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**The cycle of  
veterinary  
antibiotics  
in the ecosystem**

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The cycle of veterinary antibiotics in the ecosystem

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## Abstract

In this literature review we focus on the dispersal of the most frequently used antibiotic classes for veterinary purposes and its residues in the ecosystem.

In summary, antibiotics are poorly absorbed in the gut of animals (Jjemba, 2002). As a consequence a large part of the initial compound is excreted. From the application in livestock to manure, the range of excreted antibiotic residues lies between 17-90%. Part of these residues enters the environment through direct application on the field or through run off from the storage. After excretion the residues can also be re-transformed to the initial compound, and thus become active again (Sarmah, 2006).

Composting of manure/slurry can reduce the concentration of antibiotics dramatically with 54 to 99 % depending on the different physicochemical properties of the antibiotics and the type of manure, though some antibiotics do not degrade at all. When no composting takes place, the reduction is low (12% for tetracycline after incubation at room temperature).

The degradation rate of antibiotic compounds in the soil is dependent on the physicochemical properties of the antibiotic, soil pH, temperature, soil organic matter and soil minerals and irrigation level. With so many different properties, the amount of antibiotics found back in soil is different for every situation. It is important to know what is left in the soil, because even after 40 years antibiotic resistant genes are present in the soil.

In the soil (resistant) antibiotic compounds can have an effect on the micro biota. Manure contaminated with antibiotics reduces the bacteria to fungi ratio in the soil, as the microbial biomass. Furthermore, antibiotic compounds will leach to groundwater or runoff to surface water where they can enter the sediment or biofilms, which act as sinks.

From the aquatic environment (resistant) antibiotic compounds can spread by water currents and (aquatic) organisms. Finally, (resistant) antibiotic compounds can be taken up by plant crops. The incidence of infections by antibiotic (multi-)resistant bacteria, like ESBL and MSRA, is increased and has implications for human health care. To assess this risk the acceptable daily intake concentration of antibiotics is measured. Nevertheless we have to be careful with the administration of veterinary antibiotics and future research has to focus on diminishing the usage of veterinary antibiotics, since more and more bacteria are becoming (multi-)resistant and are causing infections that are harder to treat.



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## Introduction

In the Netherlands the number of production animals was estimated in 2012 on 3.9 million cows (of which almost 1.5 million dairy cows and 0.9 million veal calves), 1.5 million sheep and goats and 12.4 million pigs (of which 2.0 million piglets < 6 weeks) and 96.9 million chicken (44.5 million broilers and 35 million layers) (CBS, 2013). With such amounts of production animals on limited land ( $41.5 \times 10^3 \text{ km}^2$ ) infectious diseases are a serious risk and threat with both a huge economic and social impact. Prevention of disease outbreaks and their consequences is therefore essential. In order to ensure this, antibiotics are used therapeutically to treat infectious diseases. However, in addition, antibiotics are in some countries also used sub therapeutically (herd or flock treatment), prophylactic and still in some countries as growth promoters to ameliorate the feed efficiency (Chee-Sanford, 2009; Dolliver, 2005; Phillips 2004). Examples of prophylactic antibiotic use are: dry-cow therapy, use after surgery, before transportation, during potential outbreaks and stressful conditions (MSU, 2012). In Europe, the use of antibiotics as antimicrobial growth promoters was totally banned first in Sweden in 1986 (Barton, 1998) followed by Denmark in 1999 (WHO, 2003). In the Netherlands the use of antimicrobial growth promoters was banned partly in 1999 and totally in 2006, when it was completely banned in Europe (Mevius, 2009). To date the U.S. still uses antimicrobial growth promoters, however they are starting to review and monitor the usage of veterinary antibiotics (Johnson, 2011; Cray, 2005). The main reasons for the widespread use of antibiotics are: ease of use compared to alternatives and the wide promotion of antibiotics as the solution to many problems. Further antibiotics are cheap, especially compared to alternatives (Rahamat-Langendoen, 2008).

### History of antibiotics

The introduction of antimicrobial drugs has had an enormous influence on our society. Till the introduction of the first antimicrobial drugs, infectious diseases like pneumonia, tuberculosis, diarrhea and diphtheria were considered the main causes of death in children and adults. Though microorganisms were discovered in 1675 by the Dutchmen Antonie van Leeuwenhoek, it lasted till the late 19th century, before Robert Koch correlated the existence of microscopic pathogens to the development of various diseases. This discovery led to the introduction of antiseptic procedures, including sanitation and hygiene, which reduced mortality due to postsurgical infections and several infectious diseases enormously. In 1911 the first chemical antimicrobial drug, Arsphenamine, was introduced by Ehrlich and Hata for human use against syphilis (Zaffiri, 2012). The next important discovery was the accidental discovery of penicillin, an antibacterial substance from the fungus *Penicillium notatus*, by Fleming in 1928 (Sarmah, 2006). From that date on antibiotics were extensively used for human purposes and succeeded in conquering many diseases. In the early 1940s the name antibiotic was introduced (Waksman, 1947). From the 1940s and 1950s on, antibiotics were also widely used in animal production systems (Hume, 2011; Wise, 2007).

### Disadvantages of antibiotic use

Besides the positive health effects of antibiotics in treating bacterial infections and diseases, a negative side effect occurred. Already in the early 40ties, shortly after the introduction of the antibiotic penicillin, the first signs of resistance to this antibiotic agent were reported (Abraham and Chain, 1940). In the last decades it became clear that antibiotic resistance is becoming a widespread risk to human health. As a result of the use of antibiotics, bacteria in humans and

animals may develop resistance against these antibiotics, so that they are able to survive exposure to specific antibiotics (Barie, 2012) thus complicating the healing.

### **Development of antibiotic resistant bacteria and genes**

Antibiotic resistance in bacteria can develop in two different ways: through vertical or horizontal gene transfer. Vertical gene transfer is the inheritance of genes, i.e. intrinsic resistance. The genes are passed on by duplication and division of the bacteria. Horizontal gene transfer is the transfer of genes between bacteria, between different strains of bacteria, from bacteria to eukaryotes, even from bacteria to animals, to the environment or to humans (Andam *et al.*, 2011; Dunning Hotopp, 2011).

The genes are present in plasmids, integrons and transposons of the DNA and can be transferred in three different ways, namely

- a. via cell-to-cell contact, which is called conjugation (Bories *et al.*, 2008),
- b. by viruses, called transduction, and
- c. as naked DNA from the environment, called transformation (Taylor *et al.*, 2011).

The process of conjugation may be viewed as a 'non-species-specific-process' (Seveno *et al.*, 2002). Horizontal gene transfer is sometimes referred to as acquired resistance. The mechanisms by which horizontal gene transfer is acquired could spontaneously happen in a bacterial cell by mutation (Taylor *et al.*, 2011).

