg/L]	EC_{50} for increase in resistance	MEC surface water [µg/L]	Max. ratio of MEC to NOEC for resistance development[a]	Effect concentration in ecotoxicity tests with bacteria / cyanobacteria [µg/L][b]
Knapp 2008	Oxytetra-cycline	mesocos ms fed with lake water	sum of normalized amount of 4 resistance genes	250 µg/L	increase in sum of normalized resistance genes from approx. $5*10^{-5}$ to $15*10^{-5}$	50	n.d.	<0.002-0.014 (Dutch agricultural ditches, Montforts et al. 2007)[r] Nd-1.34 (US rivers, Lindsey et al. 2001, Monteiro and Boxall 2010, Arikan et al. 2008)[r] Nd-4.2, average 0.004 (Chinese rivers, Luo et al. 2011)	0.03 (US rivers) 0.084 (Chinese river tributaries)	207 (EC_{50}, *Microcystis aeruginosa*, Holten Lützhøft et al. 1999) 32-7000 (EC_{50}) / 3-780 (NOEC), 9 species of cyanobacteria, Ando et al. 2007)
Knapp 2008	Oxytetra-cycline	mesocos ms fed with lake water	selection rate (first order rate constant for the increase of the sum of resistance genes in time)	20 µg/L	increase in the selection rate from 0.015 to approx. 0.025	5	n.d.	See above	0.3 (US rivers) 0.84 (Chinese river tributaries)	See above

a: MEC values represent maximum surface water concentrations from row "MEC surface water"

b: all cyanobacterial tests and bacterial tests with mixed communities as reported in the Wikipharma database (www.mistrapharma.se/wikipharma-13497291), and additional literature

3.3.4 Antibiotic effects at environmentally realistic concentrations

The abovementioned four studies, in which threshold concentrations could be defined, showed that NOECs were a factor of 1.2-157 higher than maximum surface water concentrations in Europe and the US. In river tributaries in close proximity to outflow from a Chinese pig farm, in which high quantities of tetracyclines were measured, higher maximum MECs were found (4.2 and 1.9 μg/L for oxytetracycline and tetracycline, Luo et al. 2011), resulting in MEC/NOEC ratios of 0.006-0.84. One study in which no NOEC could be established because an effect occurred at the lowest tetracycline concentration tested found a significant increase in the percentage of tetracycline resistant bacteria at 0.5 μg/L (Quinlan et al. 2011), which is 3.8 times lower than the highest observed MEC in Chinese rivers. Moreover, antibiotic concentrations can be considerably higher in hot spots such as sewage sludge and manure (see table 4, and also table 7), although the concentrations measured in these hot spots cannot be directly compared to aqueous NOECs due to possible decreases of the bioavailable concentrations of antibiotics in these compartments due to sorption to solid phases. In addition, close to production site effluents in China, oxytetracycline concentrations exceeded effective concentrations by far (Li et al. 2008a). Thus, for tetracyclines, environmental concentrations in other regions and potentially also concentrations in manure and sewage sludge can approach effective concentrations, while surface water concentrations in well-mixed European rivers are less likely to contain tetracycline antibiotics at concentrations shown to affect resistance in the abovementioned studies. However, the number of studies summarized in table 6 is much too small to extrapolate to all antibiotic compounds and environmental compartments, given that only tetracyclines and ampicillin were studied, and that only aquatic tests were conducted.

Most of the other studies summarized in 3.2 have used antibiotic concentrations that are considerably higher than environmentally realistic concentrations. Moreover, a small number of publications did not find significant effects at concentrations about 60 times higher than measured environmental concentrations (Engemann et al. 2006; Čermák et al. 2008; Engemann et al. 2008). Also, three investigations under realistic concentrations did not find effects (Rodríguez-Sánchez et al. 2007; Duffy et al. 2011, Berglund 2014). Notably, one publication reported on effects of ceftiofur under a realistic scenario: when soil was saturated with calf urine containing 50 ug/L ceftiofur (a realistic concentration for the first days after application of ceftiofur to calves), the survival of a ceftiofur-resistant *E. coli* strain which was spiked to the microcosms was significally prolonged during two months, despite quick reductions in bioavailability (Subbiah 2012).

Next to studies on the direct effect of antibiotics, information can also be gained from studies on the transmission of resistant bacteria or resistance genes from manure or sewage to the environment. It has been found in a number of studies that the diversity of resistance genes in environmental samples impacted by manure or sewage is at maximum as high as the diversity in the sources (manure, sewage), which is taken as an indirect evidence for the fact that there is little selection of resistance by antibiotic

residues in the receiving environments – otherwise, one could expect selection of additional resistance genes (see 3.2.1).

The results from Table 6 can also be compared with collections of ecotoxicological data. One recent compilation (www.mistrapharma.se/wikipharma-13497291) lists effective concentrations of tetracycline, chlortetracycline and oxytetracycline on cyanobacteria in the µg/L range, which is slightly lower than the effective concentrations reported in Table 6. However, due to the low number of investigations reported in Table 6, it is difficult to state whether NOECs derived from cyanobacterial or bacterial ecotoxicological tests are generally protective for effects on resistance.

While direct experimental evidence for selective effects of antibiotic residues in the environment are limited, recent publications suggest indirect evidence for such a role of antibiotic residues. First, Tello et al. (2012) modelled the percentage of bacterial species possibly exposed to antibiotic residues at concentrations high enough to pose a selective pressure, using collections of antibiotic sensitivities. They assumed that concentrations >MIC_{50}, the median minimum inhibitory concentration of one bacterial species, would represent a selective pressure for resistance development. As 50% of the bacterial population of that species would be growth-inhibited at that concentration, a (more) resistant subpopulation would gain a selective advantage over these competitors. They derived the MIC_{50} from international collections of MIC values, and projected the percentage of all bacterial species that would experience a selective pressure from the single species MIC_{50}. However, the assumption that one species might experience selective pressures only at concentrations greater than the median MIC for that species might be inadequate, as selective pressures could be exerted on strains with a MIC lower than the median MIC. Still, for several antibiotics, a considerable proportion of the bacterial community was projected to be exposed to antibiotic selective pressure in environmental or animal-related compartments, when extrapolating from measured concentrations in these compartments. This held true for ciprofloxacin in river sediments and swine faeces lagoons and tetracycline in liquid manure and farmed soil.

Second, Gullberg et al. (2011) experimentally derived antibiotic concentrations that represent a selective pressure, based on the idea that selective pressures might occur at antibiotic concentrations that are lower than the abovementioned MIC. Methodologically, they performed growth competition experiments with pairs of isogenic (genetically similar) bacteria, of which one possessed a resistance gene, at a range of antibiotic concentrations to define the so-called “minimum selective concentrations (MSC)”. At concentrations higher than the MSC, antibiotics lead to a slight growth advantage of the resistant strain which could lead to enrichment in resistant organisms. They found that the MSC can be as low as 15 µg/L (tetracycline) and 100 ng/L (ciprofloxacin), which is 100-1000 times lower than the MIC_{50} determined in Tello et al. (2012). In similar growth competition experiments on maintenance of a plasmid carrying several resistance genes, the same group again found MSC concentrations lower than MIC concentrations for antibiotics. Furthermore, when several antibiotics were combined at concentrations lower than their MIC values, their selective effect increased in a synergistic manner, and the presence of heavy metals

also contributed to the overall selective effect (Gullberg et al. 2014). Selective effects at subinhibitory concentrations have also been detected by Mogre et al. (2014), who studied experimental evolution in *E. coli* and found that concentrations of 20-25% the MIC led to mutations conferring resistance to the aminoglycoside kanamycin. Taken together with the first publication (Tello et al. 2012), an effect of antibiotic residues on resistance development or proliferation at environmentally realistic concentrations appears even more likely. However, it might be more difficult than suggested by Tello et al. (2012) to extrapolate these results to environmental compartments in which significant sorption can occur. This is due to the fact that MIC and MSC assays are often performed in liquid media, in which the bioavailability of the antibiotics might be much higher as less sorption can occur than in soil, sediment or manure.

Table 7 summarises the minimum selective concentrations (MSC) determined by Gullberg et al. (2011) and the MIC_{50} and NOEC values of the total bacterial population determined by Tello et al. (2012), together with an overview of the measured environmental concentrations in aquatic environments for the two antibiotics for which MSC and MIC_{50} data have been published. For reference, concentrations in solid or semi-solid matrices are also included. However, as mentioned, the bioavailability of both tetracycline and ciprofloxacin in these matrices is probably much lower, such that the MSC and MIC_{50} values cannot directly be translated to the existence of a selective pressure in these environments.

For ciprofloxacin, WWTP effluent concentrations measured in several studies exceed the lowest determined MSC concentration, illustrating that the concentrations of ciprofloxacin in WWTP effluent might indeed reach concentrations sufficient to select for resistant bacteria.

Table 7: Comparison of minimum selective concentrations (MSC), community MIC_{50} and NOEC values for resistance, and measured environmental concentrations, all in µg/L [a]

Compound	MSC (minimum selective concentration)	MIC50 (median of minimum inhibitory concentrations)	NOEC for resistance	Measured aqueous environmental concentration, WWTP effluent	Measured aqueous environmental concentration, surface water	Measured environmental concentration, non-aqueous hotspot [µg/kg]
Ciprofloxacin	0.1 - 2.3	100	8	<0.020-0.251 (Monteiro and Boxall 2010) – several European countries, USA and Canada 0.007-2.37 (Verlicchi et al. 2012) – several countries 0.43 (influent) -0.072 (Golet et al. 2002) - Switzerland 0.28-0.45 (Luo et al. 2011) - China 31 000 (Larsson et al. 2007)– surface water under the influence of antibiotic production sites in India	Nd – 0.03 (Monteiro and Boxall 2010) - Italy and USA 0.06 (Bergmann 2011)- Germany Nd-1.7 (Luo et al. 2011) – Chinese river	340 (Luo et al. 2011) – Chinese animal faeces lagoons
Tetracycline	15	1585	145	Nd-1.00 (Monteiro and Boxall 2010) 0.11 (Watanabe et al. 2010)	Nd – 0.11 (US rivers, Kolpin et al. 2002, Arikan et al. 2008) [r] Nd-1.9, average 0.003 (Chinese rivers, Luo et al. 2011)	4.0 (German manure, Hamscher et al. 2002) [m] 23 (Austrian manure, Martinez-Carballo et al. 2007)[m] 0.71 (0.1-46.0) (German manure, Hölzel et al. 2010) 0.132, 66.0 (manure, Bergmann 2011)[m] 0.11 (US manure lagoon, Watanabe et al. 2010)

a: For more detail on the measured concentrations, consult Table 4. MSC, MIC_{50} and NOEC values taken from Tello et al. (2012) and Gullberg et al. (2011).

l: Observed in manure lagoon sediment

m: Maximum values

r: minimum to maximum values

In summary, there is only a very limited number of studies at environmentally relevant concentrations. While three studies found NOEC values for antibiotic effects on resistance at concentrations by a factor of 1.2-157 higher than maximum measured environmental concentrations, a number of other studies did not find effects at concentrations considerably higher than measured concentrations. Selective effects of one antibiotic were however noted in a realistic scenario (Subbiah et al. 2012. In addition, extrapolations from MIC collections suggest that antibiotics such as ciprofloxacin could exert a selective pressure at environmentally relevant concentrations. Thus, further studies using dose-response designs are required to determine effects of environmentally relevant concentrations of antibiotics on environmental resistance.

3.4 Conclusions

As shown through a literature review, antibiotics at sufficient concentrations can affect resistance development and dissemination in the environment. Effects on different endpoints, including the number of phenotypically resistant bacteria, the diversity and quantity of resistance genes and on the frequency of horizontal transfer of resistance genes have been found. In addition, introduction of manure and sewage can increase the amount of resistance found in the environment. In many studies, it is difficult to differentiate between the effect of resistant bacteria and the effect of antibiotic residues that enter the environment with manure and sewage.

Due to the limited number of studies with a concentration-response design, it is currently not possible to derive threshold values for antibiotic effects on the level of resistance in the environment. Overall, too few studies have used antibiotics at environmentally realistic concentrations. In summary, it cannot currently be established whether or not antibiotics might exert effects on resistance at environmentally relevant concentrations, while these effects cannot be excluded.

4 Test methods and testing concepts for resistance development and dissemination in the environment

In this section, test methods suitable for the detection of antibiotic resistance in the environment are shortly presented. Further, test designs will be discussed that are suitable for the derivation of threshold concentrations of antibiotics favouring resistance. The test methods will then be analysed for their suitability for use in regulatory assessments. Finally, research needs will be established.

4.1 Test methods

In the following, test methods that have been used in studies on the occurrence of antimicrobial resistance in the environment are briefly introduced, and an illustrative application is described.

4.1.1 Resistance profiling of bacterial isolates

Background: Resistance is studied in a collection of bacterial isolates, i.e. bacterial cells belonging to one species or one group of species with specific characteristics. These are tested for their ability to grow in the presence of antibiotics (phenotypic test), as most antibiotics act by inhibiting bacterial growth.

Principle: Bacteria are isolated from environmental matrices by means of culture techniques (growth on culture media). Solid matrices are mostly shaken with diluents to prepare an initial suspension, while bacteria in liquid matrices can be concentrated on filters if needed. Then, the (diluted) initial suspensions or the filters are placed on solid (or liquid) culture media and kept at conditions favourable for multiplication of bacteria until growth is visible. If selective media are used, only bacteria that belong to certain taxonomic affiliations or bacteria with certain phenotypic properties are isolated. Non-selective media serve for the isolation of bacteria of broader diversity. The bacterial isolates are then screened for the occurrence of resistance towards one or multiple antibiotics, most often by culturing the isolates on media with antibiotic susceptibility discs.

Endpoint % of isolates being resistant to a specific antibiotic

Example for application: Li et al. (2010)

In effluent from an oxytetracycline-producing plant as well as the receiving river (upstream and downstream), bacterial isolates were obtained with general, non-selective microbiological media (often used in aquatic microbiology). The isolates obtained were tested for their resistance pattern, and the % resistance was compared between the different sampling points. A significant influence of the effluent on river resistance was established.

4.1.2 Derivation of minimum inhibitory concentrations (MICs) of isolates

Background: Resistance is studied in a collection of bacterial isolates, i.e. bacterial cells belonging to one species or one group of species with specific characteristics. For these

isolates, the lowest concentration of antibiotics that inhibits their growth is determined (phenotypic test), as most antibiotics act by inhibiting bacterial growth.

Principle: Bacteria are cultured and isolated as above. Then, minimum inhibitory concentrations (the lowest concentration of an antibiotic that inhibit the growth of the tested bacterium) of one or various antibiotics are determined for each isolate through the use of broth microdilution techniques or antibiotic sensitivity discs. From the MIC values collected from all isolates, the concentrations that can inhibit 50% or 90% of the whole collection can be derived.

Endpoint: MIC values for the individual isolates, and MIC50 or MIC90 (minimum inhibitory concentration for 50 or 90% of the bacterial population)

Example for application: Li et al. (2010)

In effluent from an oxytetracycline-producing plant as well as the receiving river (upstream and downstream), bacterial isolates were obtained with general microbiological media (often used in aquatic microbiology). The MICs for 10 antibiotics were then analysed in the isolates. Significantly higher MIC50 and MIC90 values were found in the downstream river communities compared to the upstream communities.

4.1.3 Enumeration of resistant bacteria by selective plating

Background: The concentration of resistant bacterial isolates in an environmental compartment is determined and often compared to the total concentration of isolates of this species or group of species.

Principle: Bacteria are cultured and isolated as above. The media are additionally spiked with an antibiotic, only allowing resistant bacteria to grow. As control, the number of non-resistant bacteria is often determined as well (by use of the same media without antibiotic supplement).

Endpoints a) total counts (concentrations) of resistant bacteria in environmental media, b) percentage of resistance over total bacteria in environmental bacteria

Example for application: Munoz-Aguayo et al. (2007)

River water chemostats were set up with diluted nutrient broth as nutrient source and supplemented with three different oxytetracycline concentrations. A diluted general, unspecific medium (1/10 LB) supplemented with oxytetracycline was used to derive counts of resistant bacteria. A higher total number of resistant bacteria and a higher percentage of resistant bacteria among total bacteria were found at the two higher concentrations.

4.1.4 Detection of resistance genes in bacterial isolates

Background: Similar to “Resistance profiling of bacterial isolates”, with the difference that the isolates are tested for the presence of resistance genes (genotypic analysis) rather than for their ability to grow in the presence of antibiotics (phenotypic assay).

Principle: Bacteria are isolated from environmental matrices by means of culture techniques (growth on culture media). Solid matrices are mostly shaken with diluents

to prepare an initial suspension, while bacteria in liquid matrices can be concentrated on filters if needed. Then, the (diluted) initial suspension or the filters are placed on solid (or liquid) culture media and kept at conditions favourable for multiplication of bacteria until growth is visible. If selective media are used, only bacteria that belong to certain taxonomic affiliations or bacteria with certain phenotypic properties are isolated. Non-selective media serve for the isolation of bacteria of broader diversity. DNA is isolated from the isolates retrieved, and a screening for the presence of resistance genes by PCR follows. Most often, several different resistance genes are screened.

Endpoint: % of isolates carrying a specific gene

Example for application: Agerso (2005)

Gram-positive and Gram-negative bacteria were isolated from fecal samples from pig farms as well as from manured soils. In these isolates, the presence of a number of tetracycline resistance genes was analysed by PCR. tet genes were frequently found in isolates that also harboured class I integrons. However, in 67 of the tested 81 Gram-negative isolates, none of the three analysed *tet* genes could be found, although they were phenotypically resistant to tetracycline.

4.1.5 Qualitative detection of resistance genes in environmental DNA

Background: The gene pool of the total bacterial community is investigated through extraction of DNA, which is evaluated for the presence / absence of resistance genes.

Principle: DNA is isolated directly from environmental matrices (such as soil or aqueous bacteria concentrated on a filter), often including steps such as bead beating, precipitation of proteins and other cell debris, and DNA purification. The DNA is intended to reflect the total gene pool of the bacteria present in the sample (total community DNA). The community DNA is then screened for the presence of resistance genes by polymerase chain reaction (PCR), yielding information on the presence or absence of a specific gene at the given detection limit.

Endpoint: % of samples carrying a specific gene / total number of genes detected in a sample

Example for application: Schmitt et al. (2006)

Soil microcosms were set up with and without manure and additional tetracycline antibiotics (5 concentrations tested). After DNA extraction, the presence of 13 tetracycline resistance genes was tested by PCR. While only a limited number of tetracycline resistance genes was found in soil without manure, nearly all genes were found in manure itself and in soil samples amended with manure. The effect of additionally spiking with antibiotics was negligible.

4.1.6 Quantitative detection of resistance genes in environmental DNA

Background: The gene pool of the total bacterial community is assessed through extraction of DNA, in which the levels of resistance genes are analysed quantitatively.

Principle: Total DNA is isolated from environmental compartments as above. The presence of resistance genes in the community DNA is then analysed by real-time PCR, allowing for a quantification of the gene abundance. The abundance is often normalized to the total DNA content (e.g. through analysis of genes generally present in bacteria, such as 16s ribosomal RNA genes), in order to derive the relative gene abundance.

Endpoint: absolute / relative resistance gene quantities per sample unit

Example for application: Knapp et al. (2008)

Lake water mesocosms were set up and spiked with 4 different oxytetracycline concentrations (5 – 250 µg/L). In DNA extracted from the mesocosm water at different timepoints, the quantities of 5 tetracycline resistance genes were analysed. The sum of the abundance of all tetracycline genes increased with time.

4.1.7 Detection of resistance genes by means of arrays

Background: The presence of a multitude of resistance genes is investigated in the gene pool of the total bacterial community.

