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Chemical Fate of Sulfadiazine in Soil: Mechanisms and Modelling Approaches

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Abstract

Sulfadiazine (SDZ) belongs to one of the largest classes of antibiotics applied in Europe in animal husbandry. Exposed to the environment by the release of manure as fertilizer to agricultural soils or directly by the excrements of grazing animals sulfonamides may affect soil microorganisms and soil functions, and may enhance the spread of bacterial resistance. In order to asses the impact and the risk of SDZ on soil organisms a fate model approach for prediction of temporally resolved pore water concentrations has been developed. Pore water constitutes the easily available fraction in soil which serves as interface variable between chemical fate and microbiological effect models.

Before combining all processes in one model it is necessary to develop mathematical descriptions for single processes and quantify the influence of substance and environmental parameters. Thus, in a first step, relevant chemical processes of the veterinary antibiotic sulfadiazine (SDZ) after medication to pigs were identified in manure and manure-amended soil. Models to describe the chemical fate were developed, parameterized and integrated with growth inhibiting effects on soil bacteria. Based on experimental data sensitive parameters and correlations between microbial processes and substance fate were analyzed.

Sulfadiazine is a dissociating compound and its environmental fate as well as its effect on microorganisms is strongly influenced by the pH value of the surrounding solution. The dissociation equilibrium is determined by solution pH and the dissociation constant pK_a of the substance from which the fraction of each species (anion, neutral, cation) can be calculated. SDZ is partly metabolized to and excreted as N^4-acetyl-sulfadiazine (Ac-SDZ) which reacts back to SDZ during storage of the manure prior to application and in the manure-amended soil. Substantial amounts of the hydroxylated metabolite (OH-SDZ) were also identified in pig manure and manure-amended soils but seem to remain unaffected by chemical transformation processes on the investigated timescale.

Reversible equilibrium sorption in manure and soil depends on matrix properties such as water content, organic carbon content, mineral surfaces, pH etc. Previously measured equilibrium sorption coefficients are in the range of 1-10 L kg^{-1}. In this work a relatively small value of 0.56 L kg^{-1} has been estimated for two investigated soils. However, estimations using this apparent partition coefficient K_d^* show that more than 80% of SDZ mass is sorbed to the solid matrix in investigated soils Kaldenkirchen, a silty sand, and Merzenhausen, a sandy loam. Based on existing

literature data for different sulfonamides it was shown that an independent prediction of sorption coefficients is still not possible as the widely used K_{OC} concept does not fully explain the observed variations in K_d values.

The chemical fate model was developed on the basis of available experimental data in manure amended soil. It considers all dynamic fate and transformation processes as first order kinetics. Model scenarios clearly show that dissipation of SDZ and its metabolites in soil is dominated by a reversible translocation from the available fraction into the residual fraction. This process may be based on a physical entrapment of the compounds with subsequent sorption. The irreversible formation of bound residues (BR) proved to be of importance for sulfadiazine leading to a significant reduction of the available antibiotic fraction. It is still not fully understood which mechanistic processes are responsible for the observed BR formation. Latest experimental results indicate a covalent binding between the amino group of the sulfonamide and humic substances in soil (Bialk and Pedersen, 2008). This suggests that formation of bound residues in soil can be restricted to SDZ and OH-SDZ which is confirmed by the model.

Uptake and bioaccumulation of sulfonamides in bacterial cells is one link between chemical fate and antibiotic effect of the compounds and is simulated by a dynamic model describing the transport of neutral and ionic compound fractions through the cell wall as a diffusion-like process. Comparison with effect data underlines that dissociation in dependence of intra- and extracellular pH determines substance accumulation. The approach has been extended by including the enzymatic reaction that is affected by sulfonamides, namely the production of DHP within the folic acid cycle. Effects are modelled in terms of competitive inhibition of the enzyme DHPS and are integrated with the chemical fate of SDZ on a molecular scale. In dependence of enzymatic parameters (V_{max}, K_m) the applied substance concentration which results in a 50% inhibition of the DHP production can be estimated.

Combining estimations on equilibrium sorption with the chemical fate model pore water concentrations of SDZ can be simulated kinetically and compared to effective concentrations which are exceeded for several days after manure application.

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Chapter 1

Aim and Scope

Sulfadiazine (SDZ) is one of the oldest and still widely used sulfonamides, a group of synthetically produced antibiotics, which was introduced in 1939 (Vree and Hekster, 1987). Gerhard Domagk, who was the director of Bayer's Institute of Pathology and Bacteriology, studied the antibiotic effect of prontosil, a dye containing sulfamyl, which protected mice from streptococcus. However, this product only showed its antibiotic action in the living animal. In 1935, a French research team at the Institute Pasteur in Paris discovered that prontosil is just the *prodrug*, i.e. the substance itself is in an inactive form and is metabolized in the body into an active compound, in this case into sulfanilamide. Since then, several molecules based on the effective sulfonamide group R-SO_2NH_2 have been developed to improve antibiotic activity and tolerance, i.e. lower dissociation constant to improve solubility and, consequently, to avoid crystallisation in the kidneys (Vree and Hekster, 1987).
Since 2006 the use of antibiotics as food additives and growth promotors is forbidden in European animal husbandry[1]. Nevertheless, in several European countries sulfonamides still constitute one of the largest groups of antibiotic compound classes (Thiele-Bruhn and Aust, 2004). In Germany, for example, they come in third with still increasing use volume after tetracycline and β-lactam antibiotics applied in veterinary medicine (Schneidereit, 2006).

[1]Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Official Journal of the European Union L 268/29-43.

1.1 Mode of Action of Sulfonamides

Sulfonamides act very effectively against bacterial or protozoal infections often in combination with trimethoprim. They are bacteriostatic compounds meaning that they inhibit bacterial cell growth reversibly. Based on their structural analogy to para-aminobenzoic acid (pABA) which is necessary for the synthesis of folic acid sulfonamides are competitive antagonists in the enzymatic reaction of pABA with dihydropteroate synthase (DHPS) and inhibit the production of dihydropteroate (DHP). If this key step is missing the lack of folic acid inhibits the bacterial cell to generate nucleic acids (DNA) and, thus, to divide in the presence of sulfonamide. Therefore, sulfonamides can only act against microorganisms which cannot take up folic acid but have to synthesize it on their own using endogenous compounds. Gram positive and negative coccoi, gram negative rods like *E. coli*, but also chlamydiaceae and protozoans belong to this group of microorganisms. This also explains why mammalian cells which depend on dietary supply of folate are not affected by sulfonamides (Vree and Hekster, 1987; Alexander et al., 1990). The bacteriostatic effect of sulfonamides becomes visible after a few hours of latency, i.e. when the pool of folic acid is depleted.
The combination with trimethoprim potentiates the antibiotic effect since trimethoprim inhibits the second step of the folic acid synthesis by acting against the dihydrofolate reductase. In addition, fewer resistances are generated if the combination of the two antibiotics is applied.

1.2 Environmental Relevance

Antibiotics are defined as “low molecular weight microbial metabolites” that at low concentrations inhibit the capability of microorganisms to reproduce as well as inhibit the growth of an individual cell (Lancini and Parenti, 1982; Thiele-Bruhn, 2003). Their properties (high stability and water solubility as well as high “toxicity” against certain organisms) make them feasible to act very effectively against bacterial infections or infections by protozoans even at low doses. Excess molecules of the parent compound and the metabolites are completely excreted from the body after a short time of residence. Therefore, residual concentrations of sulfonamides and their metabolites can be found in the environment which may be very mobile, persistent and effective/toxic. Consequently, urine of the treated animal contains a mixture of parent compound and transformation products (Thiele-Bruhn and Aust, 2004;

Boxall et al., 2002). Therefore, the most important emission pathway of sulfadiazine and its main metabolite N^4-acetyl-sulfadiazine (Kreuzig and Höltge, 2005) into the environment is the excretion of urine of livestock animals including both, grazing livestock and the use of liquid manure as fertilizer to agricultural land (Thiele-Bruhn, 2003; Boxall et al., 2003; Kay et al., 2005a). Emissions mainly occur to surface waters and soil via aquaculture, intensive livestock treatments, and runoff from agricultural soils, whereas releases of veterinary medicines into the atmosphere and impacts of emissions from treating pets as well as direct disposal of unused or expired products and waste containers are considered less relevant (Boxall et al., 2002).

Hardly any information is available about the ecological toxicity of antibiotics (Thiele-Bruhn, 2003) which is necessary to address possible adverse effects of antibiotics in the environment. Antimicrobial substances are of particular interest due to the possible dispersion of bacterial resistance (Sithole and Guy, 1987; Kay et al., 2005a; Göbel et al., 2005) in the environment triggered by emission of the compounds. Resistant microbial populations are already wide-spread in the environment and also present in soils since soil organisms produce several antibiotics and appropriate avoidance strategies. The ongoing use of antibiotics in livestock and the application of manure to agricultural soils, however, may further contribute to this problem (Pils and Laird, 2007; Heuer and Smalla, 2007). Both, the antibiotics and resistant organisms may be transferred to and accumulate in the food chain and thus reduce the success of pharmacotherapy of animals and humans (Rhodes et al., 2000; Kumar et al., 2005).

The application of contaminated manure enhances the distribution of bacteria which obtain an evolutionary advantage by resistance genes. There are at least two different ways of "developing" resistances: Some microbes have its own genes that impart resistance by mutation; other antibiotic-susceptible bacteria receive resistance genes via plasmids (packets of genetic material) from already resistant bacteria and incorporate them into their own chromosomes (Josephson, 2006). For sulfonamides, different resistant genes could be isolated from *Escherichia coli* found in pigs and even in humans also indicating a possible transfer via the food chain (Infante et al., 2005; Hammerum et al., 2006). Especially in environments influenced by agriculture an increased occurrence of sul-genes was detected (Pruden et al., 2006). In addition, the appearance of the sulfonamide in the environment may affect the composition of microbial communities and, accordingly, ecological functions.

Burkhardt and Stamm (2007) investigated the fate and depth distribution of three

sulfonamides in a loamy grassland soil and detected sulfonamide concentrations in pore water close to the soil surface of 20 to 50 μg L^{-1} which are in the same range as effect concentrations (Böhm, 1996). Accordingly, Christian et al. (2003) determined sulfonamide concentrations in soils of 15 μg kg^{-1} (d.w.) seven month after manure application. From soil, sulfonamides may leach into groundwater (Hamscher et al., 2005) or may be transported into surface water via runoff (Kay et al., 2005a,b). Nevertheless, observed concentrations in groundwater and runoff are low (Blackwell et al., 2007). This is confirmed by investigations of Kreuzig et al. (2005) who point out that runoff of sulfonamides can be neglected after tillage of the agricultural soil. In order to evaluate influences of soil properties and environmental conditions on the chemical fate of sulfonamides, however, a deeper knowledge of underlying mechanisms is necessary. Finally, risk assessment has to include both parent compound and its metabolites. Otherwise, the environmental effects of sulfadiazine and of veterinary medicines in general, might be underestimated (Boxall et al., 2003; Göbel et al., 2005).

1.3 Summary of Experimental Investigations

1.3.1 Joint Research Project

This thesis was developed within a research project funded by the German Research Foundation (DFG) called "Veterinary Medicines in Soils: Basic Research for Risk Analysis". The aim of the investigations was to elucidate the fate and effects of sulfadiazine and its metabolites in homogenized soil systems. This thesis is based on the experimental results obtained by project partners within the research project. A brief summary of the experiments is given in this chapter.

During a feeding experiment sulfadiazine was applied to pigs and manure was collected daily. Incubation experiments with fresh (3 weeks after production) and aged manure (after 6 month storage) were carried out in two different soils in the absence of plants and macrofauna under controlled laboratory conditions, i.e. constant temperature and moisture conditions. The applied manure was completely incorporated into the investigated soils and the chemical fate of SDZ and its two main metabolites was analytically followed for 218 days. Simultaneously, biological effects of sulfadiazine on soil microorganisms and soil functions were determined in both test soils. This included the investigation of influences of the antibiotic itself and of manure application on the microbial community structure by analyzing DNA sequences. In

addition, the antibiotic effect on functional diversity of soil microbes was elucidated by measuring soil respiration and impacts on nitrogen turnover (N-mineralization, nitrification, denitrification etc.). Further studies differentiated whether the application of the sulfonamide or the input of nutrient and resistant bacteria via manure affect abundance, diversity and transfer of antibiotic resistances in soil bacteria (Kotzerke et al., 2008; Heuer et al., 2008; Schauss et al., 2008).

1.3.2 Feeding experiment

^{14}C-labelled as well as non-labelled SDZ was administered to four growing pigs and manure was collected daily. SDZ and its metabolites were determined and quantified in homogenized manure samples. Besides the two main metabolites N^4-acetyl-sulfadiazine (Ac-SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) (Fig. 1.1) two minor metabolites were recovered, i.e. N-acetyl-4-hydroxy-sulfadiazine and N-formyl-sulfadiazine which were neglected in further investigations since they accounted for less than 2% of the total radioactivity in manure. During manure storage under aerobic and anaerobic conditions at 20°C up to 165 days, concentrations of SDZ, Ac-SDZ and OH-SDZ were analyzed to follow dissipation and transformation processes of the antibiotics in manure (Lamshöft et al., 2007; Spiteller, 2007).

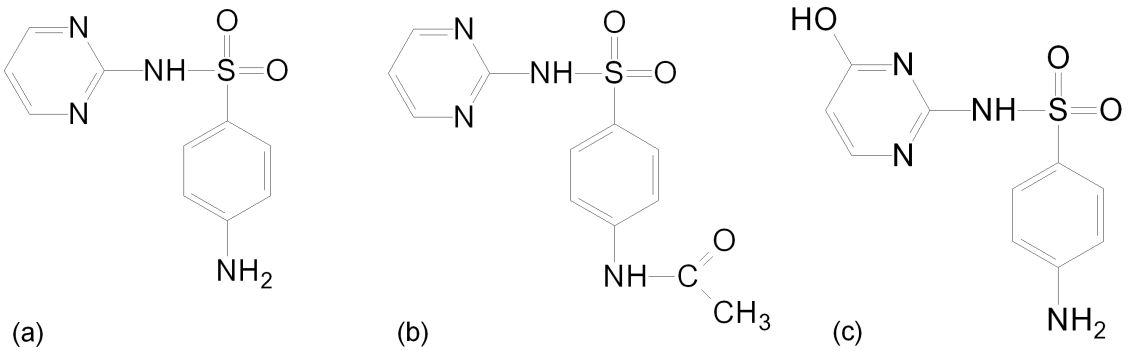

Figure 1.1: Chemical structures of (a) sulfadiazine (SDZ) and its main metabolites (b) N^4-acetyl-sulfadiazine (Ac-SDZ) and (c) 4-hydroxy-sulfadiazine (OH-SDZ).

1.3.3 Investigated soils

Within the central experiment of the research project two typical German soils, namely silty sand from Kaldenkirchen (pH 5.7) and sandy loam from Merzenhausen (pH 6.8) were used for application of manure resulting from the feeding experiment.

Table 1.1: Soil parameters of the Kaldenkirchen and Merzenhausen sampling site.

	Kaldenkirchen	**Merzenhausen**
	silty sand	*sandy loam*
pH	5.7	6.8
soil density (kg L^{-1})	1.57	1.63
porosity	0.456	0.440
water content (kg kg^{-1})	0.072	0.115
CEC (mval/100 g)	-	11.5
OC (%)	0.39	0.52
sand (%)	78.1	1.7
silt (%)	18.9	75.1
clay (%)	3.0	23.1

All subsequent investigations on the chemical fate and biological effects of sulfadiazine were conducted in these homogenized soil samples. Important properties of the two soils are summarized in Table 1.1.

1.3.4 Central fate experiments

Homogenized soil samples were amended with fresh (3 weeks after production) and aged (6 month storage) manure from pigs treated with ^{14}C-SDZ. Soil moisture was adjusted to 30% of the maximum water holding capacity (WHC_{max}) resulting in a water content of 7.2% and 11.5%, respectively. Transformation and sequestration processes were observed in the two different soils. A sequential extraction method was developed by Amelung and Kaupenjohann (2007) and applied to the homogenized soil samples resulting in three different sulfonamide fractions that were extractable with (i) $CaCl_2$, (ii) methanol (MeOH) and (iii) by applying high temperatures (microwave extraction for 15 minutes with acetonitrile/water at 150°C) only (residual fraction). Each fraction was investigated by LC/MS to quantify the amounts of SDZ, Ac-SDZ and OH-SDZ. Finally, bound residues defined as the fraction not extractable by any of the methods applied are identified by measuring the radioactivity in the extracted soil sample (Amelung and Kaupenjohann, 2007). These time-dependent extraction data served as the basis for the estimation of apparent sorption coefficients K_d^* (Chapter 3) and for the process model developed in this thesis (Chapter 4).

1.4 Objectives

The overall objective of this thesis is to develop a mechanistic simulation model predicting temporally resolved concentrations of sulfadiazine and its metabolites in soil pore water and the subsequent uptake of the antibiotically active compounds by microorganisms. Before combining all processes in one model, however, it is necessary to understand the mechanisms of relevant processes and to quantify the influence of substance and environmental parameters. Therefore, important processes governing the chemical fate of sulfadiazine and its main metabolites in manure and manure-amended soil shall be identified and described mathematically. Partition coefficients, transformation rates and other process variables shall be estimated from experimental results of project partners. Influences of environmental conditions and substance properties shall be figured out and the chemical fate shall be linked to biological effects. The pore water fraction determines bioaccumulation, toxicity and leaching potential and thus, represents the interface between chemical fate and biological effect of sulfadiazine. For prolonged contact times in soil, available SDZ in pore water and consequently efficiency of the veterinary medicine is reduced, e.g. by sorption, diffusion into micropores and/or transformation processes.

This thesis is subdivided into specific chapters which are structured according to following processes. Since sulfonamides belong to the group of dissociating compounds, the pH value is expected to play an important role for their environmental fate (Chapter 2). The sorption behaviour of SDZ and its metabolites will be reviewed and investigated based on available data. Model approaches for sorption of organic substances in soil shall be evaluated on their appropriateness to describe sulfadiazine sorption in manure and soil (Chapter 3). A system and data analysis helps to understand observed transformation and sequestration kinetics of SDZ in manure and manure-amended soils under different environmental conditions (Chapter 4). Finally, a link between the chemical fate and the antibiotic effect on soil microbes shall be realized by combining the fate model with a mathematical description of the uptake of the antibiotic compound into the bacterial cell and the subsequent enzymatic inhibition of the folic acid cycle (Chapter 5). This is the first approach that successfully integrates both, fate and effect, into a consistent model describing the whole process chain from manure application to soil until the uptake of the relevant sulfonamide fraction into the bacterial cell and the subsequent enzymatic inhibition of bacterial growth. Such a model would constitute a valuable tool in the process of risk analysis and risk assessment of such compounds.

Chapter 2

Speciation of Sulfonamides

2.1 Investigated Substances

Within the research project the focus was on the environmental fate and effect of sulfadiazine, a bacteriostatic substance which belongs to the group of sulfonamides. Since only few data on the environmental fate and effects of sulfadiazine (equilibrium sorption, transformation, sequestration, enzymatic inhibition) are available and chemical structure of substances within the sulfonamide group is similar, experimental results on sorption, transformation and sequestration behaviour and on effective concentrations of other sulfonamides are included in the theoretical discussion of this thesis in order to allow for deeper insight into general mechanisms. Table 2.1 summarizes some basic properties of relevant sulfonamides.

2.2 Speciation

Many antibiotic compounds such as sulfonamides dissociate or protonate in dependence of the pH value of the surrounding medium and their specific dissociation constants (pK_a). The ionized species may show a different distribution behaviour and reactivity in comparison to the uncharged neutral form (Thiele-Bruhn, 2003; Holten Lützhoft et al., 2000). This already points out the necessity to include sulfonamide speciation in environmental fate modelling (Gao and Pedersen, 2005). Thiele-Bruhn (2005) showed that effective doses on the microbial iron(III)-reduction in soils vary depending on the speciation of sulfonamides. Therefore, with regard to fate and effect it is important to know, to what extent anions and/or cations are formed in a given environmental surrounding.

Table 2.1: Important substance parameters for 13 sulfonamides.

	Acro.	**CAS**	**m. w.**	**pK$^a_{a1}$**	**pK$^a_{a2}$**	**K^a_t**	**log K$_{OW}$**
sulfaguanidine	**SGD**	57-67-0	214.2	2.72	11.82	13182.6	-1.22[b]
sulfanilamid	**SAM**	63-74-1	172.2	-	10.1	11749.0	-0.6[h]
sulfapyridine	**SPY**	144-83-2	249.3	2.72	8.56	1318.3	0.35[f]
sulfisomidine	**SID**	515-64-0	278.3	-	7.6	n.a.	n. a.
sulfadimidine/ sulfamethazine	**SDM**	57-68-1	278.3	2.65	7.58	631.0	0.89[f] / 0.27[b]
sulfathiazole	**STZ**	72-14-0	255.3	2.62	7.37	239.9	n. a.
sulfamethoxy-pyridazine	**SMP**	80-35-3	280.3	-	7.2	n. a.	n. a.
sulfamerazine	**SMR**	127-79-7	264.3	-	7.0	575.4	0.2[h]
sulfadimethoxine	**SDT**	122-11-2	310.3	2.65	6.82	537.0	1.63[f]
sulfadiazine	**SDZ**	68-35-9	250.3	2.49	6.48	147.9	-0.09[f]
sulfachloro-pyridazine	**SPZ**	80-32-0	284.7	1.88[c]	6.0[d]	n. a.	n. a.
sulfamethoxazole	**SMX**	723-46-6	253.3	1.74[c]	5.7[e]	n. a.	0.89[g]
sulfisoxazole	**SIX**	127-69-5	267.3	-	5.0	166.0	1.01[h]

n. a. - not available; [a] pK$_a$ and K$_t$ data from Sakurai and Ishimitsu (1980) except otherwise noted; [b]Holm et al. (1995); [c]Lin and Lu (1997); [d]Mengelers et al. (1997); [e]Vree and Hekster (1987); [f]Thiele-Bruhn and Aust (2004), Sarmah et al. (2006); [g]Garten Jr. and Trabalka (1983); [h]Hansch et al. (1995)

Sulfonamides are characterized by two pK$_a$ values: The lower one indicates protonation of the amino group whereas the other one signifies deprotonation of the SO_2NH moiety (Ingerslev and Halling-Sørensen, 2000). The fractions of the three possible species (acidic, neutral, basic) can be estimated as follows:
Given are the proton transfer reactions of the acidic species SH_2^+ to the neutral species SH (i.e. the sum of the uncharged and possibly zwitterionic form) and of the neutral species to the anion S^- with the dissociation constants $K_{a_1}=\frac{[SH][H^+]}{[SH_2^+]}$ and $K_{a_2}=\frac{[S^-][H^+]}{[SH]}$. The fraction $\alpha_{SH_2^+}$ of the cationic species is calculated according to

Schwarzenbach et al. (2003) describing the equilibrium in acid-base reactions:

$$\alpha_{SH_2^+} = \frac{[SH_2^+]}{[SH_2^+] + [SH] + [S^-]} \tag{2.1}$$

$$= \frac{1}{1 + [SH]/[SH_2^+] + [S^-]/[SH_2^+]} \tag{2.2}$$

$$= \frac{1}{1 + K_{a_1}/[H^+] + K_{a_1} \cdot K_{a_2}/[H^+]^2} \tag{2.3}$$

$$= \frac{1}{1 + 10^{pH - pK_{a_1}} + 10^{2 \cdot pH - pK_{a_1} - pK_{a_2}}} \tag{2.4}$$

The fractions of the other two species are derived accordingly resulting in:

$$\alpha_{SH} = \frac{1}{1 + 10^{pK_{a_1} - pH} + 10^{pH - pK_{a_2}}} \tag{2.5}$$

and

$$\alpha_{S^-} = \frac{1}{1 + 10^{pK_{a_1} + pK_{a_2} - 2 \cdot pH} + 10^{pK_{a_2} - pH}} \tag{2.6}$$

To be more precise we also have to investigate whether the molecule of sulfadiazine may exist as a zwitterionic species, i.e. as a molecule which is both negatively and positively charged at the same time. If the pK_a-values act in the order of classic ampholytes which means if the acidic pK_a-value is smaller than the basic pK_a-value, a zwitterion can exist. First, with increasing pH the acidic pK_a-value affects the deprotonatable group of the molecule in a way that the cation which predominates at lower pH-values is additionally charged negatively. This mechanism results in a zwitterion. If the pH value further increases the basic pK_a-value causes the protonatable group to release its hydrogen molecule and it becomes negatively charged. As far as ampholytes are concerned there exist detailed illustrating publications (e.g. Pagliara et al. (1997)).

However, if the pK_a-values are located close to one another and an intramolecular charge exchange may occur, zwitterions can even exist in the opposite case, i.e. the acidic pK_a-value is larger than the basic one. This situation applies to sulfadiazine and to sulfonamides in general and was investigated by Sakurai and Ishimitsu (1980). These authors have calculated tautomeric constants of selected sulfonamides describing the concentration ratio of zwitterionic and uncharged species. As the neutral fraction represents the sum of uncharged and zwitterionic species, which show deviating environmental behaviour because of their different charge distribution, the equations above have to be supplemented for the distinction of the neutral fraction α_{SH} using the tautomeric constant K_t:

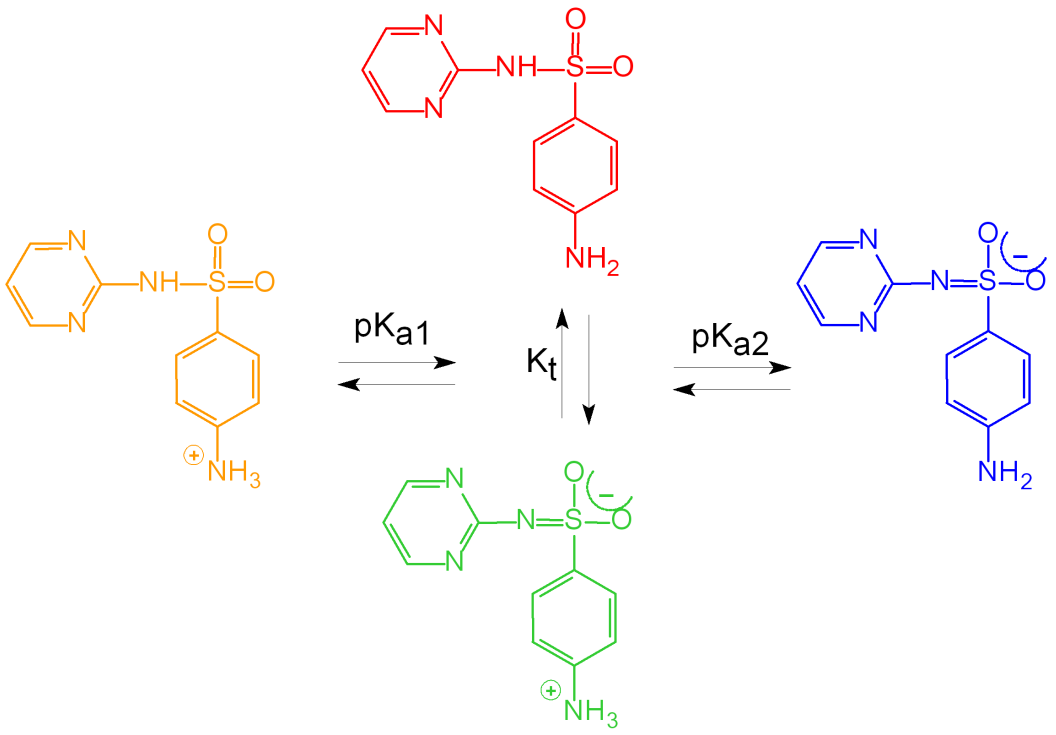

Figure 2.1: Scheme of sulfadiazine ionisation in equilibrium.