### **Antibiotic resistant bacteria in humans and animals**

Examples of antibiotic resistant bacteria are extended-spectrum  $\beta$ -lactamase (ESBL) forming bacteria (Cohen Stuart *et al.*, 2012), methicillin-resistant *Staphylococcus aureus* (MRSA) (Allen *et al.*, 2010), fluoroquinolone-resistant campylobacter's and *Salmonella Typhimurium* DT104 (Barton, 1998). Infections of gram-positive bacteria and of some gram-negative bacteria, e.g. *Escherichia coli*, *Klebsiella pneumonia* and *Citrobacter freundii*, are treated with beta-lactam antibiotics, a widely used human and veterinary antibiotics in the Netherlands with a low toxicity (Aminov & Mackie, 2007). *E. coli* is a virulent gut commensal, which can cause urine tract infections. When *E. coli* becomes resistant these infections cannot be treated anymore. MRSA is a virulent skin and soft tissue bacteria. When MRSA bacteria infect a wound (e.g. after an operation) it will be more difficult to cure the infection, thus resulting in more severe injuries or even worse. For this reason hospitals in The Netherlands have a very strict MRSA protocol, to protect patients from this. Campylobacter's being fluoroquinolone-resistant are not only dangerous for resistance transfer from animals to humans, the resistance can be transferred to other pathogens as well, like *Salmonella Typhimurium* (Barton, 1998). *Salmonella Typhimurium* is resistant against multiple antimicrobials (Seveno *et al.*, 2002). Due to the multi-drug resistance of *Salmonella Typhimurium* DT104, it caused several epidemics in animals since 1994 and is the most successful serovar of *Salmonella* in humans. All these bacteria are a major source of resistant strains and infections in humans and resistant strains have significant implications for human health care.

The prevalence of ESBL in the total human population is estimated on 8,5% (VU MC, 2011). For MRSA this is 0,1 %. However, in high risk groups, e.g. farmers having close contact with pigs or veal calves, the MRSA prevalence is much higher. In conventional pig farms (n=202), 68,3% of the farms were tested positive for MRSA, 29% of the farmers were positive and 14% of their family members living at the farm were positive (Wagenaar en van der Giessen, 2009). In the Dutch health care system all high risk persons are screened for the presence of MRSA. Forty percent of all MRSA isolates collected at hospital admission is livestock associated MRSA-*spa* type C398. From all MRSA

detected in hospitals, 26% occur in people working in close contact with animals and comprises thus the largest risk group in hospitals.

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### **Aim of the review**

In this literature review we focus on the dispersal of the most frequently used antibiotic classes for veterinary purposes and its residues in the ecosystem. Next to that, we pay attention to the potential route of the antibiotic resistant bacteria and genes in the ecosystem. The goal of this review is to describe the potential effect the use of veterinary antibiotics may have on the ecosystem and ultimately on human health.

# 1 Cycle of veterinary antibiotics in the ecosystem

In Figure 1.1 possible links between sources and the dispersal of antibiotics and its resistance are schematically presented. The cycle starts with the administration of veterinary antibiotics in livestock. In the gastro-intestinal tract, antibiotics are partially degraded (Sarmah, 2006). Both antibiotic residues and antibiotic resistant bacteria may be present in manure or slurry. The gastro-intestinal route is one route of entrance of antibiotic residues and antibiotic resistant bacteria to enter the ecosystem. Other possible pathways are via i) animal products (i.e. dairy products, meat products and bones), ii) air distribution of dust and skin particles with bacteria, iii) through direct contact with animals.

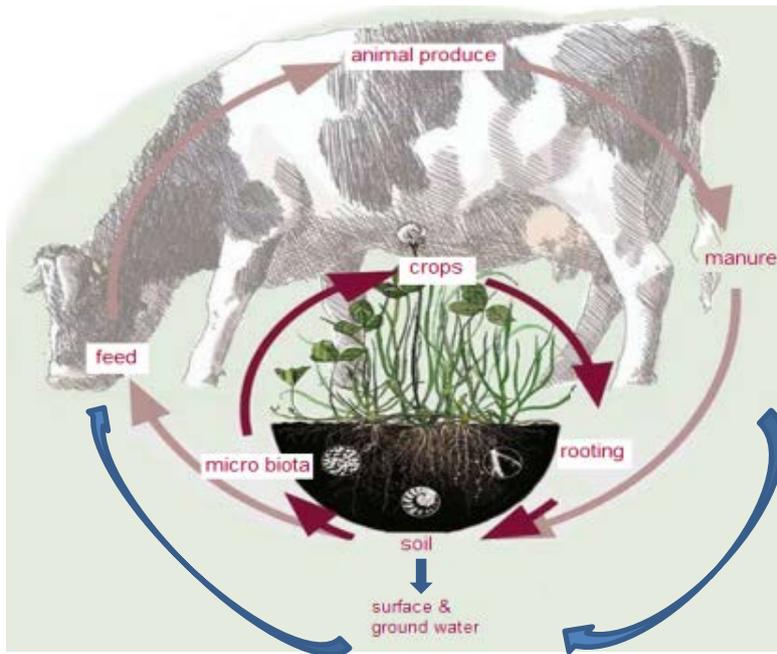


Figure 1.1. Dispersal of antibiotic residues, antibiotic resistant bacteria, and genes from veterinary antibiotic administration throughout the ecosystem.

Antibiotic residues and antibiotic resistant bacteria can enter the environment via a) direct entry, b) indirect entry or c) leaching or run-off. Direct entry consists of fecal shedding of cattle on grassland while the cattle graze or use grassland as an outdoor run. Indirect entry of antibiotics occurs during land application of manure or slurry as fertilizer. Leaching and runoff is the entrance from manure/slurry storage into the soil (Kemper, 2008). In the soil, antibiotic degradation, runoff, and sorption to soil particles may take place. Subsequently, both surface water and ground water may contain antibiotic compounds.

The entry of antibiotic residues and antibiotic resistant bacteria in the soil could have an effect on soil micro biota. Plants may take up some of the antibiotic compounds, which still may have an intact antibiotic mechanism. Thus, it potentially can influence animals and humans consuming these plants. Livestock may carry residues of antibiotics and resistant bacteria not only because of direct administration of antibiotics. Through the consumption of contaminated feed they also come indirectly in contact with antibiotics. The same counts for humans who may consume contaminated meat or plant products.

Associated with the use of antibiotics is the development of antibiotic resistance. Livestock and products may carry resistant bacteria, because of the direct administration of antibiotics and the development of resistance after administration. Other ways of getting in contact with antibiotic resistance is through the intake of contaminated feed or the breathing of air containing antibiotic resistant bacteria.

There are even natural reservoirs already present in the environment which could be a source of further antibiotic resistance spread. Therefore it is important to understand the complete cycle in order to prevent a further development and spread of antibiotic resistance within our ecosystem.

## 2 Veterinary antibiotics

From the 1940s and 1950s on, antibiotics are widely used in the animal husbandry (Hume, 2011; Wise, 2007). Over the years a continues increase could be observed. The total sales of veterinary antibiotics in the Netherlands in 2011 was 338 tons, and further decreased in 2012. This was a decrease compared to the previous years, showing that the growing awareness about the risks of antibiotic use have led to a more restricted antibiotic use. The peak in the sales was in 2007, with a total sales of 560 tons (Bondt, 2012). The difference in antibiotic sales over the years can mainly be ascribed to the difference in sales of tetracyclines (Figure 2.1)