Principle: Total community DNA is extracted from the samples. With the help of micro- or macroarrays, on which probes for a multitude of resistance genes are spotted, resistance genes can be detected in a semi-quantitative way.

Endpoint: Number (and signal intensity) of detected resistance genes

Example for application: Patterson et al. (2007)

Pig faecal samples were collected in five European countries, and DNA was extracted from all samples and hybridized to a membrane on which probes for 23 tetracycline and 12 macrolide resistance genes were spotted. The presence of resistance genes was expressed as % of the intensity of 16S (a measure for the total bacterial DNA present). The % of tet(W), the gene that was encountered in the greatest number of samples, was lowest in Norwegian pig herds with low use of antibiotics, intermediate in organic herds from other countries, and highest in conventional or intensively-reared pig herds from other European countries.

4.1.8 Analysis of clone libraries

Background: The presence of functional resistance genes is investigated in the gene pool of the total bacterial community.

Principle: Total community DNA is extracted from the samples. With the help of plasmid (or fosmid) hosts, the DNA is transferred in small pieces to easily culturable bacteria. By screening of these bacteria for phenotypical resistance, an identification and analysis of yet unknown genetic elements encoding for resistance is possible.

Endpoint: number of new resistance mechanisms

Example for application: Allen et al. (2008)

Soil from a remote location in Alaska was used to extract DNA and prepare clone libraries (714 000 *E. coli* cells with plasmids containing pieces of the soil DNA). The clone library was screened for *E. coli* cells with resistance to beta-lactam antibiotics, and 14 clones were found which expressed beta-lactamase genes. These were characterized and found to be only distantly related to beta-lactamase genes found in clinical isolates.

4.1.9 High-throughput sequencing (metagenomic analyses)

Background: The presence of a multitude of resistance genes is investigated in the gene pool of the total bacterial community.

Principle: Total community DNA is extracted from the samples. With the help of new sequencing techniques, large amounts of genetic sequences of a length of 100-400 basepairs are obtained from the sample and screened against databases of resistance genes.

Endpoint: percentage of one resistance gene in the total DNA

Example for application: Kristiansson et al. (2011)

Sediment samples of a river receiving effluent of >90 pharmaceutical production plants were taken (upstream and downstream of the production plants) and total community DNA was extracted. The total DNA (the metagenome) was sequenced by high-throughput next generation sequencing. The abundance of several resistance genes increased by 0.8 to 4% from the upstream to the downstream location.

4.1.10 Extrapolation from published distributions of antibiotic sensitivity

Background: The mimimum inhibitory concentrations (MIC values) determined for many clinical isolates are used to derive selective concentrations

Principle: For several human pathogens and commensals, MIC values of individual clinical isolates are collected in international databases (e.g. the EUCAST, European Committee on Antimicrobial Susceptibility Testing, MIC distribution database). From these MIC values, species sensitivity distributions (SSDs) can be derived that collate data on antibiotic sensitivities of all strains. Predicted environmental concentrations can be compared with these SSDs, yielding the percentage of environmental bacteria encountering antibiotic concentrations beyond what their wild-type normally tolerates. Thus, the selective pressure of an antibiotic at that concentration can be predicted.

Example for application: Tello et al. (2012)

Species sensitivity distributions (SSDs) were calculated using MIC values of bacterial species included in the EUCAST database, and environmental concentrations of three antibiotics were compared with the SSDs constructed using the median MIC values for each species. It was assumed that concentrations above the median MIC might exert a selective pressure on the respective bacteria. The environmental compartments with the highest "potentially affected fraction" (thus, the proportion of bacteria encountering concentrations higher than their median MIC) are manure and river sediments. According to such estimations, the predicted environmental concentration

in soil (PECsoil), above which Phase II testing has to be performed according to the VICH (2000) and EMEA/CVMP (2008) guidance (100 μg/kg dry weight; see section 6.1), causes some 2-54% of bacteria encountering concentrations beyond their median MIC (depending on the antibiotic).

4.1.11 Evaluation of horizontal gene transfer of resistance genes

Background: The presence of mobile resistance genes (mostly resistance genes contained on plasmids) and the frequency of their transfer are investigated.

Principle: Soil (or slurry) is mixed with a bacterium (recipient) that can be selectively cultured (e.g. through a fluorescent marker and a combination of antibiotic resistance genes). After some time, the mixture is diluted and plated on agar plates allowing for the growth of the recipient with addition of an extra antibiotic. The recipient can only grow on these plates, if it has taken up genetic material (e.g. a plasmid) that contains a resistance mechanism for the extra antibiotic. By counting the original recipient and the transformants (the cells that have received additional genetic material), the frequency of horizontal transfer can be calculated.

Endpoint: Frequency of horizontal gene transfer (occurrence of transformants per recipient added)

Example for application: Heuer and Smalla (2007)

Soil microcosms were set up and received sulfadiazine at two different concentrations as well as manure from antibiotic-untreated pigs. After mixing a soil extract with a bacterial recipient (an *E. coli* strain marked with green fluorescent protein), bacteria were allowed to mate on a filter overnight. Recipient *E. coli* cells that have taken up a plasmid from soil or manure-borne bacteria were then enumerated by plating serial dilutions on plates that are selective for the recipient strain, amended with sulfadiazine. The number of transconjugation events was determined by counting the bacteria on selective plates with and without sulfadiazine.

4.1.12 Derivation of minimum selective concentrations (MSC)

Background: By competition experiments using similar resistant and non-resistant bacteria, the lowest concentrations of antibiotics exerting a selective pressure can be identified.

Principle: An isogenic pair of bacteria, of which one carries a specified resistance gene, is constructed. A 50:50 mixture of both bacteria is allowed to grow with a range of antibiotic concentrations. The MSC is determined as the concentration at which the resistant bacterium starts to outcompete the non-resistant counterpart.

Endpoint: MSC (minimum selective concentration)

Example for application: Gullberg et al. (2011) (but no application in environmental media yet)

4.2 Testing concepts – use of concentration-response designs

In regulatory ecotoxicology, test methods often include defining concentration-response relationships, i.e. define the dependence of an observed effect on concentrations of the causative agent. This is helpful for 1) determining concentrations with an expected effect that might still be tolerable (e.g. the EC_{10}, i.e. the effective concentration causing 10% effect, or the NOEC, i.e. the no adverse effect concentration, (European Commission 2011)), or 2) to show that no intolerable effects are to be expected at environmentally realistic concentrations. The same principle is followed in the authorization of veterinary and human pharmaceuticals when evaluating the 'safe window' between intended effect and adverse effects. To this end, a number of doses are evaluated in animal experiments and adverse effects are noted (e.g. (EMEA/CHMP 2010)).

This principle can also be used to investigate the dependence of an increase in resistance in environmental bacteria on the antibiotic concentration. Such a design ultimately would allow for deriving threshold concentrations of antibiotics that are expected to increase transmission or build-up of resistance. However, few experimental investigations have so far applied a concentration-response design. These are discussed in more detail below.

One investigation with a full concentration-response design including antibiotic concentrations with little effect on resistance is a study on the increase of tetracycline resistance genes in aquatic mesocosms (Knapp, 2008). Triplicate river water mesocosms were established and inoculated with pristine river water from an environmental study area and additionally amended with oxytetracycline (0, 5, 20, 50 and 250 µg/L). Oxytetracycline was spiked every 2-3 days in order to maintain its concentration. 5-6 times over 60 days, the abundance of six tetracycline resistance genes was determined in total community DNA. An increased amount of resistance genes (normalized to 16S genes) was found in the 250 µg/L treatment. As resistance also increased over time, most clearly so in the highest treatment, the authors fitted a first-order kinetic to the increase of the sum of all tetracycline resistance genes over time for each concentration. When plotting the first-order rate constants against the oxytetracycline concentrations, it appeared that this 'selection coefficient' indeed increased with increasing concentrations. The significance of these differences is not clearly stated, however, it can be inferred from the graphs shown that a statistically significant effect occurs at 250 µg/L and probably at 20 µg/L, but not at lower concentrations. The conditions of this experiment are relatively similar to environmental conditions with respect to the nature of the microcosms and size and physical parameters of the treatments. The concentrations applied were well-monitored, and the experiment duration was longer than the duration of the other two studies (see below). Interestingly, gene levels were quite variable, and for some concentrations, major changes only occurred at day 40 of the treatment. To conclude, the oxytetracycline concentration of 50 µg/L could be regarded as NOEC for the amount of resistance genes, while effects on the selection rate (the significance of which is not clearly stated) were already apparent at 20 µg/L. Maximum detected concentrations of oxytetracycline in surface waters are in the range of 0.14 µg/L (see section 3.5).

Stepanauskas et al. (2006) investigated the role of antibiotics and metals for the occurrence of antibiotic resistance in surface water microcosms. These were established using surface water of Sawannah River with its indigenous microflora. Additional spiking with possible sources of resistant bacteria (e.g. sewage effluent) was not performed. 7 Days after spiking the microcosms with a range of tetracycline, ampicillin and metal concentrations, the percentage of isolates resistant to 300 mg/L tetracycline or 100 mg/L ampicillin was determined among 10 randomly isolated bacteria from each of 3 replicate microcosms per treatment. Statistically significant effects of tetracycline were noted at 30 mg/L (increase from 0% resistance at 0, 0.03, 0.3, and 3 mg/L tetracycline to 42% tetracycline resistance). Also, tetracycline had a significant effect on cross-resistance to ampicillin and gentamycin at 3 mg/L and higher (increase from 3-20% ampicillin resistance at the lower concentrations to 63% ampicillin resistance at 3 mg/L). Exposure to ampicillin at 10 mg/L, but not at 0.01, 0.1, or 1 mg/L, significantly increased ampicillin resistance (from <24% to ca. 85%) and gentamycin resistance. It has to be noted that the number of isolates tested in this study (30 per concentration) is at the lower end, limiting the power of the study to detect significant effects. On the other hand, the antibiotic resistance increased in most cases in a concentration-dependent matter. With respect to the test concentrations, antibiotics were only added once, and their concentration had decreased to 20-60% of the nominal concentration by day 7 (conflicting information given in the paper). To conclude, concentrations of 0.3 mg/L tetracycline and 1 mg/L ampicillin can be regarded as NOEC for resistance in the endpoints studied in this paper. From visual interpolation of the data, EC50 concentrations are estimated to be between 3 and 30 mg/L for ampicillin and gentamycin resistance caused by tetracycline exposure, and between 1 and 10 mg/L for ampicillin resistance caused by ampicillin exposure. Maximum concentrations encountered in surface water are in the range of 0.14 μg/L for tetracycline, there is no data available on environmental concentrations of ampicillin (see 3.5). Finally, concluding from the strong increase of resistance observed in microcosms spiked with cadmium and nickel, the authors argue that metal contamination might equally promote antibiotic resistance in the environment, at least in industry and mining-impacted environments.

Munoz-Aguayo et al. (2007) also made use of river water microcosms. In this case, microcosms were set up by amending river water inocula with diluted nutrient broth. The microorganisms were first allowed to grow, and after addition of 8 and 800 μg/L of chlortetracycline, were left for 10 days in chemostats. Chlortetracycline was spiked twice daily in order to maintain the concentrations, which was verified by ELISA. Six replicate microcosms were set up in time for the control and the 8 μg/L level. Each day, the counts of total bacteria and bacteria resistant to 16 mg/L chlortetracycline) were determined. The total counts of chlortetracycline resistant bacteria and the ratio of resistant to total bacteria were statistically significantly increased in the 800 μg/L treatment, but not in the 8 μg/L treatment. Absolute numbers of chlortetracycline resistant bacteria were around 10^6 (SD around 1) in the control and 8 μg/L treatment and around 10^8 for the 800 μg/L treatment, concentrations thus had increased by a factor of approximately 100. Also, the diversity of tetracycline resistance genes was analysed in total community DNA. While between 2 and 5 out of the 9 investigated

genes were found in the controls, a higher diversity was found both in the 8 µg/L treatment (between 3 and 5) and 800 µg/L treatment (between 4 and 6 genes). While the concentrations of chlortetracycline were well controlled in this experiment, the use of chemostats amended with growth medium makes it difficult to translate these findings to natural ecosystems. To conclude, the concentration of 8 µg/L chlortetracycline could be regarded as NOEC according to this study, while at 800 µg/L, the counts of resistant bacteria were increased 100 fold. Maximum concentrations of chlortetracycline found in surface water amount to 0.69 µg/L (note that other tetracycline antibiotics can also contribute to selective pressures for tetracycline resistance).

Heuer and Smalla (2007, 2009, 2011) also use a concentration-response design for their studies of sulfadiazine effects on resistance in soil microcosms co-amended with manure. However their studies only include two concentrations that both have significant effects on resistance (as measured by qPCR for two sulfonamide resistance genes, cultural assays, and an increase in the frequency of transformation events), and thus do not allow to deduce threshold levels. Last, Schmitt et al. (2006) use 4-7 different concentrations of oxytetracycline, which include predicted environmental concentrations and significantly higher concentrations as well as a control without antibiotic amendment. The study endpoint was the number of detected tetracycline resistance genes. However, as samples were co-amended with manure, all samples already contained nearly all resistance genes after manure spiking, such that no additional effect of the antibiotic could be noted.

4.3 Evaluation of test methods

In order to evaluate how far current test methods for the derivation of effects of antibiotics on resistance in the environment could be applied in a regulatory context, the following list of parameters has been evaluated (based on selections of method parameters for ecotoxicological tests, e.g. Winding 2005):

General parameters

6. ability to specifically detect resistance in environmental bacteria versus resistance in manure or sewage borne (human or animal) bacteria
7. general applicability (need for pre-information)
8. relevance: coverage of the total environmental community

Methodological parameters

9. limit of detection
10. specificity of the endpoint
11. sensitivity (here: effect size of distinguishable effects)
12. reproducibility

Test acceptance

13. standardisation

14. validation / quality controls

Practical aspects

15. Costs (material)

16. test throughput

17. complexity of test method

18. 1need for specialized equipment

By investigating the literature indicated in 4.1, and by an additional literature search coupling the respective test methodologies with the above test parameters, the abovementioned parameters were evaluated for each test method.

Only limited information is available for many of the methodological parameters. This is most likely caused by the fact that the methods introduced in 4.1 are applied in scientific investigations rather than in a regulatory context. For some of the methodological parameters, information can be retrieved for related test methods used for other matrices. This especially holds true for culturing and molecular analyses of pathogenic bacteria, for which test methods for food matrices have often been evaluated in detail. As food matrices can have a complexity which is comparable with environmental matrices, the results will generally be comparable.

In general, two types of tests can be distinguished, namely those based on culturing of bacteria and those based on molecular analyses in DNA extracted from the total community. Often, test parameters differ between cultural and molecular analyses. Most importantly, while only a minority of bacteria can be assessed by culturing (often as low as 1-10% in environmental matrices (Staley and Konopka 1985), molecular analyses in total community DNA represent the majority of the population. On the other hand, molecular analyses require knowledge of the genetic basis for a specific resistance. The design of PCR primers is based on known sequences of resistance genes, and resistance genes can only be identified with high-throughput sequencing by their similarity to existing resistance gene sequences. These methods are thus only applicable if detailed information on resistance mechanisms already exists. Notably, new resistance genes are discovered every year even for 'old' compounds such as tetracyclines, and new resistance genes will continue to appear due to evolution under selective pressure. Thus, the application of molecular methods requires a careful evaluation of existing knowledge and a choice of the resistance genes to be monitored in a particular case. In contrast, culture-based tests, which mostly identify phenotypic resistance (i.e. growth on media containing antibiotics), can be applied without any information on the genetic basis of resistance. Last, if culture media are carefully chosen (e.g through reducing the carbon content), "environmental" bacteria can be preferentially cultivated, while molecular analyses based on total community DNA will also include intestinal, non-environmental bacteria. However, the selectivity of media for "environmental" bacteria is only insufficiently validated.

With respect to the limit of detection, quantitative molecular methods show slightly higher limits of detection than cultural methods. This is mostly caused by the fact that

only small amounts of DNA extracts can be used in a PCR reaction. The limit of detection for culturable and molecular analyses in aqueous matrices depends on the nature of the sample (the amount of sample that can be collected by filtration until the filter clogs). The limit of detection of methods that are based on collections of isolates is difficult to define. In general, it depends on the sampling effort – when a higher number of isolates is analysed, resistance can be identified at a lower limit of detection, e.g. at 1% of the population if 100 isolates are tested. When the absolute detection limits of quantitative PCR in soil are recalculated into the percentage of bacteria that is resistant (assuming that soil generally contains some 10^9 bacteria g^-1), a detection limit of 103 genes g^-1 amounts to roughly 0.0001% of the population and is thus considerably lower. The reproducibility of the determination of resistance in isolates is generally not well studied in environmental matrices, as isolate collections are generally not replicated. For quantitative cultural analyses, the reproducibility has been investigated for (non-resistant) pathogens in food matrices. In general, the reproducibility of quantitative PCR methods is higher than the reproducibility of most other methods. The sensitivity, which is defined here as the effect size required for a significant effect at typical experimental conditions (100 isolates or 3 replicate analyses by enumeration or quantitative PCR) approximately amounts to an increase in the resistance percentage in 100 isolates by 20%, or an increase in the quantity of culturable resistant bacteria or resistance genes by roughly a factor 10.

With respect to standardisation, environmental methods for analysis of resistance are generally poorly standardised (with the exception of determining some pathogenic or commensal organisms in water samples by cultivation). Still, standardised tests do exist for the determination of resistance or MIC values in isolates (ISO 2006; Clinical and Laboratory Standards Institute 2012; European Committee on antimicrobial susceptibility testing 2013), however only for a limited number of, often pathogenic, species. A standardised method also exists for DNA extraction from soil (ISO 2010). However, the breakpoint concentrations at which isolates of environmental species should be regarded as resistant to a specific antibiotic are undefined, and also for well-studied pathogens, there is an ongoing discussion which breakpoints should be used (clinical breakpoints or epidemiological cut-off values). Further, the outcome of any test based on cultivation greatly depends on the choice of cultivation media. Cultivation methods aimed to capture a greater amount of species adapted to the environment (by usage of media with low nutrient content and lower culturing temperatures) might retrieve an unknown amount of intrinsically resistant species and therefore would require careful standardization. Cultivation techniques that are optimized for enteric species are often more standardised (e.g. for water samples), but fail to detect environmentally-adapted species. Moreover, appropriate quality controls are often lacking. For some cultural techniques, quality controls exist (e.g. recommendations on resistant strains that can be used as positive control for culture media selective for resistant organisms). In molecular analyses, quality controls are often limited to PCR controls with known resistant strains (positive control) and non-DNA controls (contamination control).

While a detailed analysis of isolate collections can require significant resources (e.g. for microtiter plates with antibiotics for MIC determinations, or for multiple PCR analyses of single isolates), quantitative methods (enumeration of resistant bacteria by selective plating or quantification of resistance genes) are more resource friendly, also with respect to the test throughput. Culture methods generally have a low to medium complexity and do not require specialized equipment (with the exception of plate readers for high-throughput determination of MIC values in microtiter plates). Molecular analyses can require dedicated equipment for DNA extraction from environmental matrices and a quantitative PCR machine, which however should be regarded as standard for molecular laboratories. The method most heavily relying on specific equipment is the high-throughput analysis of clone libraries. This method is also relatively complex, together with high-throughput sequencing, which requires bioinformatic skills for data interpretation.

One recently developed method should explicitely be mentioned as it could be applied in a regulatory context, when more experience has been gained with its application in environmental matrices: the determination of mimimum selective concentrations (MSC) (Gullberg 2011). In contrast to the abovementioned methods, it does not test the development of resistance, but it determines the lowest concentrations, at which antibiotics exert a selective pressure and favour a given resistant strain. This is achieved through growth competition assays of a resistant and an isogenic non-resistant bacterium. This method seems to sensitively determine effective concentrations of antibiotics (see section 4.3.4). However, it has not yet been applied in environmental matrices. Another disadvantage for a regulatory use is its technical complexity, as new isogenic pairs of resistant and nonresistant bacteria have to be constructed for each antibiotic or at least antibiotic mechanism of action.