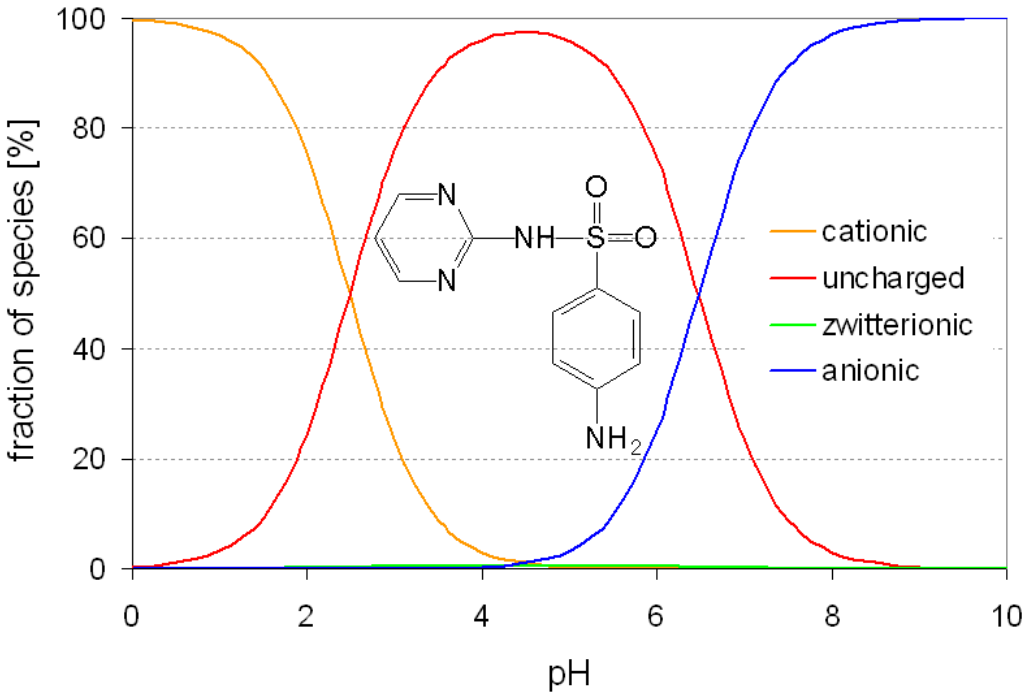

Figure 2.2: Fractions of ionic and uncharged forms of sulfadiazine in dependence of pH (pK_{a1} = 2.49, pK_{a2} = 6.48, K_t = 147.9, Sakurai and Ishimitsu (1980)).

$$K_t = \frac{[SH^0]}{[S^-H^+]} = \frac{\alpha_{SH^0}}{\alpha_{S^-H^+}} \quad (2.7)$$

$$\alpha_{SH^0} = \frac{\alpha_{SH}}{1 + 1/K_t} \quad (2.8)$$

$$\alpha_{S^-H^+} = \alpha_{SH} - \alpha_{SH^0} \quad (2.9)$$

α_{SH^0} and $\alpha_{S^-H^+}$ are the fractions of the uncharged and the zwitterionic form, respectively.

The complete dissociation scheme of sulfadiazine is shown in Figure 2.1 which illustrates the transitions from one ionisation state to another in equilibrium. In Figure 2.2 the speciation of sulfadiazine in dependence of the pH value is given over the full range of possible values.

The fraction of the zwitterionic species of sulfadiazine is negligible ($< 0.7\%$) compared to the neutral form due to its large tautomeric constant. Thus, this species is considered to be of minor importance and is not explicitly distinguished from the neutral form in the model. However, small fractions of a species can affect the overall sorption if strong sorption to specific binding sites occurs. This was pointed out by Gao and Pedersen (2005) for the adsorption of sulfamethazine to clay minerals

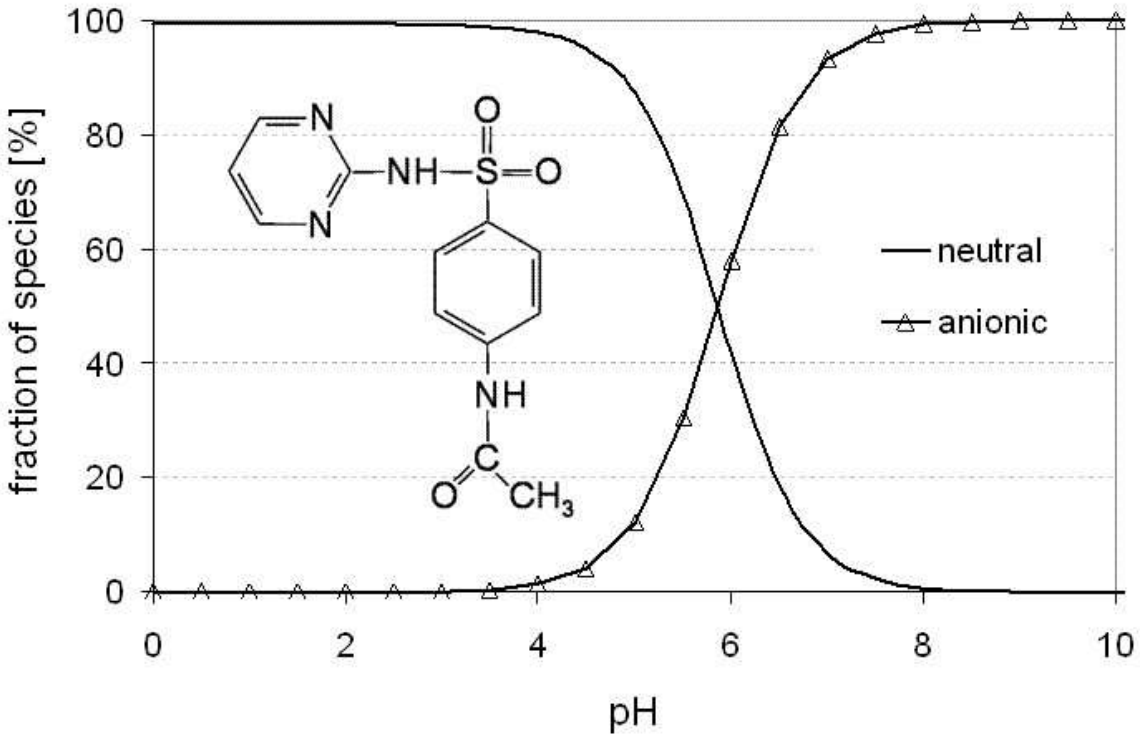

Figure 2.3: Fractions of neutral and anionic molecule of N^4-acetyl-sulfadiazine in dependence of pH ($pK_a = 5.86$, Vree and Hekster (1987)).

at low pH values. The cationic form dominates the adsorption up to a pH of 4.6, although this species constitutes only 0.45% of total sulfamethazine at this pH. A similar effect of the zwitterionic form, however, was not observed by the authors.
In order to completely assess the environmental risk of sulfadiazine metabolites of the parent compound have also to be considered. The N^4-acetyl-metabolite is characterized by a single dissociation constant of pK_a=5.86 (Vree and Hekster, 1987) which indicates deprotonation of the SO_2-NH-moiety. The protonation of the amino group as observed for the parent compound sulfadiazine is not possible because the amino group is blocked by acetylation. Thus, in dependence of the surrounding pH, N^4-acetyl-sulfadiazine may exist as neutral and anionic species (Figure 2.3). The second major metabolite is the 4-OH-metabolite that is formed by hydroxylation at the sulfonamide's pyrimidine ring. The hydroxy-sulfadiazine may be additionally deprotonated at the OH-group. However, since the respective dissociation constant is far below the environmental relevant range (pK_a <2, Mason (1958)) this can be neglected. Unfortunately, the dissociation constants specifying protonation of the amino group and deprotonation of the SO_2NH moiety are not known and it is thus assumed that they are close to the respective pK_a-values of the parent compound.

Chapter 3

Sulfonamide Equilibrium Sorption to Soil

3.1 Introduction

Sorption is one of the most important processes hampering transport of chemicals to groundwater or surface water and also affecting transformation reactions and effects on soil microbes (Schwarzenbach et al., 2003). Therefore, an extensive knowledge on parameters affecting the sorption of antibiotics to soil is a necessary prerequisite for risk and exposure assessment. For sulfonamides, which partly dissociate in dependence of the actual pH, the different species-specific sorption mechanisms have to be distinguished. These mechanisms depend on soil composition and chemical properties resulting in different apparent sorption coefficients for each investigated soil and sulfonamide. Besides the pH ionic strength, clay minerals and organic matter content and composition may play a critical role for the sorption of sulfonamides to the soil matrix. Several investigations on sulfonamide sorption to soil (Thiele-Bruhn and Aust, 2004; Ter Laak et al., 2006; Langhammer, 1989; Drillia et al., 2005), soil fractions (Thiele-Bruhn et al., 2004) and clay minerals (Gao and Pedersen, 2005) have yet been conducted. Reported sorption coefficients of sulfonamides are in the range of 0.6 to 7.4 L kg^{-1} (Sarmah et al., 2006) which translates into a mass fraction of 50% to 95% of the compound that is sorbed under average conditions in natural soils. Therefore, knowledge of the apparent K_d, i.e. the "visible" sorption coefficient, is not sufficient, because it cannot be transferred to other soils. Gao and Pedersen (2005) investigated the sorption of three sulfonamides to three clay minerals over a large pH range from 3.5 to 9.3 and estimated species-specific sorption coefficients by

fitting the results to a model explicitly considering the contributions of the cationic, zwitterionic, uncharged and anionic species to the apparent sorption coefficient. Although the results varied across the different clay minerals and can thus not be used to describe soil sorption of sulfonamides in general, the investigation clearly shows the necessity to include speciation in environmental fate modelling. In this work, a mechanistic model simultaneously considering speciation and sorption of sulfonamides to available sorption data of sulfonamides in soil matrices is applied. The model is used to identify sensitive parameters under average conditions in natural soils.

3.2 Theory

3.2.1 Compartment soil

In contrast to air and water, soil does not constitute a homogeneous compartment but is of heterogeneous composition combining the lithosphere, the hydrosphere, the atmosphere, and the biosphere by formation of the pedosphere. Thus, it is the upper animated layer of the earth´s surface that is not saturated by water (Trapp and Matthies, 1998). The German Federal Soil Protection Act (*Bundesbodenschutzgesetz § 2*)[1] defines the soil by means of its functions, i.e. the soil is the upper layer of the earth´s crust including the soil solution and the soil air (but not the groundwater) which fulfils the following functions:

- natural functions as basis of life and habitat for soil organisms, plants, animals and humans, as part of the ecosystem including water and nutrient cycles, and as a medium for degradation, balancing and development resulting from filtering, buffering and transformation properties particularly with regard to the protection of groundwater

- functions as archive for natural and cultural history

- functions as raw material deposit, area of settlement and recreation, location of agriculture and forestry, of commercial and public use, traffic, provision and disposal

[1]Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit, BBodSchG 1998, http://bundesrecht.juris.de/bundesrecht/bbodschg/

After all, soil is characterized by interactions between solid minerals, water, gases and dissolved inorganic and organic substances (Korte, 1992). Therefore, according to Trapp and Matthies (1998) the fate and transport of a contaminant in soil is influenced by several processes:

- diffusion and dispersion in gas and water filled pores
- advection with soil water and leaching to groundwater
- bioturbation by earthworms, mice and soil organisms
- sorption to soil solids (minerals and organic matter)
- sorption to dissolved organic matter (DOM)
- volatilization into the atmosphere
- transformation and degradation
- uptake by plant roots

Soil composition with respect to organic matter content and composition of the mineral fraction (sand, silt, clay) has an influence on the actual sorption capacity of a given soil. Other important soil properties are porosity, water content, specific surface area (of minerals mainly) and cation exchange capacity.
The specific surface area is the sum of all boundary layers between solid and liquid or solid and gaseous phases and is measured in $m^2\ g^{-1}$. It ranges from a few $m^2\ g^{-1}$ up to 500 $m^2\ g^{-1}$ and increases with rising content of clay, expandable minerals and organic substances. The surface charge, a parameter which plays an important role when investigating polar or ionizable compounds, depends on the pH value: Sorption of cations increases with increasing pH, whereas sorption of anions increases with decreasing pH. Consequently, the proportion of the soil´s charge mainly results from the type and amount of its sorbents. In temperate climate, soils are characterized by clay minerals and a negative net charge over the whole range of pH from 3 to 8 as the negative charge always exceeds the positive. If the number of positively charged groups equals the number of negatively charged groups there is only minimal interchange activity. This point is called charge zero point. The cation exchange mechanism describes sorption via electrostatic bindings. The sum of all exchangeable cations (in $cmol_c\ kg^{-1}$) is called cation exchange capacity (CEC) and increases with increasing pH. The potential CEC (CEC_{pot}) is measured at pH-values of 7 to 7.5,

the highest possible value in soils of humid climates, i.e. the effective CEC (CEC_{eff}) is lower than CEC_{pot} when the pH value falls below 7. For a single cation its contribution to the cation film increases with increasing concentration in the equilibrium solution. The exchange approximates asymptotically a threshold which is the CEC. However, in soils different cations compete for exchange sites. Therefore, the contribution of a cation in the film under ecological conditions depends on its contribution in the equilibrium solution and its ability to compete for sorption sites compared to other cations.
Finally, as far as chemicals are concerned that dissociate to anions or cations in a pH range typical of central European soils, i.e. 3 to 8, the pH value is an important influencing factor for sorption processes. As pollutants are not ecologically effective until desorption and transfer to the soil solution occured, reversibility of sorption processes is also of great importance (Schachtschabel et al., 1998).

3.2.2 Sorption mechanisms and modelling approaches

Environmental modelling is a common approach for risk assessment in science and policy, i.e. it supports the evaluation and prediction of a substance's behaviour in the environment under the assumption of certain environmental conditions and substance properties. Such a model is not only restricted to prediction, but also allows for deducing mechanisms and structures of the represented system. In this context, the equilibrium partitioning of organic substances is of specific interest for analyzing the fate and transport of sulfadiazine in soil (Schwarzenbach et al., 2003), as the mechanistic background of the involved sorption processes is not yet fully understood.
The relationship between the equilibrium concentrations of the substance sorbed to the soil matrix (C_s, g kg^{-1} soil) and the concentration dissolved in solution (C_w, g cm^{-3} solution) can be expressed by the Langmuir model that assumes a limited sorption capacity Q due to a limited surface:

$$C_s = Q \cdot b \cdot C_w/(1 + b \cdot C_w) \tag{3.1}$$

with factor b representing the affinity of the surface for the solute. Under these restrictive assumptions the Langmuir isotherm is only observed in a few cases (Delle Site, 2001), e. g. for the adsorption of polycyclic aromatic hydrocarbons from water onto activated carbon (Walters and Luthy, 1984).

Experimental data, however, is often best fitted by the Freundlich model (Weber Jr. et al., 1992) which assumes neither homogeneous energies nor restricted sorption.

$$C_s = K_f \cdot C_w^n \tag{3.2}$$

K_f is the Freundlich coefficient and the exponent n is a measure of nonlinearity. A special case of this model occurs for $n = 1$ which leads to a linear relationship between C_s and C_w. In the low concentration region, the Freundlich isotherm also obeys to a quasi-linear relationship. Thus, for environmental conditions this linear approach is often used to describe sorption of a solute to sediment or soil by the partition coefficient K_d between soil matrix and water as follows:

$$C_s = K_d \cdot C_w \tag{3.3}$$

The K_d value can be determined by measuring the linear part of the adsorption isotherm or, as nonpolar substances are mainly sorbed to the organic matter of the soil matrix, it can be estimated from the octanol-water partition coefficient K_{OW} and the organic carbon content (Trapp and Matthies, 1998). However, this estimation does not consider polar interactions that may be important for antibiotics and neglects the complexity and diversity in soil components and their interactions with organic solutes. Nor, does it provide a deeper insight into sorption mechanisms and influencing factors. Hence, Weber Jr. et al. (1992) proposed the Distributed Reactivity Model (DRM) which contributes to the significance of "different distributions of sorption reactions and mechanisms for different solute-solid combinations" combining components of different linear (l) and nonlinear (nl) sorption:

$$C_{s_t} = x_l \cdot K_{D_t} \cdot C_w + \sum_{i=1}^{m} (x_{nl})_i \cdot K_{F_i} \cdot C_w^n \tag{3.4}$$

C_{s_t} is the total sorbed substance equilibrium concentration, x_l is the summed mass fraction of solid phase showing linear sorption, K_{D_t} is the mass-averaged partition coefficient for the summed linear components, C_w is the equilibrium concentration in solution, and $(x_{nl})_i$ is the mass fraction of the i^{th} nonlinearly sorption process each described by the Freundlich model. For practical purposes, the number of distinguishable nonlinear components will not exceed 1 or 2 (Weber Jr. et al., 1992).
An even more mechanistically based model called polyparameter linear free energy relationships (PP-LFERs) or cavity model was introduced by Goss and Schwarzenbach (2001). The single parameter relationships like the often applied K_{OC} approach are only valuable for one single compound class and do not allow any inference on

the involved mechanisms in order to understand the variability of sorption behaviour between substance classes or between different natural organic phases. Based on this situation Goss and Schwarzenbach (2001) developed a concept that takes all partitioning interactions of organic compounds into account by specific parameters. In this way, mechanistic insight and understanding of the various interactions that influence the environmental distribution of organic chemicals is provided. Thus, the partitioning K_{i12} of a substance *i* between two bulk phases *1* and *2* which accounts for van der Waals forces and H-bonding take the following form:

$$ln\,(K_{i12}) = a_{12} \cdot CA_i \cdot vdW_i + b_{12} \cdot V_i + d_{12} \cdot HA_i + e_{12} \cdot HD_i + c_{12} \qquad (3.5)$$

CA_i is a measure for the contact area between *i* and the phases, V_i is the molar volume of *i*, HA_i and HD_i are quantitative descriptors of the H-accepting and H-donating properties of the interacting partners of *i*, and a_{12} to e_{12} are regression parameters dependent on the properties of the phases. Once determined for a certain surface (which in fact is a quite laborious effort), such a PP-LFER allows to describe sorption of an organic chemical without any additional chemical input parameter. However, this model has not been tested and adapted for use with ionic compounds or at fairly high concentrations (Goss and Schwarzenbach, 2001) and is, thus, not yet applicable for substances like sulfonamides dissociating in an environmentally relevant pH range.

Besides others, Breivik and Wania (2003) successfully applied the concept of PP-LFERs to multimedia fate models, as many chemicals of environmental concern like modern pesticides and pharmaceuticals show a partitioning behaviour different from those of the pollutants for which single parameter approaches work quite well. Nguyen et al. (2005) also showed that the PP-LFER approach surpasses other current methods which aim at estimating the equilibrium partition coefficient K_{OC} of organic compounds between water and the natural organic matter in soils. Only very little effort has yet been put on modelling the sorption of antibiotics to soils, neither using the classical approach nor by applying the PP-LFER concept. Figueroa et al. (2004) fitted sorption isotherms for the zwitterion of tetracycline antibiotics to clay to the Langmuir model. The results are restricted to the indication that pharmaceutical sorption interactions with clay are controlled "by the ionic functional groups of the base compound structure within a pharmaceutical class". Another approach was pursued by Wehrhan et al. (2007) investigating the transport behaviour of sulfadiazine in soil. The "concentration dependent, rate-limited and possibly irreversible sorption processes" were fitted by a kinetic model with two reversible at-

tachment/detachment sites and one irreversible sorption site. The soil concentration profile, though, could not be described by this approach. This may be due to the experimental setup, because the authors did not analytically distinguish between the parent compound and possible transformation products so that the observed radio-label signal includes sulfadiazine and its metabolites. However, the fate of the individual compounds in the soil column may be quite different and data do not describe the transport of sulfadiazine alone. Thus, the model does not provide any mechanistic insight as underlined by Goss and Schwarzenbach (2001) and Breivik and Wania (2003).

Usually, modelling approaches describing the fate of organic chemicals in soil combine sorption and degradation behaviour. Liu et al. (2007) developed a mass balance model including equilibrium sorption to the solid matrix, an irreversible kinetic sequestration process and biodegradation by soil microorganisms following Monod kinetics. Simulation results were in good agreement with data from biodegradation batch tests with benzene. Saffih-Hdadi et al. (2003) simultaneously modelled the fate of the pesticide parathion and its highly toxic metabolite paraoxon underlining that both, the parent compound and the metabolite, have to be considered for risk assessment especially if the transformation product is active or even more toxic than the pesticide itself. Both substances were assumed to sorb into a weak sorption phase followed by a strong sorption phase which might be irreversible. These approaches indicate that a distinction between sulfadiazine and its metabolites is required in order to analyse and model the effect of its exposure to soil. Furthermore, in a detailed investigation dissociation of single species which underlie different sorption processes in dependence of their charge should be explicitly considered. In this way, deeper mechanistic insight and an improved risk assessment is possible even with a small data set (as compared to the PP-LFER approach) combining matrix and compound properties.

3.2.3 Sorption behaviour of sulfonamides

Sorption of sulfonamides is mainly driven by the aromatic amino group which also forms covalent bonds (bound residues) with phenolic humic substances (Bialk et al., 2005; Bialk and Pedersen, 2008). Since for the metabolite N^4-acetyl-sulfadiazine (Ac-SDZ) this functional group is replaced, weaker sorption and bound residue formation is expected as compared to its parent compound. The chemical moiety of the amino group also determines the antibiotic effect of the sulfonamides which means

that adsorption might inhibit the antibiotic effect (Thiele, 2000). Sorption equilibrium of sulfonamides in soil is reached very fast after several hours (Thiele-Bruhn, 2003; Langhammer, 1989). In equilibrium, the distribution between solid and liquid phase can be described by the partition coefficient K_d representing the ratio between equilibrium concentrations in solids and aqueous solution. Batch experiments indicate sorption nonlinearity for sulfonamides (Thiele-Bruhn and Aust, 2004; Wehrhan et al., 2007) best fitted by Freundlich isotherms. However, in a concentration range of 350 to 1500 μg kg^{-1} linear sorption isotherms have been reported (Zimmermann, 2006).

Sorption to soil. Tolls (2001) summarized K_d values of sulfonamides in different soils varying from 0.6 to 4.9 L kg^{-1}. Langhammer (1989) observed that K_d values of a given compound differ considerably in diverse soils and that these variations cannot be solely explained by differences in organic carbon content. Boxall et al. (2002) investigated the effect of pH on sorption of sulfachloropyridazine in the pH range from 4.6 to 7.8. Measured sorption coefficients increased from less than 1 L kg^{-1} to 12 L kg^{-1} with decreasing soil pH. Recalling that the fraction of the anion increases with increasing pH sorption of the anionic sulfachloropyridazine appears to be significantly weaker than that of the neutral form. The cationic species can be neglected within the investigated pH range. Also, Kahle and Stamm (2007) reported that sorption of sulfathiazole to organic matter and minerals decreases with inreasing pH. In analogy, Kurwadkar et al. (2007) showed that apparent sorption coefficients increased significantly (about one order of magnitude) with increasing pH for sulfamethazine and sulfathiazole in three different soils and pH variations between 2.3 and 8.0. The resulting sorption coefficients for each of the sulfonamide species (cationic, neutral, anionic) were consistent in all three soils and were determined by an empirical model. They indicated strongest sorption for the cationic form followed by the sorption strength of the neutral and the anionic sulfonamide molecule. Accordingly, Gao and Pedersen (2005) point out that anionic species of sulfonamides do not participate in sorption interactions, whereas the cationic form dominates adsorption to clay minerals even at pH values at which this form covers only 0.45% of the investigated compound. The zwitterionic form, however, does not seem to influence sorption behaviour of sulfonamides.

Sorption of sulfonamides to soil organic matter (SOM) is generally stronger than to soil minerals (Thiele-Bruhn, 2003), and depends on composition of SOM. Previous studies indicate (Thiele-Bruhn and Aust, 2004; Thiele-Bruhn et al., 2004) that

sorption of sulfonamides is also influenced by the molecular structure and by physicochemical properties of the substance in dependence of pH, by functional groups at organic surfaces, and by cavities in the structure of soil organic matter. Several interactions between substance and soil can be involved in the sorption process of the ionic and neutral species (Liu and Yu, 2005). Sorption can be attributed to vander-Waals forces and hydrogen bonding forming complexes between the sulfonamide and soil organic matter (Thiele-Bruhn et al., 2004) as well as covalent binding, surface complexation in case of mineral adsorbents (Schwarzenbach et al., 2003) and electrostatic interactions in case of ionic and polar species.

Sorption to manure. In general, the adsorption of antibiotics to manure that is rich in organic matter is strong (Thiele-Bruhn, 2003; Delle Site, 2001) and attributed to ionic interactions and hydrogen bonds (Tolls, 2001). Numerous carboxylic and phenolic components which may act as exchange sites for sulfonamides result in a significantly stronger sorption to manure than to soil (Thiele-Bruhn and Aust, 2004). Burkhardt and Stamm (2007) determined sorption coefficients in manure (pH 8.1) ranging from 30 to 40 L kg^{-1} for sulfathiazole and sulfadiazine which is about one order of magnitude above reported K_d values in soil. But what happens when manure is amended to soil? Manure contains high levels of ammonia increasing the pH value of the soil solution after addition. Besides an increase of the cation exchange capacity (CEC) (Schachtschabel et al., 1998) the species distribution of the compound is altered. Boxall et al. (2002) related the mobilizing effect of manure on sulfonamides in soil to the pH effect of the alkaline manure favouring the anionic species.
Increasing mobility of sulfonamides has also been observed in the presence of dissolved organic matter (DOM). DOM is suspected to act as a co-solvent that enhances concentrations in the dissolved phase by sorption of the substrate (in this case sulfonamides) to DOM. Manure contains N-heterocyclic hydrocarbons which can compete for adsorption sites at soil particles with N-heterocyclic sulfonamides but a competitive effect would only be visible if available adsorption sites were almost saturated with the competitors from manure. Thus, for typical manure application rates K_d values for sulfachloropyridazine were almost constant for different DOM concentrations (Boxall et al., 2002). In addition, despite the rather fast initial sorption of sulfonamides to soil after manuring Stoob et al. (2007) found that sulfonamides are still present in the soil after a long time period.

3.3 Materials and Methods

3.3.1 Applied model approaches

Equilibrium sorption. Equilibrium sorption was estimated based on fundamentals of sorption theory (Schwarzenbach et al., 2003) considering characteristic physico-chemical parameters of sulfonamides and relevant soil properties. The apparent sorption coefficient K_d^* is given by the total equilibrium concentrations in the sorbed phase ($C_{tot,sorb}$) and the dissolved phase ($C_{tot,diss}$) and is expressed as the mass fraction weighted average of the contributions of the cationic, neutral and anionic species (eq. 3.6). As the zwitterionic fraction of sulfonamides is known to be small under environmental conditions (Sakurai and Ishimitsu, 1980), it is not explicitly considered.

$$K_d^* = \frac{C_{tot,sorb}}{C_{tot,diss}} = \alpha_c \cdot K_c + \alpha_n \cdot K_n + \alpha_a \cdot K_a \tag{3.6}$$

α_i is the fraction of the cationic (c), neutral (n) and anionic (a) species, and K_i the respective species-specific sorption coefficients. These sorption coefficients representing the interactions between a single species and the solid matrix can be assumed to be nearly constant within each soil even for different pH values. Thus, for a specific soil or manure sample the apparent K_d depends on the species distribution as a function of pH and pK_a and the ratio of the species-specific distribution coefficients. However, depending on the matrix properties the latter are not constant for different soils. To allow for transferring from one soil to another the species-specific interactions with the solid matrix have to be identified and quantitatively estimated.
The cationic species undergoes electrostatic interactions with negatively charged mineral surfaces which could be related to the cation exchange capacity (CEC) of the soil. The anionic species can be attracted by the soil matrix via surface-bridging mechanisms. Sorption of the neutral molecule is most probably due to unspecific van-der-Waals interactions or hydrogen bonding. The former depends on the total fraction of soil organic matter, whereas the latter also accounts for H-donor and H-acceptor properties of the soil matrix and the compound. It is desirable to independently estimate the species-specific sorption coefficients of sulfonamides for a given soil. However, information that allows for establishing quantitative relationships using soil and substance properties is not yet available. For this reason, equation 3.6 was used to conduct a sensitivity analysis for the contributions of the different species to the apparent K_d^*. A number of scenarios were simulated for soils Kaldenkirchen (pH 5.7) and Merzenhausen (pH 6.8) which are described in Chapter

1.3. As the pK_{a2} of SDZ is between the average pH values of the two soils, they reflect conditions that result in a significantly different speciation of SDZ, i.e. the neutral species dominates in soil Kaldenkirchen (86%) whereas in soil Merzenhausen SDZ mainly exists as anion (68%). The estimated cationic fraction is very small in both soils ($< 0.1\%$). Consequently, if neutral and anionic species of sulfadiazine exhibit significantly different sorption strength this might be reflected by diverging equilibrium sorption observations for the two soils.