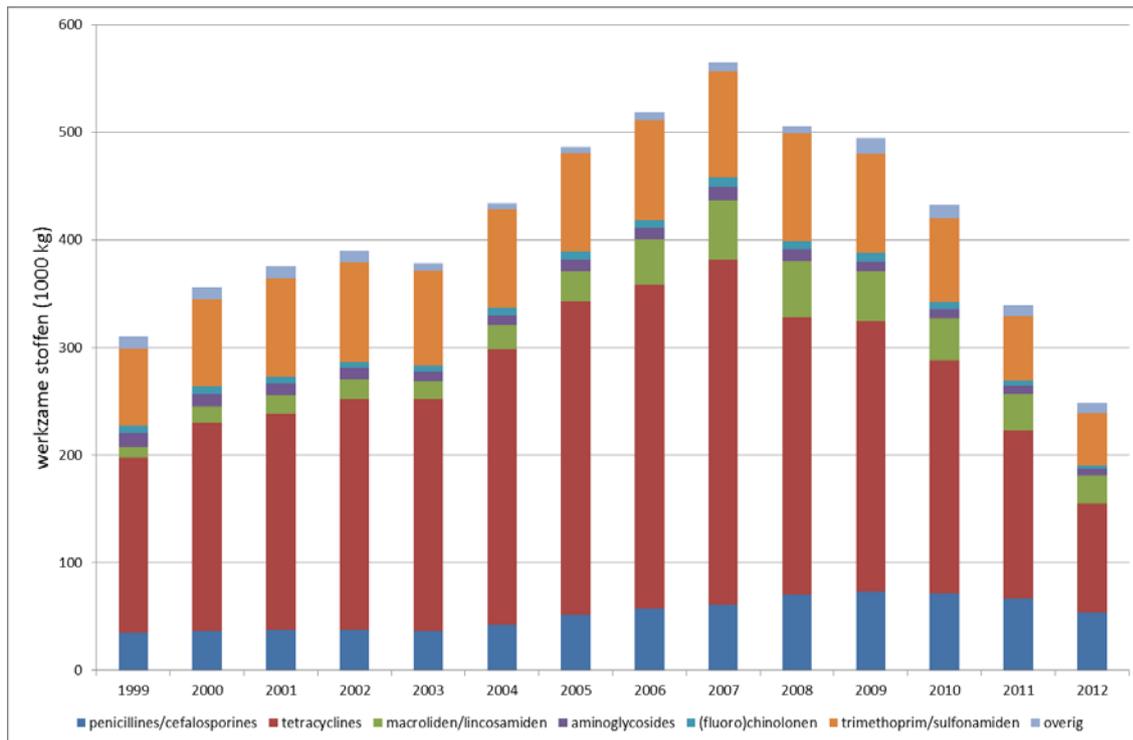


Figure 2.1. Veterinary antibiotic sales from 1999-2012, based on data from FIDIN (1999-2011) and SDA.

In Table 1, the most frequently used antibiotic classes for veterinary purposes are summarized, with the tetracycline's and the  $\beta$ -lactam antibiotics, comprised of both penicillin's and cephalosporins on top (Bondt, 2012). Also included are examples of their active ingredients, their total sales in tonnes in The Netherlands (Mevius *et al.* 2009; Kemper 2008; Bondt *et al.* 2012) and information whether this antibiotic is also used in humans (Geenen, 2010).

### **Properties of veterinary antibiotics**

In a number of studies the chemical and physical properties of multiple antibiotics are summarized (Chee-Stanford, 2009; Dolliver, 2005; Sarmah, 2006; Thiele-Bruhn, 2003; Tolls, 2001). In Table 1 the characteristics are summarized for the most commonly used veterinary antibiotics in The Netherlands.

Table 1. Antibiotic class, usage in humans and total veterinary sales in tonnes in 2011 (Bondt et al., 2012), as well as active ingredients, degradation half-life, water solubility and sorption coefficient are presented (Sarmah et al., 2006; Thiele-Bruhn, 2003; Tolls, 2001).

Antibiotic class	human usage	sales (tonnes)	active ingredient	degradation half-life (d)	water solubility (mg L <sup>-1</sup> )	Kd value (L kg <sup>-1</sup> )
Aminoglycosides	yes	7	streptomycin	na	5-200	na*
Penicillins <sup>a</sup>	yes	66 <sup>b</sup>	benzylpenicillin	10-50	5-200	na
Cephalosporins <sup>a</sup>	yes	"		10-50	5-200	na
Fluoroquinolones	yes	5	enrofloxacin	>50	5-200	>200
Glycopeptides	yes		vancomycin	na	>200	na
Macrolides		34	tylosin	<10	0-20/>200 <sup>d</sup>	5-200
	yes		erythromycin	10-50	0-20	na
			oleandomycin	10-50	0-20	na
Polyethers			monensin	>50	0-20	5-200
Sulfonamides		58	sulfamethazine	>50	5-200	<5
	yes		sulfadiazine	>50	5-200	<5
Tetracyclins		157	chlortetracycline	10-50	>200	>200
	yes		tertacycline	10-50	>200	>200
	yes		oxytetracycline <sup>a</sup>	10-50	>200	>200
Trimethoprim <sup>c</sup>	yes			10-50	5-200	<5

\*na = data not available.

a Beta-lactam antibiotics;

b the total sales for both penicillin's as cephalosporin's

c in combination with sulfonamides

d 0-20 (Thiele-Bruhn, 2003) and >200 (Sarmah et al., 2006, Schlusener & Bester, 2006)

Characteristics of antibiotics are:

- The degradation rate. The half-life of the antibiotics in days. A high degradation rate is defined as degradation in 10 days or less, mediate degradation is 10-50 days and a low degradation rate is a degradation of 50 days or more.
- Water solubility. The range of the water solubility is >200 mg L<sup>-1</sup> for high, 5-200 mg L<sup>-1</sup> for mediate and 0-20 mg L<sup>-1</sup> for low solubility. The ranges overlap, because the values for some antibiotics were so divers that they would range from low to high, therefor it was decided to score them as mediate.
- The Kd value is the sorption coefficient of the antibiotics to soil particles. The Kd ranges from >200 L kg<sup>-1</sup> (high), 5 to 200 L kg<sup>-1</sup> (mediate) and 0 to 5 L kg<sup>-1</sup> (low).

From the data in Table 1 it can be concluded that the antibiotic compounds are different in their properties. However there are opposed findings in the literature, mainly caused by differences in the way the antibiotics were studied (Sarmah et al., 2006; Thiele-bruhn, 2003).

The veterinary antibiotics which pose the highest risk for the ecosystem, are the antibiotics which

- are used in the highest amounts,
- have a low degradation rate,
- have a high water solubility, hence they can enter the aquatic environment easily and
- have a low Kd value, so are bioavailable for a longer period.

As shown in Table 1, none of the antibiotics presented has all of these characteristics. However, caution is required as part of the information is still lacking.

## 3 Antibiotic residues and antibiotic resistant bacteria in manure and slurry

### 3.1 Antibiotic administration

The method of administration of antibiotics in livestock depends on the species and the reason for use. Antibiotics are administered orally via feed or drinking water, when it is used for control treatments. Administration through injections is mostly used for therapeutic purposes (Hout, 2011).

### 3.2 Slurry/manure storage

Antibiotics are poorly absorbed in the gut of animals (Jjemba, 2002). As a consequence a large part of the initial compound is excreted. The levels of excretion reported are 30-90% (Heuer, 2011; Sarmah, 2006), 17-76% (Arikan, 2009) or up to 70% (Dolliver, 2005). After excretion the residues can also be re-transformed to the initial compound, and thus become active again (Sarmah, 2006).

Most excrements are stored as a mixture of feces and urine (slurry) and a small part is mixed with straw and stored as manure. Slurry or manure are used to fertilize the soil (Dolliver, 2008). During slurry/manure storage antibiotics are degraded, which accounts for a major part of the antibiotic loss. There are three main pathways of antibiotic degradation:

1. through mineralization (the degradation of organic substances by micro-organisms) there could be a complete elimination of the antibiotics,
2. the antibiotics can be partially transformed and
3. in the environment the degraded compounds can persist (Schlüsener & Bester, 2006).

The method of manure storage, covered or unprotected, affects the level of antibiotic resistant bacteria and antibiotic residues that potentially can leach or runoff into the environment. In the Netherlands however, all slurry pits are covered and manure and compost heaps have a fluid tight surface, and runoff water is stored (VROM, 2012).