Table 8 summarizes the parameters for the single test methodologies.In Table 9, an evaluation of the parameters has been performed. To this end, each parameter has been evaluated in 5 different categories (++, +, +-, -, –).

In summary, this evaluation shows that all test methodologies have advantages and disadvantages – there is no methodology that is clearly superior to all others. Still, a distinction can be made between cultural and molecular methods, as discussed above. When limiting the comparison to cultural methods, selective plating of resistant bacteria scores favourably with respect to the detection limit and practical aspects. Among the molecular methods, quantitative PCR scores favourably with respect to reproducibility and sensitivity. With respect to relevance and general applicability, cultural and molecular methods seem to balance their advantages and disadvantages.

4.4 Research needs

From the evaluation above, the following research needs can be identified with respect to single test methods:

- Information on methodological parameters, such as the reproducibility and limit of detection of several methods, is lacking for environmental matrices
- An evaluation of the potential of the test methods for standardisation is required

- If an evaluation of resistance in environmental bacteria is envisaged, the selectivity of common media used for preferential cultivation of environmental bacteria should be investigated
- Suitability of MSC based assays for analyses in environmental media should be evaluated

As discussed in 4.3.3, concentration-response designs have rarely been used in analyses of the effect of antibiotics on resistance in the environment. Thus, there is a need for studies with an extended concentration range including both concentrations with and without effects, in order to derive threshold values for antibiotic effects.

However, these threshold concentrations will also depend on the studied time frame. The time needed to observe an increase in resistance in the environment is deemed to depend on the mechanism leading to an increase in resistance (horizontal gene transfer, mutation, preferential survival of resistant species), and is insufficiently known.

The main research needs with respect to test designs are therefore:

- Evaluation of the time-frame required for an emergence / increase of resistance in environmental bacteria for at least the most typical mechnisms leading to an increase in resistance
- Application of concentration-response designs in order to define thresholds of antibiotic effects.

4.5 Conclusions

Culture-based methods (isolation of phenotypically resistant bacteria) and PCR-based assays are widely used methods for an analysis of antibiotic effects. For an application in regulatory assays, culture-based assays and quantitative PCR of resistance genes bear promise, as they complement each other with respect to precision, sensitivity and community coverage. However, it would be desirable to gather more information on relevant method parameters, including the sensitivity and reproducibility of test methods. Recently developed methods (including MSC assays) might bear promise for the identification of selective antibiotic concentrations. With respect to test designs, the application of concentration ranges and the identification of an optimum test duration is advisable.

Table 8: Evaluation of the suitability of test methods for the presence or development of antibiotic resistance within an environmental risk assessment

Test system	Resistance profiling of bacterial isolates	Derivation of MICs of isolates	Enumeration of resistant bacteria (selective plating)	Detection of resistance genes in bacterial isolates	Qualitative detection of resistance genes in environmental DNA	Quantitative detection of resistance genes in environmental DNA	Analysis of clone libraries	High-throughput sequencing	MIC predictions	Qual. / quant. resistance gene detection in RNA
Specific for resistance in species adapted to the environment?	possibly, if selective media are used that exclude enteric bacteria				no, as the host of the resistance genes is unknown					
Generally applicable (no need for pre-information)	yes			no - resistance gene sequence needed for primer design			yes	no - resistance gene sequence needed for data analysis	no - existing MIC data needed	no - resistance gene sequence needed for primer design
Relevance	small: only a small part of total environmental bacteria is culturable				high: contribution of non-culturable bacteria but underrepresentation of total resistance due to focus on known genes		high: contribution of non-culturable bacteria, but underrepresentation of total resistance due to lack of promotors	Very high: contribution of non-culturable bacteria and simultaneous detection of all known genes, but underrepresentation of total resistance due to focus on known genes	unknown: although relevant on theoretical grounds, no proof for relevance for environmental strains	high: contribution of non-culturable bacteria, but underrepresentation of total resistance due to focus on known genes

Test system	Resistance profiling of bacterial isolates	Derivation of MICs of isolates	Enumeration of resistant bacteria (selective plating)	Detection of resistance genes in bacterial isolates	Qualitative detection of resistance genes in environmental DNA	Quantitative detection of resistance genes in environmental DNA	Analysis of clone libraries	High-throughput sequencing	MIC predictions	Qual. / quant. resistance gene detection in RNA
Limit of detection	dependent on sampling effort - with 100 isolates per sample, 1%	MIC distributions can be obtained from almost all culturable bacteria	in soil: roughly 10-100 / g soil (examples for specific bacteria: (Johannessen et al. 2005; Forslund et al. 2011) // in water, roughly 10-100 / L (Harwood et al. 2005)	dependent on sampling effort - with 100 isolates per sample, 1%	medium: genes detectable at about 102-105 copies / g soil (Agersø et al. 2004; Agersø et al. 2006) or about 1000 / L water (Barkovskii and Bridges 2011)	medium: genes detectable at 103-105 copies / g soil (Barkovskii and Bridges 2011), or greater than 103 copies / L water (Okabe et al. 2007)	dependent on sampling effort. The percentage of resistant clones can often be low (Schmieder and Edwards 2012)	dependent on the number of sequences per sample - with 10 000 sequences per sample, between 0.01 and 0.1% of total DNA. Increasingly lower limits of detection will be achieved through advancements in sequencing technology	theoretically high	unknown - no applications yet
Specificity	depending on the degree of naturally occurring resistance, and on the culture media used				high, unless primers are not specific enough		high		theoretically high	high, unless primers are not specific enough

Test system	Resistance profiling of bacterial isolates	Derivation of MICs of isolates	Enumeration of resistant bacteria (selective plating)	Detection of resistance genes in bacterial isolates	Qualitative detection of resistance genes in environmen-tal DNA	Quantitative detection of resistance genes in environmen-tal DNA	Analysis of clone libraries	High-throughput sequencing	MIC predictions	Qual. / quant. resistance gene detection in RNA
Reproduci-bility	unknown for environmental samples. When performed with isolates from unspecific media, potentially highly variable due to heterogeneity within the bacterial community		unknown for environment -tal samples, in food matrices with well-defined species approx. <1 log unit (Schulten et al. 2000; Scotter et al. 2001; De Buyser 2003)	unknown for environmental samples. When performed with isolates from unspecific media, potentially highly variable due to heterogeneity within the bacterial community	potentially high, but seldomly tested	high (< 0.5 log unit) (Koike et al. 2007; Travis et al. 2011)	unknown	unknown for environmental samples	not relevant	unknown - no applications yet
Sensitivity: effect size of significantly distinguish-able effects	depending on number of isolates screened (with 100 isolates	depending on the number of isolates screened	often about 1 log unit difference (factor 10)	depending on number of isolates screened and number of antibiotics tested - with 100 isolates, increase in % carriage of one gene by some 20%	depending on number of genes screened (with 10 genes, change in approx. 6 genes)	often about 1 log unit difference	unknown - no comparisons between two samples yet	in the existing publications, an increase in resistance% by <= factor 5 (Kristiansson et al. 2011; Port et al. 2012)		theoretically high

Test system	Resistance profiling of bacterial isolates	Derivation of MICs of isolates	Enumeration of resistant bacteria (selective plating)	Detection of resistance genes in bacterial isolates	Qualitative detection of resistance genes in environmen-tal DNA	Quantitative detection of resistance genes in environmen-tal DNA	Analysis of clone libraries	High-throughput sequencing	MIC predictions	Qual. / quant. resistance gene detection in RNA
Standardisa-tion	standardised tests existing for resistance profiling (CLSI) and cultivation of intestinal bacteria, but not of environment al bacteria	standardised tests existing for MIC derivation and cultivation of intestinal bacteria, but not of environment al bacteria	standardised tests existing for cultivation of intestinal bacteria, but not of environment al bacteria	no standardised methods for PCR detection	no standardised methods for PCR from environmental samples existing. Standards for DNA extraction published	no standardised methods for PCR from environmental samples existing. Standards for DNA extraction published	no standardisation			no standardised methods for PCR from environmental samples existing.
Validation / quality controls	quality controls exist for MIC testing of intestinal bacteria		none existing	positive / negative controls are common during PCR			no standardisation			Pos. and neg. controls are common during PCR and cDNA preparation
Cost effectiveness (material)	10-100 E / sample	50 - 500 E / sample (depending on number of antibiotics tested - here, around 8)	ca. 10 E / sample	100 - 1000 E / sample (depending on number of antibiotics tested - here, 5)	< 10 E / sample	ca. 10 E / sample	>1000 / sample?	> 2000 E / sample	0	ca. 30 E / sample

Test system	Resistance profiling of bacterial isolates	Derivation of MICs of isolates	Enumeration of resistant bacteria (selective plating)	Detection of resistance genes in bacterial isolates	Qualitative detection of resistance genes in environmen-tal DNA	Quantitative detection of resistance genes in environmen-tal DNA	Analysis of clone libraries	High-throughput sequencing	MIC predictions	Qual. / quant. resistance gene detection in RNA
Approximate test throughput [a]	10 / week (with 100 isolates)	10 / week (with 100 isolates)	30 / week	10 / week	50 / week (for 5 genes)	50 / week (for 5 genes)	1 / month	1 / month (quicker upon availability of sequencer and existence of data analysis pipelines)		30-50 / week
Complexity of test method	low	medium	low	medium	medium	medium	high	high (data analysis)	medium	medium - high
Need for specialized equipment	no - general microbiology lab techniques	general microbiology lab (+ specialized plate reader)	no - general microbio-logy lab	general molecular microbio-logy lab techniques	general molecular microbio-logy lab techniques (+ DNA isolation apparatus)	general molecular microbio-logy lab techniques (+ DNA isolation apparatus)	high-throughput screening techniques	access to high-throughput sequencer	no	general molecular microbiology lab techniques (+ DNA isolation apparatuses)
Comments	breakpoints seldomly established for environment al isolates									

a At the throughput shown, analysis time consists mainly of hands-on time (apart from high-throughput sequencing)

Table 9: Summary of evaluation of the suitability of test methods for the presence or development of antibiotic resistance within an environmental risk assessment

Test system	Resistance profiling of bacterial isolates	Derivation of minimum inhibitory concentrations (MICs) of isolates	Selective plating of bacteria	Detection of resistance genes in bacterial isolates	Qualitative detection of resistance genes in environmental DNA	Quantitative detection of resistance genes in environmental DNA	Analysis of clone libraries	High-throughput sequencing
Specificity for resistance in environmental bacteria	+-	+-	+-	+-	--	--	--	--
General applicability (no need for pre-information)	++	++	++	--	--	--	++	--
Relevance	--	--	--	--	+	+	+	++
Limit of detection	-	-	++	-	+	+	-	+
Specificity	+-	+-	+-	+-	+	+	+	+
Reproducibility	+-	+-	+-	+-	+-	++	+-	+-
Sensitivity: Effect size of distinguishable effects	+	+	+-	+	-	+-	+-	+-
Standardisation	+	+	+	--	--	--	--	--
Validation / quality controls	+-	+-	--	-	-	-	--	--
Cost effectiveness (material)	+-	+-	++	-	++	++	--	--
Test throughput	+-	+-	+	+-	+	+	--	--
Complexity of test method	++	+-	++	+-	+-	+-	--	--
Need for specialized equipment	++	+	++	++	++	+	--	++

5 Review on the evaluation of resistance development in the current risk assessment of veterinary and human pharmaceuticals

Current approaches for the evaluation of acquired antimicrobial resistance (AMR) in the risk assessment of veterinary and human pharmaceuticals were reviewed mainly based on EM(E)A guidance documents (sections 5.1 and 5.2). In addition, further relevant documents were identified (sections 5.3 – 5.6). Although this is beyond the original scope of the work package, approaches for antimicrobial resistance assessment for biocides (section 5.7) and genetically modified microorganisms (section 5.8) were included in the evaluation.

We have focussed on approaches, considerations and methods that may be useful for the assessment of resistance development within environmental risk assessment (ERA) procedures (e.g. as the indicated test methods might be useful or as an analogous approach might be adopted in the ERA). An overview of the identified methods and approaches is given in Table 10.

5.1 EM(E)A guidance: veterinary medicine

5.1.1 VICH GL 27

Antimicrobial resistant zoonotic and non-zoonotic bacteria can be transferred from food-producing animals to humans. Therefore, the potential of veterinary antimicrobial products to select for resistance development in bacteria of human health concern shall be considered when evaluating the safety of antimicrobials used in food-producing animals. Guidance on this issue is provided by VICH GL 27 (EMEA/VICH 2004). This 'Guidance on pre-approval information for registration of new veterinary medicinal products for food producing animals with respect to antimicrobial resistance' recommends studies / data for characterising potential resistance development in the animal when using the antimicrobial product. The recommended basic information includes data on:

- Antimicrobial class
- Mechanism and type of antimicrobial action (incl. characterisation as bacterio¬static or bactericidal)
- Antimicrobial spectrum of activity based on minimum inhibitory concentration (MIC) tests with a variety of microorganisms, preferably determined using validated methods:
- MICs for target animal pathogens (from efficacy section of dossier)
- MICs for food-borne pathogens and commensal microorganisms (relevant bacteria should be isolated from main target animal species; recent isolates should be included)

- Antimicrobial resistance mechanism(s), information on molecular genetic basis of resistance (if no information is available, information from analogues may be provided)
- Occurrence and rate of transfer of antimicrobial resistance genes (evaluated e.g. based on Lorian, 1996)
- Occurrence of cross-resistance (phenotypic and, if possible, genotypic description)
- Occurrence of co-resistance (phenotypic and, if possible, genotypic description)
- Pharmacokinetic data

Additional information that may be submitted includes

- *In vitro* mutation frequency studies
- Antimicrobial activity in intestinal tract

With the exception of the reference to Lorian (1996), more detailed information on recommended test methods is lacking.

5.1.2 VICH GL 36

Possible effects of residues of veterinary antimicrobials on the human intestinal flora are addressed in VICH GL 36, which has recently been revised (VICH 2012). This guideline outlines a stepwise approach for determining the need for a microbiological acceptable daily intake (ADI) and offers test options. The ADI is the level of intake that could be ingested daily over the entire lifetime without appreciable health risk. When establishing a microbiological ADI, the following two microbiological endpoints of public health concern should be considered based on EMA/VICH (2011):

- Disruption of the colonisation barrier (a function of the normal intestinal flora limiting colonisation of the colon by exogenous microorganisms and overgrowth by indigenous, potentially pathogenic microorganisms)
- Increase of the population(s) of resistant bacteria, i.e. bacteria that are resistant to the considered antimicrobial drug or other antimicrobials. This may be due to previously sensitive bacteria acquiring resistance or an increase in the percentage of bacteria with reduced sensitivity to the drug. Although a literature survey did not provide evidence of human health effects associated to an increased percentage of resistant bacteria in the human intestinal flora, such effects cannot be excluded.

A microbiological ADI has to be derived if

- Residues of the drug and/or its metabolites, which are active against representatives of the human intestinal flora (based on MICs for relevant intestinal bacteria), reach the human colon and remain microbiologically active

If there is no scientific justification for waiving testing, the no-observable adverse effect concentrations (NOAECs) and no-observable adverse effect levels (NOAELs) for disruption of the colonisation barrier and emergence / increase of resistance have to be

determined. With regard to an increase in the population(s) of resistant bacteria in the human colon, the following considerations are relevant:

- The organisms of concern in the intestinal tract and the resistance mechanism of the drug should be taken into account
- Data on the prevalence of resistance in the human intestinal flora and on variations within and between individuals is useful for developing criteria for evaluating the emergence / increase of resistance
- Changes in the population(s) of resistant organisms during pre-treatment, treatment and post-treatment can be evaluated by enumeration techniques, phenotypic and molecular methods
- The time required for resistance development depends on the type of antibacterial, the type of resistance mechanism and on how this mechanism evolves (e.g. by transfer of genes between cells or by mutations). Short-term tests with pure cultures (e.g. MIC tests) and faecal slurries are not appropriate to determine a NOAEC for an increase in resistance
- Continuous and semi-continuous cultures and fed-batch cultures of faecal bacteria can be used to evaluate the effects of long-term exposure

Given that further research is required to evaluate the reliability and validity of the test systems mentioned, VICH GL 36 does not recommend any specific test system. Instead, the guideline offers test options.

5.1.3 Concept paper for a guideline on antimicrobial risk assessment

In a recent 'Concept paper for a guideline on antimicrobial risk assessment', EMEA/CVMP (2013) outlines that there is a requirement for risk assessment guidance beyond the foodborne risks covered by VICH GL 27. For instance, public health risks may be caused by the use of antibiotics in companion animals (e.g. meticillin-resistant Staphylococcus aureus; MRSA) and to the presence of resistant bacteria or resistance gene in the 'surrounding environment'. Given that current knowledge on the potential risks caused by the latter exposure route is poor, EMEA/CVMP (2013) state that 'it is likely too early to include such elements in any risk assessment guideline'. It is assumed that antimicrobial resistance related public health risks caused by the use of of an antibiotic in veterinary medicine will be 'semi-quantifiable at best'.

5.2 EM(E)A guidance: human medicine

The 'Guideline on the evaluation of medicinal products indicated for treatment of bacterial infections' (EMA/CHMP 2011), which has replaced EMEA/CPMP (2004), specifies which microbiological and clinical data are required for the marketing authorisation. These data should include information on:

- The precise mechanism of action of the antibacterial
- *In vitro* antibacterial activity against recent relevant clinical isolates based on MIC tests:

    - For commonly encountered pathogens, several hundred isolates should be tested. These should include microorganisms that are resistant against single / multiple classes of antibacterial agents
    - For organisms with rarely encountered mechanisms of resistance, at least 10 isolates per species should be tested
- The potential for induction of resistance expression (either temporary or permanent) by exposure to the test antibacterial
- An estimate of the rate of selection of resistant mutants and how concentration above the MIC may affect or prevent mutations (due to their unknown relevance to the clinical situation, *in vitro* data should not be included)
- *In vitro* antibacterial activity of major metabolites
- Mechanisms of resistance present in organisms, for which the MIC is unusually high
- Cross-resistance within the respective class of antibacterials and in different classes of antibacterial agents
- potential for associated resistance (e.g. when organisms resistant to other drug classes are resistant to the test antibacterial due to multidrug efflux pumps or impermeability of the outer membrane, or when several resistance determinants are co-transferred)
- Apart from the recommendation that laboratory-determined rates for resistance selection should generally not be included, EMA (2011) contains little information on test methods.

Based on these data the probability of encountering pathogens resistant to the antibacterial drug should be assessed. In addition, emergence of resistance should be monitored during 3-5 years after approval of the drug. In case of concern due to the emergence of resistances, monitoring may have to be extended. Information on emerging resistance, changing patterns of resistance and new mechanisms of resistance should be notified to the CHMP.

5.3 Other European documents

Risks related to antimicrobial resistance have to be considered in the risk-benefit analysis. Authorisation can only be granted if risks are acceptable / can be mitigated sufficiently (e.g. EMA/CVMP 2011).

Monitoring / surveillance programmes are in place for antimicrobial resistance in humans (the European Antimicrobial Resistance Surveillance Network) and in zoonotic bacteria in food producing animals. In the 'Action plan against the rising threats from antimicrobial resistance' (EC 2011), harmonisation between human and veterinary surveillance systems and a strengthening of these systems is requested. An improvement of monitoring programmes for AMR is also one of the objectives of the German antibiotic resistance strategy (Bundesministerium für Gesundheit 2011).

5.3.1 EFSA (2008)

The EFSA 'Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance' (EFSA 2008) provides guidance for antibacterial resistance assessment of bacterial strains that are used as food / feed additive.