3.4 Results and Discussion

3.4.1 Analysis of available literature data

Equation 3.6 describes the contribution of the cationic, neutral and anionic species to the apparent sorption coefficient K_d^*. Most of the sulfonamides are dominated either by the neutral or by the anionic fraction under natural pH conditions as shown in Chapter 2. The estimated species distribution for five sulfonamides in the two different soils Kaldenkirchen (K) and Merzenhausen (M) is shown in Figure 3.1. It can be seen that the neutral fraction is higher in soil K except for SPY which does not show significant dissociation in both soils.

Estimation of species-specific sorption coefficients for SPZ. For sorption of sulfachloropyridazine (SPZ) to soils two independent data sets are available (Boxall et al., 2002; Ter Laak et al., 2006). Boxall et al. (2002) investigated the effect of pH on sorption of sulfachloropyridazine (SPZ) onto the solid matrix of a clay loam and a sandy loam in a pH range of 4.6 to 7.8. Measured sorption coefficients were in the range of 12 L kg^{-1} to less than 1 L kg^{-1} and showed a clear tendency of decreasing with increasing soil pH. As the neutral fraction of sulfachloropyridazine also decreases with increasing soil pH in the two soils (Fig. 3.2a), this indicates that overall sorption is dominated by interactions of the neutral molecule with the soil matrix. If the contributions of the cationic fraction are considered to be negligible, a bivariate linear regression of K_d^* versus α_n and α_a delivers estimates for the species-specific sorption coefficients K_n and K_a. At high pH values where the anionic species constitutes to almost 100% of total SPZ the respective K_d^* should approximately equal K_a. Regression analysis for the two data sets resulted in significantly different values for K_n (7.2 L kg^{-1} for sandy loam and 14.0 L kg^{-1} for clay loam) and K_a (0.6 L kg^{-1} sandy loam, 1.2 L kg^{-1} clay loam). K_n is approximately one order of magnitude

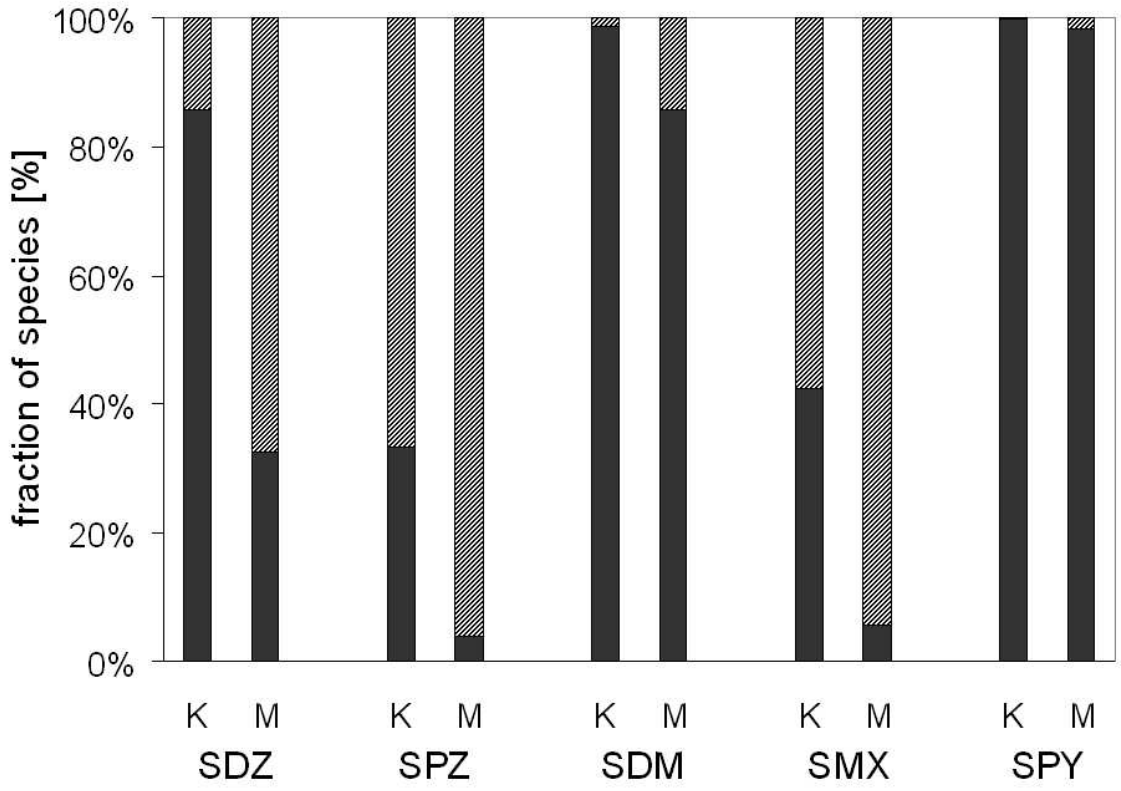

Figure 3.1: Calculated speciation of five sulfonamides (SDZ: sulfadiazine, SPZ: sulfachloropyridazine, SDM: sulfamethazine (=sulfadimidine), SMX: sulfamethoxazole, SPY: sulfapyridine) in the two soils of Kaldenkirchen K (pH 5.7) and Merzenhausen M (pH 6.8). The neutral fraction (%) is given in black, the anionic fraction is given in diagonally stripes. Relevant substance properties are presented in Table 2.1.

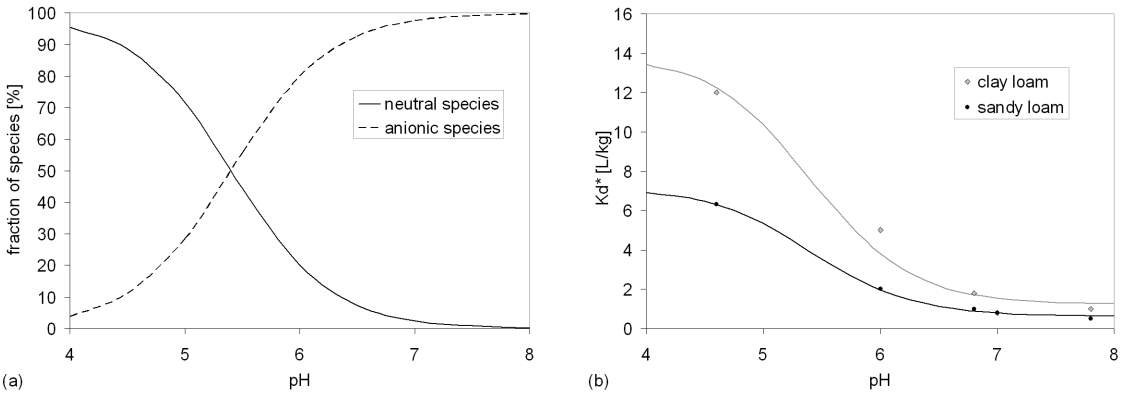

Figure 3.2: (a) Calculated fractions of the neutral and the anionic species [%] of sulfachloropyridazine (pK_{a1}=1.88, pK_{a2}=5.4, Lin and Lu (1997)) as a function of pH. (b) Measured (symbols) and estimated (lines) equilibrium sorption coefficients K_d^* (L kg^{-1}) in a clay loam and a sandy loam, respectively, as a function of pH (data from Boxall et al. (2002)).

(factor 10) larger than K_a. Additionally, both partition coefficients are larger for the clay loam as compared to the sandy loam supporting that species-specific sorption coefficients vary between different soils and matrices. Figure 3.2b shows that this simple estimation is able to describe the pH-dependency of the observed partition coefficients.

Ter Laak et al. (2006) published sorption coefficients of sulfachloropyridazine (SPZ) in 11 different soils accomplished by important soil parameters (pH, OC, CEC) which permits to investigate possible correlations of sulfonamide sorption behaviour to matrix properties. Fitting equation 3.6 to these sorption data by a least-square-method (LSM) results in distribution coefficients of $K_n = 15.3$ L kg^{-1} $\pm$ 97.4% and $K_a = 1.6$ L kg^{-1} $\pm$ 231% with a small r^2 of 0.32. This indicates that sorption of sulfonamides to soil cannot be explained by overall species-specific sorption coefficients. Cationic sorption is negligible ($K_c = 0$ L kg^{-1}). However, since the cationic fraction is small (less than 3%) in all investigated soils this term is very insensitive to the optimization routine.

K_n and K_a derived from Boxall et al. (2002) are both significantly different within the two investigated soils indicating that specific soil properties play an important role for sorption of SPZ. This is corroborated by the large standard deviations obtained above for the data set of Ter Laak et al. (2006). Thus, species-specific sorption coefficients cannot be transferred between different soils without taking the variability of matrix properties into account.

According to Tolls (2001) several mechanisms are involved in sorption of veterinary pharmaceuticals to soils. He proposes to differentiate between sorption to organic matter and mineral constituents, ion exchange and reactions like H-bonding and complexation. Therefore, it is of particular interest to gain additional information on the involved sorption mechanisms and determining parameters of the sulfadiazine species in order to transfer sorption data from one soil to another.

The cationic contribution to overall sorption is assumed to be related to the cation exchange capacity (CEC) of the soil matrix. A common approach to estimate sorption of organic molecules to solid material is the K_{OC}-concept. Here, it is assumed that sorption is proportional to the organic carbon content (OC) and therefore the sorption coefficient K_d^* can be estimated by a normalized partition coefficient between organic carbon and water (K_{OC}) (Trapp and Matthies, 1998). However, the validity of this concept for veterinary pharmaceuticals in particular and for many polar organic compounds in general is doubtful (Thiele-Bruhn and Aust, 2004; Tolls,

2001). The concept assumes sorption to be dominated by van der Waals interactions with soil organic carbon neglecting hydrogen bonding forces.
Nevertheless, this is tested and it is assumed that K_n and K_a are attributed to van-der-Waals interactions proportional to the organic carbon content, ($K_{OC,n}$ and $K_{OC,a}$, respectively) and other interactions, e.g. H-donor-acceptor forces and/or ion exchange ($K_n^{'}$ and $K_a^{'}$).

$$K_d^* = \alpha_c \cdot CEC \cdot K_{CEC} + \alpha_n \cdot \left(OC \cdot K_{OC,n} + K_n^{'}\right) + \alpha_a \cdot \left(OC \cdot K_{OC,a} + K_a^{'}\right) \quad (3.7)$$

Fitting equation 3.7 to the sorption data of Ter Laak et al. (2006) results in distribution coefficients of $K_{OC,n}$ = 283.7 L kg^{-1} ± 12.4% and $K_{OC,a}$ = 61.0 L kg^{-1} ± 77.5% with r^2 = 0.86. Cationic sorption proved negligible again. The contribution of $K_n^{'}$ and $K_a^{'}$ also turns out to be negligible according to the model fit. In Figure 3.3 measured and estimated sorption coefficients calculated with the two equations (eq. 3.6 vs. eq. 3.7) in the eleven different soils are compared. It can be seen that the introduction of the additional parameter OC (eq. 3.7) improves the agreement of the estimated values with data from experiments for most of the data points. However,

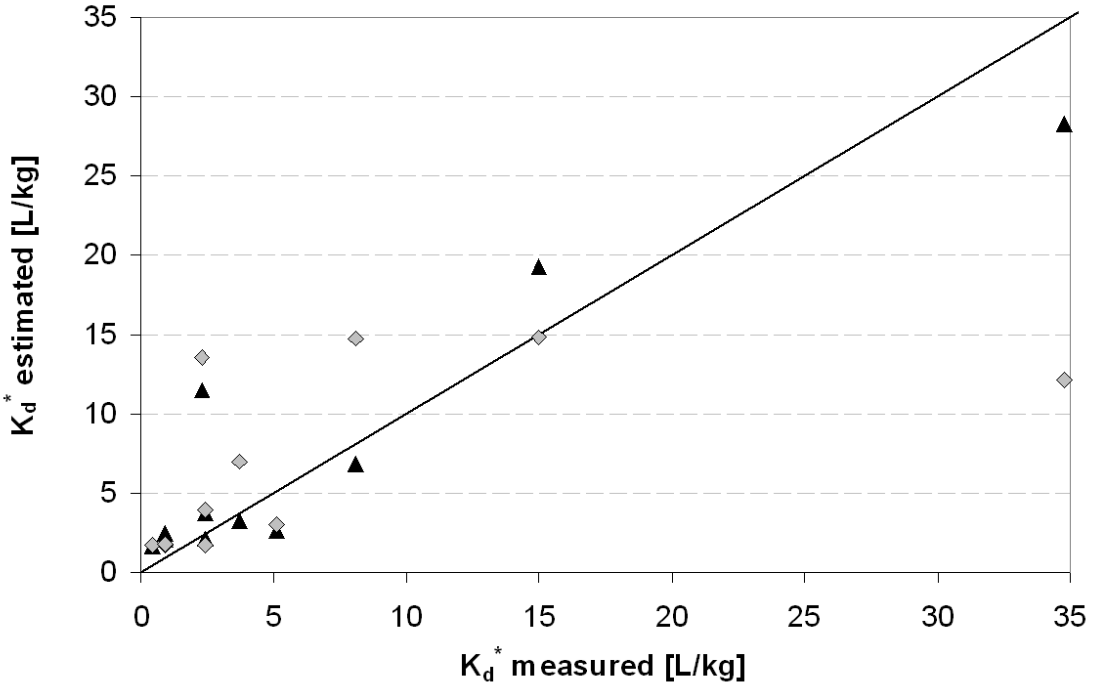

Figure 3.3: Estimated versus measured (Ter Laak et al., 2006) apparent sorption coefficients of SPZ calculated with species specific sorption coefficients (eq. 3.6, grey diamonds) and with additional matrix properties (eq. 3.7, black triangles) in eleven different soils.

overall sorption can equivalently be described with $K_a^{'}$ alone leaving out the additional term ($OC \cdot K_{OC,a}$). This results in an only slightly worse fit ($r^2 = 0.83$) with almost the same estimated K_d^* values (data not shown in Fig. 3.3). Nevertheless, there are still obvious deviations that clearly show the need for more experimental data to enable deeper mechanistic insight.
After all, the analysis of SPZ sorption data corroborates the assumption that cationic sorption does not contribute significantly to overall sorption of sulfonamides under environmental pH conditions, since the cationic fraction is negligible. It can also be concluded that anionic sorption of SPZ - and probably other sulfonamides as well - is significantly weaker than neutral sorption (one order of magnitude). An investigation by Gao and Pedersen (2005) of the sorption of three sulfonamides (sulfamethazine, sulfamethoxazole, sulfapyridine) to clay minerals corroborates the small contribution of the anionic species to overall sorption. They concluded that the anionic species does not participate in sorption interactions with clay minerals which is justified by electrostatic repulsion of the anion by the negatively charged surfaces. Approximation of SPZ sorption to different soils by OC-normalized distribution coefficients $K_{OC,i}$ does not fully explain the observed variation in partitioning of SPZ and is thus only a first step towards a generally applicable equation for the independent estimation of sulfonamide sorption in soils from substance and matrix properties.

SDZ sorption data. Literature data for K_d^* of SDZ are scarce and are restricted to a narrow pH range (6.9 - 7.5). Thus, it is not surprising that variation of the observed sorption coefficients is also small (1.4 to 2.8 L kg^{-1}) (Thiele-Bruhn and Aust, 2004; Thiele-Bruhn et al., 2004; Sarmah et al., 2006). With the assumption that the cationic species does not contribute significantly to the overall sorption, upper and lower boundaries for K_n can be estimated. The upper limit is given by the assumption that sorption of the anion is also negligible and sorption is solely explained by the neutral fraction ($K_d^* \geq \alpha_n \cdot K_n$). The lower limit makes use of the estimated ratio of 0.1 for K_a/K_n derived above from SPZ literature data. Inserting this ratio into equation 3.6 results in $K_d^* \leq K_n \cdot (\alpha_n + 0.1 \cdot \alpha_a)$. Applying these estimations to available literature data, results in a range of 6.4 L kg^{-1} to 22.9 L kg^{-1} for the K_n of SDZ (Table 3.1).

Table 3.1: Measured apparent sorption coefficients (K_d^*) and estimated ranges of neutral sorption coefficient K_n assuming that the cationic fraction is negligible and, for minimum estimation, sorption of the anion is smaller than of the neutral species ($K_a = 0.09 \cdot K_n$) and, for maximum estimation, that anionic sorption strength is negligible ($\alpha_n \cdot K_n \leq K_d^*$). Data from [1]Thiele-Bruhn et al. (2004) and [2]Thiele-Bruhn and Aust (2004).

matrix	**pH**	$\mathbf{K_d^*}$ **(L kg^{-1})**	α_n	α_a	K_n (L kg^{-1}) **min**	**max**
[1]clay fraction of silt loam 1	6.9	2.82	0.275	0.725	8.1	10.2
[1]silt loam 1	7.0	1.99	0.232	0.768	6.4	8.6
[1]sand fraction of silt loam 1	7.4	1.44	0.107	0.893	7.3	13.4
[2]silt loam 2	7.5	2.0	0.087	0.913	11.2	22.9

3.4.2 Sensitivity analysis of the sorption behaviour of SDZ in the investigated soils

The pK_{a2} value of SDZ is close to neutral and thus, fractions of neutral and anionic molecules are very sensitive to pH changes in the environmentally relevant range. Sulfadiazine (pK_{a2} 6.48) is mostly neutral (86%) in soil Kaldenkirchen (pH 5.7), whereas in soil Merzenhausen (pH 6.8) the anionic fraction prevails (68%). The minor role of the anionic fraction to sulfadiazine sorption in these two soils can be confirmed by a sensitivity analysis. Figure 3.4 displays the apparent sorption coefficient K_d^* in dependence of the sorption strength of the anionic and cationic species relative to the neutral species expressed as the ratio of the species-specific sorption coefficients. It can be seen that under these circumstances a significant effect of anion sorption on K_d^* requires higher sorption strength of the anion compared to the neutral molecule ($K_a > K_n$) which is in contrast to the findings derived from literature data above. The cationic species in the two soils is rather small ($< 0.1\%$) due to the low dissociation constant pK_{a1} which is responsible for protonation of the amino group. Thus, it can only be of importance for the overall sorption, if the cationic sorption coefficient (K_c) exceeds the neutral one (K_n) by a factor of at least 100. Figure 3.4 shows that K_d^* values increase for $K_c/K_n > 100$ in Kaldenkirchen soil, whereas in Merzenhausen soil even a ratio of 1000 does not significantly increase overall sorption. This sensitivity analysis corroborates the minor role of the cationic species for the overall sorption of sulfonamides to soils under natural conditions. However, this also implies that the

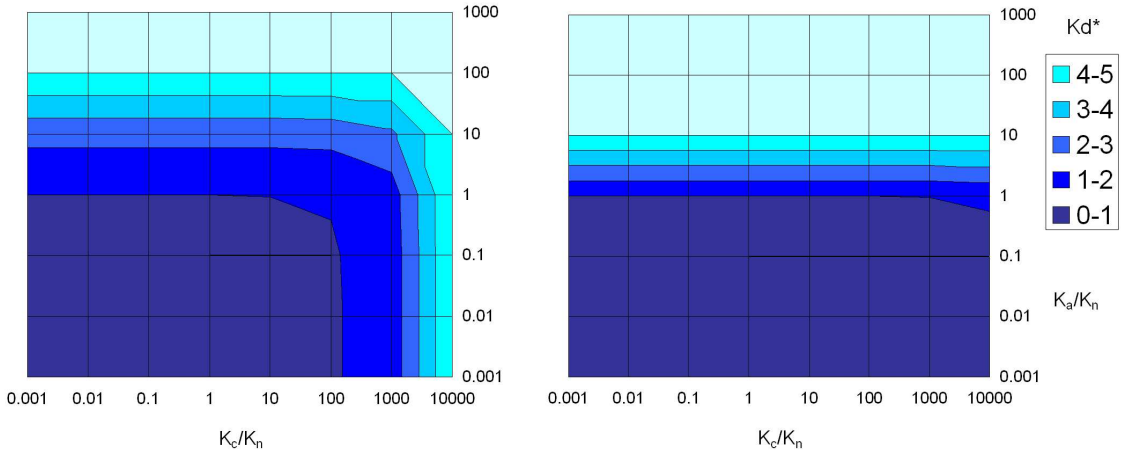

Figure 3.4: Effect of relative sorption strength of anionic (a) and cationic (c) sulfadiazine species on apparent K_d^* in soils Kaldenkirchen (*left*) and Merzenhausen (*right*). K_n was arbitrarily set to 1 L kg^{-1}.

sorption coefficient K_c cannot be reliably estimated from existing sorption data due to its insensitivity.

3.4.3 Estimation of apparent sorption coefficients

The experimental determination of sorption coefficients is usually performed with soil samples that have been spiked with the substance of interest. Aliquots of the spiked sample are then equilibrated with deionized water or $CaCl_2$ solution. After an appropriate equilibration time the aqueous solution is separated from the solid matrix (often by centrifugation) and analysed. This fraction is assumed to contain the dissolved equilibrium concentration. Correctly, the equilibrium concentration on the adsorbent had to be determined independently after extraction. However, this is often omitted and a simple mass balance calculation is used to estimate the amount of substance sorbed.

Within the research, the two manure-amended soils were sequentially extracted at specific dates with $CaCl_2$ solution followed by methanol (Amelung and Kaupenjohann, 2007). The results can be used to get a first estimation of the sorption behaviour of SDZ and its metabolites in the two soils. It is assumed that the substance mass in the $CaCl_2$ extract represents the dissolved fraction in equilibrium

with the fraction reversibly sorbed to the soil matrix during extraction. The distribution coefficient K_d^* (L kg^{-1}) of this equilibrium process is given by the ratio between the concentration on the solid matrix (μg kg^{-1} dry soil matter) and the concentration in the $CaCl_2$ extract. Assuming further that methanol exhaustively extracts the remaining reversibly sorbed substance fraction from the solid matrix after $CaCl_2$ extraction the apparent sorption coefficients of SDZ, Ac-SDZ and OH-SDZ can be estimated from available extraction data (Amelung and Kaupenjohann, 2007). The concentration on the solid matrix is then calculated by the substance mass in the methanol extract divided by the dry weight of the extracted soil sample. The concentration of the dissolved fraction is given by the substance mass in the $CaCl_2$ extract divided by the total volume of the solution. The latter is composed of the volume of the $CaCl_2$ solution added to the soil sample and, if fresh and not dried soil samples are investigated, of the soil water of the sample.
Experimental data indicate that the total mass in the extracts decreased with time. Nevertheless, the calculation described above can be performed for all soil samples since in each extraction step the actual equilibrium concentration of the $CaCl_2$ solution and the solid matrix were determined by independent analytical procedures. Table 3.2 shows the resulting K_d^* values calculated from the extraction data. Only data points where both concentrations were above the quantification limit have been used. For each soil and substance the variation between the single data points is equally small with a coefficient of variation between 16% and 35%. Large variation is only observed for the two smallest data sets (n=3) with individual data points close to the quantification limit. Mean K_d^* values are at the low end of literature values for SDZ in different matrices, e.g. K_d^* of SDZ in soil-slurry mixture 1.18 L kg^{-1} (Thiele-Bruhn and Aust, 2004), K_d^* of SDZ in soil 2.0 L kg^{-1} (Thiele-Bruhn et al., 2004). This indicates that the above assumptions are valid and the apparent distribution coefficients can be reliably estimated with the approach. Calculated K_d^* values for SDZ and OH-SDZ are very similar in the two soils indicating that the

Table 3.2: K_d^* (L kg^{-1}) (mean ± standard deviation) calculated from subsequent extraction experiments (Amelung and Kaupenjohann, 2007).

	SDZ	**OH-SDZ**	**Ac-SDZ**
Kaldenkirchen	0.56 ± 0.09 (n=7)	0.16 ± 0.04 (n=5)	0.40 ± 0.09 (n=4)
Merzenhausen	0.57 ± 0.12 (n=6)	0.15 ± 0.05 (n=3)	0.26 ± 0.07 (n=3)

effect of the different speciation (neutral fractions are 86% and 32%, respectively) on sorption is balanced by the difference in the soil matrices. If we recall that for sulfonamides K_a is approximately ten times smaller than K_n, the K_n value in soil M is estimated to be a factor of three larger than in soil K. This is in line with the larger fraction of organic carbon (0.52% vs 0.39%) and the significantly higher clay contents (23.1% vs 3.0%) of soil M resulting in a larger surface area available for binding processes.

3.4.4 Effect of speciation and sorption on sulfonamide concentration in soil solution

An important point is the availability of a compound which is a prerequisite for uptake by organisms and thus, in case of sulfonamides also for their inhibitory effect (Zarfl et al., 2008b). Generally, the available fraction of a compound in soil is the dissolved fraction in the soil solution. If it is assumed that environmental conditions (e.g. pH, temperature, water content) are more or less constant over time the relative distribution between the cationic, neutral and anionic species is constant in a given system. In the two investigated soils, concentrations in soil solution (C_w) only depend on sorption of the neutral molecule (K_n) and the anion (K_a) since the cation is not able to persist under the respective pH conditions. In order to elucidate the effect of speciation and sorption on the actual concentration in soil solution, the dissolved sulfadiazine concentration C_w (in mol $\cdot$ L^{-1}) can be predicted by using the following equation:

$$C_w = \frac{C_{tot}}{\frac{wcont}{\rho_W} + K_d^* \cdot (1 - wcont)} \tag{3.8}$$

C_{tot} is the total sulfonamide concentration in soil in mg $\cdot$ kg^{-1}, *wcont* is the water content (w/w), and ρ_W is the density of water (kg $\cdot$ L^{-1}). This equation is valid if substance distribution into soil air can be neglected and the assumption of instantaneous equilibrium holds, i.e. additional kinetic losses can be excluded. Equation 3.8 is used to estimate SDZ concentrations in soil solution of Kaldenkirchen and Merzenhausen (properties see Table 1.1) using estimated K_d^* values (Table 3.2). After application of 2.8 mg kg^{-1} SDZ (44% of the total radioactivity (Lamshöft et al., 2007; Schmidt et al., 2008)) to investigated soils the pore water concentrations of SDZ are calculated to be 4.7 mg L^{-1} in soil Kaldenkirchen and 4.5 mg L^{-1} in soil Merzenhausen. Although estimated K_d^* values are small and pore water concentrations are high the fraction of the sorbed sulfadiazine in relation to the total SDZ

amount applied to soil accounts for more than 80% due to a low water content. The concentration of dissolved sulfadiazine is essential for the antibiotic effect on soil microorganisms. Tappe et al. (2008) determined pH dependent growth inhibitory concentrations (EC_{50}) of SDZ to range from 0.5 mg L^{-1} (pH 7) to 2.57 mg L^{-1} (pH 5) for *Pantoea agglomerans*, a very sensitive soil bacterium, and from 2.85 mg L^{-1} (pH 5) to 15.4 mg L^{-1} (pH 7) for *Pseudomonas aeruginosa*. Thus, under the applied test conditions concentrations of SDZ are predicted to be in the effective range for sensitive soil bacteria like *P. agglomerans* whereas the dissolved SDZ fraction is probably too small to affect bacteria like *P. aeruginosa*. Consequently, antibiotic effects were observed to a smaller extent in experiments with SDZ applied soil samples than with in-vitro laboratory experiments.
Applied K_d^* values are at the low end of literature data which explains the relatively high pore water concentrations. However, even with these low K_d^* values the fraction of sorbed sulfadiazine accounts for more than 80% of total SDZ in soil due to the low water content during the experiments (7.2%, w/w and 11.5%, w/w). This means that on the one hand larger partitioning coefficients would decrease actual pore water concentrations possibly leading to a reduction of effectiveness. On the other hand the sorbed fraction constitutes an easily available reservoir that can maintain the pore water concentration level for longer periods of time.