#### Unprotected storage

In a study by Dolliver *et al.* (2007) the amount of runoff and degradation of three antibiotics (chlortetracycline, monensin, and tylosin) from an unprotected manure pile was measured under two different weather conditions. The different weather conditions consisted of a high and a low precipitation level. The concentrations of the antibiotics in the runoff increased with higher initial concentrations of the antibiotics in the manure for both conditions. The relative antibiotic losses in the runoff ranged from 0.2 to 0.6% with lower runoff water losses and from 1.2 to 1.9% with higher runoff water losses (Dolliver, 2008). It is hypothesized that the percentage of antibiotic losses is this low due to strong adsorption of the antibiotics in manure/slurry (Dolliver, 2008; Tolls 2001).

#### Protected storage

Leaching and runoff of antibiotics is positively correlated with the amount of precipitation (Dolliver, 2008). The expectation is that protected manure storage reduces the amount of runoff and leaching into the ecosystem even further, however it is not yet discussed in the literature.

### 3.3 Composting

Composting manure can also help to reduce extractable antibiotics. Composting is an aerobic process of breaking up of organic material by microorganisms. Arikan *et al.* (2009) studied the process of composting of cattle manure. They found that composting reduces the concentration of chlortetracycline

residues with 98% in 30 days. The same researchers had found in an earlier study that the concentration of oxytetracycline in cattle manure, due to composting for 35 days, was reduced with 99%, while oxytetracycline in manure incubated at room temperature was reduced with only 12% (Arikan, 2007). In another study the degradation rates of the antibiotics chlortetracycline, monensin, tylosin and sulfamethazine during 35 days of composting of turkey litter were respectively >99%, 54%, 76% and 0%. Sulfamethazine did not degrade at all (Dolliver, 2005). These differences in degradation rates are due to different physicochemical characteristics. The characteristics are degradation half-life, water solubility and the sorption coefficient.

### 3.4 Slurry/manure application

It is hypothesized that due to a high number of microorganisms in manure the biodegradation of antibiotics in soil is increased (Thiele-Bruhn, 2003). The antibiotics in feces and urine go directly into the soil (or water). The impact of weather and soil conditions (temperature, humidity) are unknown. Further study of this direct entry is necessary to find the implications for soil and plants. Indirect entry of antibiotics in the soil consists of land application of manure/slurry as fertilizer to enrich crops with trace elements, like N, P and K (Dolliver & Gupta, 2005). By law, in the Netherlands slurry has to be injected into the soil or has to be covered immediately after application. The method of spreading slurry might affect leaching to groundwater or runoff into surface water.

### 3.5 Antibiotic resistant bacteria

Not only antibiotic residues were measured during composting, some studies also looked at antibiotic resistant genes. A reduction of erythromycin resistance gene abundance in swine manure compost was found by Chen *et al.* (2007).

### 3.6 In summary

The percentage of antibiotic compounds found between application in livestock and soil is schematically given in Figure 3.1. From the application in livestock to manure, the range of excreted antibiotic residues lies between 17-90%. Part of these residues enters the environment through direct application on the field or through run off from the storage. Composting of manure/slurry can reduce the concentration of antibiotics dramatically with 54 to 99 % depending on the different physicochemical properties of the antibiotics and the type of manure, though some antibiotics do not degrade at all. When no composting takes place, the reduction is low (12% for tetracycline after incubation at room temperature).

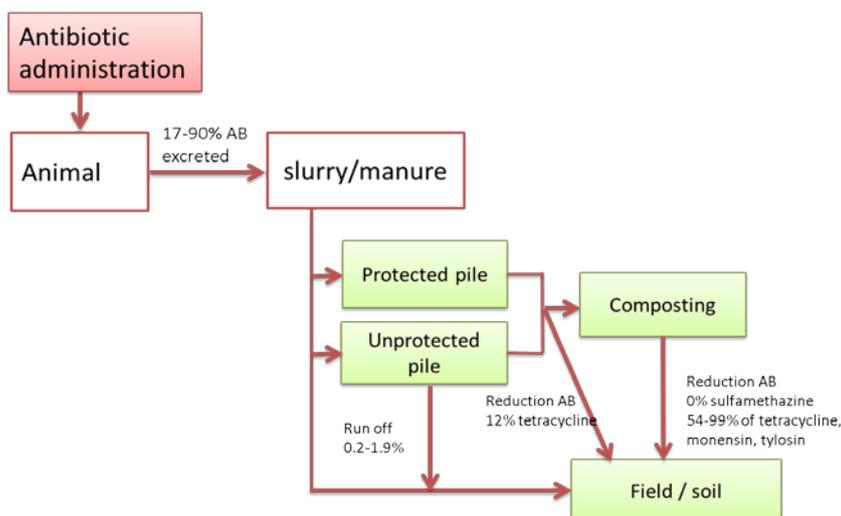


Figure 3.1 Schematic figure of antibiotic loss during storage and composting

## 4 Pathways of antibiotic residues and antibiotic resistant bacteria into the terrestrial and aquatic environment

In this chapter the pathways, degradation rate, and the fate of veterinary antibiotics, its residues, and antibiotic resistant bacteria and genes in agricultural soil will be discussed.

### 4.1 Degradation of antibiotics in soil

The fate of antibiotics in soil is mainly influenced by the physicochemical characteristics of the antibiotics (Table 1), by soil pH, soil organic matter, soil minerals, temperature and other weather conditions (Seveno, 2002; Thiele-Bruhn, 2003). In agricultural soils antibiotic residues can be found even at levels more than 10 meters deep (Aminov, 2007).

Christian *et al.* (2003) studied the residues of the most used antibiotics in liquid manure and in soil after application of contaminated manure in Northwestern Germany. They found that macrolides and sulfonamides, especially sulfamethazine, were highly stable in both substrates, whereas tetracyclines were never detected due to their high sorption coefficient. Beta-lactams and fluoroquinolones were sporadically detected. In a degradation experiment of 120 days with sandy loam soil at a temperature of 20 °C the half-lives of the macrolides erythromycin, oleandomycin, and tylosin were respectively 20, 27, and 8 days (Schlüsener & Bester, 2006). Tylosin is rapidly degraded whereas oleandomycin is the most stable antibiotic of the three. The antibiotic class of  $\beta$ -lactams (penicillins and cephalosporins) degrades rapidly, because of the unstable lactam ring present in this class. Due to this fast degradation these antibiotics are rarely found in the environment (Thiele-bruhn, 2003). Tetracyclines have high sorption coefficient ( $K_d$ ) values and adhere to soil particles, so they do not migrate easily. However 50% of chlortetracycline is degraded after 30 days and for oxytetracycline the half-life ranges from 18 up to 79 days (Sarmah *et al.*, 2006). Sulfonamides are water-soluble and migrate easily (Hu *et al.*, 2010). The sulfonamide, sulfachloropyridazine, has a low  $K_d$  value, so is highly mobile in soils and when the soil pH decreases sorption to soil particles will increase (Boxall *et al.*, 2002). The availability of antibiotic residues is thus highly dependent on the type of antibiotic and the type of soil.

There are multiple literature reviews available on the sorption coefficients, degradation rates and the chemical and physical properties of veterinary antibiotics (eg. Sarmah *et al.*, 2006; Thiele-Bruhn, 2003).

### 4.2 Influence of antibiotics on soil microbiology

Manure is used as a fertilizer, because it is rich in trace elements, like N and P. Hence, it will not only replenish soil nutrients, but also increases microbial growth and activities (Hammesfahr *et al.*, 2008). Microbial activity and earthworms provide the organic matter decomposition and nutrient stabilization of soils (Aira & Domínguez, 2009; Aira *et al.*, 2007, 2009). Earthworms provide also the aeration of soils and are the ecosystem engineers because they alter physicochemical properties of the soil and microbial communities (Aira *et al.*, 2009).