While for intrinsic (or natural) resistance, the potential for horizontal spread is considered to be low, acquired resistance that is mediated by added genes has a high potential for horizontal transfer. On this basis, the following assessment is suggested:

- When all bacterial strains within a taxonomic group are resistant to an antibacterial (based on MICs), resistance may be intrinsic. In the absence of published information, the structural nature and genetic basis of resistance has to be analysed.
- If single strains have MICs above the cut-off level (or microbiological breakpoint for the respective species), this may be due to acquired resistance. The genetic basis of resistance has to be analysed.
    - If acquired resistance is due to added genes (e.g. plasmids or transposons), the potential of horizontal transfer is high and the risk is considered as not acceptable.
    - If acquired resistance is due to genomic mutation, the risk of horizontal transfer is assumed to be low and the risk is generally considered as acceptable.

5.4 Codex Alimentarius

The Codex Alimentarius Commission (CAC) was established by the FAO and the WHO and has 180 member governments including the European Community (Prater 2012). The Codex ad hoc Intergovernmental Task Force on Antimicrobial Resistance has developed 'Guidelines for risk analysis of foodborne antimicrobial resistance' that focus on human health risks (CAC 2011). According to CAC (2011), possible sources of information for the risk assessment are amongst others:

- Data generated within surveillance programmes on the use of antimicrobials and the prevalence of foodborne antimicrobial resistance
- Studies on interactions between microorganisms and their environment in the food production process (e.g. faeces, sewage)
- *In vitro* | *in vivo* studies of AMR microorganisms / determinants
- Studies on the resistance selection potential of antimicrobial agents
- Data on the link between usage of the antimicrobial substance and resistance (especially regional data)

- Data on the link between resistance and virulence and / or fitness of the microorganisms

Hazard identification shall be based on a review of literature and on data from surveillance programmes. Specific strains / genotypes of foodborne microorganisms shall be identified, which may cause a risk by a specific combination of food / AMR microorganism or AMR determinants and / or antimicrobial agents to which resistance is expressed. Information on the biology of the resistant microorganism or the AMR determinant in the relevant environment / niche is considered as useful.

As far as possible the risk profile shall include information on:

- Growth and survival of foodborne AMR microorganisms during the food production to consumption process
- Possible inactivation in foods (e.g. due to pH)
- Resistance mechanism, location of AMR determinants
- Cross-resistance and / or co-resistance to other antimicrobials
- Transferability / frequency of transfer of AMR determinants
- Prevalence of AMR determinants in human and non-human microflora

Exposure assessment shall include information on:

- Resistance selection pressure (i.e. detailed information on use of the antimicrobial)
- Rate of resistance selection in commensal and zoonotic microorganisms after administration of the antimicrobial
- Proportion of the microorganisms that are resistant to the antimicrobial
- Resistance transfer rates between microorganisms
- Other possible sources of foodborne AMR (e.g. biosolids, wastewater, manure)
- Survival capacity and redistribution of foodborne AMR microorganisms during the food production to consumption process

CAC (2011) does not recommend specific test methods.

For hazard characterisation, a qualitative description or a semi-quantitative / quantitative model may be used to link the exposure level to a probability of subsequent disease cause by AMR resistant microorganisms. Hazard characterisation should include dose-response relationships if available.

Scoring systems are suggested for qualitative exposure assessment, hazard characterisation and risk characterisation.

CAC (2011) suggests considering the inclusion of animal feed, feed ingredients, biosolids, wastewater, manure and other waste-based fertilisers in surveillance programmes.

In 2005, the CAC has issued a 'Code of practice to minimize and contain antimicrobial resistance'. Although this code does not include environmental issues, it is stated that the persistence of resistant microorganisms in the environment might have to be addressed when aiming to reduce antimicrobial resistance. CAC (2005) suggests further research on the magnitude of transfer of resistance between environmental microorganisms.

5.5 OIE

The ad hoc group of experts on antimicrobial resistance of the World Organisation for Animal Health (Office International des Epizooties, OIE) has also developed procedures for assessing potential risks of antimicrobials caused by the veterinary use of antibacterials (see e.g. Vose et al. 2001). In the current version, risks to human health and animal health are addressed (OIE 2012). Many elements of these procedures are similar or identical to the assessments according to CAC (2011; cf. section 6.4), APVMA (2007) and FDA/CVM (2003; section 6.6). Terminology corresponds to FDA/CVM (2003). Some issues shall be mentioned in the following.

Risk is defined as infection of humans / animals with microorganisms that have acquired resistance to the respective antimicrobial used in veterinary medicine, and the consequent loss of therapeutic efficacy in humans / animals.

Interestingly, the exposure assessment performed for analysis of both human and animal health risks shall consider the "cycling of resistant micro-organisms between humans, animals and the environment". However, not further information is provided how this complex task should be accomplished. In the exposure assessment performed for analysis of animal health risks, exposure of wildlife to resistant microorganisms shall be addressed (again, without further specification).

OIE (2012) does not include any information on test methods.

5.6 Other non-European documents

5.6.1 APVMA (2007)

The 'Veterinary manual of requirements and guidelines' of the Australian Pesticides and Veterinary Medicines Authority (APVMA 2007) contains a chapter specifying the requirements with regard to antibacterial resistance in the context of applications for registration of veterinary antibacterials. This chapter refers to VICH GL 27 (EMEA/VICH 2004; see above) and to FDA/CVM (2003; see below). An antimicrobial resistance risk assessment is required for assessing possible public health risks caused by AMR human pathogens.

A qualitative risk assessment is required first, with the option of subsequently performing a quantitative risk assessment. Data requirements according to APVMA (2007) largely correspond to those in VICH GL 27 and include information on:

- Occurrence and mechanism of transfer of AMR
- Rate of transfer of AMR between microorganisms

- In case of point mutation: mutation rate

Hazard shall be characterised with regard to

- The mechanisms of resistance in relevant microorganisms
- Details of microbial resistance patterns in relevant microorganisms *in vitro*:
    - MICs against relevant microorganisms
    - Estimated rate of resistance development (e.g. based on *in vitro* studies with microorganisms passaged in the presence of the test antibacterial)
- Details on resistance patterns in relevant microorganisms that have emerged with use of the antibacterial product or related substances

Scoring systems are suggested for characterising potential exposure, the probability of hazard, and the impact in susceptible humans (negligible, low, medium, high).

The impact characterisation shall include:

- A description of the relationship between the frequency / magnitude of exposure of humans to foodborne AMR microorganisms and the severity / frequency of the impact
- An estimation of the critical threshold of exposure required to cause an infection in susceptible humans

The overall assessment includes a risk-benefit analysis.

5.6.2 FDA/CVM (2003)

The FDA/CVM guidance on 'Evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern' describes FDA's approach to assess human health risks caused by foodborne AMR bacteria. Most of the relevant elements of FDA/CVM (2003) have already been mentioned in the section on APVMA (2007), which is partly based on this FDA/CVM guidance. Some additional points are detailed in the following.

According to FDA/CVM (2003), risk is evaluated qualitatively. If information on a relevant factor is lacking, the most conservative estimate (i.e. worst case) is assumed with regard to this factor.

The first step is a 'release assessment', i.e. an estimation of the likelihood that the antimicrobial drug results in emergence or selection of AMR bacteria in the animal. This release assessment is based on information on the drug's characteristics (e.g. antibacterial mechanism, spectrum of activity), pharmacokinetics/-dynamics, resistance mechanism, occurrence and rate of transfer of resistance. This also includes studies on:

- The rate of resistance development in food-borne bacteria of human health concern following the proposed use of the drug
- The decline of resistance in food-borne bacteria of human health concern after cessation of therapy

The subsequent exposure assessment deals with the probability of human exposure to foodborne bacteria of human health concern. It is not specific to the drug product. The 'consequence assessment' focuses on human health consequences. The results from release, exposure and consequence assessment are integrated in the risk estimation. A matrix for integration of the qualitative outcomes of the different assessment steps is included in FDA/CVM (2003).

5.7 Antimicrobial resistance assessment for biocides

Although this is beyond the scope of chapter 5 it should be mentioned that the evaluation of antibiotic resistance is also discussed with regard to biocides (SCENIHR 2009). There is evidence that the use of specific active substances in biocidal products may contribute to an increased occurrence of antibacterial resistance in bacteria. This is due to the fact that some mechanisms involved in resistance to biocides also confer resistance to antibiotics (e.g. efflux pumps and changes in permeability of the cell envelope; Russell 2003, Sheldon 2005 as cited by SCENIHR 2009). Thus, the selective pressure exerted by sublethal levels of biocides may promote resistance to both biocides and antibiotics. This is of greatest concern, if this cross-resistance is encoded by mobile genetic elements.

SCENIHR's 'Assessment of antibiotic resistance effects of biocides' (2009) focuses on the relevance of antibiotic resistance for human and veterinary medicine. Some considerations that may be useful for the ERA of antibacterials shall be outlined in the following.

Bacterial resistance to biocides can be evaluated by determining minimum bactericidal concentrations (MBCs), bactericidal activity (lethality of the normally used concentrations of the biocides) and inactivation kinetics following exposure to the biocide.

For an assessment of the risk of selecting for antibacterial resistance the following information is required for each biocide and application:

- In-use concentration and residual concentration of the active substance
- Stability of the active substance (and thus, its activity)
- Specific environmental factors (including exposure time, temperature, pH, nutrient level, the type of bacterial community)
- Change in microbial population
- Type of resistance determinant (mobile genetic element or not)
- Potentiation or antagonism with other substances (e.g. components of a formulation)

SCENIHR (2009) differentiates between the direct hazard (selection and dissemination of resistant bacteria) and the indirect hazard (transfer of mobile genetic elements to other, naturally susceptible, strains, e.g. commensal flora).

Currently, no standard protocols are available for evaluating antimicrobial resistance induced or selected by biocides (i.e. to determine the minimal concentration of a biocide that is able to select or trigger the emergence or expression of antimicrobial resistance). Therefore, SCENIHR (2009) strongly recommended to develop such protocols for a qualitative assessment of biocide induced resistance and cross-resistance. These protocols should combine repeated exposure to sublethal (including residual) biocide levels with available standardised antibiotic susceptibility tests.

SCENIHR (2009) stressed that the introduction of surveillance programmes for monitoring levels of bacterial resistance and cross-resistance is required.

5.8 Antimicrobial resistance assessment for genetically modified microorganisms

In its technical report on 'approaches to risk assessment in the area of antimicrobial resistance, with an emphasis on commensal microorganisms', EFSA (2011a) has identified a synergy between the approaches for antibacterial resistance assessment for (1) additives and products used in animal feed and (2) genetically modified organisms (see also EFSA 2011b). For risk assessment of genetically modified microorganisms (GMM), the required data include information on:

- Presence of indigenous mobile genetic elements
- The capacity to exchange genes:
    - Inherent capability to transfer or acquire DNA
    - Possible presence of plasmids, and specificity (host range) of these plasmids,
    - Presence of genes encoding resistance / tolerance to antimicrobials, heavy metals or toxins, especially if related to mobile genetic elements such as conjugative transposons, prophages, integrons and mating factors
- Organisms with which exchange of genetic material occurs under natural conditions
- Presence of introduced genes for antimicrobial resistance (including the presence of marker genes coding for antimicrobial resistance) and specification if these gene are located on mobile genetic elements (for evaluation of the potential for transfer, see above)

For genetically modified microorganisms and their products, an environmental risk assessment (ERA) is performed that depends on the category of the GMM / GMM product.

For category 1 products (defined substances / mixtures of such substances, from which GMMs and introduced genes have been removed; e.g. amino acids, vitamins) and category 2 products (complex products, from which GMMs and introduced genes have been removed; e.g. cell extracts, enzyme preparations), the ERA is limited to a demonstration of the absence of viable GMMs and recombinant DNA in the product. If these are absent, exposure is considered negligible.

For category 3 products (products, in which no GMMs capable of multiplying/ transferring genes are present, but which contain newly introduced genes; e.g. heat-inactivated starter cultures), the likelihood of transfer of recombinant DNA to other microorganisms and potential consequences of such a horizontal gene transfer are assessed. This includes an assessment of:

- Quality and location of recombinant DNA (chromosome / mobile element)
- Environments into which recombinant DNA may be released (e.g. faeces, manure, wastewater, surface water, soil)
- Stability of recombinant DNA in the relevant environments
- Presence of indigenous microorganisms as possible recipients in horizontal gene transfer
- Consequences of horizontal gene transfer:
    - As worst case scenario, the new properties of the microorganism with the acquired resistance are assessed on a theoretical basis considering potential selective advantages in the specific environment.
    - The possibility of pathogenicity to humans, animals and plants and interference with ecosystem functions is evaluated.

For category 4 products (consisting of / containing GMMs capable of multiplying/ transferring genes; e.g. live starter cultures for fermented foods), fate and effects of the DNA are evaluated as described above for category 3 products. In addition, survival, proliferation potential and possible effects of the GMM are evaluated. This includes:

- A characterisation of the GMM receiving environments
- A characterisation of the physiological properties (e.g. intrinsic antimicrobial resistance) allowing the GMM to compete and survive in the respective environment(s)
- An evaluation of interactions of the GMM with abiotic / biotic environment (incl. microorganisms, plants, animals)
- An evaluation of factors contributing to degradation or stabilisation of recombinant DNA in the respective environment(s)
- A characterisation of potential recipients for recombinant DNA and an assessment of the likelihood of horizontal gene transfer
- Environmental and health consequences of a possible horizontal gene transfer
- Effects of the GMM on plants and animals

Table 10: Overview of approaches and methods of potential use for the environmental risk assessment of antibacterial resistance based on current risk assessment guidance documents

Document (abbreviated title)	Background / objective	Approach / method useful for the environmental risk assessment	Reference
VICH GL 27	Antimicrobial resistance assessment for veterinary antimicrobials for food producing animals with regard to human health risks	Basic information requirements including: • Mechanism of antibacterial activity • Spectrum of activity for target and non-target organisms (based on MIC tests) • Occurrence and mechanism of resistance • Rate of transfer of resistance • Occurrence of cross-resistance and co-resistance	EMEA/VICH (2004)
VICH GL 36	Assessment of possible effects of residues of veterinary antimicrobials on the human intestinal flora	• Stepwise approach for determining the need to derive a microbiological acceptable daily intake (ADI) • Consideration of background resistance • Changes in resistance during pre-treatment, treatment and post-treatment: evaluated using enumeration techniques, phenotypic and molecular methods • Considerations on time required for resistance development (short-term testing not appropriate)	VICH (2004), EMA/VICH (2011)
Guideline on evaluation of medicinal products for treatment of bacterial infections	Microbiological and clinical data requirements for marketing authorisation of antibacterials in human medicine	Information requirements including: • Mechanism of antibacterial activity • *In vitro* antibacterial activity (based on MIC tests) • Potential for temporary / permanent induction of resistance • Rate of resistance selection • Mechanism of resistance • Occurrence of cross-resistance and associated resistance / co-resistance	EMA/CHMP (2011)

Document (abbreviated title)	Background / objective	Approach / method useful for the environmental risk assessment	Reference
Guidelines for risk analysis of foodborne antimicrobial resistance	Antimicrobial resistance assessment or veterinary antimicrobials for food producing animals with regard to human health risks	Information requirements including: • Growth and survival of AMR microorganisms during food production to consumption process • Resistance mechanism, location of AMR determinant • Cross-resistance, co-resistance • Transferability and frequency of transfer of AMR determinant • Prevalence of resistance, rate of resistance selection Scoring systems for qualitative exposure assessment, hazard characterisation and risk characterisation	CAC (2011)
Veterinary manual of requirements and guidelines	Antimicrobial resistance assessment for veterinary antimicrobials with regard to human health risks	Information requirements including: • Mechanism of resistance • Occurrence and mechanisms of resistance • Rate of resistance development Scoring systems for qualitative assessment of exposure, hazard and impact in susceptible humans. Estimation of critical threshold to cause an impact in humans.	APVMA (2007)
Evaluating the safety of antimicrobial animal drugs with regard to effects on bacteria of human health concern	Assessment of human health risks caused by foodborne AMR bacteria	Information requirements including: • Rate of resistance development • Decline of resistance after cessation of treatment Qualitative risk evaluation (matrix for integration of the qualitative outcomes of the different assessment steps)	FDA/CVM (2003)
Update of criteria used in assessment of bacterial resistance to antibiotics	Antibacterial resistance assessments of bacterial strains used as feed additive	Stepwise procedure to differentiate between intrinsic (natural) and acquired resistance Classification as resistant: based on cut-off level (microbiological breakpoint) for respective taxonomic group Estimation of risk based on potential of horizontal transfer (low for genomic mutations, high for mobile genetic elements)	EFSA (2008)

Document (abbreviated title)	Background / objective	Approach / method useful for the environmental risk assessment	Reference
Assessment of antibiotic resistance effects of biocides	Assessment of risks with relevance to human and veterinary health	Assessment of the risk of antibacterial resistance requires information on: • In-use concentration and residual concentration of the active substance • Stability of the active substance • Specific environmental factors (including exposure time, temperature, pH, nutrient level, type of bacterial community) • Changes in microbial populations • Type of resistance determinant • Potentiation / antagonism with other substances	SCENIHR (2009)
Technical report on approaches to risk assessment in the area of antimicrobial resistance Scientific opinion risk assessment of genetically modified microorganisms and their products for food and feed use	Risk assessment (including environmental risk assessment) of genetically modified microorganisms and their products for food and feed use	Environmental risk assessment of genetically modified microorganisms includes assessment of: • Presence of mobile genetic elements • Capacity to exchange genes • Stability of recombinant DNA in environment • Physiological properties of genetically modified microorganism relevant to survival in the environment • Presence of microorganisms as possible recipients in horizontal gene transfer • Consequences of horizontal gene transfer (potential selective advantages, possible pathogenicity, interference with ecosystem functions)	EFSA (2011a, b)

5.9 Examples for the derivation of microbiological ADI values

In the human food safety assessment of veterinary antimicrobials, an ADI is derived based on toxicological or microbiological studies (generally chronic toxicity studies with mammals). Microbiological studies comprise evaluations of the disruption of the colonisation barrier and of the increase in the population of resistant bacteria (e.g. based on EMA/VICH 2011, see section 6.1 and Figure 4). For deriving the ADI, a safety factor is applied to the most sensitive effect level, i.e. the no observed effect level (NOEL) or no-observable adverse effect level (NOAEL).