3.4.5 Dynamic distribution ratios

Some investigations indicate that equilibrium sorption of sulfonamides includes an additional kinetically limited sorption process which follows the fast initial phase (Kreuzig and Höltge, 2005; Langhammer, 1989). In newer literature, the term "dynamic K_d values" has been used to describe sulfonamide sorption to manure, organic matter or soil (Kahle and Stamm, 2007; Amelung and Kaupenjohann, 2007). In fact, the observed increase in distribution ratios between solution and solid matrix cannot be equalled with the equilibrium distribution coefficient K_d^* which describes the concentration ratio of reversibly sorbed substance concentration and solved concentration in **equilibrium**. In contrast, this parameter includes the kinetic sequestration process which is usually assigned as formation of "non-extractable" residues. In soil science, the complexity of the compartment soil (Chapter 3.2.1) poses a challenge to determine single processes and its parameters. Soil solution, air and solid matrix (consisting of varying fractions of sand, silt, clay and organic carbon) represent an intricate structure in which the compound underlies several processes simultaneously.

As far as sorption of sulfonamides is concerned this means that a fast equilibrium process and different binding processes following slower or irreversible kinetics cannot be distinguished with one single measurement in time. After application of the antibiotic compound to the soil sample and with increasing time the extractable fraction may be additionally reduced by kinetically controlled translocation and/or binding processes that can be either reversible or irreversible (bound residues). In order to distinguish between the different fractions, Amelung and Kaupenjohann (2007) developed a sequential extraction procedure which allows for a deeper insight into underlying mechanisms. This is discussed in detail in the following Chapter 4.

Chapter 4

Transformation Reactions and Formation of Bound Residues[1]

4.1 Introduction

Transformation reactions in manure. Veterinary antibiotics are prone to different metabolism reactions in the treated animal after administration. These processes mainly lead to more polar structures that exhibit enhanced water solubility favouring excretion via urine. Main metabolites of sulfadiazine (SDZ) metabolism in pigs are long known to be N^4-acetyl-sulfadiazine (Ac-SDZ) and 4-hydroxy-sufadiazine (OH-SDZ) (Vree and Hekster, 1987). These two main metabolites were also identified in manure collected from pigs fed with ^{14}C-SDZ (Lamshöft et al., 2007).
During manure storage the parent compound and its excreted metabolites may be further transformed or completely mineralized. Degradation of sulfonamides during manure storage has been observed to proceed very slowly (Langhammer, 1989; Boxall et al., 2003). For example, 40% of sulfadimidine and 60% of sulfathiazole were unchanged after 5 weeks of manure storage under aerobic conditions (Langhammer, 1989). The most important transformation in manure is de-acetylation of Ac-SDZ back to SDZ (Grote et al., 2004). This transformation was also observed for several acetylated sulfonamide metabolites in wastewater treatment plants (Göbel et al., 2007). The OH-SDZ metabolite has been shown to be stable in manure under aerobic as well as anaerobic storage conditions (Heuer et al., 2008). Other metabolites of minor importance have also been identified in manure, e.g. OH-N^4-acetyl-SDZ (Lamshöft et al., 2007).

[1] This chapter is in preparation for publication in Zarfl et al. (2008a).

Transformation reactions in soil. Experimental investigations on formation of CO_2 from radio-labelled SDZ and its metabolites in manure-amended soils revealed that less than 2% of the applied substance is mineralized (Kreuzig and Höltge, 2005; Schmidt et al., 2008) even after 218 days. In analogy, biodegradation of sulfamethoxazole in test schemes according to ISO standards was observed to be negligible (Gartiser et al., 2007a,b). Thus, mineralization of sulfonamides in soil is of minor importance. Though the FOCUS working group on degradation kinetics 2006[2] includes "products that are in bound residues" into one degradation sink, the evolution of CO_2 is the only appropriate endpoint for ultimate biodegradation (Dörfler et al., 1995; Matthies et al., 2008). Whereas no hints for acetylation of sulfonamides in the environment exist, rapid de-acetylation has been observed for sulfamethoxazole (Höltge and Kreuzig, 2007). Ac-SDZ seems to be a metabolite which is only produced in the SDZ-treated animal and not in the environment whereas hydroxylation of SDZ to OH-SDZ in soil has not yet been explicitly described.

Non-extractable residues and formation of bound residues. The issue of non-extractable and bound residues first appeared in the context of risk assessment of pesticides (Roberts et al., 1984). Here, both termini are equally applied assuming that the non-extractable fraction completely belongs to the fraction of bound pesticide residues. According to the European directive 91/414/EEC[3] "non-extractable residues present in the soil [...] are chemical species (parent compound and metabolites, or fragments) [...], that cannot be extracted by methods which do not significantly change the chemical nature of these residues". This definition particularly aims at the covalently bound fraction. Decreasing extractability is often described as "aging" or sequestration (Amelung and Kaupenjohann, 2007). This can be attributed to the formation of bound residues (BR) in which the compound is covalently bound to the soil matrix (e.g. to humic materials (Bialk and Pedersen, 2008)), but also to storage in cavities not accessible for extraction solvents (non-extractable residues, NER) or to incorporation into microbial biomass (Charnay et al., 2004). In

[2]FOCUS, 2006. Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 version 2.0.

[3]European Economic Community, Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market (Official Journal of the European Communities L230, 19.08.91. p. 1).

the following it will be clearly distinguished between *non-extractable residues* (NER) as a "technical" term based on analytical methods and *bound residues* (BR) as a "mechanistical" term related to the compound's fate. Bound residues describe the fraction of a compound which is not available for extraction and uptake by organisms without changing the chemical nature of the matrix. Formation of BR is thus considered to be an irreversible sequestration process on the observed time scales. Assuming that bound residues cannot be extracted at all by any extraction method this fraction constitutes the lower boundary for the non-extractable residues. NER are defined technically as the substance fraction which is not extracted when applying a certain extraction procedure to the investigated sample. This means that the extraction procedure used determines the amount of non-extractable residues and has to be specified together with the result.
Another problem is that bound residues may be formed from the parent compound or from metabolites or degradation products with differing fractions depending on substance and soil properties. This leads to a great uncertainty as far as the regulatory significance is concerned (Craven and Hoy, 2005). In particular, it is unclear if the parent compound is immobilized directly or if the reaction proceeds via intermediates that may be one of the known or unknown metabolites.

Bound residue formation from SDZ. Experimental results indicate that formation of BR from SDZ can be excluded during manure storage (Spiteller, 2007). In soil and manure-amended soil experimental data show a fast decrease of extractable SDZ and metabolites with half-lives of less than seven days (Kreuzig and Höltge, 2005; Schmidt et al., 2008; Amelung and Kaupenjohann, 2007). It has also been observed that application of stronger extraction procedures (e.g. higher temperatures) enhance the recovery of sulfonamides from spiked and manure-amended soils (Stoob et al., 2006; Amelung and Kaupenjohann, 2007). Nevertheless, in the long run there is a decrease in recovery even with the more effective extraction methods that is most likely due to the formation of covalently bound residues. However, the mechanisms that are responsible for emergence of NER and formation of BR are still not fully understood (Matthies et al., 2008). Much stronger formation of bound residues was observed in non-sterile soils than in sterile soils implicating an essential influence of microbial activity on bound residue formation (Kreuzig and Höltge, 2005). Bialk and Pedersen (2008) investigated the bound residue formation of sulfonamides with humic acids in the presence of a fungal enzyme (peroxidase). They suggest covalent binding between the amino group of the sulfonamide and humic substances that

result in a deactivation of the antibiotic effect. In this case, Ac-SDZ cannot participate in sequestration of sulfadiazine, whereas the hydroxylated metabolite with its unchanged amino group may contribute to the bound residue formation. Since hydroxylation does not alter the NH_2-moiety, the various OH-metabolites are also still biologically active (Nouws et al., 1985) and thus of large interest to be included in risk assessment. Thus, all of the various interconnected processes affecting fate and effect of sulfonamides in soils have to be considered simultaneously to give a complete picture.

The objective of this study is to elucidate the mechanisms affecting the observed decrease of extractable SDZ and its metabolites over time in manure-amended soils. Experimental data on the fate of SDZ in manure and manure-amended soils are analyzed to identify the most relevant processes and reaction pathways. Influences of manure application on the chemical fate of SDZ shall be identified.

4.2 Methods

4.2.1 Model development

Sulfonamide dynamics in manure and manure-amended soils are determined by a number of different overlying processes that might influence each other and thus have to be considered simultaneously in the model approach. The speciation equilibrium of the compounds depends on the dissociation constants (pK_a) and on solution pH. The fraction of each species (cation, neutral, and anion) can be calculated as described in Chapter 2. Transformation reactions from Ac-SDZ to SDZ and from SDZ to OH-SDZ may occur during manure storage prior to application and in the manure-amended soil. Since experiments in soil with and without manure did not clearly show the effect of additional nutrients and microbial activity from the manure on apparent reaction rates (Spiteller, 2007; Schmidt et al., 2008) a possible biomass influence on transformation kinetics is neglected. Reversible equilibrium sorption of the compounds to solid matrices in manure and soil is described by the apparent distribution coefficient K_d^* which depends on the substance speciation and on soil properties (Chapter 3). By the sequential extraction experiment three different fractions were determined (Amelung and Kaupenjohann, 2007) that have to be assigned to process variables of the model. Here, we assume that the amount of substance on the $CaCl_2$ extract and the methanol extract is an appropriate measure for the total substance mass in soil solution and the sorbed phase that has not yet

been subject to sequestration. The remaining residual fraction (RES) constitutes the amount that has been sequestered by a reversible process requiring stronger conditions for extraction. Bound residues constitute an ultimate sink for the chemicals and their formation is regarded as an irreversible process in the model. Bialk and Pedersen (2008) found that the covalent coupling of sulfonamide antibiotics most likely proceeds between the amino group of the compound and humic substances. Since Ac-SDZ is acetylated at this amino group the capability of the metabolite to form such covalent bindings is inhibited and the respective pathway is not considered in the model. The complete model structure for the fate of SDZ and its metabolites Ac-SDZ and OH-SDZ in manure-amended soil is shown in Figure 4.1.

Model assumptions. Sulfadiazine and its metabolites can be regarded as an additional carbon substrate for degrading organisms. However, compared to the total

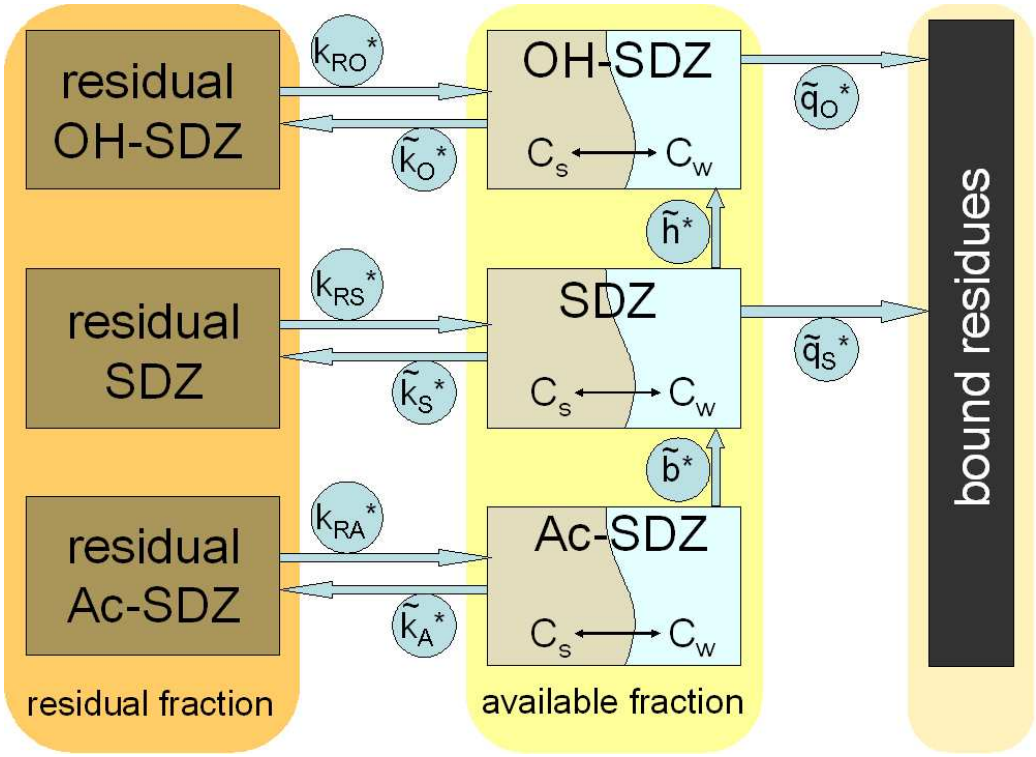

Figure 4.1: Model structure for transformations of SDZ and its metabolites Ac-SDZ and OH-SDZ in manure and soil. Processes include equilibrium sorption between dissolved (C_w) and sorbed (C_s) state, reaction from Ac-SDZ back to SDZ with an apparent bulk rate constant $\tilde{b}^*$, hydroxylation from SDZ to OH-SDZ with an apparent bulk rate constant $\tilde{h}^*$, kinetically controlled translocation with apparent bulk rate constants $\tilde{k}^*$ and apparent rate constants k^*, and formation of bound residues (BR) with an apparent bulk rate constant $\tilde{q}^*$.

mass of carbon substrates in soil the compounds only serve as secondary substrates and are transformed by "co-metabolism" (Matthies et al., 2008). Thus, all transformation reactions of SDZ, Ac-SDZ and OH-SDZ including the formation of BR are assumed to follow first order kinetics. For the sake of simplicity, equilibrium sorption is not explicitly included in the model. Although only the dissolved fraction in soil solution is available for transformation, uptake, sequestration and BR formation, the model uses the sum of dissolved and equilibrium sorbed fraction (called available fraction) as central state variable for sulfadiazine and its main metabolites. This is justified, because the concentration in soil solution is related to the total concentration by a constant factor which is determined by the apparent partition coefficient K_d^* and bulk soil properties such as dry soil density and volumetric water content. Thus, all rate constants estimated below for the available fraction are bulk rate constants (indicated by $\tilde{}$) that implicitly consider the specific sorption equilibrium in the investigated soil. Moreover, all rate constants have to be looked at as apparent rate constants (indicated by *) that implicitly consider the speciation of SDZ and its main metabolites.

According to the model structure given in Figure 4.1 the following system of ordinary differential equations describes the fate of SDZ and its two main metabolites in manure and manure-amended soil:

$$\frac{dS}{dt} = \tilde{b}^* \cdot A + k_{RS}^* \cdot RS - \left(\tilde{h}^* + \tilde{k}_S^* + \tilde{q}_S^*\right) \cdot S \tag{4.1}$$

$$\frac{dA}{dt} = k_{RA}^* \cdot RA - \left(\tilde{b}^* + \tilde{k}_A^*\right) \cdot A \tag{4.2}$$

$$\frac{dO}{dt} = \tilde{h}^* \cdot S + k_{RO}^* \cdot RO - \left(\tilde{q}_O^* + \tilde{k}_O^*\right) \cdot O \tag{4.3}$$

$$\frac{dRS}{dt} = \tilde{k}_S^* \cdot S - k_{RS}^* \cdot RS \tag{4.4}$$

$$\frac{dRA}{dt} = \tilde{k}_A^* \cdot A - k_{RA}^* \cdot RA \tag{4.5}$$

$$\frac{dRO}{dt} = \tilde{k}_O^* \cdot O - k_{RO}^* \cdot RO \tag{4.6}$$

$$\frac{dBR}{dt} = \tilde{q}_S^* \cdot S + \tilde{q}_O^* \cdot O \tag{4.7}$$

S, A, and O are the concentrations of SDZ, Ac-SDZ and OH-SDZ in the available fraction, RS, RA, and RO are the respective concentrations in the residual fraction, and BR is the fraction of bound residues which cannot be further differentiated. All concentrations are given in μmol kg^{-1} dry soil to assure consistency of the model simulations. $\tilde{b}^*$ is the apparent bulk rate constant for the transformation from Ac-SDZ back to SDZ, $\tilde{h}^*$ is the apparent bulk rate constant describing hydroxylation

from SDZ to OH-SDZ, $\tilde{q}^*_S$ and $\tilde{q}^*_O$ are the apparent bulk rate constants for the formation of bound residues from SDZ and OH-SDZ, respectively. The apparent bulk rate constants $\tilde{k}^*_S$, $\tilde{k}^*_A$ and $\tilde{k}^*_O$ describe the transfer kinetics into the residual fraction and k^*_{RS}, k^*_{RA} and k^*_{RO} are the apparent rate constants for the reverse process.

Model parameterization and sensitivity analyses. Independent data for the kinetic rate constants are not available from the literature. Storage experiments with contaminated manure from feeding experiments under aerobic and anaerobic conditions revealed that OH-SDZ remains unchanged and formation of bound residues in manure can be neglected ($< 2\%$) (Spiteller, 2007). A rough estimation of the rate constant for the de-acetylation of Ac-SDZ to SDZ in manure as the predominant process was made from the data. As far as the fate of SDZ in soil is concerned, all kinetic constants were deduced by fitting the model to the experimental data from Amelung and Kaupenjohann (2007) in soils from Kaldenkirchen and Merzenhausen. Also, the model was fitted to adapted data with a reduced amount of OH-SDZ in the residual fraction resulting from a correction according to the the mass balance (subsection 1.3.4) and compared with results derived from the original data set. Least-squares fitting was performed with the computer software Scientist for Windows Version 2.01[4]. Initial conditions ($t = 0$ d) for the state variables S, A, O, RS, RO and BR were inversely calculated and optimized in analogy to the rate constants since measured data are only available for day 1. The initial condition RA for the residual fraction of Ac-SDZ was set to zero due to insensitivity of the fitting procedure against the small values of Ac-SDZ measured in the residual fraction. Scenario analyses were conducted to identify transformation pathways of minor importance that can be omitted in the model without deteriorating model performance.
For model comparison the model selection criterion (MSC) was chosen which does not only consider deviations from experimental data but also the number of fitted parameters. This criterion is a modification of the *Akaike Information Criterion* (Akaike, 1976) representing the information content of the parameter set p resulting from the fit of the model to n observations:

$$MSC = LN\left(\frac{\sum_{i=1}^{n} w_i \cdot \left(Y_{obs,i} - \bar{Y}_{obs}\right)^2}{\sum_{i=1}^{n} w_i \cdot \left(Y_{obs,i} - Y_{calc,i}\right)^2}\right) - \frac{2 \cdot p}{n} \tag{4.8}$$

The data set was weighted with the respective inverse value (i.e. $w_i = Y^{-1}_{obs,i}$) in order to consider deviations from data with low values equally to data with higher

[4]http://www.micromath.com

values. MSC is the largest for the most appropriate model. To verify the validity of the model approach data from another independent experiment were compared with simulated data using the same parameter set derived from fitting.

4.3 Results and Discussion

4.3.1 Fit to manure extraction data

The simplified model approach describing the chemical fate of sulfadiazine in manure includes the reaction of Ac-SDZ back to SDZ but neglects all other transformation processes. This results in the following reduced system of ordinary differential equations:

$$\frac{dS}{dt} = \tilde{b}^* \cdot A \tag{4.9}$$

$$\frac{dA}{dt} = -\tilde{b}^* \cdot A \tag{4.10}$$

With initial conditions $S(0) = S_0$ and $A(0) = A_0$ the analytical solution is as follows:

$$S(t) = S_0 + A_0 \cdot \left(1 - e^{-\tilde{b}^* \cdot t}\right) \tag{4.11}$$

$$A(t) = A_0 \cdot e^{-\tilde{b}^* \cdot t} \tag{4.12}$$

This solution is fitted to measured concentrations of SDZ and Ac-SDZ during manure storage under anaerobic conditions at 20°C (Spiteller, 2007). The apparent bulk rate constant $\tilde{b}^*$ is determined resulting in a half life of 30 days (r^2=0.996). Data points and model simulations are shown in Figure 4.2 demonstrating the continuous decrease of Ac-SDZ whereas the concentration of SDZ increases. These observations are comparable to experimental results from Grote et al. (2004) which lead to a half life for reaction of Ac-SDZ to SDZ of 15.5 days under aerobic conditions.

4.3.2 Fit to extraction data of soils amended with fresh manure

The general fate model was fitted to data sets from experiments in soils Kaldenkirchen and Merzenhausen (Amelung and Kaupenjohann, 2007) with goodness-of-fit criteria of $r^2 = 0.99$ and MSC = 4.2 - 4.6. Various scenarios assuming different model structures showed that a best fit for SDZ, Ac-SDZ and OH-SDZ in the three fractions

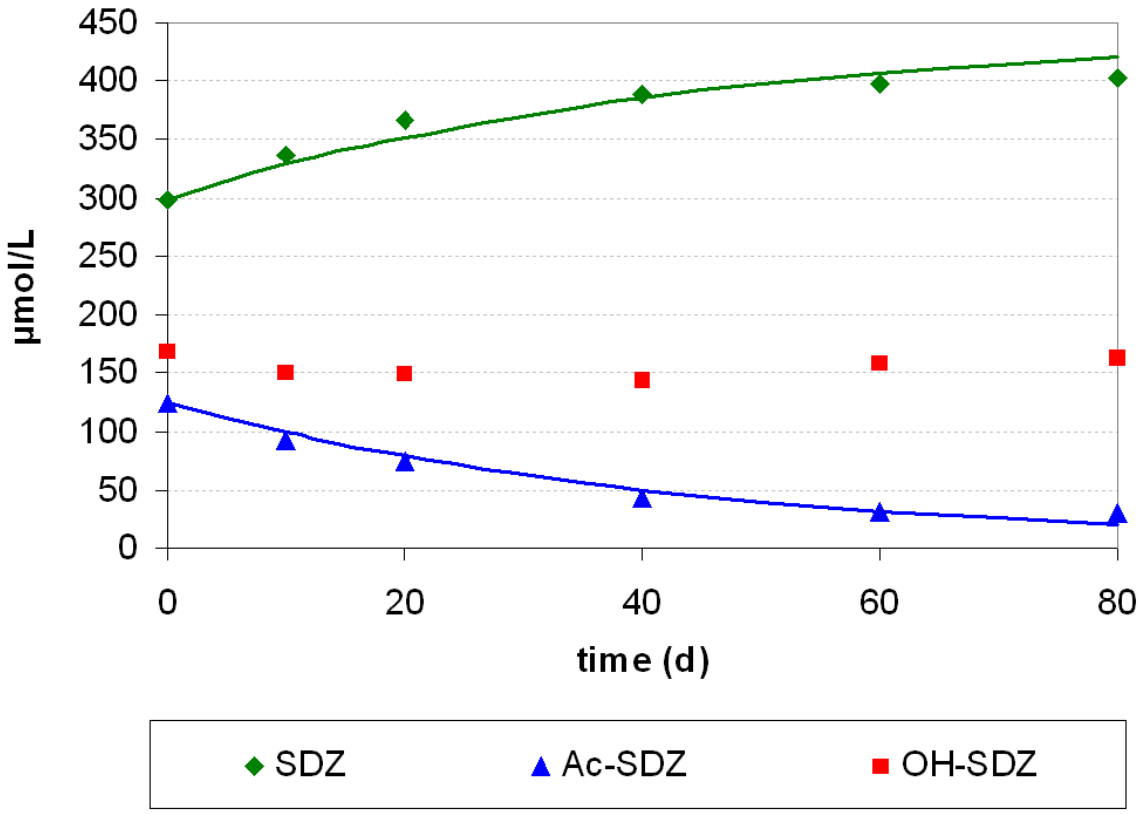

Figure 4.2: Measured data (Spiteller, 2007) of sulfadiazine (SDZ), N^4-acetyl-sulfadiazine (Ac-SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) and simulated concentrations (lines) of SDZ and Ac-SDZ in **manure**. Model results are based on a least-squares fit to experimental data.

(available, residual, bound residues) could be achieved assuming SDZ and OH-SDZ to form bound residues out of the available fraction and not via the residual fraction. In both soils which were amended with fresh manure kinetic constants resulting from a least-squares fit were of the same order of magnitude (Table 4.1). Half-lives for the de-acetylation were estimated to be four to five days which is a factor of four faster than observed in manure under aerobic conditions (Grote et al., 2004). Hydroxylation of SDZ is negligible in both soils as estimated rate constants are small ($\tilde{h}^* \rightarrow 0$). This is consistent with observations in manure where neither formation nor degradation of OH-SDZ occurred during storage (Schmidt et al., 2008; Heuer et al., 2008).

These results suggest that soil type does not affect the chemical fate of SDZ and its metabolites significantly. Figures 4.3 and 4.4 demonstrate measured and simulated concentrations of SDZ, Ac-SDZ and OH-SDZ after fitting the model to extraction data from Amelung and Kaupenjohann (2007). It can be seen that the model fit is

Table 4.1: Kinetic rate constants resulting from a least-squares fit of the model (Figure 4.1) to extraction data of soils Kaldenkirchen and Merzenhausen (data from Amelung and Kaupenjohann (2007)) after application of **fresh** (3 weeks) manure.

parameter	**Kaldenkirchen**	**Merzenhausen**
(d^{-1})	*fresh manure*	
$\tilde{b}^*$	1.86E-01	1.97E-01
$\tilde{h}^*$	3.98E-33	6.31E-18
$\tilde{k}_S^*$	6.46E-02	1.11E-01
k_{RS}^*	4.62E-03	6.11E-03
$\tilde{k}_A^*$	2.08E-02	8.89E-03
k_{RA}^*	5.84E-02	1.06E-03
$\tilde{k}_O^*$	4.69E-02	3.87E-02
k_{RO}^*	3.39E-03	2.32E-03
$\tilde{q}_S^*$	6.94E-02	9.13E-02
$\tilde{q}_O^*$	6.84E-03	3.13E-03

able to explain the data well. An important result is the fact that the relocation of the three compounds in the residual fraction must be reversible to explain the available data. The transfer of SDZ, Ac-SDZ and OH-SDZ into the residual fraction is of the same order of magnitude for all of the three compounds indicating that this transfer may be a diffusion-controlled physical entrapment. Relocation, however, is one order of magnitude slower for SDZ and OH-SDZ, but remains in the same range for Ac-SDZ. Since SDZ and OH-SDZ form bound residues they may also be sequestered more strongly in the residual fraction whereas the reversibility for Ac-SDZ is not decelerated by additional binding processes in the residual fraction. However, the processes governing sequestration of Ac-SDZ proved to be insensitive to the model outcome because of the small amounts of Ac-SDZ present.

Experimental data also suggest that transfer of the compounds into the residual fraction occurs on a similar timescale as compared to bound residue formation. Thus, these sequestration processes dominate the substance's fate. In the long run, Ac-SDZ disappears rather fast, whereas SDZ and the hydroxylated metabolite can still be found in the available fraction seven month after manure application to soils. Since these two substances are both antibiotically active they may affect soil microbial organisms and soil functions over a long timescale.