Hammesfahr *et al.* (2008) studied the effect of the antibiotic sulfadiazine, a sulfonamide, on the soil microbiology of a sandy and a loamy soil. They studied the soil microbial biomass by measuring the phospholipid fatty acids (PLFA) concentration. The results of this study showed that the amount of extractable sulfadiazine was different in the two soil types. This can be explained by different properties of the soil. The sandy soil had a lower pH, lower organic carbon content and a lower clay

and silt content than the loamy soil. Particularly, the  $K_d$  values in clay soils are higher than in sand soils. This could explain the finding that bio-available sulfadiazine decreased faster in the loamy soil. Furthermore, the total PLFA concentration and the ratio of gram-positive and gram-negative (gram<sup>+</sup>: gram<sup>-</sup>) bacteria differed comparable between the two soil types. The decrease of total PLFA by addition of manure and sulfadiazine with more than 4 days of incubation time was comparable. The bacteria to fungi ratio and the microbial biomass were decreased by addition of manure and sulfadiazine. The same researchers studied the effect of different liquid pig manure concentrations with different sulfadiazine concentrations on the soil microbial structure in a sandy soil type. They found that the microbial biomass was increased with addition of manure, though it decreased with application of manure with sulfadiazine (Hammesfahr *et al.*, 2011). So the microbial biomass is reduced when the antibiotic sulfadiazine is artificially added to manure. Manure stored for six months compared to fresh manure both contaminated with sulfadiazine, has an effect on the soil microbial biomass (total PLFA concentration). The total PLFA was reduced after 29 days of manure storage. Stored contaminated manure reduced the bacteria to fungi ratio as well (Hammesfahr *et al.* 2011a). The disadvantage of stored manure as fertilizer is the reduction of microbial biomass in soil, composed to a higher reduction of antibiotic concentration during storage of manure.

#### 4.3 Antibiotic resistant bacteria in soil

The use of manure or slurry containing antibiotic residues is expected to induce resistance in the soil bacteria as well. The mobilization of genes (horizontal gene transfer) is higher in manure-

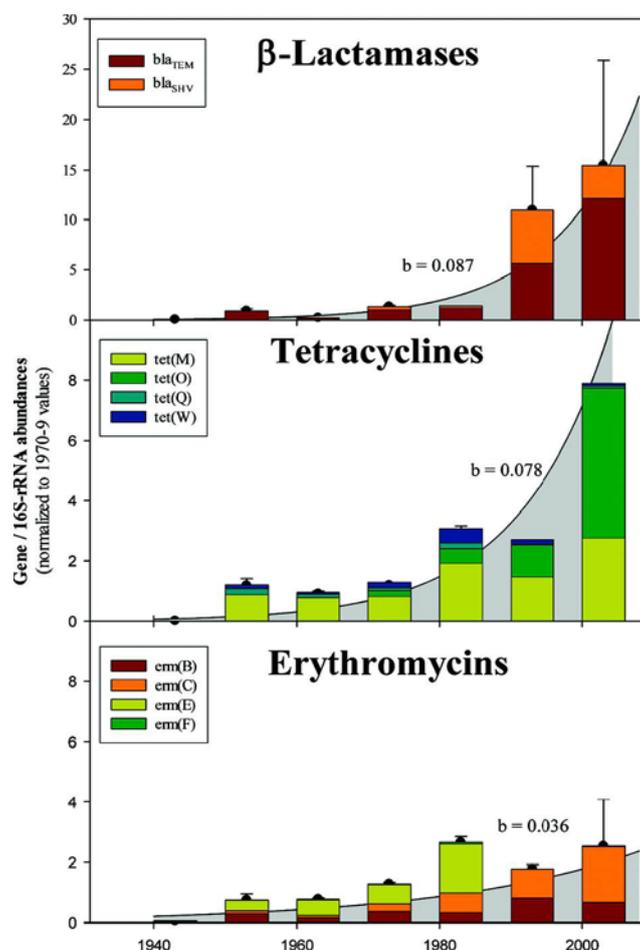


Figure 4.1. The relative increase of antibiotic resistance genes in five rural soils in The Netherlands from 1940 to 2008 (Knapp *et al.*, 2010).

amended soils than in non-manure-amended soils (Seveno *et al.*, 2002), which implicates that gene transfer occurs more often in manure-amended soils. Moreover, the abundance of antibiotic resistant genes is increased in soils over the years. In a study by Knapp *et al.* (2010) in the Netherlands, antibiotic resistance genes for ESBLs, tetracycline's, erythromycins and glycopeptides were determined in five rural soils in the Netherlands, archived from 1940 to 2008. For all antibiotic classes studied and especially for tetracycline the amount of antibiotic resistant genes have significantly increased in a range of 2 to 15 times between 1970-1979 and 2008 (Figure 4.1). An important finding in this study was that irrigation water seems to be more important than manure use for the abundance of antibiotic resistant genes in archived soils. Therefore it is advised to perform more research on the long-term impact of irrigation water quality and drainage (Knapp *et al.* (2010). They came to this conclusion, because two of the investigated sites had a greater increase in antibiotic resistant genes than the third. The three sites were all fertilized with manure and were all irrigated. The main difference, however, was that one site had a well-drained sandy soil, whereas the other two sites were irrigated with contaminated freshwater. The importance of water as a sink for bacteria is discussed in the next chapter.

The presence of antibiotic resistant bacteria is not limited to agricultural land where manure is used. Even in pristine environments antibiotic resistance occurs, because of the occurrence of natural reservoirs of antibiotic resistance genes in the environment (Allen *et al.*, 2010; Heuer *et al.*, 2011). The question arises what the ecological reasons are for antibiotic resistant genes to be present in a natural environment. It is hypothesized that those genes are useful in cell processes, like survival and growth, homeostasis, detoxification, regulation of multiple pathways, virulence and as chemical signal agents (Monier *et al.*, 2011). Whether the source of antibiotic resistant genes in the pristine environment is related to entrance of those genes via manure application and runoff is unknown.

#### **4.4 Other environments**

##### **Surface and ground water, sediments and biofilms**

Veterinary antibiotics can enter the aquatic environment through leaching and runoff from manure storage and land application of manure. The amount of veterinary antibiotics which access the aquatic environment depends on the amount of precipitation (Dolliver & Gupta, 2005; Taylor *et al.*, 2011) and other physicochemical properties (Table 1). The most mobile antibiotics will be found in surface and ground water more often than antibiotics with a high  $K_d$  value (Taylor *et al.*, 2011). In a study by Christian *et al.* (2003) the amount of antibiotic residues was measured in the surface water in eight rivers through urban and rural regions in Northwestern Germany. They did not find a significant amount of antibiotic residues from animal husbandry in the surface water. However, for sulfadimidine the amount of antibiotic residues could have originated from agricultural input. When antibiotic derivatives enter aquatic systems, the sediment becomes the main sink of antibiotic residues and antibiotic resistant genes (Taylor *et al.*, 2011), because it is rich in nutrients (Seveno *et al.*, 2002). Antibiotic compounds can affect important biological processes, like denitrification in aquatic environments and the resistance of bacteria can become more prevalent through the transfer of antibiotic resistant genes, especially in eutrophic environments (Costanzo *et al.*, 2005). These findings could have implications for the water quality that is used for drinking water, recreation or irrigation.