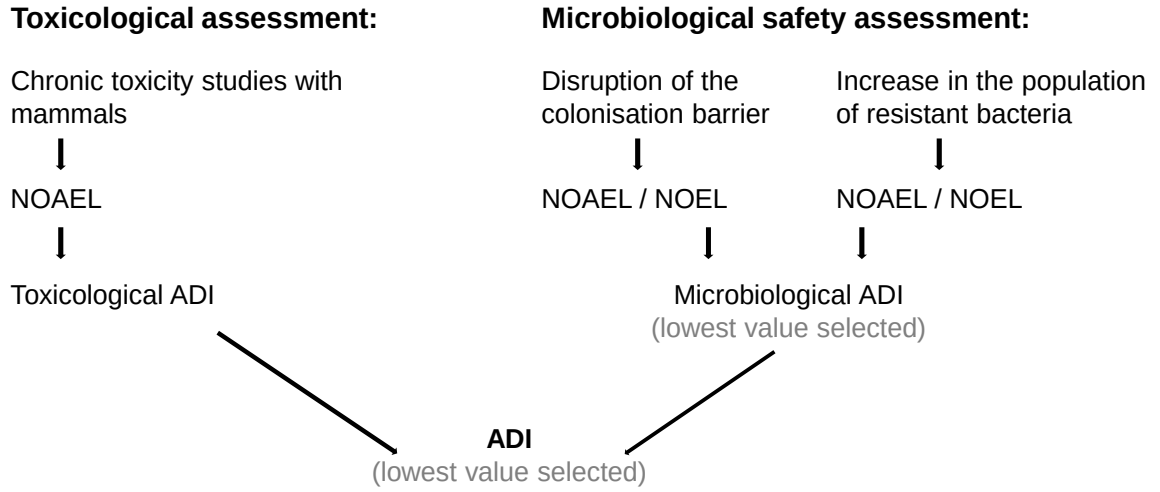

Fig. 4: Derivation of the acceptable daily intake (ADI) in human food safety assessments of veterinary pharmaceuticals based on Yan (2012), modified (NOAEL: no-observable adverse effect level; NOEL: no observed effect level)

The 'ADI list' of the Australian Government (2012) compiles information on the acceptable daily intakes for substances of agricultural and veterinary use. In this list, it is specified for many substances on which study the ADI is based and why the applied safety factor was chosen. However, more detailed information (e.g. on experimental details) is not provided. For the majority of the included substances, the ADI is based on chronic toxicity studies with mammals. Yet, there are a few examples for ADI values based on effects on gastrointestinal bacteria (i.e. on the potential for disruption of the colonisation barrier), and there is one example for an ADI based on toxicity to the intestinal microflora and an increase in the proportion of resistant bacteria. For chlortetracycline, a study with oxytetracycline in humans was used to derive the ADI given that both compounds are similar with regard to their structure and microbiological potency. As reported by WHO (1999), this study was performed with healthy human volunteers who received therapeutic (2 g/d) and lower doses of oxytetracycline for 7 days. A dose of 2 g/day led to an increased level of resistant Enterobacteriaceae in the faeces, and to increased colonisation by yeast. At 20 mg/day, susceptible strains of intestinal microflora were affected (it is not stated if increased resistance was observed at this concentration). At 2 mg/day, the level of resistance was not affected. Based on the NOEL of 2 mg per adult and day, which corresponds to 30 µg/kg bodyweight (bw) and day, and a safety factor of 10 an ADI of 3 µg/kg bw was

obtained for for chlortetracycline (Australian Government 2012) and for chlortetracycline, oxytetracycline and tetracycline (WHO 1999; see also below).

Some further information on the derivation and revision of a so-called group ADI for chlortetracycline, oxytetracycline and tetracycline is provided by WHO in the 'Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives' (1999). Due to incomplete absorption from the gastrointestinal tract, tetracycline concentrations in the intestine are high and the intestinal flora may be affected as it is known from experience in human medicine. In addition to the abovementioned study with oxytetracycline, a chemostat experiment with tetracycline is described. In this *in vitro* continuous bacterial culture system, dosages were equivalent to 0.025, 0.25 and 2.5 mg/kg bw and day. At 2.5 mg/kg bw and day, the percentage of resistance *E. coli* strains increased from <20% to >50% after 24 h and >60% after 48 h of exposure, but declined to approx. 35% after 6 days. At 0.025 and 0.25 mg/kg bw and day, no effects on the percentage of resistance were observed. The other microbiological study endpoints were not affected at the tested tetracycline concentrations. Based on this study and literature findings it was concluded that (1) selection of resistant bacteria is the most sensitive endpoint for tetracyclines, and (2) in view of the high sensitivity of this endpoint and the low variation between individuals, no safety factor is required. Thus, the abovementioned ADI of 3 µg/kg bw was increased to 30 µg/kg bw (WHO 1999).

Although no studies on resistance development were evaluated, the following consideration made by WHO (2012) shall be mentioned. In this report, ADI values for amoxicillin and the aminocyclitol antibiotic apramectin were derived. It was argued that the development of resistance is unlikely, because the majority of levels of both substances in the target tissue are lower than the lowest MIC_{50} values for representative intestinal microorganisms. Thus, a microbiological ADI was established based on the MIC_{50} for colonisation barrier disruption and the potential for an increase in resistance was not further evaluated.

5.10 Examples for assessments of human health risks caused by foodborne antimicrobial resistance

The procedures for assessing human health risks caused by foodborne antimicrobial resistance are relatively new and have not yet been widely used, a fact that is mainly due to their complexity. The assessment includes elements developed for evaluating potential risks of chemicals given that the hazard is related to the veterinary use of antimicrobials. It includes risk assessment methods developed for foodborne bacteria and for infectious diseases (McEwen 2012a, b), i.e. microbiological food safety assessment methods. Such assessments integrate exposure data, which are e.g. obtained from studies on food contamination and consumption, and dose-response curves for the respective pathogenic or commensal microorganism. Usually, they focus on a single pathogen and a single food or a limited number of foods. The evaluation of human health risks caused by foodborne antimicrobial resistance is even more complex. This is due to factors such as the transfer of resistance determinants between microorganisms (CAST 2006; see also below). Generally, clinical endpoints are evaluated, e.g. an

increased duration or severity of a bacterial infection due to reduced treatment options, or an increased number of days of hospitalisation (Bailar & Travers 2002, McEwen 2012a, b). Quantitative assessments express risk as probability of the adverse outcome (e.g. illness). In qualitative assessments, the risk is classified e.g. as non-acceptable / acceptable (or high / low; CAST 2006).

In many cases, it is difficult or impossible to quantify exposure of humans to a foodborne hazard. In addition, the uncertainty associated to dose-response curves is often high (CAST 2006), and the magnitude of adverse effects is controversial (McEwen 2012b). Together with gaps in the understanding of the interactions between microorganisms, animals and humans, this leads to a considerable uncertainty in the assessments (Bailar & Travers 2002, McEwen 2012b).

Examples for assessments of human health risks caused by foodborne antimicrobial resistance have been summarized by Bailar & Travers (2002) and McEwen (2012a). Most assessments were performed for antimicrobials that are already in use, i.e. are retrospective. The majority of the assessments are of qualitative nature. In most cases, the quantitative assessments do not or only to a limited extent account for complex factors such as multiple exposure pathways, transfer of resistance between microorganisms, co-selection and cumulative effects of antimicrobial use in multiple species (McEwen 2012a).

Many assessments are based on an outcome-attribution approach, where an estimate of antimicrobial resistance-related illness in humans (derived from surveillance data) is attributed to the occurrence of resistant microorganisms in food or to antimicrobial use in the animals (Bailar & Travers 2002, McEwen 2012a). For example, FDA/CVM (2001) performed a quantitative assessment of the human health risk resulting from ingestion of foodborne fluoroquinolone resistant *Campylobacter* spp. A model was developed to correlate the prevalence of fluoroquinolone resistant *Campylobacter* spp. in chickens with the prevalence of fluoroquinolone resistant *Campylobacter* spp. infections in humans. The required data were obtained from surveillance programmes and literature. In the model, it is assumed that fluoroquinolone resistance in *Campylobacter* spp. on the chicken carcass has been caused by fluoroquinolone use in chicken. Factors such as the inter-species spread of resistance were not considered (FDA/CVM 2001, Bailar & Travers 2002, McEwen 2012a).

In the following, an example for an assessment according to FDA/CVM (2003) is presented in more detail. Hurd et al. (2004) assessed potential human health risks caused by foodborne bacteria (*Campylobacter* spp. and *Enterococcus faecium*) resistant to the macrolides tylosin and tilmicosin (a semisynthetic derivative of tylosin). The assessment is based on the FDA/CVM guidance (2003). A quantitative deterministic model was developed for estimating the probability of clinical treatment failure in average U.S. inhabitants affected by tylosin or tilmicosin resistant *Campylobacter* spp. or *Enterococcus faecium* and treated with a macrolide antibacterial. The modelling approach is based on a chain of steps or key events from the initial event to the hazard (the event hazard or fault scenario). Probability was estimated at each step. Quantitative data were not available for all steps. Where data were missing,

conservative estimates were used. The assessment focused on swine, poultry and nondairy beef cattle, for which tylosin and tilmicosin are approved in the U.S., and on foodborne transmission of an antimicrobial resistance determinant. As the assessment was considered as initial evaluation according to FDA/CVM (2003), it did not include a detailed evaluation of uncertainty. The key events considered in the assessment of Hurd et al. (2004) are summarized in Figure 5.

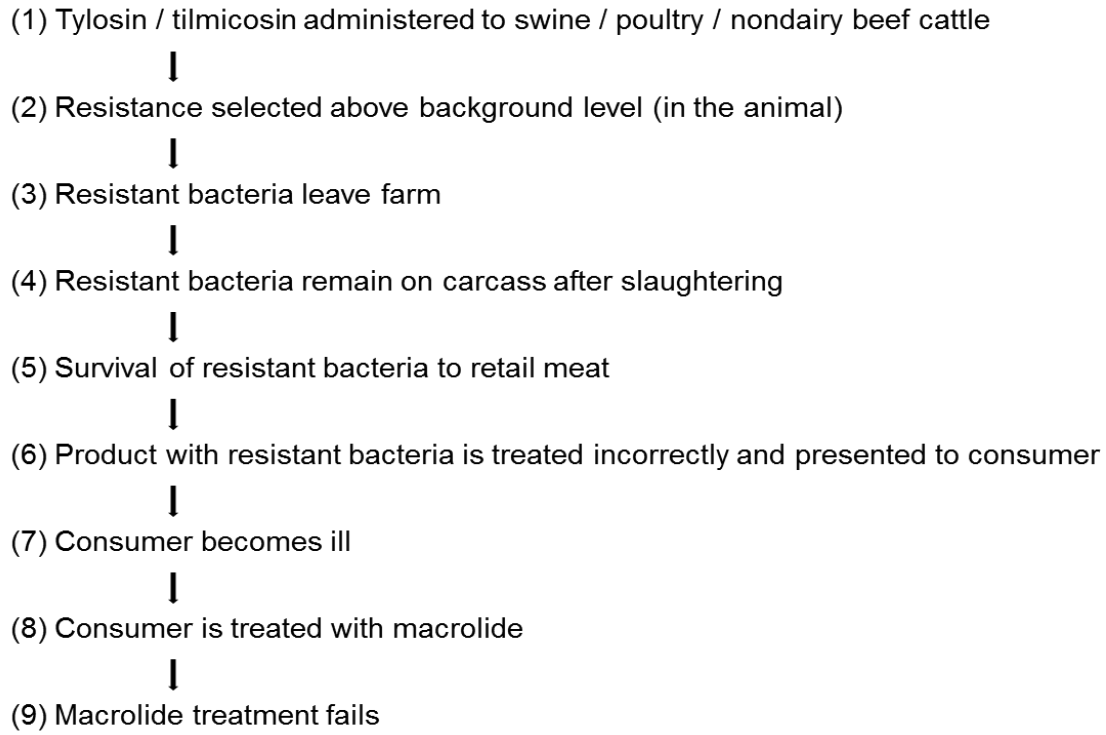

Fig. 5: Steps (key events) considered in the assessment of Hurd et al. (2004).

Only the first two steps, which theoretically might be relevant for the present project, are further described in this report. In the first step (administration of tylosin / tilmicosin to the animals), all uses of the two macrolides were considered (i.e. preventive, therapeutic and growth control). Estimates of the numbers of animals receiving tylosin and tilmicosin were based on commercial surveys.

The selection of the resistance determinant (step 2) was estimated based on:

a) The reported prevalence of *Campylobacter* spp. and *E. faecium* in the animals. For *E. faecium* this was conservatively assumed to be 100%, for *Campylobacter* spp. estimations were based on survey data.

b) The background level of resistance of *Campylobacter* spp. and *E. faecium* to the two macrolides, which may be intrinsic or due to previous use of tylosin, tilmicosin or other antibacterials that select for cross-resistance. The background percentage of resistant bacteria was estimated as 1-10% for *Campylobacter* spp. for the different animals based on survey data. For *E. faecium*, a conservative estimate of 11-30% was used.

c) The probability of mutation or acquisition of a resistant determinant and subsequent survival of a resistant strain. For *Campylobacter* spp., a 3% probability of resistance development was used based on survey data on resistance levels in humans. For *E. faecium*, a conservative estimate of 100% was used because of the absence of data.

For further information on steps 3 to 9 see Hurd et al. (2004). Overall, very low probabilities of human treatment failure were estimated (< 1 in 14,000,000 for *Campylobacter* spp., < 1 in 3,000,000,000 for *E. faecium*) that are mainly due to the low (*Campylobacter* spp.) or extremely low (*E. faecium*) probability that infections would be treated with macrolides.

Based on the reviews of Bailar & Travers (2002) and McEwen 2012a and the two examples described above, the available assessments of human health risks caused by foodborne antimicrobial resistance appear to be of limited use with regard to the development of an approach for the environmental risk assessment of antibacterial resistance. This is due to the following facts:

1. Most assessments (given their retrospective nature) use surveillance data on resistant microorganisms rather than experimental data on the increase in resistance as starting point.
2. The assessments do generally not consider the transfer of resistance between microorganisms.
3. The assessments retrospectively focus on specific pathogen/resistance combinations, while an assessment performed for authorization has to be more generic, i.e. address all relevant microorganisms and resistance determinants.

If a human health risk assessment is intended for the development and transfer of resistance in the environment, such an assessment is complicated by the limited information on human exposure through the environment and the lack of dose-response curves for human colonisation by uptake mechanisms other than uptake with food. Such risk assessments are useful to characterise the role of environmental exposure for specific pathogen/resistance combinations, for which enough data are available to perform a risk attribution, and for which environmental exposure is deemed most relevant.

5.11 Conclusions

From the current evaluation it is obvious that so far concern related to risks caused by antimicrobial resistance is primarily related to human and (to a lower extent) to animal health issues (cf. Table 10; see also EMA/CVMP 2010, 2011).

Persistence (CAC 2005) and dissemination of antimicrobial resistant bacteria in the environment (EMA/CVMP 2010) and transfer of resistance between humans, animals and the environment (OIE 2012) are considered as relevant with regard to human and animal health. However, in the evaluated documents no further information is provided how this issue should be addressed. Since this topic is not considered in the current legislation, EMA/CVMP (2010) recommended further developing the legislation.

Possible effects of AMR microorganisms or AMR determinants on the environment are currently only considered in the approach for antibacterial resistance assessment for genetically modified microorganisms.

In most cases, the evaluated documents do not contain recommendations with regard to specific test systems. This appears to be mainly due to the fact that further research is required for evaluating reliability and validity of test systems (see e.g. EMA/VICH 2011).

As detailed in section 5.10, available assessments of human health risks caused by foodborne antimicrobial resistance appear to be of limited use with regard to the development of an environmental risk assessment approach for antibacterial resistance. These retrospective human health risk assessments do not use experimental data on the acquisition of resistance or increase in resistance as starting point, but are based on surveillance data. Such data are generally not available for a prospective assessment and are in most cases lacking for the environment.

However, the evaluated documents contain a number of approaches and considerations that are useful for the assessment of resistance development within ERA procedures of pharmaceuticals. With regard to an experimental approach and the derivation of effect concentrations, especially VICH GL 36 (EMA/VICH 2011) and the examples identified in section 5.9 appear useful.

6 Proposals for the evaluation of the development of resistance in the environment in the current risk assessment of veterinary and human pharmaceuticals

In the previous sections, it has been investigated whether there is sufficient evidence that residues of antibiotics in the environment can increase the occurrence or transfer of antibiotic resistance in the environment. It has been concluded that while direct evidence is only present for such effects in worst-case situations (such as surface water in close proximity to outlets of wastewater of antibiotic production sites) but not at environmentally relevant antibiotic concentrations in soil or in bigger streams, such effects cannot be precluded. A range of test systems for antibiotic resistance has been evaluated with regard to the potential for inclusion in environmental risk assessment. In the coming section, it is discussed whether the environmental risk assessment of antibiotics should be amended with requirements to investigate effects on environmental antibiotic resistance, and how such an amendment could be set up. This section is started with a short overview of the risk assessment process of veterinary and human pharmaceuticals.

6.1 Environmental risk assessment procedures for human and veterinary pharmaceuticals

Compared to the environmental risk assessment (ERA) frameworks for chemicals, biocides and pesticides, ERA procedures for pharmaceuticals are relatively new. A first draft guidance addressing both human and veterinary pharmaceuticals was developed in the early nineties, but this concept of a single document for human and veterinary medicines was abandoned later (cf. Koschorreck & Apel 2006). A note for guidance on the ERA for veterinary pharmaceuticals was published in 1997 (EMEA/CVMP 1997), a discussion paper on the ERA of human pharmaceuticals in 2001 (EMEA 2001). The current European procedure for ERA of human pharmaceuticals (EMEA/CHMP 2006) and the ERA procedure for veterinary pharmaceuticals, which has been harmonised between the European Union, the U.S.A. and Japan (VICH 2000, 2005; for the EU supported by EMEA/CVMP 2008), are tiered approaches with action limits between Phase I and Phase II. In the following, the main elements of these procedures are summarised.

The ERA of human pharmaceuticals starts with a simple exposure assessment for the aquatic environment (Phase I). If the predicted environmental concentration in surface water (PEC_{SW}) is below 0.01 µg/L and there are no other environmental concerns such as effects on reproduction, no further assessment is required. If PEC_{SW} is ≥ 0.01 µg/L, a number of physico-chemical, environmental fate and aquatic effect studies are required in Phase II Tier A of the ERA. The risk for surface water and groundwater is evaluated by comparing the respective PEC with the predicted no effect concentration (PNEC). If a risk is indicated, further studies have to be carried out in Phase II Tier B. In addition, the bioconcentration factor has to be derived, if lipophilicity is high (log K_{OW} is ≥ 3). Effects on terrestrial organisms have to be evaluated in case of high sorption coefficients (log K_{OC} values ≥ 4). If an unacceptable risk for the environment is identified, precautionary and safety measures have to be evaluated, but the marketing authorisation cannot be refused (EC 2004, EMEA/CHMP 2006).

Effects of human pharmaceuticals on bacteria are evaluated in an activated sludge respiration inhibition test according to OECD test guideline (TG) 209 (OECD 2010) in Phase II Tier A (see also overview in Table 11). If the ratio of the PEC_{SW} to the PNEC derived in this test is > 0.1, fate of the pharmaceutical and / or its metabolites and effects on microorganisms have to be further evaluated in Phase II Tier B. With regard to the effects tests EMEA/CHMP (2006) refers to standardised tests with single microbial species (e.g. *Pseudomonas putida*) as mentioned in EC (2003). If the ratio of the refined PEC derived in Phase II Tier B to the PNEC for microorganisms is > 1, further studies of antimicrobial effects are recommended, but no guidance is provided regarding specific test methods. For pharmaceuticals with a K_{OC} > 10 000 L/kg that are not readily biodegradable, a soil microorganism nitrogen transformation test (TG 216, OECD 2000) is required.

In addition to these bacterial tests, a growth test (TG 201, OECD 2011) with cyanobacteria instead of the growth test with green algae is required in Phase II Tier A for antimicrobials (EMEA/CHMP 2006).

In phase I of the ERA of veterinary pharmaceuticals, potential exposure is assessed based on the intended use of the product. An initial worst-case exposure assessment is performed for soil or effluent from aquaculture facilities. No further assessment is required (1) for products used to treat intensively reared or pasture animals, if the predicted concentration in soil is below 100 µg/kg dry weight, and (2) for products used in confined aquaculture facilities, if the predicted concentration in effluent from such facilities is below 1 µg/L. These action limits do not apply to parasiticides (VICH 2000). In Phase II Tier A of the ERA, data have to be generated on physico-chemical properties and environmental fate. Ecotoxicity studies are required for aquatic and, if the product is used to treat terrestrial animals, terrestrial organisms. The resulting PNECs are compared to the PECs for the respective environmental compartment. If a risk is identified for one or more species, higher tier testing with these species is required in Tier B. A bioconcentration study has to be performed if log K_{OW} is ≥ 4. If an unacceptable risk is identified in Tier B, risk mitigation measures have to be evaluated. Environmental risks are considered in the risk-benefit analysis. If the risks exceed the benefits of a product, the marketing authorisation can be refused (EC 2001, VICH 2004).