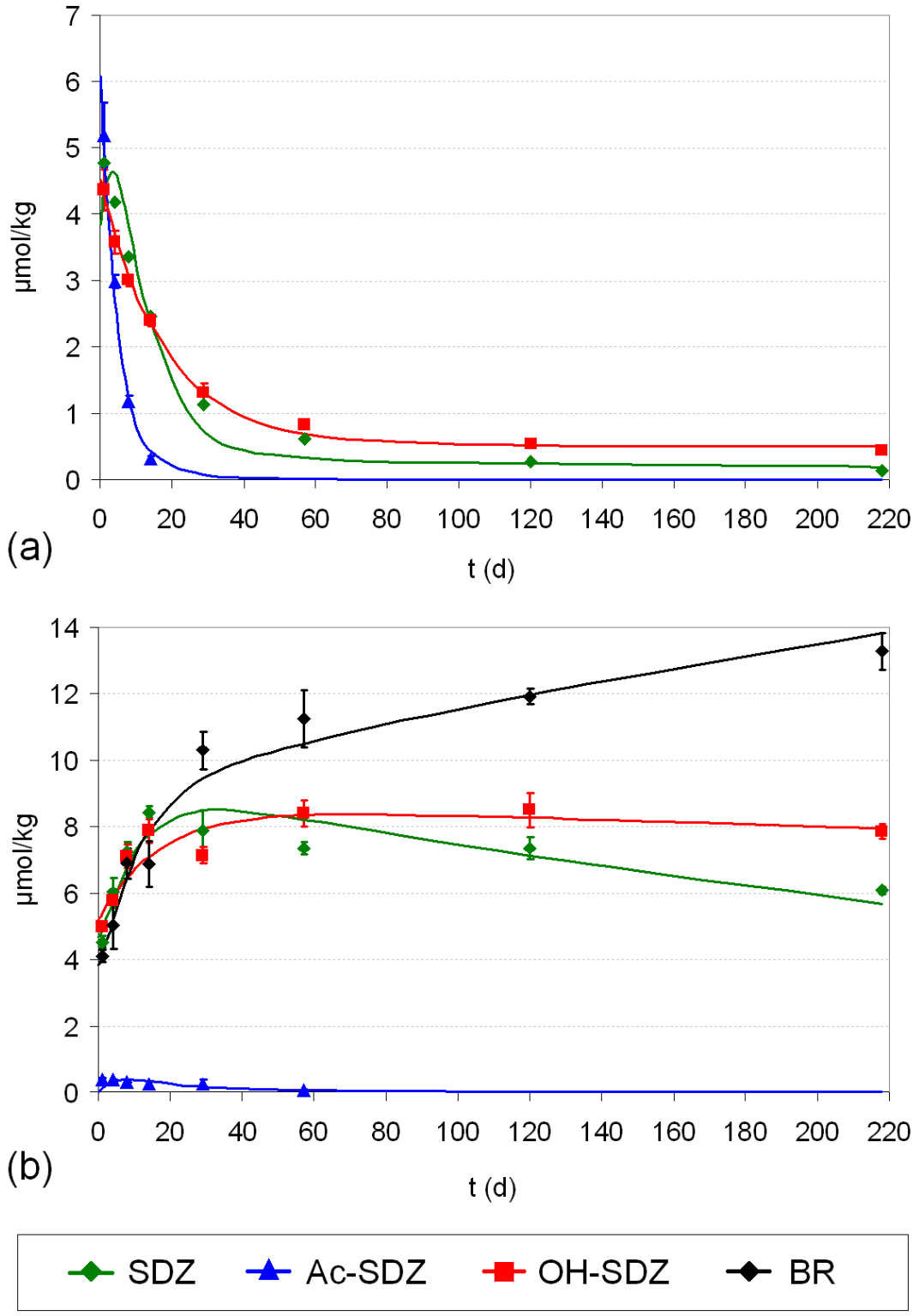

Figure 4.3: Measured (Amelung and Kaupenjohann, 2007) and simulated concentrations ($\mu mol\ kg^{-1}$, *d.w.*) of sulfadiazine (SDZ), N^4-acetyl-sulfadiazine (Ac-SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) in soil **Kaldenkirchen** amended with **fresh** manure in the (a) available and (b) residual as well as bound residue (BR) fraction. Model results are based on a weighted least-squares fit to experimental data.

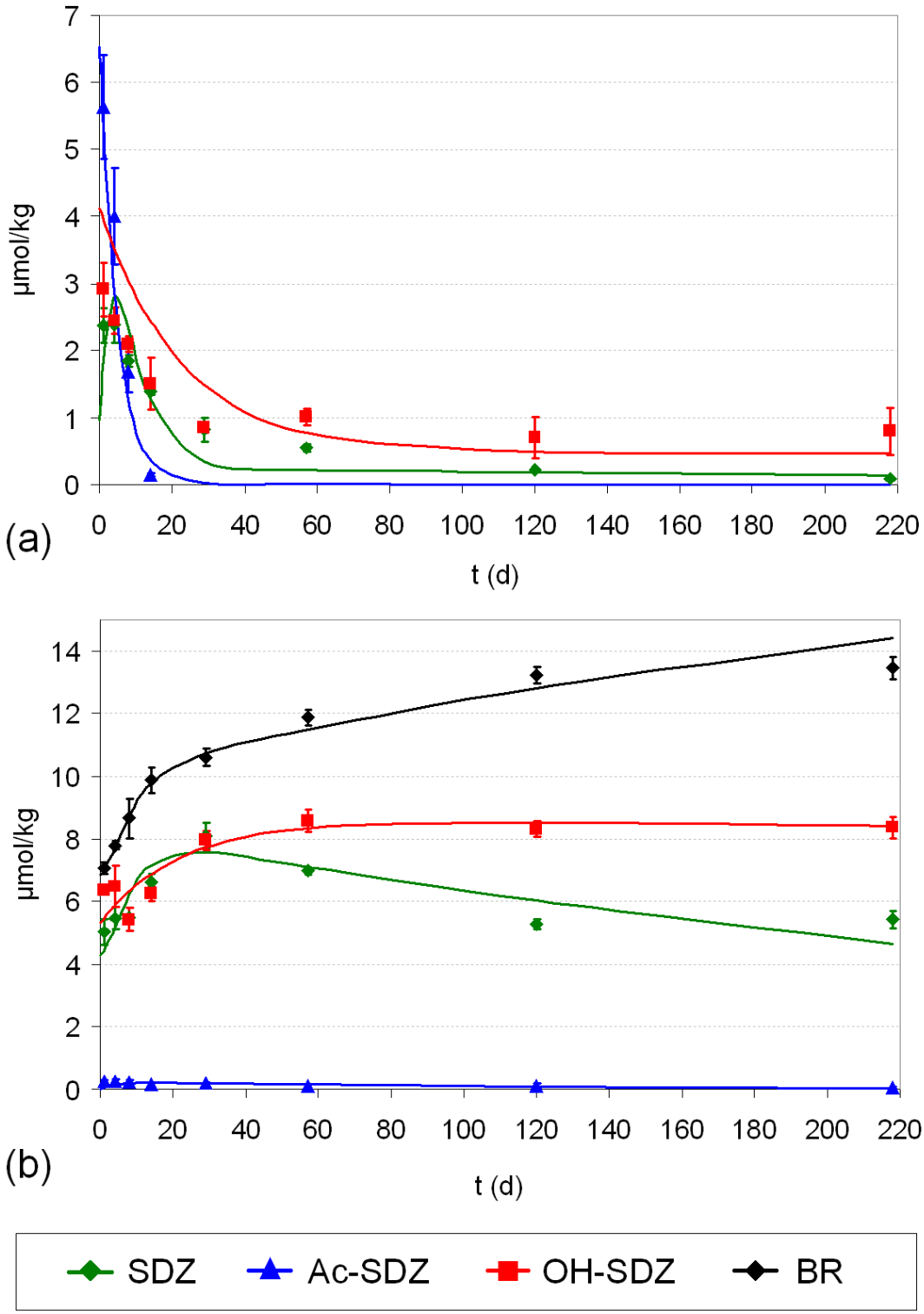

Figure 4.4: Measured (Amelung and Kaupenjohann, 2007) and simulated concentrations ($\mu mol\ kg^{-1}$ *(d.w.)*) of sulfadiazine (SDZ), N^4-acetyl-sulfadiazine (Ac-SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) in soil **Merzenhausen** amended with **fresh** manure in the (a) available and (b) residual as well as bound residue (BR) fraction. Model results are based on a weighted least-squares fit to experimental data.

Another important factor is the problem of the initial conditions. Since the first sample was taken approximately 24 hours after the start of the experiments the initial concentrations of the compounds in the fractions were not known. Data suggest that significant amounts of SDZ and OH-SDZ have to be already present in the residual and in the bound residue fraction immediately after starting the experiment indicating that this transfer is due to a fast process already occurring during sample preparation (i.e. homogenization of the soil-slurry mixture).

4.3.3 Evaluation of the model

For a first evaluation of the validity of the model outcome, independent experimental data of amending aged manure (after 6 month of storage) to soil Kaldenkirchen were used. Again, SDZ and the hydroxylated metabolite persist in the available fraction seven month after manure application to soil. In the aged manure, the amount of Ac-SDZ is negligible, because it has been completely transformed back to SDZ during storage. Thus, rate constants related to the fate of Ac-SDZ ($\tilde{b}^*$, $\tilde{k}_A^*$, $\tilde{k}_{RA}^*$) cannot be derived. However, experimental data cannot be explained by the same parameter set extracted from the experiments with fresh manure. Especially the concentrations in the available fraction are underestimated by the model between day 30 and 120 after manure application (Figure 4.5).

Thus, the model was independently fitted to the data ($r^2 = 0.98$; MSC = 4.5) resulting in a much better agreement (Figure 4.6). Kinetic parameters describing the interchange between the available and the residual fraction ($\tilde{k}_i^*$ and k_i^*) as well as the formation of bound residues via SDZ ($\tilde{q}_S^*$) are of the same order of magnitude as for the experiments with fresh manure. Again, hydroxylation of SDZ is slow, but a slower BR formation via OH-SDZ as compared to the fresh manure data is simulated (Table 4.2).

It can be seen from Figures 4.5 and 4.6 that standard deviations of measured OH-SDZ concentrations in the residual fraction were quite large (up to 50%) indicating severe analytical uncertainties. Over-estimation of the OH-SDZ caused by matrix effects could not be completely excluded and is subject to ongoing verification tests by the project partners. Indeed, the mass balance established by comparison with total radioactivity measurements (see subsection 1.3.4) revealed a significant difference in the residual fractions with too large values for the sum of SDZ, Ac-SDZ and OH-SDZ. Thus, OH-SDZ concentrations in the residual fractions were arbitrarily adapted for all experiments based on this mass balance. Adapted data from all

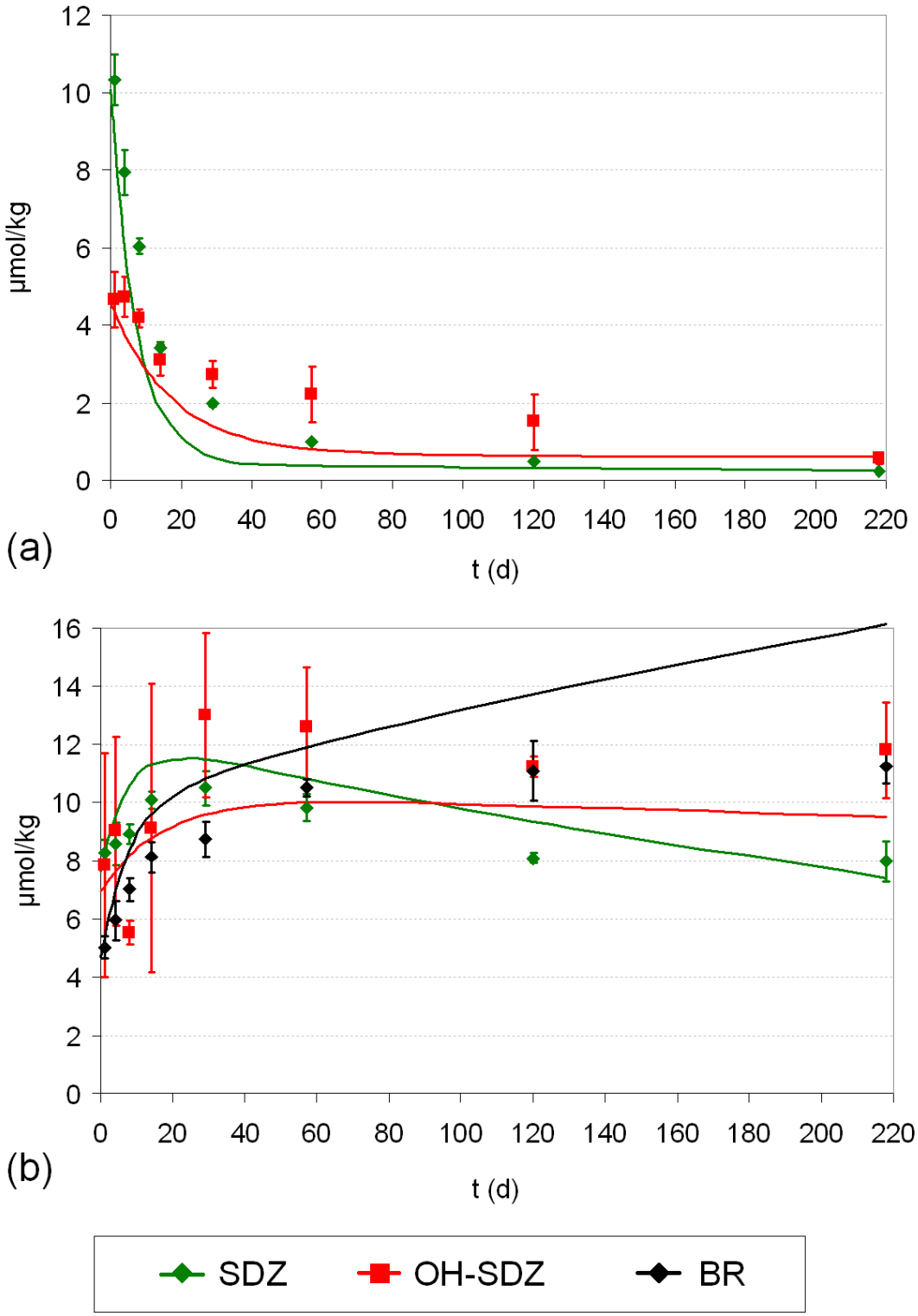

Figure 4.5: Measured data (Amelung and Kaupenjohann, 2007) and simulated concentrations ($\mu mol\ kg^{-1}$, *d.w.*) of sulfadiazine (SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) in soil **Kaldenkirchen** amended with **aged** manure in the (a) available and (b) resdiual as well as bound residue (BR) fraction. Model results are based on the data set derived from investigations of soil amended with **fresh** manure (Table 4.1).

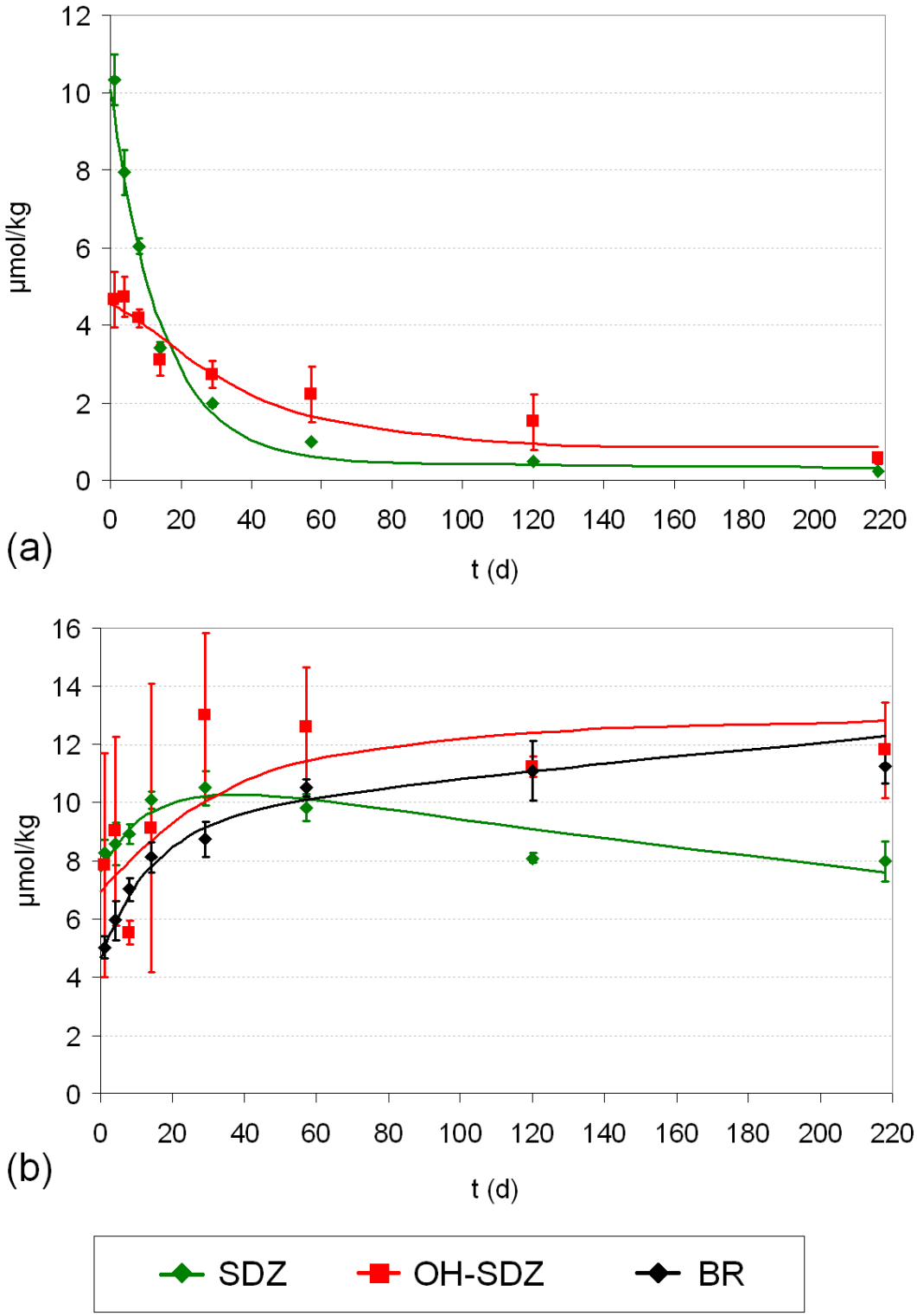

Figure 4.6: Measured data (Amelung and Kaupenjohann, 2007) and simulated concentrations ($\mu mol\ kg^{-1}$, *d.w.*) of sulfadiazine (SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) in soil **Kaldenkirchen** amended with **aged** manure in the (a) available and (b) residual as well as bound residue (BR) fraction. Modelling results are based on a weighted least-squares fit to experimental data.

Table 4.2: Kinetic rate constants resulting from a least-squares fit of the model (Figure 4.1) to original and adapted extraction data of soil **Kaldenkirchen** (original data from Amelung and Kaupenjohann (2007)) after application of fresh (3 weeks) and aged manure (6 month). The measured overestimation of OH-SDZ in the residual fraction was adapted to the mass balance on radioactivity.

parameter	**fresh manure**	**aged manure**	
(d^{-1})	*adapted data*	*original data*	*adapted data*
$\tilde{b}^*$	1.85E-01	-	-
$\tilde{h}^*$	3.98E-33	9.59E-03	6.31E-18
$\tilde{k}_S^*$	6.57E-02	2.55E-02	3.00E-02
k_{RS}^*	4.50E-03	2.92E-03	2.85E-03
$\tilde{k}_A^*$	2.08E-02	-	-
k_{RA}^*	5.76E-02	-	-
$\tilde{k}_O^*$	3.61E-02	3.35E-02	1.24E-02
k_{RO}^*	4.37E-03	2.01E-03	1.14E-03
$\tilde{q}_S^*$	6.65E-02	3.37E-02	3.61E-02
$\tilde{q}_O^*$	1.09E-02	6.31E-18	1.31E-03

three scenarios were again fitted to the model (r^2 = 0.98 - 0.99; MSC = 3.8 - 4.5). Only slight differences in the parameter values for the two experiments with fresh manure occurred (data not shown for soil Merzenhausen). In the experiment with aged manure the results show a more consistent picture if the model was fitted to the adapted OH-SDZ data in the residual fraction (Figure 4.7 and Table 4.2). Here, parameter values for SDZ and Ac-SDZ remain virtually unchanged and parameters describing the translocation of OH-SDZ into the residual fraction are of the same order of magnitude ($\tilde{k}_O^*$, k_{RO}^*). In agreement with the results of the fresh manure experiments, hydroxylation is negligible and BR formation is now predicted with a bulk rate constant similar to those of the fresh manure data. Since parameter values for the fate of SDZ and its metabolites in soil are in the same range regardless of the manure's age, it can be concluded that manure storage does not significantly influence the SDZ kinetics in soil.

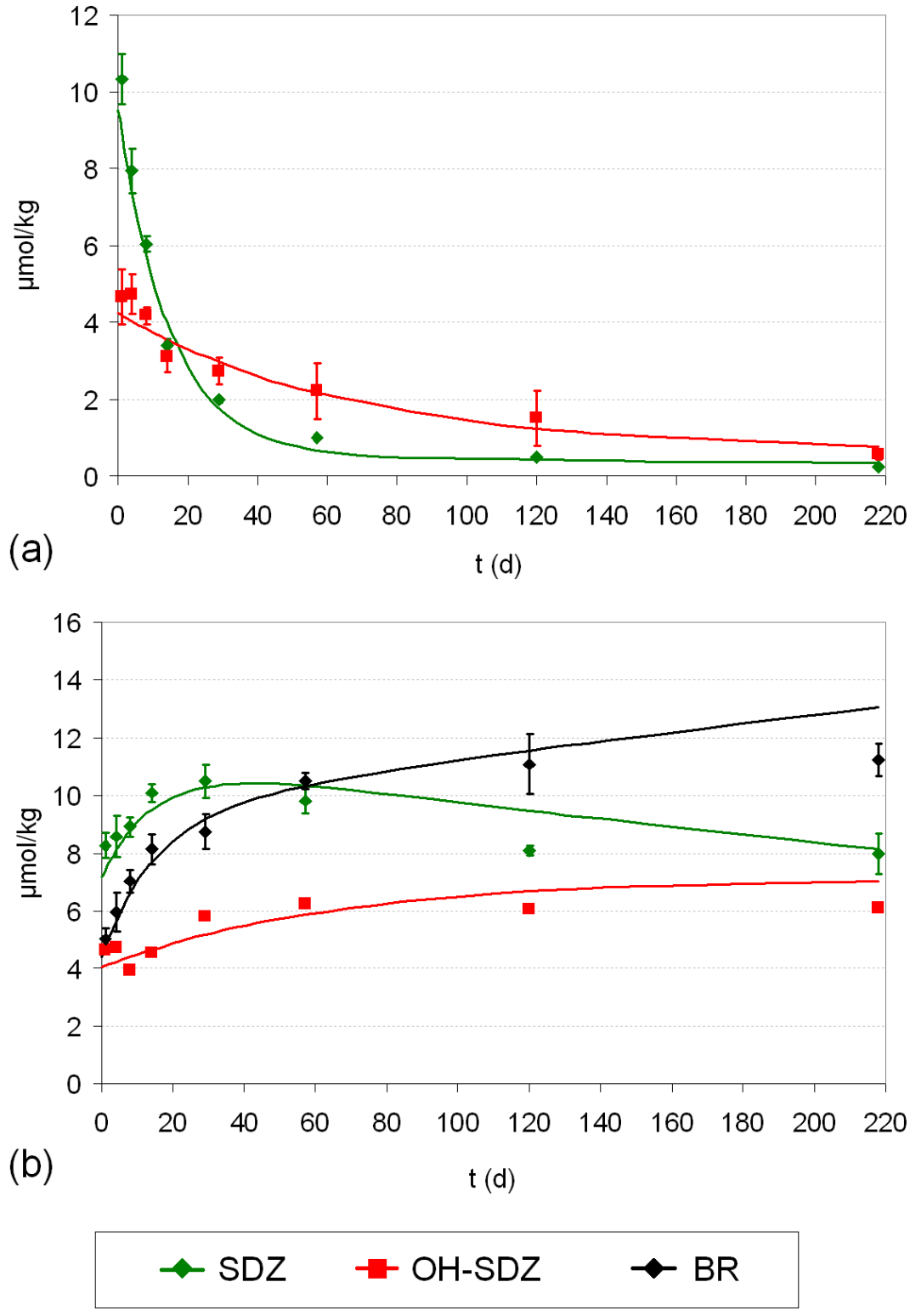

Figure 4.7: Measured data (Amelung and Kaupenjohann, 2007) and simulated concentrations ($\mu mol\ kg^{-1}$, *d.w.*) of sulfadiazine (SDZ) and 4-hydroxy-sulfadiazine (OH-SDZ) in soil **Kaldenkirchen** amended with **aged** manure in the (a) available and (b) residual as well as bound residue (BR) fraction. Modelling results are based on a weighted least-squares fit to experimental data adapted for OH-SDZ to the mass balance on radioactivity.

4.3.4 Simplification of the model

Summarizing the parameter results of the fitting procedures and according to the observed fate of SDZ and its metabolites in soils Kaldenkirchen and Merzenhausen the initial model presented in Figure 4.1 can be simplified from ten to five kinetic constants (Figure 4.8). On the observed timescale and in the investigated soils, hydroxylation of SDZ can be neglected ($\tilde{h}^* = 0$). However, a sensitivity analysis on the kinetic rate constant $\tilde{h}^*$ showed that hydroxylation may proceed up to an order of magnitude of $1E$-03 d^{-1} without deteriorating the simulation results. The respective half-life of about two years out-scored by the much faster formation of bound residues and is thus insensitive in the model. The interchange of the compounds between the available and the residual fraction occurs with the same parameter values ($\tilde{k}^* := \tilde{k}^*_S = \tilde{k}^*_A = \tilde{k}^*_O$; $k^*_R := k^*_{RS} = k^*_{RA} = k^*_{RO}$). Based on the available extraction data

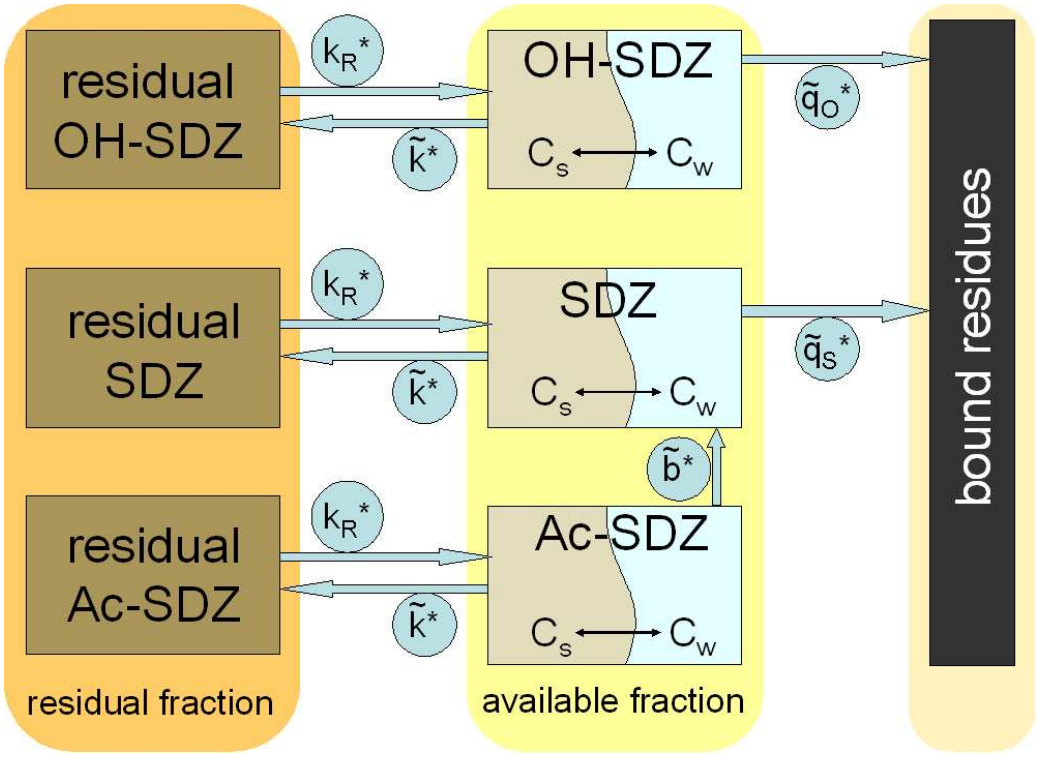

Figure 4.8: Simplified model structure for transformations of SDZ and its metabolites Ac-SDZ and OH-SDZ in manure and soil. Hydroxylation from SDZ to OH-SDZ can be neglected. Processes still include reaction from Ac-SDZ back to SDZ with an apparent bulk rate constant $\tilde{b}^*$, kinetically controlled translocation with generalized apparent bulk rate constant $\tilde{k}^*$ and an apparent rate constant k^*_R for all compounds and formation of bound residues (BR) with apparent bulk rate constant $\tilde{q}^*$.