The aquatic environment functions as a sink for both resistant as non-resistant bacteria in which they can outcompete other environmental bacteria. Bacteria can survive in water, because water provides protection against dehydration and against UV light, *i.e.* many bacteria are sensitive for

photo degradation. In addition, in water the nutrients for bacteria are easily accessed (Taylor *et al.*, 2011). The bacterial activity and gene transfer in water is dependent on nutrient level and on temperature (Seveno *et al.*, 2002). Possible threats for widespread occurrence of antibiotic resistance are that (resistant) bacteria can be transported over long-distances with water currents and that they can become airborne with water bursts (Taylor, 2011).

Next to the indirect route of antibiotic resistance entrance in the aquatic environment, a major direct route through aquaculture exist (Taylor *et al.*, 2011). The contribution of antibiotics in aquaculture to antibiotic resistance in surface water, ground water, sediments and aquatic organisms is not discussed.

### Biofilms

Another indirect route of antibiotic resistance spread is through biofilms. Biofilms have a protective matrix wherein (antibiotic resistant) bacteria can reproduce and transfer genes; hence biofilms are possibly an important reservoir for antibiotic resistant genes (Taylor *et al.* 2011).

### Air

Antibiotic residues and antibiotic resistant bacteria can also attach to air dust particles (Thiele-Bruhn, 2003). Around animal farms dust originates from feed, water, molds, bedding, feces and the skin of animals. Moreover the dust can move through the ventilation shafts of the farm (Kemper, 2008). The air distribution of dust particles with resistant bacteria could have implications for the health of farmers and the environment (Chapin, 2005; Hamscher *et al.*, 2003).

In Figure 4.2 the pathways in which veterinary antibiotic compounds can enter the ecosystem are shown.

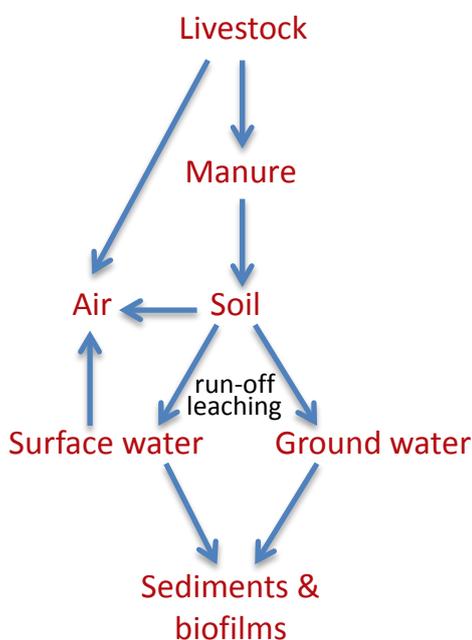


Figure 4.2. Routes of veterinary antibiotics and possible resistance into the environment.

## 5 Uptake of antibiotic residues, antibiotic resistant bacteria and genes in plants

In the previous chapters multiple studies showed that veterinary antibiotic compounds and antibiotic resistance are present in manure, soil and water. The question arises whether these compounds and bacteria can be taken up by plants or animals.

### 5.1 Plant uptake

When antibiotics are present in soil and ground water, several studies indicate that they can be taken up by arable and vegetable crops. Antibiotics are mainly taken up through water transport and passive absorption in vegetables (Hu *et al.*, 2010). The rate of uptake is dependent on the type of antibiotic and the crop. Antibiotics with a high water solubility (Table 1), like sulfonamides are assimilated more easily (Hu *et al.*, 2010). In 2005, Kumar *et al.* reported a linear dependence of the antibiotic concentration in plants on the antibiotic concentration in soil/water (Kumar *et al.* 2005). The same dependency is found in a study by Cropp *et al.* (2010). They developed a two-compartment model for plants to measure the concentration of contaminants, e.g. antibiotics, in plants as a function of time (Cropp, Hawker, & Boonsaner, 2010).

Bassil detected significant absorption of gentamycin (60 - 57 µg/kg, when applied in concentrations of 0.5 or 1.0 mg/kg, respectively) and streptomycin (50 - 17 µg/kg) only in radish, not in lettuce and carrots. The gentamycin concentration in the peel was higher than in the inside part of the root, while streptomycin was concentrated inside the root more than in the peel, but not in the leaves (Bassil, 2012). Chitescu showed that sulfamethoxazole was taken up by grass (7 - 21 µg/kg) or water cress (4 - 7.5 µg/kg); ketoconazole was taken up in much lower concentrations. Oxytetracycline was not detected in any sample (Chitescu, 2012).

Hu *et al.* studied the occurrence of antibiotics in manure, soil, surface water and vegetables in organic vegetable bases in China. Manure with antibiotic residues is used in the organic vegetable production. They detected no ciproflaxin in radish, rape, celery and coriander. Other antibiotics were detected in concentrations of 0.1 - 532 µg/kg, the highest concentrations in the highly water soluble compounds sulfadoxine, lincomycine and oxytetracycline. For lincomycine even biological accumulation was observed (higher concentrations in plant tissues compared to the soil).

Concentrations in organic vegetables were up to 4 times higher than in conventional vegetables (Hu *et al.*, 2010) The distribution of antibiotics in a plant was in the sequence leaf > stem > root, and performed (Hu *et al.*, 2010). Do Thi Cham Van found no uptake of chlortetracycline and sulfamethazin in cabbage, with input concentrations of 100, 200 and 400 µg/kg (Do Thi Cham Van, 2012). Herklotz found that carbamazepine, salbutamol, sulfamethoxazole, and trimethoprim all were taken up in cabbage, both in roots and leaves. The concentrations in roots were 98.87 ng/g carbamazepine, 114.72 ng/g salbutamol, 138.26 ng/g sulfamethoxazole, and 91.33 ng/g trimethoprim Herklotz (2010).

Roots are the main accumulation site of antibiotic compounds in plants (Migliore, Cozzolino, & Fiori, 2003). However, Hu *et al.*, reported higher accumulation rates of several antibiotics in the leaves compared to the roots of coriander, celery rape and radish (Hu *et al.*, 2010).

Gene and bacterial transfer in soil occurs mainly around plant roots where the amount and diversity of nutrients is highest (Seveno *et al.*, 2002)

Dolliver *et al.* (2007) found that sulfametazine, a sulfonamide class antibiotic, with an initial concentration of 50 mg L<sup>-1</sup> and 100 mg L<sup>-1</sup> was taken up from manure-amended soil after 45 days of growth by lettuce, corn and potato. The total sulfametazine concentration in the crops increased with respectively 14, 45 and 117%. The highest total uptake was for lettuce and corn had the lowest total uptake. However only for potato the results were significant (Dolliver *et al.* 2007). Boxall *et al.* (2006) found in an uptake study of veterinary antibiotics by carrot roots and lettuce leaves that enrofloxacin and trimethoprim were taken up by these two crops (Boxall *et al.*, 2006). However the authors concluded that the amount was not significantly great that it would cause an environmental risk.

Kumar (2005) tested corn, green onion and cabbage. All three crops absorbed chlortetracycline but not tylosin. The concentrations of chlortetracycline in plant tissues were small (2–17 ng g<sup>-1</sup> fresh weight), but these concentrations increased with increasing amount of antibiotics present in the manure (Kumar, 2005).

### **Growth effects**

Enrofloxacin was studied by Migliore *et al.* (2003) in cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), bean (*Phaseolus vulgaris*) and radish (*Raphanus sativus*). A toxic effect was merely induced by enrofloxacin with a concentration between 50 and 5000 µg l<sup>-1</sup>. The toxic effect comprised of modifying both length of primary root, hypocotyls, cotyledons and the number/length of the leaves (Migliore *et al.*, 2003).