For veterinary pharmaceuticals, the following effect studies with microorganisms are required (see also Table 11): In Phase II Tier A, a soil microorganism nitrogen transformation test (OECD TG 216) with a test duration of 28 d is performed for pharmaceuticals used for terrestrial treatments with two test concentrations (the maximum PEC and 10 x the maximum PEC). In Phase II Tier B, this test is extended to 100 d, if in the in the 28 d-test a difference of ≥ 25% is recorded in the rate of nitrogen formation between the lower test concentration and the control (VICH 2004). In case that a risk is indicated higher-tier studies (e.g. field studies) may be performed to investigate the effects of veterinary antibacterials on microbial processes in soil (EMEA/CVMP 2008).

As for human antimicrobials, cyanobacteria instead of green algae are the preferred test species in the algal growth test (OECD TG 201) when evaluating the effects of veterinary antimicrobials (VICH 2004; EMEA/CVMP 2008).

Effects on resistance are not considered in the current ERA procedures of human and veterinary pharmaceuticals (see section 6) (Ågerstrand 2015).

Table 11: Overview of tests with bacteria and cyanobacteria required in the current environmental risk assessment procedures of human pharmaceuticals according to EMEA/CHMP (2006) and of veterinary pharmaceuticals according to VICH (2005) supported by EMEA/CVMP (2008)

Required test	Test guideline (TG)	Required for human / veterinary pharmaceuticals in ERA Phase / Tier	Part of base set / required if... (Remarks)
Bacterial tests			
Activated sludge respiration inhibition test	TG 209 (OECD 2010)	Human pharmaceuticals: Phase II Tier A	Base set
Standardised tests with single microbial species (e.g. *Pseudomonas putida*)	P. putida: ISO (1995)	Human pharmaceuticals: Phase II Tier B	Required if ratio of PECSW to PNEC derived based on TG 209 > 0.1 (no specific guidance on provided by EMEA/CHMP 2006)
Soil microorganisms: nitrogen transformation test	TG 216 (OECD 2000)	Human pharmaceuticals: Phase II Tier B	Required if KOC > 10 000
		Veterinary pharmaceuticals: (1) Phase II Tier A: test duration 28 d	Required if pharmaceutical used for treating terrestrial animals
		(2) Phase II Tier B: test extended to 100 d	Required if in 28 d test difference between rate of nitrogen formation in control and the lower test concentration, which is corresponding to the PEC, is ≥ 25%
Higher-tier studies, e.g. field studies	–	Veterinary pharmaceuticals: Phase II, Tier B	If risk is indicated based on previous tests
Cyanobacterial test			
Algal growth test	TG 201 (OECD 2011)	Human pharmaceuticals: Phase II Tier A	Base set
		Veterinary pharmaceuticals: Phase II Tier A	Base set

6.2 Is an integration of a test strategy for resistance development into existing pharmaceuticals risk assessment needed?

In section 4, the role of antibiotics for an increase in the occurrence or transfer of resistance in the environment has been discussed. In this section, the possible risks of such an increase in antibiotic resistance in the environment are discussed with respect

to protection goals in risk assessment. Possible protection goals are described along with the knowledge gaps that currently hamper an analysis of the extent of the respective risk.

6.2.1 Protection goals and knowledge gaps

An increase in antibiotic resistance in the environment is relevant with regard to two protection goals: public (human) health and environmental health. Risks related to public health are caused by an increase in resistance in the environment, which could lead to an increase in human infections with resistant pathogens and, in turn, could increase treatment costs and mortality. Risks related to environmental health consist of changes in the composition of the bacterial community through an increase in resistance, which could result in decreased functioning of natural bacterial communities. These functional changes could include relevant ecosystem services. Both protection goals are discussed in more detail in the following paragraphs, including existing knowledge gaps.

Public health risks of increased antibiotic resistance in the environment

For environmental antibiotic resistance to become a public health risk, a suite of events has to occur, as outlined in Figure 6.

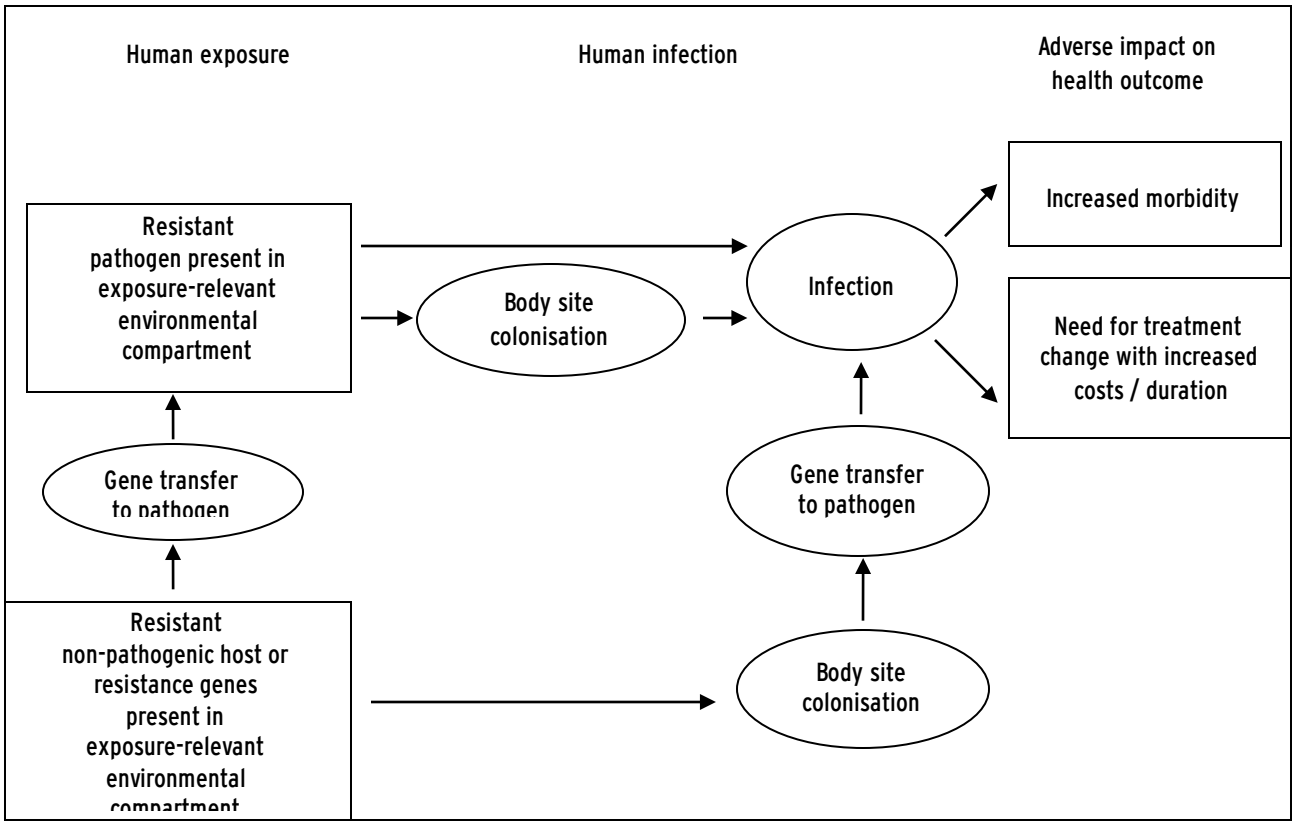

Fig. 6: Effect chain from environmental resistance to processes potentially leading to increased public health risks

First, a resistant pathogen has to be present in an environmental compartment where human exposure can occur, such as recreational water or air. Second, concentrations

have to be sufficient to cause infection, or to (temporarily) colonise body sites such as the intestines or skin. Third, the presence of the specific resistance determinant has to result in increased morbidity, or increased treatment costs (e.g. through application of different antibiotics or patient isolation requirements in hospitals). For the case of resistance genes located on a non-pathogenic bacterium, gene transfer to a pathogen must occur, either in the environment or on body sites.

Information on the likelihood of nearly all of these processes is lacking. In addition, the probabilities of these processes will depend on the specific genes and pathogens, as survival in the environment as well as the risks of colonisation and infection vary between pathogens, and as gene transfer rates depend on the bacterial hosts. The complexity of bacterial ecology in the environment and the human body also greatly increases the difficulty of obtaining meaningful data for the processes above (Wellington et al. 2013, Smith et al. 2005).

Last, while it is extremely challenging to obtain quantitative estimates of public health effects of environmental resistance, it is also difficult to compare the risk of transfer of antibiotic resistance through food or human-to-human contact with the risk of transfer through the environment. On the one hand, for the few zoonotic pathogens for which the importance of different routes of exposure has been investigated, it has been found that food consumption can play a major role (Evers et al. 2008; Domingues et al. 2012). On the other hand, exposure to pathogens through water can be significant as well (Domingues et al. 2012; Pires et al. 2012). Moreover, while the transfer of a resistance gene from an environmental bacterium to a human commensal or pathogen promoted by antibiotic residues in soils or surface waters might be a rare event, the consequences can potentially be grave, as this might lead to the formation of "new" combinations of pathogens with resistance genes of clinical relevance. Examples for a possible transfer of resistance from environmental bacteria to pathogens for example include the CTX-M genes that are now clinically relevant ESBL genes in *E. coli* (Poirel 2002, Pfeifer 2010).

Environmental health risks of increased antibiotic resistance in the environment

Many important ecosystem processes involve environmental bacteria (including, e.g. organic matter and pollutant degradation in agricultural soils and denitrification in sewage treatment plants). Changes in process rates could therefore pose risks to environmental health in the context of ecosystem functions. An increase in environmental antibiotic resistance could lead to effects on ecosystem processes if

- transfer of resistance genes to bacteria involved in such processes might affect their fitness and thereby process rates, as carriage of acquired genes can bring about fitness costs (Andersson and Levin 1999)
- changes in community composition under the selective pressure of antibiotic residues lead to the disappearance of bacteria involved in processes that are sensitive to antibiotics.

In a literature review, Mensink et al. (2007) concluded that quantitative studies on the effect of the presence of antibiotic resistance genes on the structure and function of

aquatic microbial communities are lacking, such that no conclusions can be drawn on their ecological consequences.

The function of microbial communities is – from a regulatory point of view – already addressed in the ERA of pharmaceuticals, which specifically includes a test for nitrogen transformation in agricultural soils under the influence of antibiotic residues (OECD TG 216), and a test for effects of antibiotics on respiration of sewage sludge (OECD TG 209) as detailed in section 7.1.

In view of the available test systems for effects of antibiotic residues on ecosystem functions (section 7.1) the following issue should be considered: if functional endpoints appear to be more sensitive than resistance endpoints, i.e. if antibiotic residues affect the functioning of bacterial communities at lower concentrations than the concentrations needed to result in a measurable increase of resistance, the application of functional tests might provide a simple alternative for the setup of new test systems. Further research is required to address this issue.

To conclude, the existence and the relevance of adverse effects of an increased abundance of antibiotic resistance in the environment on environmental or human health are very difficult to evaluate, and no conclusive estimate of the extent of these risks can be given at the moment. In addition, as shown in section 4, there is insufficient evidence whether antibiotic residues can increase the occurrence or transfer of antibiotic resistance in the environment. In view of these knowledge gaps, the following section addresses the question if the risk assessment of antibiotics should be amended with a test system for effects on antibiotic resistance in light of the precautionary principle.

6.2.2 Precautionary principle and precautionary approaches in other legislative acts

The precautionary principle is a guiding principle for (environmental) policy in cases where potentially adverse effects have been identified but a full assessment of the risk is not possible (Løkke 2006, Karlsson 2010). It was introduced in environmental policy in the 1970s in Germany (cf. European Environment Agency 2001, Løkke 2006) and is described in the Rio Declaration (UNCED 1993). In the EU, it has been elaborated in the 'Communication from the Commission on the precautionary principle' (Commission of the European Communities 2000). According to this communication, the precautionary principle applies when

1. the chosen level of protection of the environment or human / animal / plant health is threatened and
2. full scientific evidence of the risk is lacking or the risk cannot be fully quantified.

Uncertainties in the scientific evaluation of the potential risk may, for example, be caused by a lack of relevant data or controversies regarding available data. The precautionary principle is applied as part of the risk management process. If it is invoked, preventive measures shall be adopted that are periodically re-evaluated in view of new scientific data. In addition, research has to be performed to improve the

data base for assessing the potential risk and to ultimately complete the risk evaluation (Commission of the European Communities 2000).

With respect to antibiotic resistance in the human health risk assessment, examples exist for the application of a precautionary approach. One such example is implemented in VICH GL 36 (EMA/VICH 2011). There, a requirement for testing effects of antibiotic residues on resistance in human intestinal bacteria was introduced, while it is noted that experimental evidence of adverse effects of changes in the proportion of antibiotic resistant bacteria in the human intestinal flora on human health (e.g. prolonged antimicrobial therapy) are lacking.

6.2.3 Conclusions and recommendations

As shown in section 4, there is insufficient information to judge whether or not environmentally relevant concentrations of antibiotics can provoke an increase in resistance development or in the survival or transfer of resistant bacteria or genes with sewage sludge or manure. This is mainly caused by a lack of studies with an appropriate study design. Research needs comprise systematic investigations of concentration-response relationships between antibiotic residues and effects in environmental matrices for a number of representative antibiotics from different antibacterial classes. In addition, selective effects of other compounds classes (including heavy metals) and of compound mixtures should be addressed.

Further research is also required for evaluating if resistance endpoints are more sensitive than the functional endpoints evaluated in current environmental risk assessment procedures of human and veterinary pharmaceuticals.

In addition, knowledge gaps exist for the likelihoods of events that lead to adverse effects on public health or the environment and that are brought about by the presence of antibiotic resistance in the environment. However, a precautionary approach might be taken, especially as potential human health effects can be grave.

The final decision whether effects of antibiotic residues on environmental antibiotic resistance should be evaluated in the environmental risk assessment of pharmaceuticals requires an evaluation of the possible application of the precautionary principle. It should be based on a debate including a wide range of stakeholders such as risk assessors, specialists in infectious diseases and antibiotic resistance as well as microbial ecologists and environmental experts.

In any case, advances in test systems and test methodologies for an assessment of effects of antibiotic residues on environmental resistance will also help closing knowledge gaps through the generation of experimental data.

We therefore suggest

1. to identify suitable test designs and test methodologies to investigate the effects of antibacterials on the emergence / increase of resistance in the environment,

2. to systematically evaluate the effects of representative antibacterials from different classes on the emergence / increase of resistance in relevant environmental matrices with appropriate test designs and test methods, and
3. to analyse whether existing test systems (e.g. for ecosystem functioning) might provide more sensitive endpoints than antibiotic resistance and might thus be sufficiently protective.

Beyond these points, research needs include the following:

- Does the carriage of resistance genes have adverse effects on (the structure or) functioning of bacterial communities?
- What is the degree of human exposure to resistance via the environment (source attribution), and can the public health risks of environmental resistance be specified?

However, these research questions represent complex problems. The second research question can only be tackled for single combinations of pathogens and resistances, and such investigations are hampered by a lack of relevant data.

6.3 Evaluation of the development of resistance in the environment within the current risk assessment of veterinary and human pharmaceuticals?

The authorization procedure for antibiotics, and more specifically, the environmental risk assessment therein, provide a possible framework for an assessment of effects of antibiotic residues on resistance in the environment, mainly because it is routinely carried out for new antibiotic products entering the European (or national) markets.

Still, there are several drawbacks to such a process. First, relatively high concentrations of antibiotics in the environment might arise from situations that are not addressed in the current environmental risk assessment of antibiotics. This mainly holds true for emissions through waste water from antibiotic production sites (see section 3.5.1), but also for emissions with biogas solid waste and (probably to a minor extent) for emissions from unused drugs disposed with sewage or solid waste. Second, other pollutants might co-select for antibiotic resistance in the environment, among them biocides such as triclosan, or heavy metals (see section 5.2). Co-selective effects of substances regulated under different regulatory frameworks are however generally not addressed in the current risk assessment that is carried out for a pharmaceutical (antibiotic) product, a biocide, pesticide or an industrial chemical. Third, while an environmental risk assessment of antibiotics is mainly suited to address selective effects of antibiotics, an assessment of the transmission of antibiotic resistant bacteria or resistance genes through the environment (independent from the role of antibiotic residues for maintaining a selective pressure) seems more difficult to integrate into the product-based environmental risk assessment of antibiotics. Fourth, the highest concentrations of antibiotics are found in sewage sludge and manure, and selective effects of antibiotics are thus more likely to occur there than in the matrices addressed in the environmental risk assessment of antibiotics (soil and surface water). Still, resistant bacteria formed in sewage and manure can enter soil and surface water. The

question thus arises whether selective effects of antibiotics should rather be addressed in the compartments with the highest antibiotic concentrations, or in compartments that are seen as more relevant with respect to the protection of their biodiversity and functioning (soil and surface water).

Alternatives to an inclusion of test systems for antibiotic effects on resistance in the environmental risk assessment of antibiotics consist mainly of the following:

1. Setting of environmental quality standards for antibiotics, e.g. for surface waters (including presence and dissemination of resistance as additional endpoints)
2. Monitoring of resistant bacteria in environmental matrices, e.g. within the framework of resistance monitoring or post-marketing surveillance

The second point is addressed shortly in the following passage, followed by a discussion of possible test systems for integration into the environmental risk assessment of antibiotics.

Finally, all measures intended to reduce the usage of antibiotics and the occurrence of resistance in both animal farming and human healthcare will ultimately also reduce the presence of resistance in the environment through reducing the discharge of antibiotics and resistant bacteria from animal and human sources.

6.3.1 Post-marketing surveillance / monitoring of environmental resistance

Surveillance programmes that gather monitoring data on resistant bacteria exist in EU member states on the basis of directive 2003/99/EC (European Commission 2003b) on the monitoring of zoonoses and zoonotic agents, which requires Member States to monitor and report antimicrobial resistance in *Salmonella* and *Campylobacter* isolates from animals and food. Often, data on resistance in *E. coli* and enterococci as indicator bacteria are also gathered (e.g in the Netherlands and Denmark). For human pathogens, decision 2119/98/EC8 (European Parliament and The Council of the European Union 1998) led to the establishment of a network for the epidemiological surveillance and control of communicable diseases in the EU, and was complemented by Decision 2000/96/EC9 (European Commission 1999a) with amendment 2003/542/EC10 (European Commission 2003a) on the diseases to be progressively covered by the network. In the framework of these decisions, resistance data of human pathogens is gathered from member states and reported by the ECDC (ECDC 2012).

Environmental matrices or hot spot matrices could thus be included in national resistance surveillance systems. This has already been mentioned in CAC (2011), where it was stated that relevant matrices such as biosolids, wastewater, manure and other waste-based fertilisers should be included in antibiotic resistance surveillance programmes. An addition of environmental samples might logistically be easier in the veterinary monitoring programmes, as these routinely treat samples of different nature (food products, isolates from food producing animals).

Last, resistance monitoring systems have also been set up by producers of pharmaceuticals and might represent another possibility for inclusion of environmental compartments in resistance monitoring (de Jong et al. 2009; de Jong et al. 2012).

Post-marketing surveillance is thus another option for gathering information that is needed for risk assessments, including

- The role of cross-resistance / co-resistance
- Temporal and seasonal changes in resistance prevalence
- The proportion of microorganisms resistant to the antimicrobial agent

This information can provide a framework for the monitoring of long-term trends in resistance in the environment.