Table 4.3: Kinetic rate constants resulting from a least-squares fit of the simplified model (Figure 4.8) to adapted extraction data of soils Kaldenkirchen and Merzenhausen (original data from Amelung and Kaupenjohann (2007)) after application of fresh (3 weeks) and aged manure (6 month).

parameter	**Kaldenkirchen**	**Merzenhausen**	**Kaldenkirchen**
(d^{-1})	*fresh manure*		*aged manure*
$\tilde{b}^*$	1.85E-01	1.88E-01	-
$\tilde{k}^*$	1.21E-02	1.09E-02	1.82E-02
k_R^*	1.11E-03	1.20E-03	1.98E-03
$\tilde{q}_S^*$	6.52E-02	9.86E-02	3.75E-02
$\tilde{q}_O^*$	1.38E-02	1.13E-02	2.62E-04

the state variable for the residual fraction of Ac-SDZ may also be neglected since its amount in the corresponding extract tends to zero. Consequently, the model is insensitive when fitting the respective kinetic constants ($k_{RA}^* = \tilde{k}_A^*$) to the extraction data. Mechanistically, however, there is no evidence that Ac-SDZ should behave differently as far as the relocation into the residual fraction is concerned and thus, it is still included in the mechanistic model approach. The values of the resulting kinetic constants (Table 4.3) can be deduced by fitting the model to the adapted data without loss of goodness (r^2 = 0.97 - 0.99; MSC = 3.3 - 4.7) and thus confirm the assumptions on the fate of SDZ and its main metabolites in soil.

4.4 Conclusion

Comparison of experimental data on the fate of SDZ and its metabolites in manure-amended soil with model scenarios helped to elucidate the most relevant transformation pathways and parameters. Rapid decrease of the Ac-SDZ metabolite to SDZ and significant formation of bound residues from SDZ as well as the OH-SDZ metabolite are the dominant transformation reactions, while hydroxylation of SDZ seems to be of minor importance under environmental conditions. Besides equilibrium sorption that has not been investigated in more detail another reversible process was identified which leads to a kinetically controlled reduction of the immediate available fractions of SDZ and at least the hydroxylated metabolite. This translocation into a so-called residual fraction may serve as a long-term reservoir for degradation, uptake

into plants and soil organisms, inhibitory effects on soil bacteria and formation of bound residues.

Thus, the results indicate a possible long-term relevance of the hydroxy-metabolite. Knowledge about the fate of hydroxylated metabolites in comparison to their parent compound is scarce and is only available for pesticides like atrazine. For example, Seybold and Mersie (1996) as well as Moreau and Mouvet (1997) point out that the hydroxy functional group leads to a higher sorption of hydroxylated atrazine to soils. This is confirmed by investigations of Kruger et al. (1996) who suppose that the additional OH-group may increase binding reactions. Thus, it is essential to include individual sorption experiments for the acetyl- and the hydroxy-metabolite to elucidate the contribution of the functional groups to the environmental fate of sulfadiazine in soil. The mechanism behind the reversible translocation process has not yet been identified. An explanation may be a slow diffusion process in soil leading to a physical entrapment of the compounds in small pores. This is also indicated by similar kinetic constants for the three investigated compounds SDZ, Ac-SDZ and OH-SDZ. Two different models have already been proposed by Pignatello and Xing (1996): The first one describes an intraparticulate diffusion through organic matter (in combination with physical entrapment), whereas the second is characterized by a sorption-retarded diffusion in soil micropores. Presumably, both model approaches are involved and can explain the "residual fraction". Determination of sorption enthalpies may help to gain more insight into sorption mechanisms. As far as sequestration is concerned the developed fate model can be compared to sorption models proposed by Wehrhan et al. (2007). There, breakthrough curves describing transport of SDZ in soil columns were best fitted with a sorption model assuming three sorption sites whereof two were reversible kinetically controlled sorption sites and one was an irreversible sorption site (i.e. five kinetic rate constants). Metabolites could be neglected since SDZ was spiked to the soil columns and, according to the results presented above, seem not to be generated or degraded in significant amounts in soil. Explicitly, the general fate model described above is based on one irreversible sorption process for SDZ and OH-SDZ as well, i.e. the formation of bound residues by covalent binding of the amino group to humic acids. A reversible kinetically controlled process is the back and forth transfers of SDZ, OH-SDZ and Ac-SDZ from the available fraction into the residual fraction. A second reversible sorption process is implicitly considered by the equilibrium sorption described by the apparent partition coefficient K_d^* which is included in the apparent bulk degradation rate constants.

Consequently, the developed mechanistic fate model includes two sorption sites and three kinetic rate constants and is equivalent to the model description in the soil columns. In general, the model structure is transferable to describe the environmental fate of other sulfonamides and also of other compound classes. In dependence of the respective metabolism and sequestration behaviour state variables and kinetic rate constants would have to be adapted.

After all, the amount of solute in pore water is the central state variable to link chemical fate to the biological effects. With the developed chemical fate model the total concentration in the available fraction can be predicted over time. Using equation 3.8 the respective pore water concentration can be simply calculated from K_d^* and the actual water content. The effect for a certain end point provoked by this concentration is dependent on the regarded organism and its habitat, e.g. the antibiotic activity depends on the mode of action of the compound. The reaction of the effective compounds (SDZ and OH-SDZ) with the enzyme DHPS in the bacterial cell in competition to the natural substrate para-aminobenzoic acid interferes with the folic acid cycle and, consequently, inhibits bacterial growth. Thus, for sulfonamides the link between chemical fate and biological effect is realized by the uptake of the antibiotic from solution by bacteria and a subsequent accumulation in the bacterial cell. The applied model concept and comparison of simulation results with measured effect data is described in the following chapter.

Chapter 5

Uptake of Sulfonamides by Microorganisms[1]

5.1 Introduction

Sulfonamides interfere with the folic acid cycle in microorganisms by competing for one of the key enzymes, dihydropteroate synthase (DHPS). As a result the production of folic acid is reduced which leads to a complete inhibition of cell reproduction (Brown, 1962; Vinnicombe and Derrick, 1999). Therefore, sulfonamides are characterized as broadband antibiotics in pharmacotherapy. Use of growth regulators in livestock feeding is interdicted since 2006 according to the EC directive 1831/2003[2], but sulfonamides are still applied in large amounts in veterinary medicine (Thiele-Bruhn, 2003; Kreuzig et al., 2003; Schneidereit, 2006). Sulfonamides have to be transported into the cell to compete with p-aminobenzoic acid (pABA) for the enzyme DHPS. It is known that the antibiotic effect of sulfonamides on cell activity differs depending on substance properties and pH conditions. Mengelers et al. (1997) investigated the effect of several sulfonamides on *Actinobacillus pleuropneumoniae*, a pathogenic bacterium causing pig respiratory disease (Marsteller and Fenwick, 1999), and observed that the minimal inhibitory concentration (MIC) varied with the pK_{a2} of the substance and the extracellular pH. Thiele-Bruhn and Beck (2005) showed that effective doses for the suppression of the microbial iron(III)-reduction in soils also vary depending on the speciation of the antibiotics that is influenced

[1]Reprinted in parts from Zarfl et al. (2008b), Copyright (2007), and Tappe et al. (2008), Copyright (2008), with permission from Elsevier.

[2]Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Official Journal of the European Union L 268/29-43.

indirectly by the soil pH. Therefore, the pH-dependent uptake and accumulation of sulfonamides in bacterial cells is of particular interest not only for human and veterinary chemotherapy but also for the assessment of fate and effects of pharmaceuticals in the environment. In this chapter, the influence of the speciation on the potential intracellular accumulation of sulfonamides is investigated. For this purpose a recently published mechanistic model for the transport of chemicals into cells (Trapp and Horobin, 2005) has been adapted and supplemented. The model is based on the assumption that not only neutral molecules but also the dissociated species are able to pass the cell envelope. Critical model parameters are identified and their sensitivity on model results is tested within realistic parameter ranges. Finally, enzymatic reactions are integrated into the uptake model to simulate the competitive effect of sulfonamides on pABA and to assess the impact of the additional process within realistic parameter ranges. The aim of this investigation is to find out in how far the observed substance-specific and pH-dependent antibiotic effects can be explained by a different accumulation of sulfonamides in bacterial cells.

5.2 Theory

5.2.1 Transport into bacterial cells

To affect a bacterial microorganism, the solute substance molecules have to pass the cell envelope and enter the cytoplasm. Including cell wall and plasma membrane the cell envelope can already be regarded as an intrinsic drug barrier. As far as permeability of biological membranes is concerned four different uptake mechanisms can be differentiated (Rang et al., 2007):

- diffusion through the lipid bilayer
- diffusion through porins formed by proteins
- transmembrane transport proteins which bind a molecule on one side of the membrane, then change their conformation and leave the molecule on the other side of the membrane
- endocytose: uptake of small amounts of liquid including solved (pinocytose) or solid (phagocytose) substances.

Non-polar substances mainly diffuse through the cell membrane influenced by two physicochemical parameters, the partitioning coefficient between lipid and water

(K_{OW}) and the diffusion coefficient which hardly varies between different pharmaceuticals (Rang et al., 2007). For sulfonamides active efflux transport systems have been identified (Köhler et al., 1996) but so far no specific transport protein for sulfonamide influx is known (Schrempf, 2007). Although active transport by unspecific carrier mechanisms cannot be ruled out, uptake kinetics of organic contaminants by bacteria are usually regarded as diffusive transfer across the cell membranes (Nouws et al., 1985; Hancock, 1997; Kitahara et al., 1997; Tras et al., 1998). The ratio of substance concentrations in the cell (C_{cell}) and the surrounding medium (C_{env}) can be regarded as a measure for the maximal accumulation of the antibiotic in the cell. Equation 5.1 describes a simple relationship for this accumulation assuming that uptake is solely determined by mass flux of the neutral molecule, while charged species cannot cross the cell envelope barrier (Büttner and Büttner, 1980):

$$\frac{C_{cell}}{C_{env}} = \frac{1 + 10^{pH_{cell} - pK_{a2}}}{1 + 10^{pH_{env} - pK_{a2}}} \tag{5.1}$$

However, this is a strong assumption that is not necessarily valid (Rang et al., 2007). Recently, Trapp and Horobin (2005) published a mechanistic model assuming total uptake to be the sum of mass fluxes of the neutral and charged species of the molecule. In their model, the flux of the neutral molecule across the cytoplasmic membrane is driven by the chemical potential gradient and can be specified by Fick's first law of diffusion (Atkins (1990), p.735):

$$J_n = -P_n \cdot (a_{cell,w,n} - a_{env,w,n}) \tag{5.2}$$

where J_n is the net flux with the concentration gradient from the outside into the cell in $mol \cdot m^{-2} \cdot s^{-1}$, P_n is the permeability of the membrane for neutral (n) molecules in $m \cdot s^{-1}$, and $a_{cell,w,n}$ and $a_{env,w,n}$ are the activities of the dissolved (w) neutral molecules (n) in the cell ($cell$) and in the surrounding environment (env), respectively.

The flux of ions across electrically charged membranes is more complex since it depends on the chemical potential as well as on the electrochemical potential. The assumption of a linear potential gradient across the membrane then leads to the Nernst-Planck equation (Briggs et al., 1961):

$$J_d = -P_d \cdot \frac{N}{e^N - 1} \cdot \left(a_{cell,w,d} \cdot e^N - a_{env,w,d}\right) \tag{5.3}$$

where J_d is the net flux of the dissociated (d) species (cationic and anionic, respectively) in $mol \cdot m^{-2} \cdot s^{-1}$, P_d is the permeability of the membrane for an ion, $a_{cell,w,d}$

and $a_{env,w,d}$ are the activities of the dissolved (w) dissociated species in the cell and in the surrounding environment, respectively. The constant N is defined as follows:

$$N = \frac{z \cdot E \cdot F}{R \cdot T} \tag{5.4}$$

with z being the electric charge (+ 1 for cations, - 1 for anions), E the membrane potential, F the Faraday constant (96484.56 $C\ mol^{-1}$), R the universal gas constant (8.314 $J{\cdot}mol^{-1}{\cdot}K^{-1}$), and T the absolute temperature (K). The membrane potential E for *E. coli* is about -0.11 V (Zilberstein et al., 1984).

In general, it is assumed that the permeability of the cell envelope is significantly lower for charged molecules than for neutral molecules ($P_d < P_n$) but cannot be neglected (Escher et al., 1999). If we additionally assume that the reaction kinetics of sulfonamides in the cell is much slower than the uptake kinetic, steady-state accumulation in the cell can be estimated by solving the following system of ordinary differential equations describing the change of total substance concentration in a single cell (C_{cell}) and the total substance concentration in the surrounding matrix (C_{env}), both in $mol \cdot m^{-3}$.

$$\begin{aligned}\frac{dC_{cell}}{dt} &= \frac{A_{cell}}{V_{cell}} \cdot \Big[C_{cell} \cdot \Big(-P_n \cdot \gamma_n \cdot f_{cell,w,n} - P_c \cdot \frac{N_c}{e^{N_c}-1} \cdot \gamma_c \cdot f_{cell,w,c} \cdot e^{N_c} \\ &\quad -P_a \cdot \frac{N_a}{e^{N_a}-1} \cdot \gamma_a \cdot f_{cell,w,a} \cdot e^{N_a}\Big) + C_{env} \cdot (P_n \cdot f_{env,w,n} \\ &\quad +P_c \cdot \frac{N_c}{e^{N_c}-1} \cdot f_{env,w,c} + P_a \cdot \frac{N_a}{e^{N_a}-1} \cdot f_{env,w,a}\Big)\Big] \end{aligned} \tag{5.5}$$

$$\frac{dC_{env}}{dt} = -\frac{n_{cells} \cdot V_{cell}}{V_{env}} \cdot \frac{dC_{cell}}{dt} \tag{5.6}$$

P_c and P_a are the permeabilities of the cell membrane for the cationic (c) and the anionic (a) species, respectively, N_c and N_a are defined as above with $z = +1$ or -1, V_{env} is the total volume of the environment, A_{cell} is the cell surface, V_{cell} is the cell volume of a bacterium, and n_{cells} is the total number of bacterial cells in the environment. $f_{cell,w,i}$ and $f_{env,w,i}$ are the fractions of the dissolved cationic (c), anionic (a) and neutral (n) species of the total substance mass in the cell and in the surrounding medium, respectively. γ_i is the activity coefficient of the respective species in the cell which relates concentration to activity according to $a_{cell,w,i} = \gamma_i \cdot C_{cell}$. Replacing the terms $P_n \cdot \gamma_n \cdot f_{cell,w,n} + P_c \cdot \frac{N_c}{e^{N_c}-1} \cdot \gamma_c \cdot f_{cell,w,c} \cdot e^{N_c} + P_a \cdot \frac{N_a}{e^{N_a}-1} \cdot \gamma_a \cdot f_{cell,w,a} \cdot e^{N_a}$ by a "release velocity" k_r and $P_n \cdot f_{env,w,n} + P_c \cdot \frac{N_c}{e^{N_c}-1} \cdot f_{env,w,c} + P_a \cdot \frac{N_a}{e^{N_a}-1} \cdot f_{env,w,a}$ by an "uptake velocity" k_u in equation 5.5 leads to the following

simplified differential equation for the cell concentration:

$$\frac{dC_{cell}}{dt} = \frac{A_{cell}}{V_{cell}} \cdot [-k_r \cdot C_{cell} + k_u \cdot C_{env}] \tag{5.7}$$

Note that k_r and k_u are in units of m·s^{-1}. With $\frac{dC_{cell}}{dt} = 0$ the ratio of C_{cell} to C_{env} at steady state is given by the ratio of uptake and release rate:

$$\frac{C_{cell}}{C_{env}} = \frac{k_u}{k_r} = AF \tag{5.8}$$

This concentration ratio describes the potential accumulation of the substrate in the cells relative to their surroundings and is defined as the accumulation factor AF (eq. 5.8). It is commonly supposed that the antibiotic effect of sulfonamides is solely due to the anionic sulfonamide species (Henry, 1943), because of its close structural similarity to the original substrate p-aminobenzoate. Thus, to relate sulfonamide accumulation to growth inhibition of bacterial cells, accumulation of the anionic species in the cells is the relevant parameter. This is described by the anion accumulation factor (AAF) which relates the effective concentration (intracellular anion concentration) to the applied dose (total sulfonamide concentration in the surrounding medium).

$$AAF = \frac{C_{cell}}{C_{env}} \cdot \alpha_{a,cell} = AF \cdot \alpha_{a,cell} \tag{5.9}$$

The fraction of anion in the cell and thus also the AAF depend on the internal pH and the pK_{a2} of the substance.

5.2.2 Model parameterization and sensitivity analyses of the uptake model

Sulfonamides accumulate in the particle-free cytoplasm (=liquid phase) of the cells, as it was shown for sulfadiazine in *Escherichia coli* cells (Büttner and Büttner, 1972). Thus, intracellular sorption to lipids and other compounds can be neglected. Furthermore, external and intracellular pH values are constant over time and speciation is in equilibrium described by equations 2.1-2.6. From equation 2.1 it follows that the cationic species can be neglected as the pK_{a1} of sulfonamides is several orders of magnitude below environmentally relevant pH values. Permeabilities of the cell envelope are estimated analogously to Trapp (2004) from the octanol-water partition coefficient (K_{OW}) and a combination of estimated cell envelope thickness Δx for bacteria (20 nm) and an average diffusion coefficient D for neutral organic compounds

in biomembranes of 10^{-14} $m^2 \cdot s^{-1}$(Lieb and Stein, 1971):

$$\log P_n = \log K_{OW} + \log\left(\frac{D}{\Delta x}\right) \approx \log K_{OW} - 6.3 \tag{5.10}$$

The permeability of biomembranes for organic ions is approximately 3.5 orders of magnitude smaller than for neutral molecules (Raven, 1975; Trapp and Horobin, 2005) so that

$$\log P_d = \log K_{OW} - 10.2 \tag{5.11}$$

Substance properties for a variety of sulfonamides were collected from the literature and are summarized in Table 2.1. Measured log K_{OW} values for several sulfonamides range from -1.22 (sulfaguanidine) up to 1.63 (sulfadimethoxine) resulting in P_n values from $3.0 \cdot 10^{-8} m \cdot s^{-1}$ to $2.1 \cdot 10^{-5} m \cdot s^{-1}$.
A reference scenario is chosen representing neutral extracellular conditions (pH = 7.0) and a typical pH value of 7.5 for bacterial cytoplasm (Zilberstein et al., 1984; Beales, 2004). The permeabilities are calculated for an intermediate log K_{OW} (0.0) resulting in values of $5.0 \cdot 10^{-7} m \cdot s^{-1}$ (P_n) and $1.6 \cdot 10^{-10} m \cdot s^{-1}$ (P_d), respectively. With the ionic strength I of the cytoplasm of *E. coli* of about 0.3 mole (Weiden et al., 1967) the activity coefficient γ_n of the neutral molecule in the cytoplasm can be estimated from the Setchenov equation to be 1.23. The activity coefficient for the monovalent cation and anion ($|z| = 1$) is calculated with the Davies approximation to 0.74 ($\gamma_c = \gamma_a$) at I=0.3 mole (Appelo and Postma, 1999). Extracellular sulfonamide concentrations were not corrected for ionic strength, i.e. activities were assumed to be equal to concentrations. Sensitivity analyses are performed for the effect of intracellular and extracellular pH as well as for the permeability difference between neutral and ionic sulfonamide species.

5.2.3 Development of the model for competitive enzymatic kinetics

As described in section 1.1 sulfonamides represent a structural analogue to para-aminobenzoic acid (pABA) which is necessary for the synthesis of folic acid. Thus, they act as competitive antagonists in the reaction of pABA with the enzyme dihydropteroate synthase (DHPS) and inhibit the production of dihydropteroate (DHP) which is a co-factor for the formation of folic acid. This enzymatic reaction can be described according to the well-known Michaelis-Menten equation for both the enzymatic reaction with pABA and the one with the sulfonamide:

$$E + S \rightleftharpoons ES \rightarrow E + DHP^* \tag{5.12}$$

$$E + P \rightleftharpoons EP \rightarrow E + DHP \tag{5.13}$$

E is the concentration of the enzyme DHPS, S and P are the concentrations of the sulfonamide and of pABA, respectively. The pABA concentration is assumed to be in steady state, i.e. the total available amount of pABA in the cell is not affected by any enzymatic reaction ($\frac{dpABA}{dt} = 0$). ES and EP are the respective concentrations of the enzyme-substrate complex and DHP^* is the DHP analogue. Additionally, for enzyme reactions it can be assumed that the total amount of enzyme is constant and that the enzyme-substrate complexes reach quasi-stationarity:
(a) $E + ES + EP = E_0$
(b) $\frac{dES}{dt} = 0$ and $\frac{dEP}{dt} = 0$
Then, the change of product and sulfonamide concentrations is described by the following kinetic equations:

$$\frac{dS}{dt} = -k_1 \cdot E \cdot S + k_{-1} \cdot ES = -k_2 \cdot ES = -\frac{dDHP^*}{dt} \tag{5.14}$$

$$\frac{dES}{dt} = k_1 \cdot E \cdot S - (k_{-1} + k_2) \cdot ES = 0 \tag{5.15}$$

$$\frac{dEP}{dt} = k_3 \cdot E \cdot P - (k_{-3} + k_4) \cdot EP = 0 \tag{5.16}$$

$$\frac{dDHP}{dt} = k_4 \cdot EP \tag{5.17}$$

With the condition (a) equation 5.15 can be solved for ES:

$$\begin{aligned}
& ES = \frac{k_1}{k_{-1} + k_2} \cdot E \cdot S = \frac{k_1}{k_{-1} + k_2} \cdot (E_0 - ES - EP) \cdot S \\
\Leftrightarrow\ & ES \cdot \left(1 + \frac{k_1}{k_{-1} + k_2} \cdot S\right) = \frac{k_1}{k_{-1} + k_2} \cdot (E_0 - EP) \cdot S \\
\Leftrightarrow\ & ES = \frac{k_1}{k_{-1} + k_2 + k_1 \cdot S} \cdot (E_0 - EP) \cdot S = \frac{(E_0 - EP) \cdot S}{\frac{k_{-1}+k_2}{k_1} + S} \\
\Leftrightarrow\ & ES = \frac{(E_0 - EP) \cdot S}{K_{m,S} + S}
\end{aligned} \tag{5.18}$$

For equation 5.16 it holds accordingly:

$$EP = \frac{(E_0 - ES) \cdot P}{K_{m,P} + P} \tag{5.19}$$

$K_{m,S}$ and $K_{m,P}$ are the Michaelis constants for the sulfonamide and for pABA, respectively.
Inserting the right-hand side of equation 5.19 into equation 5.18 leads to:

$$\begin{aligned} ES &= \frac{\left(E_0 - \frac{(E_0 - ES)\cdot P}{K_{m,P}+P}\right)\cdot S}{K_{m,S}+S} \\ &= \frac{(K_{m,P}+P)\cdot E_0 - (E_0 - ES)\cdot P}{(K_{m,P}+P)\cdot(K_{m,S}+S)}\cdot S \\ &= \frac{(E_0\cdot K_{m,P} + E_0\cdot P - E_0\cdot P + ES\cdot P)\cdot S}{K_{m,P}\cdot K_{m,S} + K_{m,P}\cdot S + K_{m,S}\cdot P + P\cdot S} \end{aligned}$$

To solve for ES, all terms including this variable are separated on the left-hand side of the equation resulting in:

$$\begin{aligned} ES \quad\cdot\quad & \left(1 - \frac{P\cdot S}{K_{m,P}\cdot K_{m,S} + K_{m,P}\cdot S + K_{m,S}\cdot P + P\cdot S}\right) \\ &= \frac{E_0\cdot K_{m,P}\cdot S}{K_{m,P}\cdot K_{m,S} + K_{m,P}\cdot S + K_{m,S}\cdot P + P\cdot S} \\ \Leftrightarrow ES &= \frac{E_0\cdot K_{m,P}\cdot S}{K_{m,P}\cdot K_{m,S} + K_{m,P}\cdot S + K_{m,S}\cdot P + P\cdot S - P\cdot S} \\ &= \frac{E_0\cdot K_{m,P}\cdot S}{K_{m,P}\cdot K_{m,S} + K_{m,P}\cdot S + K_{m,S}\cdot P} \end{aligned}$$

Replacing ES in equation 5.14 with this term leads to:

$$\begin{aligned} \frac{dS}{dt} &= -\frac{dDHP^*}{dt} = -k_2\cdot ES = -\frac{k_2\cdot E_0\cdot K_{m,P}\cdot S}{K_{m,P}\cdot K_{m,S} + K_{m,P}\cdot S + K_{m,S}\cdot P} \\ &= -\frac{V_{max,S}\cdot S}{K_{m,S} + S + \frac{K_{m,S}}{K_{m,P}}\cdot P} \end{aligned} \tag{5.20}$$

Analogously, insertion of equation 5.18 in equation 5.19 leads to:

$$\frac{dDHP}{dt} = \frac{V_{max,P}\cdot P}{K_{m,P} + P + \frac{K_{m,P}}{K_{m,S}}\cdot S} \tag{5.21}$$

$V_{max,S}$ and $V_{max,P}$ are the maximum velocities for the enzymatic reaction of the sulfonamide and of pABA, respectively, and the terms $\frac{K_{m,S}}{K_{m,P}}\cdot P$ and $\frac{K_{m,P}}{K_{m,S}}\cdot S$ characterize the respective competition between pABA and sulfonamide for the enzyme DHPS. Equation 5.20 describing the concentration change of the sulfonamide by the enzymatic reaction can now be amended to the uptake model as additional loss term for C_{cell} in equation 5.7 where the sulfonamide concentration S reflects the effective

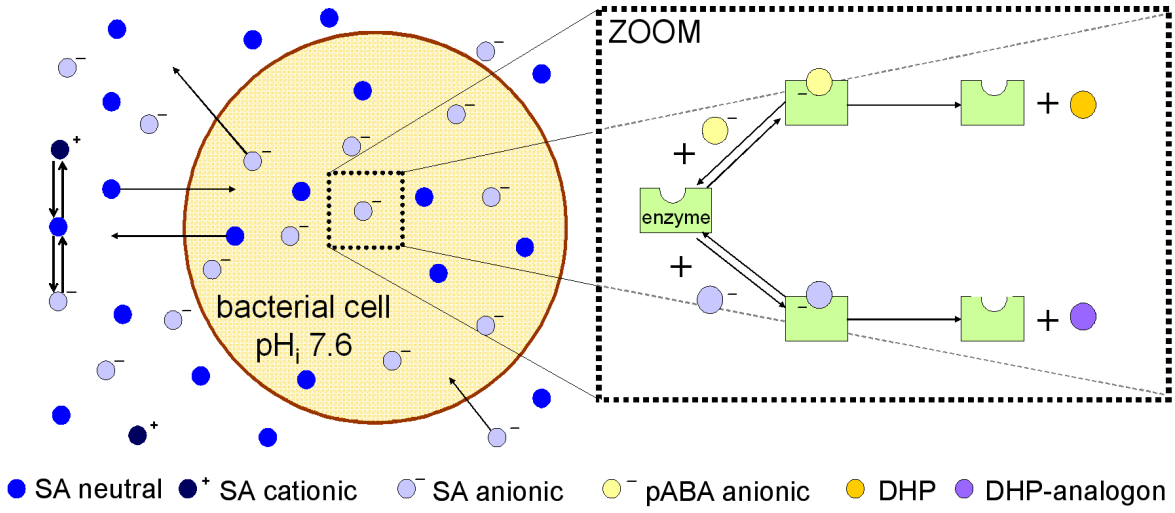

Figure 5.1: Structure of the model integrating uptake of sulfonamides into the bacterial cell and the subsequent enzymatic reaction in competition to pABA.

anionic fraction α_a, only. The steady state of the uptake model which is described by the relation of uptake and release rate (AF) changes with the integration of the enzymatic equations to:

$$AF_e = \frac{C_{cell}}{C_{env}} = \frac{k_u}{k_r + \frac{V_{cell}}{A_{cell}} \cdot \frac{V_{max,S} \cdot \alpha_a}{K_{m,S} + \alpha_a \cdot C_{cell} + \frac{K_{m,S}}{K_{m,P}} \cdot P}} \tag{5.22}$$

In this way, an additional dependency of AF_e on the intracellular sulfonamide concentration (C_{cell}) is introduced. The AF (without considering the enzymatic reaction), in contrast, remains unaffected by the amount of applied substance concentration (5.8). Figure 5.1 illustrates the integration of the uptake model with enzymatic reactions of pABA and the sulfonamide.