Ocsana (2013) investigated the effects of nine antibiotics (amoxicillin, ampicillin, penicillin G, ceftazidime, ceftriaxone, tetracycline, doxycycline, ciprofloxacin and erythromycin) on foliage photosynthesis and photosynthetic pigment content in wheat. Net assimilation rate was inhibited most strongly by ciprofloxacin and cephalosporins, mainly due to reductions in stomatal conductance. Photosynthetic electron transport rate was affected by penicillins, cephalosporins and tetracyclines. Contents of photosynthetic pigments, chlorophylls and carotenoids, were most strongly reduced in treatments with tetracyclines, ciprofloxacin and erythromycin (Ocsana, 2013).

### **Transfer of resistance**

Bezanson *et al.* (2008) found that antibiotic resistance in oxidase-positive bacteria could horizontally transfer to raw vegetables, like lettuce, spinach and alfalfa (Bezanson, MacInnis, Potter, & Hughes, 2008).

## **5.2 Dispersal by animals**

In this section the spread of antibiotic resistance through animals is discussed. Animal uptake can be caused by the uptake of resistant bacteria and or antibiotics in air, water, soil, feed (milk, animal products (fishmeal) and plants).

Earthworms have bacteria on their diet (Aira, 2008), thus earthworms present in manure and soil can ingest antibiotic resistant bacteria and spread the resistance.

Widespread transfer of antibiotic resistance could easily occur by birds, especially from birds that feed on manure-amended pastures. Blanco *et al.* (2009) found that pig slurry amended pastures have the most antibiotic resistant types and that these are found in the faeces of the red-billed chough (*Pyrrhocorax pyrrhocorax*).

Aquatic organisms are next to birds another main means for transportation of antibiotic resistant bacteria over long-distances. Through aquatic systems antibiotic residues, antibiotic resistant genes and antibiotic resistant bacteria can in addition be taken up by animals by drinking the water or eating contaminated aquaculture. This occurs especially in filter feeding animals like bivalves (Taylor, 2011). Resistant bacteria can become particularly widespread, when migrating birds eat contaminated aquaculture (Allen, 2010).

Biofilms are not only present in marine and fresh water bodies, but also on the inside of wastepipes, irrigation pipes and water pipes in homes and farms. Bacterial contamination can occur of water and in this way the bacterial resistance can pass on biofilms in the water pipes (Veldman, 2012).

### 5.3 Implications for human health

Especially, farm personnel are an exposed population group to antibiotic resistant bacteria through air distribution of dust particles and direct contact with livestock. In The Netherlands, there is a quarantine period for farmers when they have to go to the hospital. In Dutch dairy farms MRSA is isolated from humans and animals (Feßler, 2012) and around 25% of Dutch pig farm personnel are carriers of MRSA (Rahamat-Langendoen, 2008).

Furthermore, food can be contaminated with antibiotic resistant bacteria or genes. Conventional and organic retail chicken meat from Dutch supermarkets were contaminated with ESBL forming *E. coli*, respectively with 100% and 84% (Cohen Stuart, 2012).

In another study the prevalence of *Salmonella* in Greek poultry meat samples was studied. Around 40% of the samples were contaminated with *Salmonella* of which 79% were antibiotic resistant (Zdragas, 2012). It is possible when animals and humans ingest it that these bacteria or genes pass to the gut micro biota (Devirgiliis, 2011) and in this way pose a threat to animal and human health. Resistance can spread to other animals or can contaminate food used for human consumption, especially in raw food products, e.g. cheese or meat (Perreten, 1997) or for food that is fermented (Ruimy, 2010). However, adequate hygiene assessment in the food industry and the house hold could diminish (resistance) food-borne pathogen outbreaks (Phillips, 2004). Commensal bacteria can as well play an important role as a reservoir for antibiotic resistant genes (Seveno, 2002; Perreten, 1997). Antibiotic resistance of gram-negative bacteria is found in both conventional and organic animal husbandry, and products from both origins had ESBL-encoding genes (Ruimy, 2010). However, organic farming has to be supported, because 15% of organic dairy farms proves that it is possible to farm without using antibiotics and in organic farming a double withdrawal time is respected in The Netherlands (Smolders, personal communication).

The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), formed a scientific expert committee to assess risks and evaluate the safety of food additives. The committee is called the Joint FAO/WHO Expert Committee on Food Additives (JECFA). They evaluate not only food additives, they also process aids (considered as food additives), flavoring agents (by functional groups), residues of veterinary drugs in animal products, contaminants and acceptable daily intake (ADI) values for veterinary drugs. The ADI value comprises the maximum daily intake level of a chemical which can be consumed daily over a lifetime without being a threat for human health. On the website of the FAO there is a link where the ADI values and other characteristics of veterinary drugs can be found. In table 2 the ADI values are given for the antibiotics discussed in Table 1. The potentially most dangerous antibiotics are

enrofloxacin and erythromycin, because the ADI value is lowest. No ADI values for the antibiotic classes cephalosporins, glycopeptides, sulfonamides and trimethoprim are given.

Dolliver *et al.* (2007) discussed the human health risk of sulfamethazine in plant crops. They concluded that the daily intake of sulfamethazine in plant tissue is far below the ADI value of 5 mg kg<sup>-1</sup> body weight and is thus not hazardous, however antibiotic resistance is not incorporated and considered in their conclusion.

In Table 2 the level of priority of risk assessment for the veterinary antibiotics is given as well. The priority of risk assessment is made by combining the potential to reach the environment, usage and toxicity profile characterization (Capleton, 2006). The potential to reach the environment is based on the method of application, the target treatment group and the metabolism of the compound.

Usage consists of the assumption

that antibiotics used in greater amount have a greater indirect exposure potential. The toxicity level is comprised of the ADI value made by JECFA, the toxic effects of the compound, biological half-life and the potential to bio-accumulate. When the priority is set as high, the veterinary antibiotic has a high potential to cause a human health risk.

Already for four antibiotic active ingredients, benzylpenicillin, monensin, sulfadiazine and trimethoprim, the priority is set as high. Due to lack of data, the JECFA was not able to set a score for several other antibiotics. In the future the number of high priority compounds may increase. Erythromycin and enrofloxacin were supposed to be a high risk given the low ADI value. Although for erythromycin no data was available and for enrofloxacin the usage and the toxicity profile were characterized as medium, while the potential to reach the environment was high. The toxicity profile of tetracycline was given as medium, although they have a high potential to reach the environment and are the most used antibiotics. The first step to ensure that veterinary antibiotics are not causing any risks, the usage of benzyl penicillin, monensin, sulfadiazine and trimethoprim must be reduced.

Table 2. The ADI values set by the JECFA for veterinary antibiotics in microgram per kilogram bodyweight and priority for risk assessment (Capleton *et al.*, 2006).

Antibiotic class	compound	ADI value (µg/kg bw)	priority for risk assessment
aminoglycosides	streptomycin*	0-50	na
penicillins*	benzylpenicillin	<30	high
cephalosporins*		na	na
fluoroquinolones	enrofloxacin*	0-2	low
glycopeptides	vancomycin*	na	na
macrolides	tylosin	0-30	medium
	erythromycin*	0-0.7	na
	oleandomycin	acceptable	na
polyethers	monensin	0-10	high
sulfonamides	sulfamethazine	5	na
	sulfadiazine*	na	high
tertracyclins	chlortetracycline	0-30	medium
	tertacycline*	0-30	medium
	oxytetracycline*	0-30	na
trimethoprim*		na	high

\* antibiotics also used against antimicrobial infections in humans (Boxall, 2006; Kemper, 2008)

## 6 Prevention of dispersal of antibiotic residues and antibiotic resistant bacteria en genes

Through transport of people, animals, animal products and crops over the whole world antibiotic resistant bacteria and genes and antibiotic residues are more easily spread. Via contaminated terrestrial and aquatic environment it can furthermore disperse by means of wildlife, water currents and aquatic organisms. In order to prevent or to diminish the transfer of antibiotic resistance, possible ways to break the cycle (Figure 1.1) are discussed.