6.4 Proposal for evaluating the development and dissemination of antibiotic resistance within the environmental risk assessment of antibiotics

6.4.1 Information available from microbiological evaluation

A number of data, which are required during microbiological evaluation of veterinary antimicrobials used in food-producing animals, or during microbiological and clinical evaluation of antimicrobials used in human medicine, is considered useful for the environmental risk assessment. These data are summarised in Table 12.

Table 12: Overview of information required during (1) microbiological evaluation of veterinary antimicrobials used in food-producing animals according to VICH GL 27 (EMEA/VICH 2004) and 36 (R) (VICH 2012) and (2) microbiological and clinical evaluation of antimicrobials used in human medicine according to EMEA/CHMP (2011), which may be useful for the environmental risk assessment.

Information		Veterinary antimicrobials used in food-producing animals	Antimicrobials used in human medicine
1	Mechanism of action	yes	yes
2	Type of antimicrobial action	yes: characterisation as bacteriostatic / bactericidal	optional: assessment of bactericidal activity
3	Antimicrobial spectrum of activity	yes: MIC tests with a wide variety of microorganisms: target pathogens (from 'Efficacy' section of dossier), food-borne pathogens and commensal microorganisms	yes: MIC tests with relevant pathogens
4	Antimicrobial activity of metabolites	optional	yes: for major metabolites
5	Antimicrobial resistance mechanism(s)	yes, "where possible": information on resistance mechanism(s) and molecular genetic basis (in the absence of data on the substance: information from analogues)	yes: investigation of mechanism of resistance in microorganisms (pathogens), for which MIC is high
6	Occurrence of resistance	yes: (a) based on MIC tests (see above); optional: data from animal studies to characterise resistance development; (b) if required, NOAEC / NOAEL of emergence / increase in resistance in intestinal micro-organisms	yes: (a) based on MIC tests (see above); (b) post-approval surveillance: assessment of emergence of resistance over 3-5 years
7	Selection of resistance	optional: *in vitro* mutation frequency studies	optional: estimate of the rate of selection of resistant mutants, if resistance is caused by single mutational event

Information		Veterinary antimicrobials used in food-producing animals	Antimicrobials used in human medicine
8	Transfer of resistance genes	yes: information on occurrence and rate of transfer of antimicrobial resistance genes (data on target pathogens, food-borne pathogens or commensal microorganisms; in the absence of data on the substance: information from analogues)	no
9	Occurrence of cross-resistance	yes: phenotypic and, if available, genotypic description	yes: (1) within the respective class of antibacterials and (b) across different classes of antibacterials
10	Occurrence of co-resistance[1] / associated resistance[2]	yes: phenotypic and, if available, genotypic description of co-resistance	yes: description of the potential for associated resistance including co-resistance
Information required according to		6b: VICH (2012), all other information: EMEA/VICH (2004)	EMEA/CHMP (2011)

1: Co-resistance: organisms resistant to other drug classes are resistant to the test antibacterial, because genetic determinants (e.g. integrons, transposons, plasmids) encoding for different, unrelated resistance mechanisms are transferred and expressed together in a microorganism (SCENIHR 2009).

2: Associated resistance: organisms resistant to other drug classes are resistant to the test antibacterial due to multidrug efflux pumps or impermeability of the outer membrane, or when several resistance determinants are co-transferred (EMEA/CHMP 2011).

6.4.2 Proposals for test systems for the development of resistance in the environment

Scope

In the following sections, principles of a test system are described which has a fourfold scope:

- Determine the extent of antibiotic resistance in an environmental compartment without the presence of antibiotics (natural background)
- Investigate whether antibiotic residues can lead to the development or increase in resistance in environmental samples
- Investigate whether antibiotic residues might increase the survival or growth of resistant bacteria entering the environment through potential hot spots of resistance (manure, sewage)
- Investigate whether antibiotic residues might increase the level of horizontal transmission of resistance genes entering the environment through potential hot spots of resistance (manure, sewage) to environmental bacteria

At least for Western Europe, human exposure to hot spots (manure, sewage sludge) is deemed less important than the role of hot spots for transmission of antibiotic resistant bacteria and / or resistance genes to environmental compartments, for which exposure

of the general population is possible (e.g. surface water or manured soils). In addition, such hot spots are not considered as environmental compartments that should be protected. The test system is therefore aimed at evaluating the role of residues in the receiving environmental compartments rather than their role in the hot spots themselves. However, in principal, similar test systems could also be developed for hot spots themselves, investigating the role of antibiotic residues in manure or sewage sludge for the development, survival or transfer of antibiotic resistance during manure storage or in the sewage treatment plant.

Principle

The test systems are based on spiking of relevant environmental matrices for human pharmaceuticals (surface water) and veterinary pharmaceuticals (soils) with a range of antibiotic concentrations. In these samples, the role of antibiotics for the development or increase in resistance is evaluated, whereas the unspiked control serves to determine the resistance background. A second set of samples additionally receives potential sources of antibiotic resistant bacteria and / or resistance genes, namely sewage sludge (human pharmaceuticals) or manure of farm animals (veterinary pharmaceuticals). In these samples, the effect of antibiotics on survival or growth of resistant bacteria present in sewage sludge or manure, and the effects on the amount of resistance genes are monitored, next to effects on the horizontal transmission of resistance. The test setup is shown in Figure 7.

The detection of resistance is suggested to be based both on cultivation assays and on molecular analyses of total community DNA. This is due to the fact that the advantages and disadvantages of both measurement strategies partially compensate.

Selection and number of test matrices – soil / water and sludge / manure

Relevant hot-spots for development of resistance to the respective antibacterial depending on its release, its physico-chemical characteristics and its stability (e.g. liquid manure, manured soil, sediments of effluent-receiving rivers). In general, for human pharmaceuticals, the main release of antibiotics and resistant bacteria is thought to occur with human sewage. The development of resistance in surface water should thus be tested. Sewage sludge fertilization represents a second route of entry for residues and resistance determinants to soils. For veterinary antibiotics, manured soil represents the environmental compartment under the greatest impact from antibiotics and resistant bacteria.

In analogy to OECD TG 216 (OECD 2000), usage of at least one sandy soil is suggested, as such soils are supposed to represent a realistic worst case for the bioavailability of antibiotics. The pH of the soil should be selected such as to represent a realistic worst case for the formation of bioavailable forms of the antibiotic (e.g. presence in uncharged, biomembrane-transferable forms, as far as possible). Soils should preferentially be sampled from a location that has not been fertilized with animal manure during the last years, in order to represent soils with a natural background of resistance.

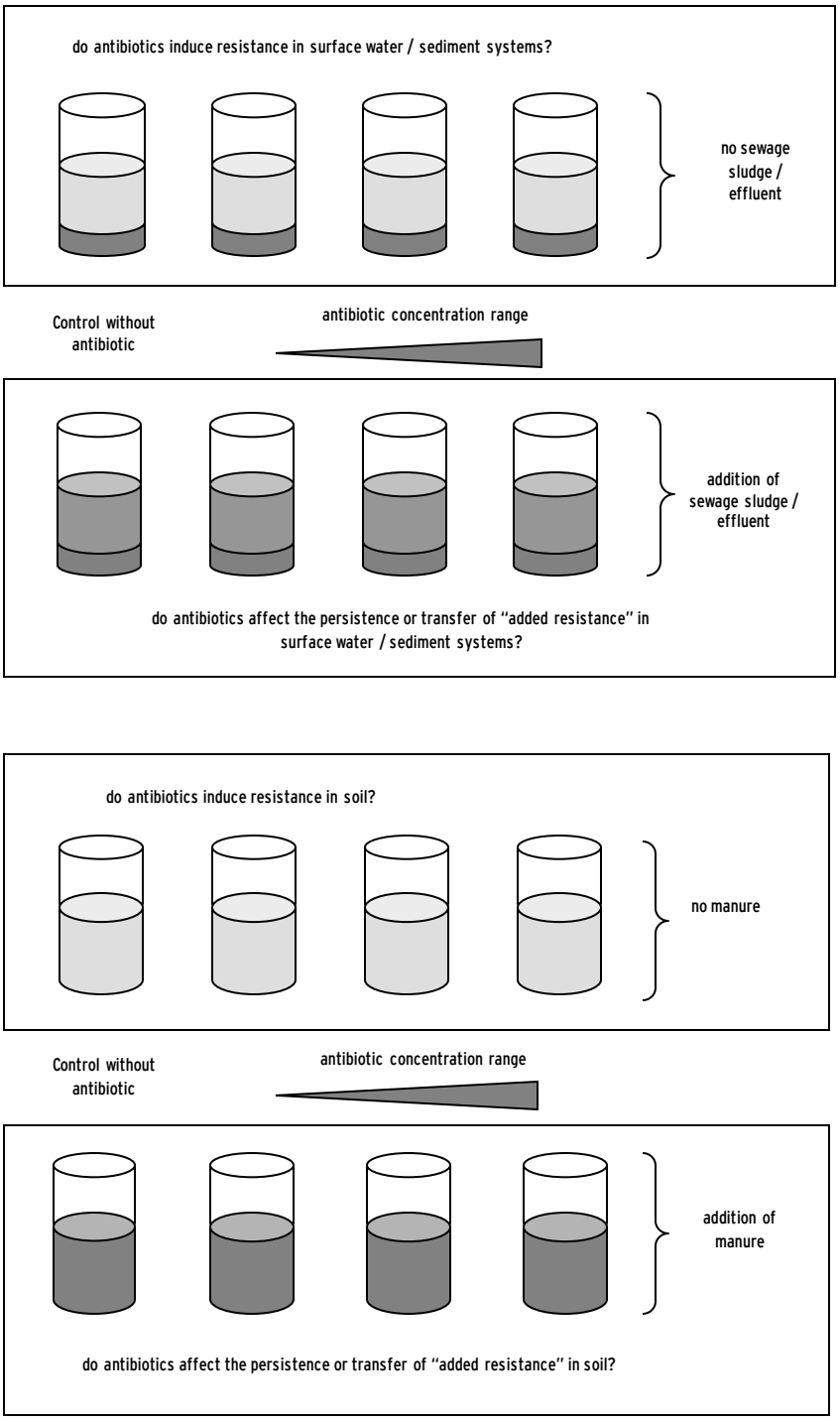

Fig. 7: Proposed test scheme for the effects of antibiotics on the induction of resistance in environmental matrices, and on the persistence or transfer of resistance added with sewage sludge or manure

With respect to surface water, test systems are suggested to be set up by use of local surface waters and sediments with their indigenous microorganisms. While most investigations so far have exclusively worked with surface water, sediment/water systems such as in OECD 308 offer the advantage of including a compartment where antibiotics with higher sorption coefficients will accumulate. In addition, bacterial

densities in / on sediment are higher than in the water column itself, increasing the chance for horizontal transfer of resistance.

With respect to hot spots, manure is suggested to be sampled from farm animals reflecting the intended use of the product. Fresh faeces stemming from animals at the end of antibiotic therapy is suggested as a worst case for the occurrence of resistant bacteria in the manure hot spot. Also, for human pharmaceuticals, soil microcosms with sewage sludge can be used, if the antibiotic residues are likely to accumulate in sludge. For water microcosms, sewage sludge should be collected from a local sewage treatment plant also treating wastewater from hospitals and care homes. However, it should be noted that standardisation of manure and sludge will be difficult to achieve.

Preparation of test substance

Solutions of the test substance can be prepared in analogy with OECD TGs 216 / 217 for soil and with OECD TG 308 for water microcosms. Thus, as far as possible, water is used as solvent (see also OECD 2000).

Test concentrations – MIC distributions as guidance

Two strategies can be followed with respect to test concentrations: on the one hand, the selection of test concentrations can be guided by the maximum worst case concentrations assumed to be present in the environmental compartment, e.g. based on the PECsurface water and PECsoil derived according to VICH (2000, 2004) and EMEA/CHMP (2006). The maximum concentrations to be tested could then be the PECsurface water and PECsoil, possibly including a safety factor. This practice is also followed in the OECD test guidelines 216, 217 and 308.

On the other hand, a concentration range can be selected, including concentrations expected to generate no effects as well as concentrations expected to generate effects (i.e. testing for a complete range of effects). Such a concentration range can enable the estimation of the critical threshold of exposure leading to a significant increase in the populations of resistant bacteria in the respective environmental compartment. In this case, a range of concentrations (at least 5, see e.g. OECD 216) should be used, including the predicted environmental concentrations. Concentrations at which effects may be expected can be derived from MIC distributions (e.g. by calculating the concentration that reflects the mean MIC_{50} or MIC_{90} for all pathogens for which MIC data exists (Singer et al. 2011; Tello et al. 2012), and by correcting this concentration for reductions of bioavailability by sorption).

While the first approach is resource economical, the second approach is superior for determining the validity of the test approach due to the fact that effects observed at higher test concentrations can serve as a kind of positive test control. In addition, the second approach yields data on effect thresholds, which help in estimating the margin of safety between environmental concentrations and concentrations leading to increases in resistance.

Further studies are needed to establish the variation in background resistance that might have implications on the number of controls and treatment replicates to be included.

Exposure duration

According to VICH GL 36, short-term tests are not appropriate to establish a microbiological ADI based on resistance development. Instead, the duration of tests intended for derivation of microbiological ADIs should be based on the type of antibacterial, the resistance mechanism, the genetic basis of this mechanism and the bacterial species (VICH GL 36). The same reasoning can be applied to testing effects of antibiotic residues on resistance in the environment. From section 4 it is noticeable that effects of antibiotic residues are sometimes seen after relatively short time frames (7 days, Stepanauskas 2006), but that effects can increase over longer time frames (e.g. over the 60 days exposure used in Knapp 2008, or during three consecutive amendments with manure of 60 days each in Heuer 2011). However, the dependence of the time frame for the development of resistance on the genetic mechanism of resistance, the antibiotic compound, or the bacterial species involved cannot be inferred from the few publications that have investigated environmental media. Thus, there is currently insufficient knowledge to propose specific incubation times, and a need for more research into the role of the abovementioned parameters in the environment. In the lack of further information, one could follow existing guidelines, which suggest testing for up to 144 days for prolonged tests in soil systems (OECD 216, 217).

For the evaluation of survival or growth of resistant bacteria or resistance genes stemming from hot spots, it has been found that the level of resistance genes originating from feedlot waste can be reduced by a factor of around 1000 after less than 10 days (e.g. Engemann 2008), such that an optimal exposure duration might be shorter. However, in soil systems, increased quantities of resistance genes have been found in manure and antibiotic co-spiked microcosms, and effects after 61 days were stronger than after 31 days (Heuer 2007). Thus, no general conclusions can be taken with respect to optimal exposure durations for studies on the disappearance or growth of resistant bacteria or genes reaching environmental compartments from hot spots.

Sampling schedule

As stated above, there is insufficient information on the role of exposure duration for the development of resistance upon exposure to an antibiotic in the environment to suggest specific exposure durations or a sampling schedule. Thus, more research is needed with respect to the development of resistance in time. In the lack of further information, sampling and determination of resistance could be performed in accordance with OECD 308, which suggests sampling at six occasions during the total incubation time, with an optional preliminary study conducted to establish an appropriate sampling regime.

In studies that evaluated the disappearance of resistance genes or bacteria stemming from sewage sludge or manure in other environmental compartments, the

disappearance / degradation kinetics have sometimes been found to follow first order (thus, following an exponential decay, e.g. Engemann 2008). An optimal sampling schedule would then consist of more intensive sampling early after establishment of the microcosms and less intensive sampling at later stages.

Analysis – proposed test systems for development and transfer of resistance

As discussed in section 5, both culturable and non-culturable methods have advantages and disadvantages. Most importantly, culturable analysis do not require knowledge on the genetic determinants of resistance and thus can also detect yet unknown resistance mechanisms, but they only target a minor proportion of the total bacterial community in the environment due to difficulties in culturing environmental bacteria on existing media. On the other hand, molecular analyses based on total community DNA represent a much bigger part of the total community, but are limited to the genetic determinants currently known. It is thus proposed to apply methods of both types simultaneously.

Culturable analyses

In general, culturable analyses can serve to identify the proportion of culturable bacteria phenotypically resistant to a given antibiotic. Culturable analyses can be targeted at specific species, or at broader groups of species. When the development of resistance in environmental organisms is investigated, usage of nonspecific media with reduced carbon content is suggested in order to increase the range of species captured by such media (Olsen and Bakken 1987).

For microcosms amended with manure or sewage sludge, it is suggested to also target intestinal species such as *E. coli* as commensal indicator species in order to evaluate the survival of potentially resistant bacteria in the environment. Investigations might be directed towards specific microbial groups, if there is reason to assume that there are organisms of particular concern.

The proportion of resistance can either be established by comparing the counts obtained on antibiotic-spiked media and antibiotic-free media (selective plating), or by analysing resistance in a number of isolates obtained from media without antibiotics (resistance profiling of isolates). While the former is less resource intensive and has a lower limit of detection, the latter probably bears the advantage of higher sensitivity (detection of smaller effects).

It is suggested to not only test resistance to the antibiotic group to which the study compound belongs, but also to other groups of antibiotics, due to the possibility of cross-resistance (see e.g. Stepanauskas 2006).

In the isolates obtained, resistance can be investigated according to standardised assays (CLSI 2011). Endpoints investigated should include the percentage of resistance of retrieved isolates, or the MIC distributions of retrieved isolates. Statistically, the difference between groups can then be determined through Fisher's exact test or chi-square statistics.

Molecular analyses in total community DNA

For the setup of analyses in total community DNA, information from other parts of the risk assessment is essential, mainly concerning the type of the resistance determinant (see section 7.3.1). If this is known, primers (and probes) for selective detection of these resistance genes can be developed. For antibiotics with resistance mechanisms that are similar to those of already existing groups, PCR methods will often already have been developed. Resistance genes can then be detected in community DNA by PCR or quantitative PCR. While detection in bacterial isolates is also possible, this would reduce the strength of molecular analyses (namely the possibility to analyse resistance genes in the total community rather than in the minor culturable part). Quantitative PCR bears the advantage of a quantitative analysis with good reproducibility. If quantitative PCR is used, normalisation to the total amount of bacteria can be performed by comparison with a PCR product, which is representative of the bacterial density, such as a 16S ribosomal RNA. In this case, both the absolute amount of resistance genes and the normalized amount should be reported.

Horizontal gene transfer

The potential to transfer resistance depends on the location of the antimicrobial resistance determinants (cf. EFSA 2008): Resistance that is carried on mobile genetic elements (e.g. plasmids, transposons) has a high potential for horizontal transfer, i.e. for distribution in the environment.

In the microcosms to which hot spot material has been added (manure / sewage sludge), the frequency of horizontal gene transfer from resistant bacteria to either environmental bacteria or to commensal bacteria should thus be evaluated.

Evaluation

For each sampling time, the proportion of resistance (and the total amount of resistant bacteria, if qPCR or selective plating is used) is calculated for each analysed antibiotic concentration (cultural methods) and each analysed resistance gene (molecular analyses), and the rate of horizontal gene transfer is determined. Concentration-response curves can be set up by use of general concentration-response functions (if the shape of the concentration-response curve is sigmoidal). From the concentration-effect relationship, an EC_{10} (or NOEC and LOEC) and EC_{50} can be derived if an upper limit of resistance has been identified. Otherwise, the concentrations that cause an increase in resistance by 10% and 100% can be reported.

The overall assessment should enable an identification of suitable risk reduction measures.

Research needs

The research needs for the suggested test methodology, as also summarised in section 5.4., are:

- Information on methodological parameters such as the reproducibility, limit of detection and inter-sample variation of several methods are lacking for environmental matrices

- If an evaluation of resistance in environmental bacteria is envisaged, the selectivity of common media used for preferential cultivation of environmental bacteria should be investigated
- The potential of test methodologies for standardisation needs to be evaluated
- The time-frame required for an emergence / increase of resistance in environmental bacteria for at least the most typical resistance determinants has to be evaluated in further research, as it may vary depending on the type of antibacterial, the resistance mechanism, the genetic basis of this mechanism and the bacterial species. Short-term tests are not appropriate to evaluate the emergence / increase of resistance (see section 7.4.2.6).