5.2.4 Parameterization of the enzymatic sub-model

In order to parameterize the enzymatic sub-model available literature on dihydropteroate synthase (DHPS) and on the impact of sulfonamides as substrates for the enzyme was investigated. For pABA kinetics enzymatic parameter values are given with $K_{m,P} = 31.5$ nmol $\cdot$ L^{-1} (Roland et al., 1979) and $V_{max,P} = 10.5$ nmol $\cdot$ L^{-1} $\cdot$ s^{-1} (Talarico et al., 1991). Assuming an intact bacterial cell the concentration of pABA is in the range of the Michaelis constant for half saturation ($K_{m,P}$) and is varied for sensitivity analysis of the model within one order of magnitude around the value for $K_{m,P}$, i.e. in the range of 3.15 to 315 nmol $\cdot$ L^{-1}. For sulfonamides respective parameters can be estimated from experimental data from Swedberg et al.

(1979), Roland et al. (1979) and Talarico et al. (1991) in relation to parameters for pABA. This results in a parameter range of $[0.45 - 0.75] \cdot V_{max,P}$ (Roland et al., 1979; Swedberg et al., 1979) for the maximum velocity $V_{max,S}$ and in $[0.05 - 0.55] \cdot K_{m,P}$ (Talarico et al., 1991; Swedberg et al., 1979) for the Michaelis constant $K_{m,S}$. Simulations of the AF_e with varying enzymatic parameter values under different sulfonamide concentrations are compared with the original AF to identify the impact of the enzymatic reaction on the intracellular substance accumulation.

5.3 Results and Discussion

5.3.1 Accumulation of sulfonamides in bacterial cells

Dynamic simulations with the uptake model revealed that steady state is reached within a few minutes for all compounds, if permeabilities are calculated for -1.2 < log K_{OW} < 1.6 according to equations 5.10 and 5.11. In analogy, Kitahara et al. (1997) observed a rapid accumulation of ciprofloxacin in cells of the widespread bacterium *Pseudomonas aeruginosa* reaching equilibrium after 3 to 38 minutes in dependence of temperature (37°C and 17°C, respectively).

To test if uptake of sulfonamides into bacterial cells dominates enzymatic reaction kinetics in the cell, velocities of both processes have to be estimated. Swedberg et al. (1979) determined maximal enzymatic reaction velocities (V_{max}) of sulfathiazole (STZ) in relation to the amount of proteins resulting in 0.265 to 0.333 nmol STZ per minute and mg protein. With a protein content of 55% of which 10% are able to transform para-aminobenzoic acid, and with a cellular mass of $2.80 \cdot 10^{-10}$ mg (Moat and Foster, 1995) V_{max} for STZ ranges from $6.5 \cdot 10^{-5}$ nmol s^{-1} to $8.2 \cdot 10^{-5}$ nmol s^{-1}. On the other hand, maximal uptake velocity is reached if equation 5.7 is maximal, i.e. if $C_{cell} = 0$ and, thus, $C_{env}(t) = C_{env}(t=0)$.

Consequently, for log K_{OW} values of sulfonamides between -1.22 and 1.6 L kg^{-1} (Table 2.1) k_u ranges from $2.1 \cdot 10^{-8}$ to $1.4 \cdot 10^{-5}$ m $\cdot$ s^{-1} resulting in a maximal velocity of STZ of $5.3 \cdot 10^2$ to $3.5 \cdot 10^5$ nmol $\cdot$ s^{-1} with $C_{env}(t=0) = 5000$ nmol (Swedberg et al., 1979) and a cellular radius of $6.0 \cdot 10^{-7}$ m (Moat and Foster, 1995). Compared to the estimations above the reaction kinetics of sulfonamides in the cell are seven to ten orders of magnitude slower than the uptake. The steady-state ratio of the total sulfonamide concentration in the cell (C_{cell}) to the applied sulfonamide dose (C_{env}) (potential accumulation factor AF) can thus be used as a measure for the maximum accumulation of sulfonamides. Only few experimental data exist on sulfonamide ac-

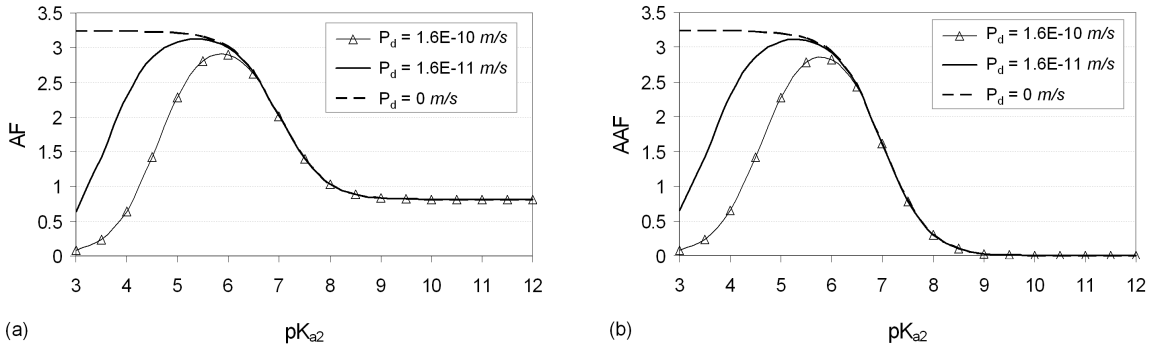

Figure 5.2: Effect of pK_{a2} and P_d on potential sulfonamide (a) accumulation factor (AF) and on (b) anion accumulation factor (AAF) in *E. coli* cells for the reference scenario (P_n=5.0 · 10^{-7} m s^{-1}, pH_{cell} = 7.6; pH_{env} = 7.0).

cumulation in bacterial cells. For *E. coli* accumulation ratios of three sulfonamides (sulfamethoxazole, sulfathiazole, sulfanilamide) from 1.4 to 2.8 have been reported (Roland et al., 1979; Büttner and Büttner, 1980). This is in good agreement with model simulations of the reference scenario (AF: 0.8 - 3.2, see Figure 5.2a).

As the antibiotic effect of sulfonamides is supposed to be solely due to the anionic sulfonamide species (Henry, 1943), accumulation of the anionic species in the cells is the relevant parameter. The anion accumulation factor (AAF) relates the effective concentration (intracellular anion concentration) to the applied dose (total sulfonamide concentration in the surrounding medium). For constant intracellular pH the anionic fraction decreases with increasing pK_{a2} values and thus, the AAF differs most from the AF in the range of higher pK_{a2} (Figure 5.2b). The accumulation predicted by the model is caused by an "ion-trapping effect" which was first described by De Duve et al. (1974). Because the external pH (7.0) is lower than the intracellular pH (7.6), the fraction of neutral species in the surrounding solution is larger than in the cytoplasm. In order to balance the resulting activity difference, the neutral species is increasingly transported into the cell, where it is partly deprotonated and trapped in form of the anion. Accumulation is largest for pK_a-values in the range from 5 to 7. Compounds with pK_{a2} values above 9 are not accumulated at all. They exhibit constant AF-values of approximately 0.8 due to the different activity coefficients inside and outside the cell (Fig. 5.2 a). For these substances, anion concentration in the cell is negligible and thus the AAF is almost zero (Fig. 5.2 b). Under such conditions no antibiotic effect of the compound should be observed.
For pK_{a2} values below 5.0 simulated accumulations depend on the assumed perme-

ability ratio for the neutral and ionic species. If ions cannot pass the membrane the "ion-trapping effect" leads to a constantly large accumulation in this pK_{a2} range. Increasing the permeability for the anion results in decreased accumulation due to the increasing potential for transport of the anions (out of the cell). In the following, the results of sensitivity analyses for the four most significant parameters determining the AAF in the model are presented: the permeability ratio of the cell envelope for the neutral and the ionic species, the extra- and intracellular pH value, and the dissociation constant pK_{a2}.

5.3.2 Sensitivity of permeability ratio

A critical parameter for the potential accumulation is the ratio of the cell wall permeability for the neutral and the ionic species. If uptake of ions is completely neglected ($P_d = 0$) like it was generally assumed for a long time (e.g. Baronofsky et al. (1984)), accumulation increases with decreasing pK_{a2} following a sigmoid curve (see Figures 5.2a, b). For the reference scenario with a permeability ratio of $10^{3.5}$ between the neutral and the ionic species, a maximum potential accumulation of 2.9 is calculated for sulfonamides with a pK_{a2} of 6.0. If the permeability for the ions is arbitrarily decreased by a factor of 10, the maximum accumulation is higher (3.1) and is shifted towards lower pK_{a2} values.

For most sulfonamides the permeability of the bacterial cell for ions is not very sensitive to the calculated AAF. This holds true for all sulfonamides with a pK_{a2} in the range, where ionic transport velocity across the cell membrane does not significantly affect the accumulation behaviour. If we allow a maximum relative deviation of 5% between simulation results for no ion permeability ($AF_{P_d=0}$) and the reference scenario (AF_{ref}, log P_d = log P_n - 3.5), i.e.

$$\frac{|AF_{ref} - AF_{P_d=0}|}{AF_{P_d=0}} < 0.05, \tag{5.23}$$

the resulting pK_{a2} limit simply calculates to pH_{cell} - 1.5 independent of the extracellular pH. As AAF and AF are linearly related, results are identical no matter whether AF- or AAF-values are compared[3]. A decrease of the permeability for the

[3]Proof:

$$\frac{|AF_{ref} - AF_{P_d=0}|}{AF_{P_d=0}} = \frac{\alpha_a \cdot |AF_{ref} - AF_{P_d=0}|}{\alpha_a \cdot AF_{P_d=0}} = \frac{|AAF_{ref} - AAF_{P_d=0}|}{AAF_{P_d=0}} \tag{5.24}$$

α_a is the anionic sulfonamide fraction in the cell which is constant in the uptake model assuming constant intracellular pH.

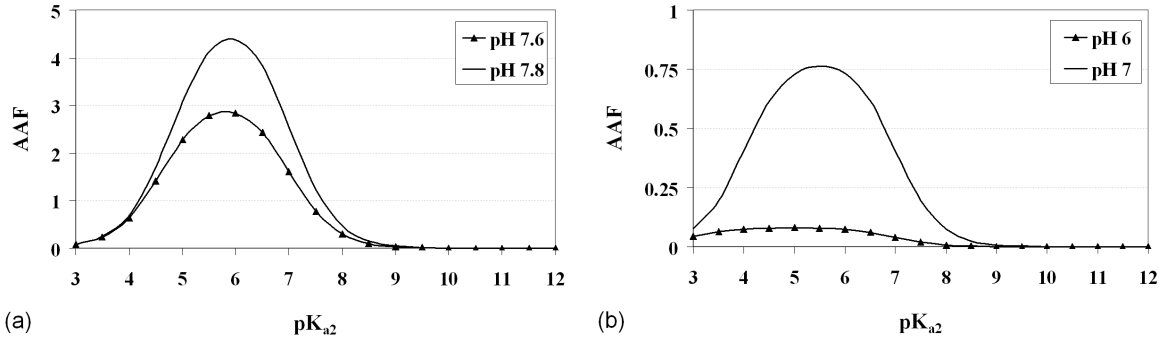

Figure 5.3: Effect of pK_{a2} and intracellular pH on anionic accumulation factor in bacterial cells for pH_{env} = 7.0. (a) *E. coli*: regulation of internal pH ; (b) *Clostridium thermoaceticum*: weak regulation of internal pH.

ions by a factor of 10 (relative to the neutral form) is propagated linearly through the model and the limit for pK_{a2} accordingly decreases to pH_{cell} - 2.5. Consequently, the intracellular pH value and the permeability ratio for the neutral and the ionic species determine for which sulfonamides anion transport could affect the accumulation behaviour. The intracellular pH in bacterial cells normally does not exceed a value of 8 (Beales, 2004) resulting in a pK_{a2}-limit of 6.5 for the default permeability assumption. For all sulfonamides characterized by a pK_{a2}-value larger than 6.5, uptake of the ionic species into bacterial cells is negligible. Although there are a number of sulfonamides with dissociation constants below this value, sensitivity to the calculated AAF is rather small and it seems difficult to confirm or reject the hypothesis that not only neutral sulfonamide molecules but also anions can pass bacterial cell envelopes on the basis of these data.

5.3.3 Influence of intracellular pH

Most bacteria are able to regulate their intracellular pH, but they differ in the range of preferred pH values depending on the pH regulation mechanisms of microbial species (Beales, 2004). For example, the enterobacterium *E. coli* keeps its intracellular pH nearly constant between 7.6 and 7.8 regardless of the external pH (Zilberstein et al., 1984). In contrast, the clostridiaceum *Clostridium thermoaceticum*, an ubiquitous bacterium primarily occurring in soils and in the intestinal tract, prefers pH values from 5 to 8 for growth and allows internal pH values to vary from 5.7 to 7.3 (Beales, 2004). Using these two microorganisms as examples the effect of intracellular pH on anionic sulfonamide accumulation is demonstrated. Figure 5.3a shows

that even a relatively small shift of 0.2 pH units in an *E. coli* cell results in different accumulation of sulfonamides with pK_{a2} values around the optimum ($\pm$ 1.5 log units). For conditions with similar extra- and intracellular pH values accumulation factors are close to 1, i.e. sulfonamides are not accumulated, as has been experimentally observed by Roland et al. (1979). In Figure 5.3b scenarios for minimum and maximum internal pH in cells of *C. thermoaceticum* are displayed. It can be seen that the AAF of sulfonamides with $pK_{a2} = 6$ increases by a factor of 10, if the intracellular pH increases from 6 to 7. Obviously, the accumulation in bacterial cells is highly sensitive to the relation between extra- and intracellular pH value. If the pH value in solution exceeds the intracellular pH value, no accumulation occurs (AAF < 1), because the neutral fraction is larger inside the cell than outside.
In these cases, the AAF is the smaller the larger the differences between extra- and intracellular pH are. For compounds with dissociation constants clearly above the intracellular pH, the AAF approaches zero.

5.3.4 Influence of extracellular pH

Excluding pH optima for growth of extremophiles the physiological pH range of organisms varies from 5 to about 9 (Madigan et al., 2003). The sensitivity of external pH on the potential accumulation of a number of sulfonamides with pK_{a2} values ranging from 5.7 to 11.8 (Table 2.1) is demonstrated in Figure 5.4. The ratio between intra- and extracellular concentration increases with decreasing pK_{a2} values and is largest for low external pH values. Sensitivity to external pH variations is larger for compounds with a small pK_{a2} value, for which little variations of external pH lead to a significant change of potential sulfonamide accumulation in the cell. This implies that the effectiveness of sulfonamides with low pK_{a2} values should be highly dependent on extracellular pH for bacteria that keep internal pH within a small pH range. Figure 5.4 shows that the sulfonamide that accumulates the most changes from sulfisoxasole (SIX) at $pH_{env} = 5.5$ to sulfachloropyridazine (SPZ) and sulfamethoxazole (SMX) at $pH_{env} = 7.0$. At $pH_{env} = 8.5$ there is no accumulation for any of the compounds (AAF $\ll 1$).This can be attributed to the difference between extra- and intracellular pH and the respective species distribution in dependence of pK_{a2}. Thus, the relative antibiotic activity of sulfonamides with different pK_{a2}-values on the same bacteria is not necessarily the same at different extracellular pH values. The membrane potential E is strongly dependent on the extracellular pH (Hirota et al., 1981), i.e. with increasing extracellular pH E changes with an average

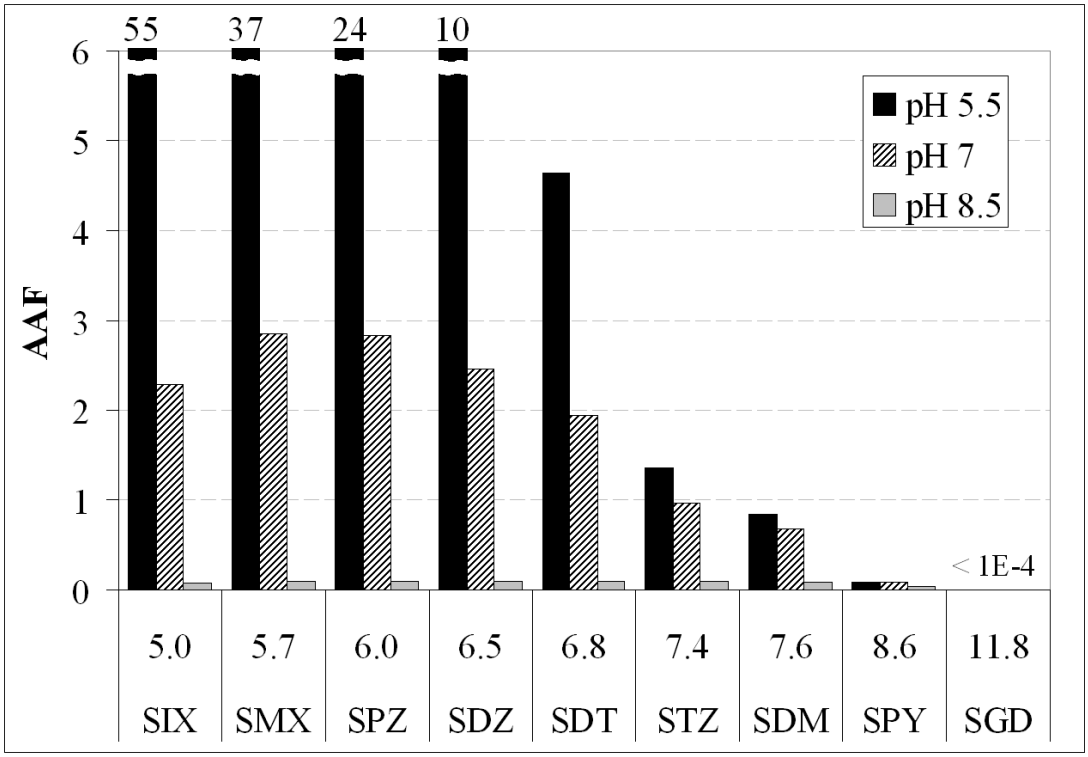

Figure 5.4: Calculated anionic accumulation factor of nine sulfonamides with different pK_{a2} in bacterial cells for an intracellular pH of 7.6 and three different extracellular pH values.

slope of -22 mV per pH unit (Felle et al., 1980). Over the environmental relevant pH range, a membrane potential of approximately -70 mV at pH 5 up to -150 mV at pH 9 can be expected. Nevertheless, since E affects only the ion uptake (equation 5.3), the steady state accumulation is relatively insensitive to variations in membrane potential. Therefore, pH effects on sulfonamide accumulation cannot be attributed to changes in membrane potential but directly to the substance dissociation behaviour.

5.3.5 Comparison of model results with effect data

Assuming that the accumulation for all sulfonamides is not significantly affected by cell metabolism and that all sulfonamides are comparably effective as enzyme inhibitor, the antibiotic effect should be the stronger the higher the accumulation in the cell is. In other words, minimum inhibitory concentrations (MIC) should be inversely proportional to the accumulation of the effective molecular species. As mentioned above, it is commonly supposed that the antibiotic effect of sulfonamides is solely induced by the anionic sulfonamide species (Henry, 1943). Therefore, MIC values (in units of μg mL^{-1}) against the gram-negative bacterium *Actinobacillus*

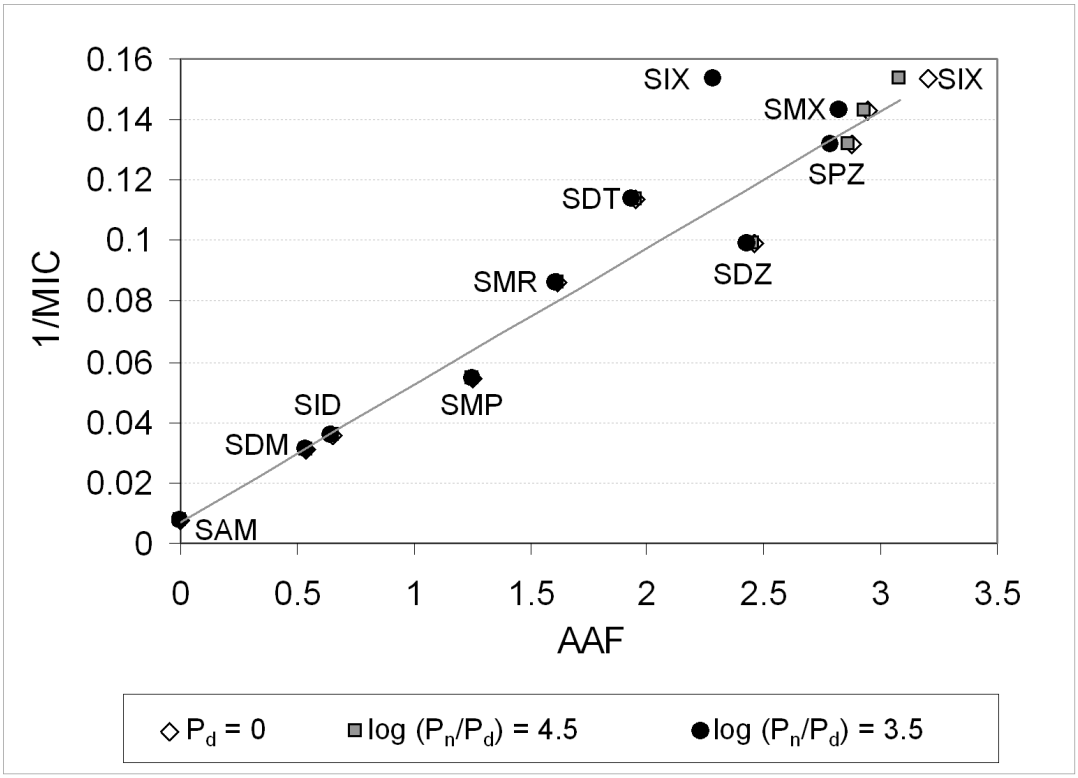

Figure 5.5: Comparison of minimum inhibitory concentration (MIC) of 10 different sulfonamides ($5.0 < \mathrm{pK}_{a2} < 10.1$) from Mengelers et al. (1997) measured at constant extracellular pH of 7.0 with the simulated anion accumulation factor (AAF).

pleuropneumoniae determined for 10 sulfonamides by Mengelers et al. (1997) are compared to the AAF representing the anionic sulfonamide accumulation in the cells. Under the assumption that all sulfonamide anions exhibit similar inhibitory effects on the enzyme DHPS the reciprocal of the MIC should be linearly correlated to the AAF. In Figure 5.5 calculated AAF-values for three different permeability ratios (anion vs. neutral form) are displayed against the reciprocal MIC values. If the hypothesized linear relationship between anionic accumulation of sulfonamides in the cell and the observed growth effect was true, all data points should be close to the line displayed in the diagram. Deviations of data points from this hypothesized linear relationship are largest for the scenario with the largest P_d-value (ratio: 103.5), whereas there is almost no difference between the two other scenarios. As only three of the ten compounds react sensitive to a change in the permeability assumption of the cell wall against the anion in the model, it is not possible to ultimately decide whether a different inhibitory effect or the different permeability ratio produce the deviation from the linear relationship.

5.3.6 pH dependent growth inhibition of soil bacterial isolates

Tappe et al. (2008) conducted growth inhibition tests of two sensitive soil bacterial isolates, *Pseudomonas aeruginosa* and *Pantoea agglomerans* against eight sulfonamides. Dissociation constants pK_{a2} of the compounds ranged from 5.7 (SPZ) to 11.7 (SGD) (Table 2.1) and additionally, the extracellular pH value was varied between 5 and 8. Since growth rates of both bacteria are pH dependent, for each of the four investigated pH values (5, 6, 7, 8) a specific growth rate was determined in the control sample (without sulfonamide) and set to 100%. The sulfonamide concentrations resulting in a 50% reduction of the unaffected growth rates from these controls were referred to as effective concentration (EC_{50}).
Again, under the assumption that inhibition is proportional to the intracellular anion concentration of the respective sulfonamide, normalized reciprocal EC_{50} values are compared to normalized anion concentrations in the cell simulated for different scenarios. Simulated intracellular anion concentrations are normalized to the maximum to enable a direct correlation between anionic sulfonamide accumulation in the cell and measured growth inhibition. The use of reciprocal effect concentrations allows for a direct comparison of measured data and observed effects in the graphical representation, i.e. the higher the column the higher is the inhibitory effect of the sulfonamide on bacterial growth. In analogy to the reference scenario of the uptake model, it is assumed that most bacteria are able to maintain an intracellular pH near neutral growing over a wide pH range (pH homeostasis) (Beales, 2004). This reference scenario is applied to experimental pH conditions and investigated sulfonamides.

Reference scenario with pH homeostasis. Data from Mengelers et al. (1997) and investigations on model parameters and model sensitivity showed that sulfonamides with lowest pK_{a2} accumulate the most in cellular cytoplasm. Model simulations (Fig. 5.6) demonstrate that highest intracellular anion concentrations can be expected at low extracellular pH values (pH 5). This is consistent with experimental results for *P. aeruginosa* which showed strongest growth inhibition for sulfonamides with small pK_{a2} and at the lowest pH value (pH 5).
Same experiments with *P. agglomerans* resulted in a different picture than for *P. aeruginosa*. The three sulfonamides with the lowest pK_{a2} (SPZ, SMX, and SDZ) effectively inhibited growth of *P. agglomerans* at all pH values tested with a clear

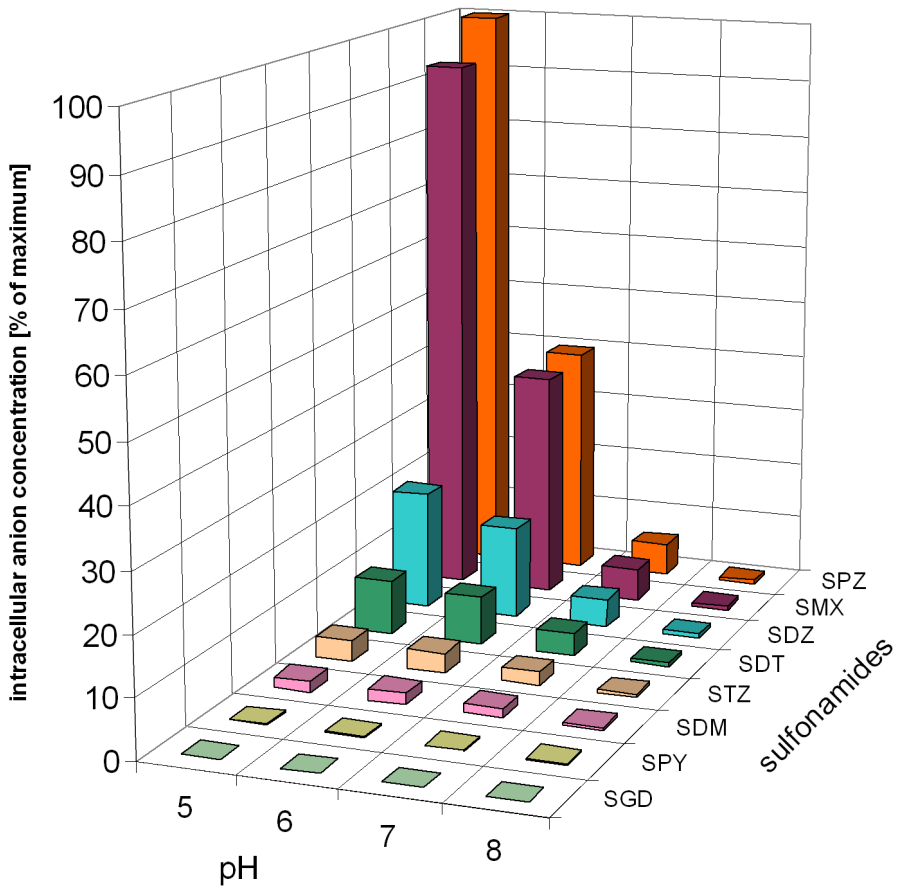

Figure 5.6: Simulated intracellular anion concentrations of sulfonamides normalized to the maximum value for better comparison with effect data. The model is parameterized according to the reference scenario with constant intracellular pH (7.5) and different extracellular pH values.

optimum at pH 7. The next 4 sulfonamides (SDT, STZ, SDM, SPY) ranked for their increasing pK_{a2} values, showed an optimum at pH 8. The sulfonamide with the highest pK_{a2} (SGD, pK_{a2}=11.69) showed no inhibition at all even at the highest concentration applied (20 mg L^{-1}). Experimental effect data are illustrated in Figure 5.7. Spearman's rank correlation coefficient r^2 between measured effect and simulated concentration data is independent of the frequency distribution of the investigated variables (measured vs. simulated data) and does not require the relationship between these variables to be linear.