The first step is the prevention of antibiotics entering the ecosystem. For the Netherlands, the most relevant step is by reduction of the usage of veterinary antibiotics. With minimizing the disease incidence in livestock, the usage of veterinary antibiotics can be decreased. Preventive management, including reduction in animal density, better feed, minimizing stress (e.g. minimizing transportation of the animals), keeping livestock under natural conditions and the restriction to only the therapeutically usage of antibiotics are possible measures to be taken. In some occasions alternatives can be used. Some examples are probiotics, prebiotic bacteriophages, bacteriocins, phytotherapeutics and herbs (Hume, 2011).

Secondly, not covered within this report, the decrease of antibiotics description for human use may help in the reduction of the entrance of antibiotics in the ecosystem. To enhance public awareness to the problems caused by antibiotic resistance The Antibiotic Awareness Day is organized by the ECDC (European Centre for Disease Prevention and Control) network. Here the focus lies on the problems caused by antibiotics due to the prescription of antibiotics for humans. In the Netherlands the human prescription is already low, but within Europe large differences exist, with the Mediterranean countries being the “top-users” (ESAC, 2011). The next measure would be to expend the focus of this day to the usage of veterinary antibiotics.

Another possibility to lower the antibiotic usage and its resistance is to change the purchasing behaviour of humans. At this moment, consumers tend to buy relatively cheap food, which motivates the food industry to produce as cheap as possible. By doing so, intensive agriculture systems with a high use of antibiotics are stimulated. Anomaly (2009) came with the idea of the pivotian tax. The consumers have to pay an extra tax on conventional produced food and this tax will be spend on the industry, which diminishes the use of antibiotics (Anomaly, 2009). In this way a whole new market will be available, namely an antibiotic-free market. Raising awareness is one way to diminish the usage of veterinary antibiotics. Some antibiotics should be exclusively used for humans to reduce the incidence of antibiotic resistance (Carlet, 2012). Although in the end the food producing sector has to change or stop the usage of those antibiotics.

Additionally, the dispersal of antibiotic compounds into the ecosystem has to be stopped. This could be established by enhancing the degradation rate of antibiotics. When antibiotic compounds are degraded before the manure is used as land fertilizer, leaching and runoff of the compounds can be prevented (Jjemba, 2002).

When veterinary drugs, e.g. antibiotics, are prescribed, withdrawal periods are set. Within the organic food production a double withdrawal time is respected. In this period nothing of the treated animal can be sold or used. These periods are meant to guarantee that at the moment of consumption the animal products do not have residues above the maximum residue limit (MRL) for that specific drug. The MRL is defined as the maximum residue limit for which no negative health effects are predicted for the consumer. The MRLs are set for veterinary and human drugs and are

set at the European level (RIVM, 2012). In and shortly after the period of antibiotic administration to the animal, antibiotic residues and antibiotic resistant bacteria are present in the excretion products of the animal, for cows in milk and manure. The milk can not be sold during the waiting period and often stored with the slurry. Yet there are no withdrawal periods set for manure/slurry storage and application. However, in The Netherlands there is a fixed period where manure can be applied on the field, namely from February till September, so in practice a large property of the manure is stored for a longer time before application (VROM, 2012). Without the withdrawal periods antibiotic residues and antibiotic resistant bacteria can enter the environment by runoff and leaching in higher concentrations. Antibiotic residues in stored, especially composted, manure are reduced up to 99% depending on the antibiotic class.

## 7 Concluding remarks & future research

Mainly through antibiotic administration in livestock and humans are antibiotic residues and antibiotic resistant bacteria and genes present in the ecosystem. Nevertheless, more research is necessary to study the prevalence of antibiotic residues and antibiotic resistant bacteria and genes (intrinsic resistance) already present in the pristine environment and whether these natural occurring bacteria influence resistance or if they are affected by veterinary antibiotics.

During the movement of veterinary antibiotics through the ecosystem, the antibiotics are degraded. However, to which extent is dependent on the physicochemical properties of the antibiotics, temperature and properties of the substrate. Different antibiotic classes have different implications for the ecosystem.

Antibiotic resistant genes are found in agricultural soils up to even 40 years after the first application of manure (Knapp, 2010). They are even found on soils never fertilized with manure. The results are in relation to the use of contaminated irrigation water. Irrigation water can leach to ground water, yet not much is known on the prevalence of antibiotic resistance deeper in the ground, in sediments or on the impact of contaminated irrigation water.

The studies discussed on the soil microbiology were all performed with the antibiotic sulfadiazine. The effects of other veterinary antibiotics on the microbial population and on different soil types have to be studied in more detail together with the impact of earthworms on the prevalence of antibiotic resistant bacteria in soils. Also the effect of manure storage has to be extensively studied to find out whether an increased reduction of antibiotic concentration or a reduction of microbial biomass in soil has a more pronounced effect on the ecosystem.

The uptake of antibiotic residues or antibiotic resistant bacteria and genes is especially dangerous for crops of which the tubers and roots are used. Different antibiotics could exist as well on plants (as dirt on the outside), but the effect of this source is not known.

The risk of antibiotic resistance to humans and animals when they come in direct contact with polluted soil, water and when they consume contaminated plants (fruits, vegetables and grains) has to be assessed. Some bacterial infection diseases in humans are caused by swimming in contaminated surface water or by drinking contaminated drinking water. If the bacteria are antibiotic resistant those infections will be harder to treat, however more knowledge is necessary to say precisely if it is a health concern.

The third route of dispersal is through air, yet there is not much data on the degradation behaviour of antibiotic residues in air.

One way to delete antibiotics from the ecosystem is to adsorb antibiotics in order to reduce environmental risks. An effective sorbent for tetracycline from the aquatic environment is studied by Xu *et al.* (2009). Drugs uptake and degradation by aquatic plants as a bioremediation technique is discussed by Forni *et al.* (2001).

In order to discover new antibiotics and to study antibiotic resistance genes metagenomic approaches are used (Devirgiliis, 2011; Monier, 2011). Metagenomic approaches have a benefit in being culture-independent. Environmental bacteria which cannot be cultured in standard media can be studied with metagenomics. It is also beneficial to study metabolic functions which are important for human health (Devirgiliis, 2011).

Since there is a difference between cattle, pig, and poultry production systems not only in the amount of antibiotic usage but as well in manure management and the application of manure, extensive research is needed to study the effect of the antibiotics used in the different production systems on the ecosystem.

To what extent the concentrations of antibiotic compounds are found in the ecosystem in which they can still pose risks for humans, animals and the ecosystem has to be studied in more detail. A characterization is necessary for the antibiotics with missing data for their risk assessment.

In conclusion, the incidence of infections by antibiotic (multi-)resistant bacteria, like ESBL and MSRA, is increased and has implications for human health care. It is thus necessary to continue monitoring and reducing the usage of veterinary antibiotics. For this reason, we have to enhance the awareness in farmers and public of antibiotic resistance in order to make sure that there are still antibiotics available to treat infections in both animals and humans in the future.

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