6.5 Summary

An increase in antibiotic resistance in the environment might be relevant for the protection goals 'human health' and 'environmental health'. With respect to human health, it is difficult to specify the contribution of environmental transmission of resistant bacteria or resistance genes to human exposure, as information on many contributing processes (such as gene transfer rates in the environment, or colonisation rates with resistant pathogens or resistant bacteria) are lacking. Due to the complexity of the environmental ecology of resistance, acquisition of such data will remain difficult, also in future. However, the final decision whether effects of antibiotic residues on environmental antibiotic resistance should be evaluated in the environmental risk assessment of pharmaceuticals requires an evaluation of the possible application of the precautionary principle.

While an analysis of effects of antibiotic residues on resistance might be included in the environmental risk assessment during the authorisation of medicinal products, disadvantages of such a strategy include:

- neglecting the effects of other pollutants (such as heavy metals) and
- not fully addressing the role of hotspots such as manure and sewage sludge for dissemination of resistance genes and for transmission of resistant bacteria and resistance genes to the environment.

Alternatives to an inclusion of additional tests in the authorisation of veterinary and human antibiotics could consist in monitoring of resistant bacteria in environmental matrices, or including effects of pollutants on presence and dissemination of resistance in the derivation of environmental quality standards.

If an inclusion of additional test systems in the authorisation of antibiotics is intended, such test systems could consist of surface water and soil microcosms that are additionally spiked with a concentration gradient of antibiotics, and with sewage sludge or manure as 'hot spot' sources of resistant bacteria. In these, the occurrence of resistance could be monitored by cultural techniques and quantitative PCR, and by an analysis of horizontal gene transfer rates.

7 Expert meeting "antibiotic resistance in the environment - inclusion in the environmental risk assessment of antibiotics?"

In this last section, discussions that were held at a one day expert meeting on the inclusion of resistance in the environment in the risk assessment of pharmaceuticals are summarised. The 24 participants included representatives of academia (specialists on microbial resistance, microbial ecologists, environmental scientists) as well as risk assessors (including risk assessors active in the authorisation of pharmaceuticals). During the expert meeting, the results described in the chapters 2-8 of this report were presented and discussed, together with presentations of research findings on closely related topics. For a list of participants and the agenda, see Annex II). The discussion was structured around specific topics (questions). The viewpoints of the participants raised during the workshop are summarised below along specific topics.

7.1 Extension of the environmental risk assessment of pharmaceuticals to effects on resistance in the environment

After a presentation of the results of the literature study on the role of antibiotic residues for the occurrence of resistance in the environment described in 4, the following question was voted on: "Is there sufficient evidence for a selective role of antibiotics to start regulating resistance effects in environmental risk assessments now?" Nine participants voted 'no', whereas 7 participants voted 'yes'.

In the discussion of this result, it appeared that some participants had voted against extra regulatory steps due to the need for further research results, while others mentioned that action could already be taken while conducting further research at the same time. While lacking evidence of the role of environmental residues for antibiotic resistance and the associated health effects, regulatory steps could still be taken on the basis of the precautionary principle. The difficulty to obtain information on the role of environmental residues of antibiotics for public health could also justify concerted regulatory action, in the sense that regulatory actions are taken until proven that the environment is not relevant. However, the protection goal and the framework in which antibiotic resistance in the environment is regulated should be clearly specified, as the discussion is often scattered. For example, the present ERA procedures for pharmaceuticals do not cover the entire life cycle, as they ignore production-related releases and disposal of unused medicine. Finally, a tolerable selection pressure still has to be defined in order to be able to interpret results gained from experimental test systems.

A specific suggestion was made for surface water used for drinking water abstraction: The International Association of Waterworks in the Rhine Catchment area (IAWR), the International Association of Waterworks in the Danube Catchment Area (IAWD) and the River Waterworks Association – Meuse (RIWA-Maas) have suggested a maximum concentration (a so-called acceptance threshold) of 0.1 μg/L for all bioactive substances (see e.g. Wirtz 2009). Such a value could be adopted at first instance and might be replaced by lower values for substances, for which there is evidence of toxicity at lower concentrations.

Further, environmental quality standards setting within the Water Framework Directive would enable systematic monitoring of pharmaceuticals, but the inclusion of antibiotics into the list of priority substances appeared unlikely to most participants.

Last, the reduction of the overall levels of antibiotics was seen as an effective risk mitigation strategy by most participants. However, it was acknowledged that use reduction is difficult to bring about.

7.2 Which test methods should be used in regulatory tests within an environmental risk assessment?

A presentation of test methods and testing concepts for the detection of resistance development and dissemination in the environment (as described in Chapter 5) was followed by a discussion. The main points mentioned during this discussion are summarised below.

A combination of qPCR and cultivation methods was deemed suitable. Experimentally, both PCR based methods for resistance genes and mobile genetic elements show clear evidence for an increase in resistance to sulfonamides in soil systems.

It was suggested that genetic endpoints (presence of resistance genes) might be more sensitive than phenotypic endpoints in detecting antibiotic effects. If resistance was defined on a genetic level rather than on a phenotypic level, discussions such as occurring in food microbiology (on the appropriateness of different breakpoints for resistance) could be avoided.

The presence of resistance on mobile genetic elements was mentioned as especially relevant. Test systems could consist of horizontal gene transfer analyses (see also 5.1.11 and 7.4.2.8). It was mentioned that horizontal gene transfer analyses have the potential to be standardised. Standardisation would for example require agreements on hosts, densities of bacterial donors added to the test systems, and checks for the toxicity of the medium. There are, however, only a handful of species that can currently be used as hosts (e.g. *E. coli* and *Acinetobacter*). Alternatively, the presence of mobile genetic elements such as integrons or plasmid transfer factors could be analysed by qPCR.

The complexity of environmental compartments and the complexity of antibiotic effects might call for simple tests to be used initially (e.g. tests that are not conducted in environmental matrices). Among such simple tests, extrapolation from MIC collections (Singer et al. 2011; Tello et al. 2012) could represent an initial approach for aquatic effects. For terrestrial systems, an application of MIC approaches might be hampered by the limited data on physicochemical properties and environmental fate in soil. In the discussion on the suitability of MIC extrapolations, it was mentioned that this approach might however underestimate effects on resistance, as minimum selective concentrations of antibiotics are proposed to be considerably lower than MIC values (see 4.3.4 and (Gullberg et al. 2011)). Thus, an assessment factor should be included, which should include the ratio between MICs and MSCs. Further research should then evaluate if this approach is sufficiently protective. Also, the lower end of the wild-type MIC distributions should be used for MIC extrapolations, rather than MIC

cut-offs for clinical resistance, in order to provide sufficient sensitivity. As disadvantage of MIC extrapolation methods, it was mentioned that MIC values are in most cases only available for rapidly growing aerobes, but not for typical intestinal anaerobes. On the other hand, the human commensal or pathogenic bacteria such as *E. coli* that are well represented in the MIC databased might be disproportionally important for an assessment of human health.

Tests of minimum selective concentrations (MSC) might represent another relatively simple test system. Experimentally, the use of nutrient-rich media for the derivation of MSC should be critically considered as extrapolation to the environment might be difficult. In the end, it should be possible to extend simple test systems like MSC tests to complex communities and more complex media, e.g. soil. In complex communities, however, MSC concentrations might be higher than in simple ones.

As alternative for tests for minimum selective concentrations as described by Gullberg et al. (2011), tests based on pollution-induced community tolerance (PICT) might be evaluated. Another test system, high-throughput screening of resistance elements (5.1.9), requires knowledge on the genetic mechanisms for resistance to a specific agent. Thus, testing of a new substance will be hampered if no previous knowledge on resistance to this compound class is available. However, high-througput screening can also detect co-selection of other resistance mechanisms and thus might still be applicable to new substances with new resistance mechanisms, if these induce co-selection.

Last, it was suggested to highlight how applicants could profit from extended testing requirements, as this could improve the acceptance of extended testing.

A presentation on existing risk assessment frameworks (cf. 6) also stimulated a discussion on test endpoints. While test results for resistance development in intestinal bacterial communities impacted by antibiotic residues have to be provided according to VICH GL 35, applicants often fail to provide test results due to the lack of standardised tests and unclear guidance. It was criticized by the participants that test results – should they be provided – are used for the derivation of an ADI value without application of a safety factor. However, the driving factors that led to the development of the guidance on microbiological ADIs could be analysed in order to provide a 'road map' for development of guidance on effects of antibiotics on resistance in the environment.

7.3 Research needs

One part of the discussion was devoted to research needs with respect to testing the role of antibiotic residues in the environment for resistance development.

It was discussed that optimisation of test methods represents an important research goal. A second goal is to determine the antibiotic concentrations that select for resistance and its different mechanisms in environmental matrices, and to determine the time needed for resistance development. Namely, while horizontal gene transfer might occur shortly after introduction of antibiotic residues, broad community changes might establish only after longer times. However, it was noted that soil with its

heterogeneity and humic acid content represents a challenging environmental compartment for such analyses. Also when comparing the results of 'selective concentrations' in soils with results from monitoring studies on concentrations of antibiotic residues in the environment, the analytical difficulties in soil and manure should not be underestimated. When applying concentration-response designs, "strange" results might occur, for example when high concentrations of residues promote compound degradation, and thereby possibly decrease effects on resistance. For MSC based systems, an extension to resistance genes harboured on plasmids and other mobile genetic elements should be studied, as their main application so far was for resistance brought about by mutation.

With respect to the antibiotics that should preferably be studied, "newer" antibiotics that still exhibit therapeutic efficacy and are used as last resort antibiotics (3rd generation cephalosporins, carbapenems) are the most relevant agents to study. In general, the choice of compounds for study should be based on their potential risks. Still, "old" antibiotics were mentioned as worthwhile to study in order to gain mechanistic insights and knowledge on selective pressures in general. Beyond antibiotics, the role of other agents such as heavy metals for co-selection of resistance was mentioned as research need.

Finally, the need for research on the contribution of resistance in the environment to clinical treatment failure was mentioned. However, it was noted that comprehensive evidence on the size of the contribution of environmental residues of antibiotics to clinical failure cannot be achieved, due to the complexity of the whole effect chain.

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9 Annex I: Details of studies summarized in table 5

Source	Compound	experimental conditions	gene targeted	method (gene-based)	bacterium targeted	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Binh 2007	amoxicillin	microcosms kept at 10 degrees in the dark	bla-TEM	PCR of bla-TEM, detection by dot-blot hybridization	unspecific agar	selective plates	ampicillin			
Stepanauskas 2006	ampicillin	23 degrees in the dark			unspecific (dilute nutrient broth as in McArthur and Tuckfield, 2000)	resistance testing of isolates	tetracycline, ampicillin			yes
Subbiah 2012	Ceftiofur	4 degrees	Ceftiofur-resistant *E. coli*	Selective plates						No (but investigated bioavailability)
Yu 2009	ciprofloxacin	around 23 degrees, outdoor, no shading			*E. faecalis*	selective enrichment and plating, MIC testing of isolates	oxytetracycline (16 ug/mL) / ciprofloxacin (4 ug/mL)			no
Subbiah 2012	Ceftiofur	4 degrees	Ceftiofur-resistant E. coli	Selective plates						No (but investigated bioavailability)

Source	Compound	experimental conditions	gene targeted	method (gene-based)	bacterium targeted	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Yu 2009	ciprofloxacin	around 23 degrees, outdoor, no shading			*E. faecalis*	selective enrichment and plating, MIC testing of isolates	oxytetracycline (16 ug/mL) / ciprofloxacin (4 ug/mL)			no
Munoz-Aguayo 2007	chlortetracycline	RT, protected from light	tet(A), tet(B), tet(C), tet(D), tet(E), tet(L), tet(M), tet(S), tet(Q)	normal PCR	*unspecific (1/10 LB)*	selective plates	chlortetracycline (16 ug/mL)			yes, ELISA
Engemann 2006	oxytetracycline	light treatment (OTC amended) and dark treatment, 30 degrees, maintained aerobically	tet(O), tet(W), tet(M), tet(Q)	real-time PCR on water filters	unspecific (Difco plate count agar)	selective plates	oxytetracycline (16 ug/mL)			yes (ELISA) for free concentrations
Engemann 2008	oxytetracycline	outdoor mesocosms	tet(O), tet(W), tet(M), tet(Q), tet(B), tet(L)	real-time PCR on water filters						ELISA, LC-MS-MS

Source	Compound	experimental conditions	gene targeted	method (gene-based)	bacterium targeted	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Knapp 2008	oxytetracycline	ambient	tet(B), tet(L), tet(O), tet(Q), tet(W), regulatory genes of Tn916 and Tn155	real-time PCR						yes, by ELISA and verified by LS-MS. Concentrations aimed to be kept at nominal concentrations
Yu 2009	oxytetracycline	around 23 degrees, outdoor, no shading			*E. faecalis*	selective enrichment and plating, MIC testing of isolates	oxytetracycline (16 ug/mL) / ciprofloxacin (4 ug/mL)			no
Li 2010	oxytetracycline	field study	23 tet genes, classI integrons	normal PCR of bacterial isolates	unspecific (R2A and TSB)	non-selective isolation and MIC determination for 10 antibiotics	tetracyclines among others			yes, by LC-MS

Source	Compound	experimental conditions	gene targeted	method (gene-based)	bacterium targeted	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Rodríguez-Sánchez 2008	oxytetracycline and gentamycin	ambient	tet group primers, 5 gentamycin primers	normal PCR	unspecific (1/10 TSB)	selective plates	tetracycline or streptomycin (10 ug/mL)	soil suspensions and bacterial isolates used as donors, E coli CV601 as recipient	oxytetracycline or gentamycin	yes, HPLC and biosensor strain
Kim 2007	tetracycline	batch reactors kept at 22-26 degrees			heterotrophs (R2A), enterics (MacConkey), lactose fermenters	selective plates	tetracycline (5 / 20 mg/L)			yes, by ELISA (average effluent concs around 30 ug/L)
Hund-Rinke 2004	tetracycline	outdoor conditions, sawn with grass	tet(A), tet(B), tet(C), tet(D), tet(E), tet(M), tet(O), tet(Q), tet(S), tet(W)	normal PCR						yes
Rysz 2004	tetracycline	25 degrees, acetate as carbon source in columns	17 tet resistance genes	PCR of resistant isolates	unspecific (TSB)	MPN and selective MPN	tetracycline (50 mg/L)			yes, by HPLC - concentrations in soil not clearly described (4% of administered dose in influent)

Source	Compound	experimental conditions	gene targeted	method (gene-based)	*bacterium targeted*	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Atoyan 2007	tetracycline	mesocosms run at 18-20 degrees			*E. coli*, fecal streptococci	selective plates (mTEC, KF streptococcus agar)	tetracycline			no
Schmitt 2006	tetracycline, oxytetracycline	microcosms kept at 25 degrees in climate chambers	13 tetracycline resistance genes and sul1, sul2, and sul3	normal PCR on soil DNA						yes, LC-MS-MS
Quinlan 2011	Tetracycline	mesocosms kept at ambient conditions			unspecific (1/2 nutrient agar)	selective plates	Tetracycline (100 mg/L)			yes
Cermak 2008	lincomycin	microcosms maintained at a daily temperature regime of 16 and 6 degrees			general (R2A) and actinomycetes	selective plates	lincomycin			no
Duffy 2011	streptomycin	field study					unspecific (LB)	enrichment of soil slurry in LB and then in AB amended LB, then isolation of colonies		n.d.

Source	Compound	experimental conditions	gene targeted	method (gene-based)	*bacterium targeted*	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Heuer 2007	sulfadiazine	microcosms run at 10 degrees in the dark	sul1, sul2, sul3	real-time PCR for sul1, normal PCR for sul2 and sul3	unspecific (diluted TSB)	selective plates	sulfadiazine 200 ug/mL	E. coli CV601gfp	sulfadiazine	no
Heuer 2009	sulfadiazine	microcosms, conditions not stated	sul 2, traN (for low GC plasmids)	real-time PCR				E. coli CV601gfp	sulfadiazine	no
Heuer 2011	sulfadiazine	microcosms kept at 15 degrees in the dark	sul1, sul2	real-time PCR						
Westergaard 2001	tylosin	aerobically at 25 degrees in the dark				unspecific (1/10 LB)	selective plating	tylosin (100 ug/ml)	no significance stated	no
Xiong 2015	Mix of enrofloxacin, ciprofloxacin and norfloxacin	Microcosms run at 20 degrees in the dark	qepA, oqxA, oqxB, aac(6')-Ib-cr, qnrS	real-time PCR						yes
Pei 2007	mix of oxytetracycline, sulfamethoxazole, tylosin and monensin	aerobic or anaerobic conditions at 4 or 20 degrees	tet(W), tet(O), sul(I), sul(II), ere(A), msr(A)	real-time PCR						Yes

Source	Compound	experimental conditions	gene targeted	method (gene-based)	*bacterium targeted*	method	resistance targeted	gene transfer: setup (recipient)	resistance targeted	Measured concentrations?
Xiong 2015b	Mix of a) 3 tetracyclines, b) 3 sulfonamides, c) 3 fluoroquinolones	Aerobic at 20 degrees in the dark	Tet(M), tet(O), tet(S), tet(Q), tet(X), tet(B/P), sul(1), sul(2), sul(3), qepA, oqxA, oqxB, aac(6')-Ib-cr, qnrS	real-time PCR						yes
Berglund 2014	mix of 11 antibiotics	Field study	12 resistance genes	real-time PCR						yes, HPLC

10 Annex II: Agenda and participants of expert workshop

10.1 Agenda

Introduction			
9:00	- 9:30	Jens Schönfeld	background
		Anja Coors	introduction round
Results of the current project			
9:30	- 9:45	Heike Schmitt	1. Antibiotics in the environment presentation literature review and discussion
9:45	- 10:45	Heike Schmitt	2. Role of antibiotics for the development and proliferation of resistance in the environment presentation literature review, conclusions and research needs coffee break discussion
External speakers			
10:45	- 11:15	Kornelia Smalla	Experience with test systems for detecting effects of antibiotics
11:15	- 11:45	Will Gaze	Application of MSC-type tests for selective concentrations of antibiotics
11:45	- 12:45		lunch
Results of the current project			
12:45	- 13:25	Karen Duis	3. Evaluation of antibiotic resistance in existing risk assessment frameworks presentation literature review, discussion
13:25	- 14:10	Heike Schmitt	4. Test methodology and testing concepts for the development and proliferation of resistance in the environment presentation literature review, conclusions and research needs, discussion
14:10	- 15:10	Heike Schmitt	5. Proposal for a test system presentation of the proposal for a test system coffee break discussion
Overall discussion			
15:10	- 15:50	all	Overall discussion

10.2 Participants

Participant	Institute
Andrew Singer	Centre for Ecology and Hydrology, Wallingford
Anja Coors	ECT Oekotoxikologie, Flörsheim am Main
Anette Küster	Federal Environment Agency, Dessau-Roßlau
Boris Kolar	Environmental Protection Istitute, Maribor // EMA, CVMP, ERA WP
Charles Knapp	University of Strathclyde
Dana Elhottova	Biology Centre of the Academy of Sciences of the Czech Republic
Heike Schmitt	Utrecht University
Jens Schönfeld	Federal Environment Agency, Dessau-Roßlau
Jiri Jirout	Biology Centre of the Academy of Sciences of the Czech Republic
Joakim Larsson	University of Gothenburg
Simone Lehmann	Federal Environment Agency, Dessau-Roßlau
Karen Duis	ECT Oekotoxikologie, Flörsheim am Main
Kornelia Smalla	Julius-Kühn Institut, Braunschweig
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