Thus, a bivariate analysis on correlation was performed indicating that measured effect data and simulated anion concentrations do not correlate significantly (n=32,

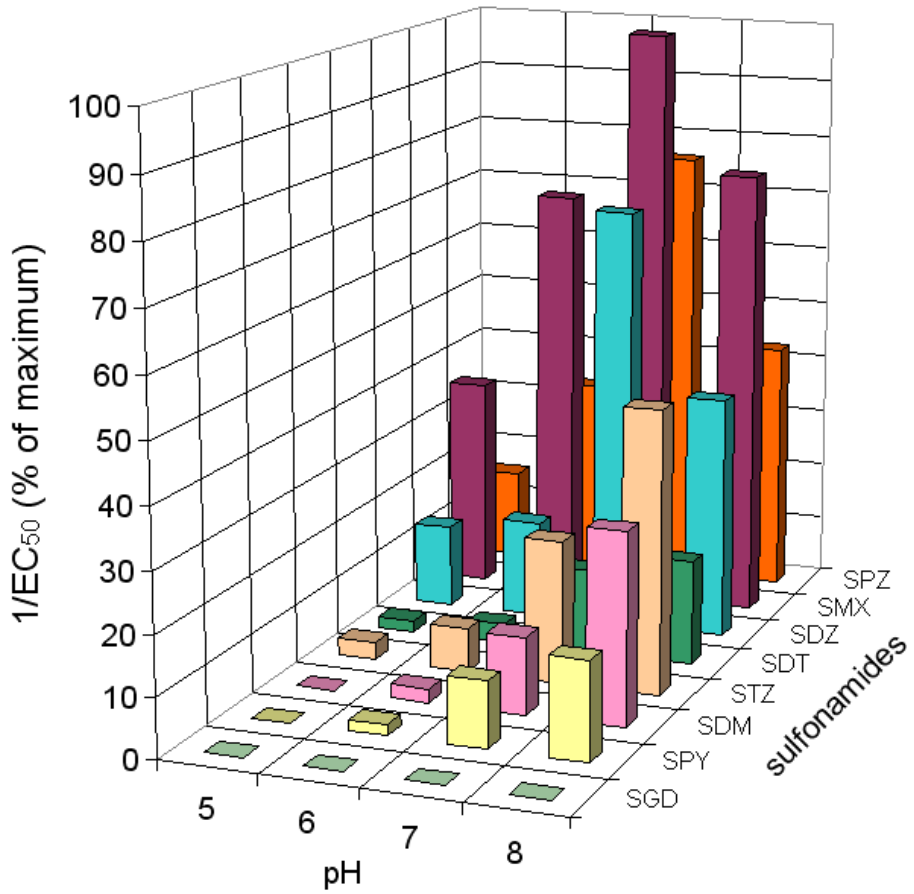

Figure 5.7: Growth inhibitory activities of sulfonamides (sorted with rising pK_{a2}) as normalized reciprocal values at different pH on *Pantoea agglomerans* (Tappe et al., 2008).

r^2(Spearman)=0.43, p level[4] = 0.02). This is probably due to the assumption of a constant intracellular pH of 7.5 independent of the respective extracellular pH.

Scenario with varying intracellular pH. The strongest inhibition of *P. agglomerans* was obtained at the highest pH values of 7 and 8. According to the model assumptions this should be associated with the highest internal anion concentration which does not comply with the assumption of a constant intracellular pH. In order to explain the discrepancy between data on growth inhibition of *P. agglomerans* and model results a consistent shift of simulated internal anion concentrations to higher pH values is necessary and requires variable intracellular pH values in the model. Under environmental conditions, this may be explained by a restricted ability of *P.*

[4]Significance of the correlation is determined with SPSS 14.0.1, SPSS Inc. Chicago, Illinois, USA

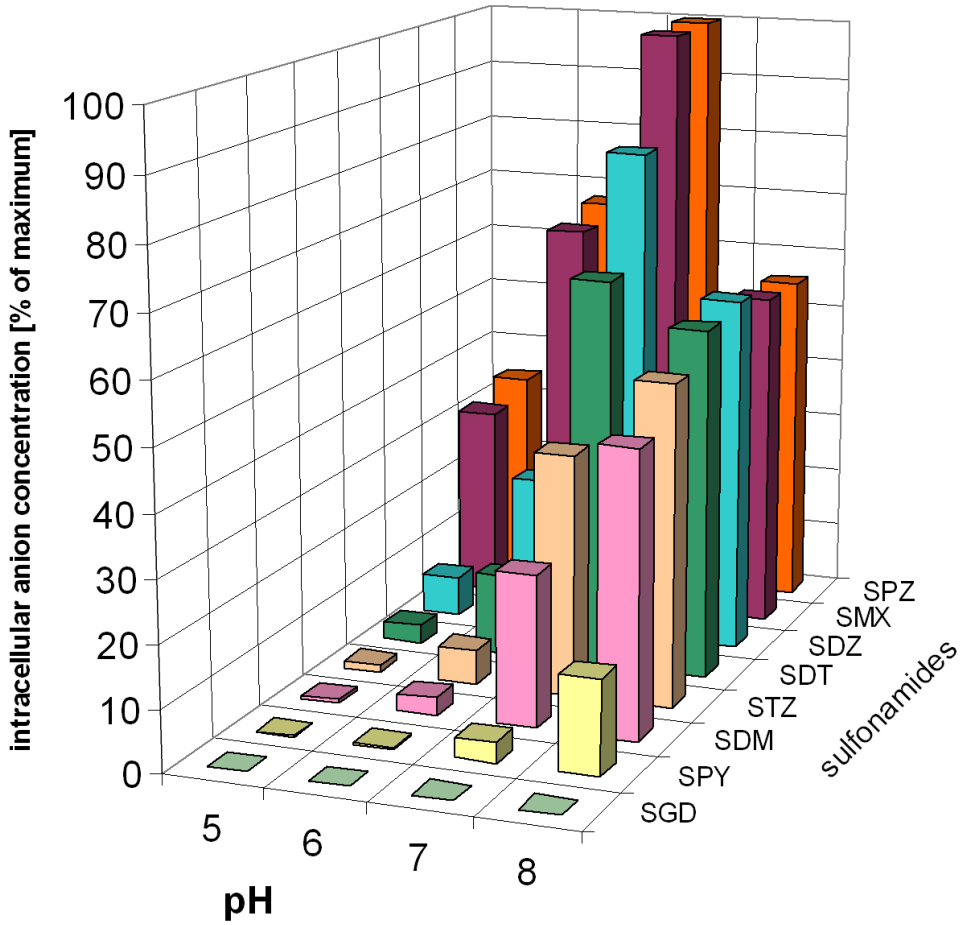

Figure 5.8: Simulated intracellular anion concentrations of sulfonamides for different extracellular pH values and variable intracellular pH values (pH_i). The respective pH_i values were inversely calculated in order to obtain the best fit to experimental effect data of *P. agglomerans* given in Figure 5.7. For extracellular pH values of 5, 6, 7, and 8 the corresponding pH_i values are 5.4, 6.0, 7.2 and 7.7.

agglomerans to maintain quasi-constant pH values against a strong gradient from inside to outside (homeostasis (Kobayashi et al., 2000)). In order to investigate if such a variable intracellular pH could explain the effect data, pH_i values were determined which fit the model results best. Applying a least-square method (LSM) the model was fitted to the data for all sulfonamides at the four different extracellular pH values and intracellular pH values were inversely calculated. For extracellular pH values of 5, 6, 7, and 8 the corresponding pH_i values are 5.4, 6.0, 7.2 and 7.7. The resulting intracellular anion concentrations predicted by the model are shown in Figure 5.8.

In contrast to the correlation analysis between simulation results assuming pH home-

ostasis and measured effect, data of *P. agglomerans* show a highly significant correlation (n=32, r^2(Spearman)=0.92, p level < 0.001) when variable intracellular pH values are assumed. The calculated pH_i values imply a very weak pH regulation in *P. agglomerans*. Thus, it is likely that a the impact on the microbial population in soil through sulfonamide action strongly depends on the way bacteria regulate their pH in cytoplasm. Measurements of pH_i and intracellular substance concentration in soil bacteria could provide further insight into underlying mechanisms determining pH- and pK_a-dependency of effects.

5.3.7 Integration of enzymatic inhibition into the uptake model

So far, a model to estimate intracellular concentrations of the sulfonamide anion in dependence of the pK_{a2} and the pH value in the cell and the surrounding medium was introduced. Observed effect data (MIC, EC_{50}) on growth inhibition can be qualitatively explained by the model. Nevertheless, the uptake model does not explicitly simulate the growth inhibitory effect of sulfonamides.

The next consequential step to an integration of chemical fate and antibiotic effect focuses on a combination of the uptake model with a kinetic model describing enzymatic processes during the folic acid cycle and its inhibition by sulfonamides. A sensitivity analysis of the enzymatic parameters Michaelis constant (K_m) and maximum velocity (V_{max}) allows for an evaluation of the impact of the additional process on model predictions.

The uptake model calculates the accumulation of the effective anionic sulfonamide fraction in a bacterial cell (ion trapping) without considering the enzymatic reaction expressed as the *anion accumulation factor* (AAF) which depends on the substance specific dissociation constant pK_{a2} of the sulfonamide and the extra- and intracellular pH value (subsection 5.3.1). The steady state of the integrated uptake and enzyme reaction model (AAF_e) additionally depends on the choice of enzymatic parameter values (K_m, V_{max}) and on the applied sulfonamide concentration (equation 5.22). Figure 5.9 compares the anion accumulation factor of different sulfonamides at pH 7.0 without (AAF) and with (AAF_e) consideration of the subsequent enzymatic reaction in dependence of the respective pK_{a2} value for *E. coli* ($pH_{cell} = 7.6$). Simulations were performed with mean values of the parameter range (reference scenario) and for the maximum and minimum parameter values, respectively (best and worst case scenarios). Figure 5.9 shows that at high sulfonamide concentra-

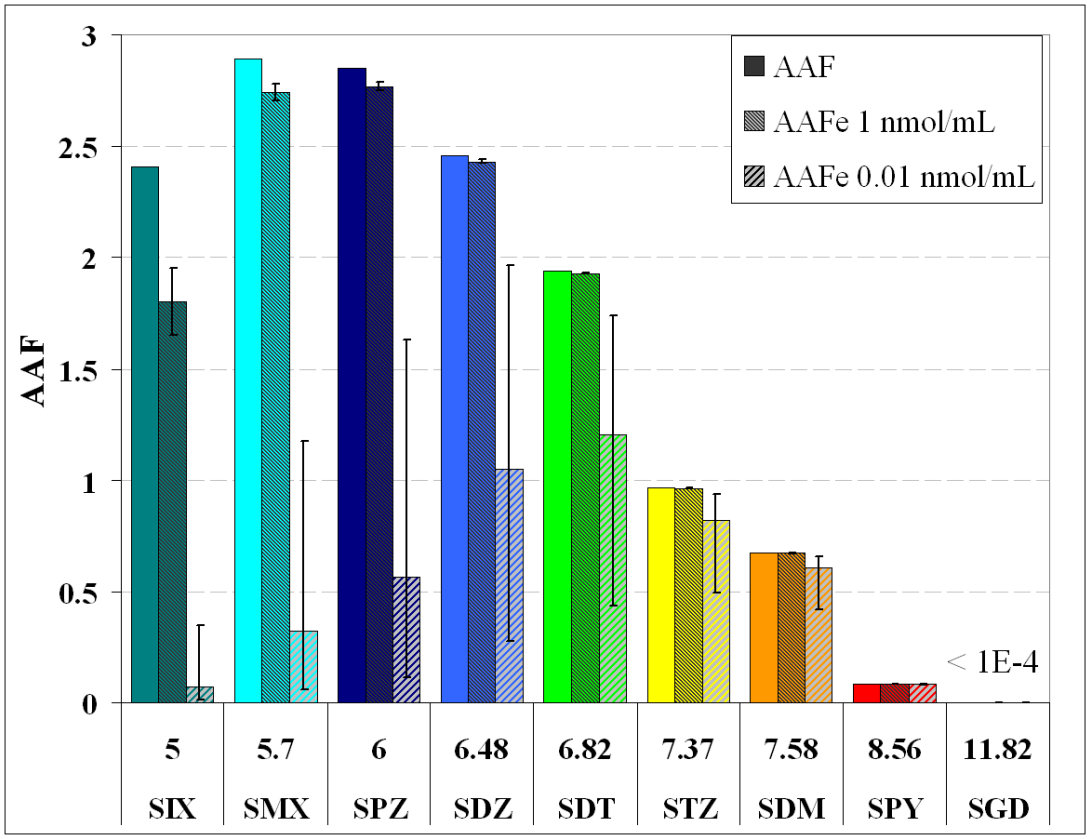

Figure 5.9: Anion accumulation factor at pH_{env} = 7.0 of different sulfonamides at two concentrations without (AAF) and with (AAF_e) consideration of the subsequent enzymatic reaction in dependence of the respective pK_{a2} value. AAF is independent of the applied sulfonamide concentration. pH_{cell} was selected for *E. coli* (7.6). Error bars indicate maximum variability in the selected parameter range.

tion the AAF_e is smaller than AAF for sulfonamides with pK_{a2} values below 6.0, with only small variability caused by the enzymatic parameters (< 5%). At low concentrations, however, the simulated AAF_e values are much lower than AAF and also react very sensitive to changes in $V_{max,S}$ and $K_{m,S}$ values. This is because the applied sulfonamide concentration is so low that the respective anionic fraction in the cell is directly transformed into the DHP analogon and cannot accumulate to a large extent. At high sulfonamide concentrations, however, intracellular anion accumulation dominates in the integrated uptake and enzymatic reaction model and results in an AAF_e close to AAF. Nevertheless, the anion accumulation factor alone does not reflect the effective concentration. Thus, according to equation 5.21 the model also delivers the amount of produced DHP. In order to determine an effective sulfonamide concentration, the amount of DHP, produced when no sulfonamide is

applied, is taken as reference value. Then, for each sulfonamide the simulated concentration is determined at which the inhibition results in a 50% reduction of the DHP-formation in the cell (DHP_{inh50}) as a reference value. Simulations are based on the assumption that all sulfonamides affect the enzymatic reaction to the same extent, i.e. enzymatic parameters for all sulfonamides are equal. Consequently, this means that the intracellular anion concentration that reduces the DHP-formation by 50% is all the same. Then, the DHP_{inh50} is the concentration that has to be applied of the specific sulfonamide to achieve this anion concentration in the cells. Figure 5.10 illustrates the correlation between the pK_{a2} value and the simulated effective sulfonamide concentration (DHP_{inh50}). Lowest effect concentrations inhibiting the DHP production by 50% are predicted for sulfonamides with pK_{a2} values between 6 and 7.5 which includes SDZ ($pK_{a2} = 6.48$). Smaller pK_{a2} values induce a strong depletion in the cell and higher values additionally result in a small intracellular anionic fraction.

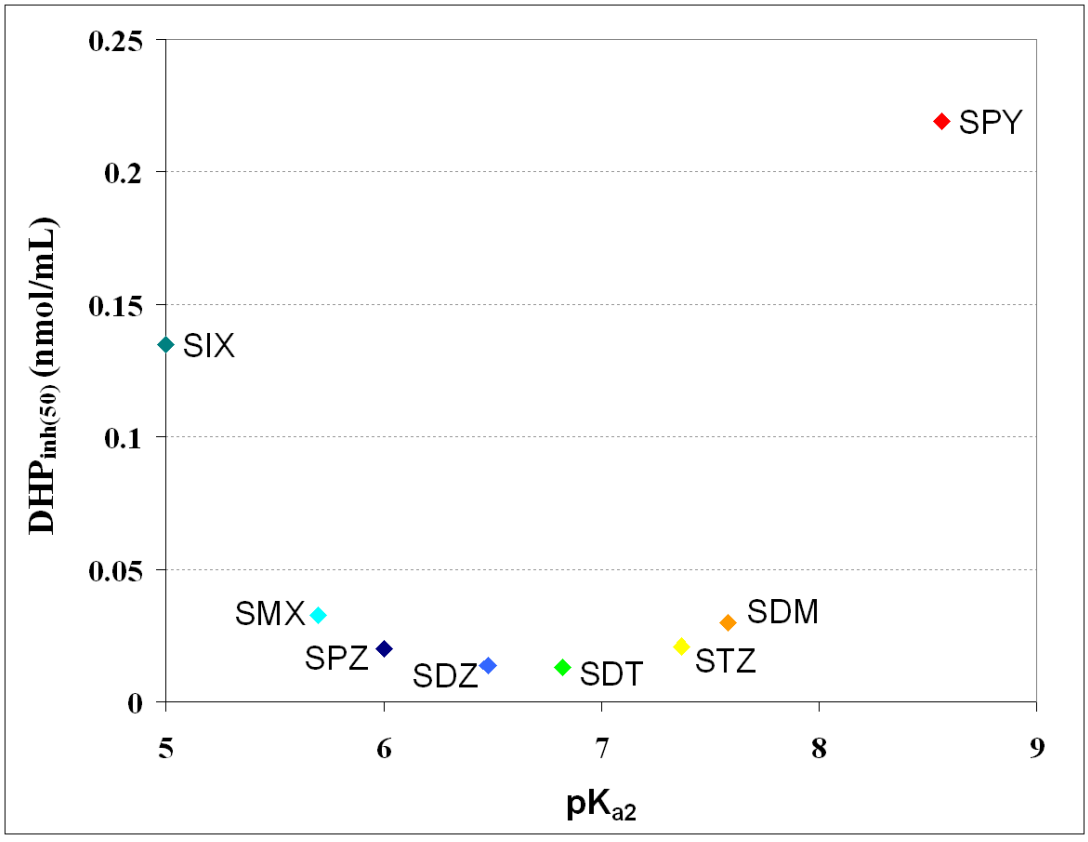

Figure 5.10: Correlation between the pK_{a2} value and the simulated sulfonamide concentration (DHP_{inh50}) at which the inhibition results in a 50% reduction of the DHP-formation in the cell. Colours for sulfonamide representation are chosen according to Fig. 5.9.

5.4 Conclusion

Potential accumulation of sulfonamides in bacterial cells is sensitive to the pH values in- and outside the cells if cell metabolic reactions do not affect the steady-state concentration in the cell. The ratio of intra- to extracellular pH and the absolute pH values determine the total sulfonamide accumulation in dependence of pK_{a2}. Intracellular pH directly affects the concentration of the effective anionic species and thus, the antibiotic inhibition of DHPS. The model allows for differentiating between the uptake of the neutral and ionic species and intracellular shift to anionic species, which is essential to produce the antibacterial effects of sulfonamides. Ranking of observed effect data agrees with simulated anion accumulation in the cell, but further experiments are necessary to confirm the proposed relationship between antibiotic inhibition and intracellular accumulation. The uptake model can be extented by the enzymatic reaction which reduces, in dependence of the amount of applied sulfonamide, the anionic accumulation in the cell. This integrated model allows to estimate (effective) sulfonamide concentrations resulting in a 50% reduction of the DHP-formation.

Experimental and modelling investigations indicate that bacteria with a strong control of intracellular pH (pH homeostasis) would be strongly inhibited at low soil pH, especially by sulfonamides with low pK_a values. However, sorption of the neutral species of sulfonamides seems to be significantly stronger than that of the anionic species (Boxall et al., 2002). As a competitive process, sorption to soil may then increase with decreasing pH resulting in a decreased availability for the organisms. Bacteria with a poor regulation, as postulated for *P. agglomerans*, would be weakly inhibited at low soil pH and exhibit strongest inhibition at pH values of 7 to 8, which is the prevailing pH range in pig manure after storage and alkalinic fermentation. Briefly summarized, it is likely that a possible impact on the microbial population in soil through the action of sulfonamides strongly depends on the way bacteria can regulate their pH_i. Measurements of pH_i and intracellular substance concentrations in soil bacteria could provide further insight into underlying mechanisms determining pH- and pK_a-dependency of effects.

In general, bioaccumulation behaviour of organic substances in food webs is mostly described by model approaches mainly based on the octanol-water partitioning coefficient (log K_{OW}) (Watanabe et al., 2005; Armitage and Gobas, 2007; Norstrom et al., 2007). Gobas et al. (2006) already pointed out that limits like log $K_{OW} > 5$ and molecular weight > 500 g $\cdot$ mol^{-1} are not appropriate indicators to exclude

membrane permeation and thus a significant bioaccumulation. The authors claim that four of ten chemicals in their BAF-BCF database fulfilling the above mentioned criteria are characterized by a BAF or BCF larger than 5000, also including certain sulfonamides.

In this thesis it was shown that for dissociating compounds pH-dependent speciation can be the decisive process determining the accumulation in bacterial cells. The log K_{OW} approach alone may lead to an underestimation of the accumulation of substances that are affected by the described ion trapping effect. This phenomenon may also play a role for bioaccumulation of ionic substances in other biota. For example, Lo and Hayton (1981) described a pH effect on the accumulation of sulfonamides in fish which can be attributed to ion trapping if the absorption rate for the neutral molecule is significantly larger (at least about two orders of magnitude) than the absorption rate of the ion. The mechanistic model described in this chapter can thus also contribute to an improvement of existing bioaccumulation models.

Chapter 6

General Discussion and Final Conclusions

This thesis is based on the investigations and results of the project FOR566 funded by the German Research Foundation (DFG) with the project title "Veterinary Medicines in Soil - Basic Research for Risk Analysis". What can finally be concluded as far as *risk* is concerned? *Risk* is the probability that a possible adverse effect occurs. In environmental sciences the risk which may arise from a specific compound includes the adverse effect and the possibility of environmental exposure of this compound. Predicted environmental concentrations (PEC) resulting from exposure modelling are compared with predicted no effect concentrations (PNEC) which are based on toxicity tests for a specific biological endpoint. If the PEC of a chemical substance exceeds its PNEC this substance is supposed to constitute an environmental risk.

Kim et al. (2007) investigated the aquatic toxicity of six sulfonamides and determined risk quotients indicating "potential environmental concern" (e.g. PEC/PNEC = 6.3 for sulfamethoxazole). In contrast to an aquatic environment, however, soil is definitely a more complex compartment comprising a gaseous phase, an aquatic phase and the solid matrix which itself varies in composition over a wide range of properties. Additionally, toxicity on the microbial community cannot be tested for a specific endpoint. Instead sum parameters like soil respiration are often consulted for an assessment of antibiotic induced effects on soil microorganisms and soil functions.

In order to pose a risk to men and environment the substance has to be biologically active and has to be available for uptake by organisms in soil. Sulfadiazine is, of

course, an active substance since it is an antibiotic compound designed to inhibit bacterial cell growth. This is most likely also the case for its metabolite 4-hydroxy-sulfadiazine since the active substance group NH_2 is not changed by hydroxylation. But what can we say about availability? Based on estimations of apparent sorption coefficients (Chapter 3) and on the mechanistic fate model (Chapter 4) the time-dependent development of the dissolved concentration of SDZ in soils Kaldenkirchen and Merzenhausen after application of fresh and aged manure can be estimated. Pore water concentrations of sulfadiazine at the beginning of the central fate experiments are calculated using equation 3.8 to be 4.5 mg L^{-1} and 4.7 mg L^{-1} in the two soils Kaldenkirchen and Merzenhausen, respectively.

Tappe et al. (2008) experimentally determined effective SDZ concentration (EC_{50}) for *Pantoea agglomerans* under different pH values in solution. Values of 2.2 mg L^{-1} and 0.5 mg L^{-1} at pH 6 and 7 are reported, respectively. These pH values are close to the pH conditions of the two soils (K: pH = 5.7, M: pH = 6.8). Hence, the EC_{50} values can be used to compare pore water concentrations in the soil with effect concentrations. The chemical fate model predicts pore water concentrations to remain above the respective EC_{50} values for the first 9 days (soil K) and 16 days (soil M) after manure application. This points out that under realistic conditions effective concentrations can be maintained for relatively long time periods. In order to complete risk assessment for SDZ an analogous estimation for the effective metabolite, i.e. the hydroxylated sulfadiazine (OH-SDZ), is necessary and possible when inhibitory concentrations are known.

The results of this thesis confirm that bound residues, which are irreversibly incorporated into the soil matrix, e.g. by covalent binding to humic compounds, have to be distinguished from a kinetically controlled translocation into a residual fraction. The formation of bound residues in soils reduces the available fraction of the compounds and it is unlikely that the bound molecules - although they persist in soil - can be re-released in an active form. However, the residual fraction may have long term effects on soil functions or organisms, because SDZ and OH-SDZ could still be found more than 200 days after application to soil. Depending on the microorganism species this fraction can still be effective and influence soil functions (Focks, 2008; Kotzerke et al., 2008). Model results indicate that the translocation of SDZ and its metabolites into the residual fraction is kinetically controlled and may thus be a diffusion-type physical entrapment process. This is a starting point for further experimental investigations on sorption of SDZ, OH-SDZ and Ac-SDZ which seems

to be stronger in the residual fraction than in the available one. The model predicts a long-term remobilization of antibiotic activity from the residual fraction.
Under environmental conditions the soil structure may be changed mechanically by human impacts. Such "disturbing" processes may again induce a mobilization of the compound. Several researchers describe a remobilization of sequestered or even bound xenobiotics by incorporation of microorganisms (Barriuso et al., 2004), bioturbation by earthworms (Gevao et al., 2001), or plant growth (Olson et al., 2007). In addition, plant growth inducing a higher microbial activity in the rhizosphere and field conditions (temperature, moisture conditions) may influence the chemical fate of the antibiotic compounds in general and sequestration dynamics in particular. Consequently, compared to laboratory experiments, the antibiotic activity in soil may even be enhanced under field conditions. Further analyses of sequestration mechanisms and the formation of bound residues may allow for an improved mechanistic understanding of the chemical fate processes. The presented modelling approach describing the fate of SDZ and its main metabolites can be easily extended for additional influence factors. Expanding the model by a mathematical description for antibiotic uptake by plants may supplement the chemical fate by an additional relevant process which leads to a contamination of the food chain. Finally, integration of the chemical fate model for homogenized soils into a transport model allows for an application to the field scale.

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