Analysis of veterinary pharmaceuticals in soil and their impact on microbial populations

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Meiner Tochter Sarah
in Liebe gewidmet

Abstract

Veterinary medicines may enter agricultural soils by the use of animal excrements as fertilizers. In this study the impact of veterinary pharmaceuticals sulfadiazine (SDZ) and chlortetracycline (CTC) on soil bacterial communities was investigated. Additionally the SDZ concentration in the soil was analysed. Microcosms containing two sorts of agricultural soils, orthic luvisol and gleyic cambisol were used. The soils were spiked with the antimicrobial agents SDZ and CTC at three different concentrations (1, 10, 50 mg/kg) and incubated for 48 or 64 days at 20 °C. SDZ and the metabolite acetyl-SDZ were extracted from the aged soil samples using pressurized liquid extraction (PLE). Acetonitrile/water (80:20 v/v) at 100 °C was chosen as the extraction mixture, solid-phase extraction (SPE) or other clean-up of the samples was not necessary. The quantification was conducted using LCMS/MS with electro spray ionisation (ESI). The recovery rates were between 82.5% and 87%. SDZ dissipated very rapidly and strongly time-dependent from both soils. Furthermore, the impact on the microbial respiratory activity was measured continuously in a respirometer (Sapromat). Changes in bacterial community structure were visualized by means of PCR-denaturing gradient gel electrophoresis (DGGE) of 16S rDNA derived from the soil samples. Additionally, growth inhibitory effects of SDZ and CTC were tested in agar diffusion tests. In microcosms with soil and antibiotics only, no effects could be observed, either on respiratory activity or on bacterial population structure. Therefore, further incubations were conducted in the presence of an additional assimilable carbon source. In the presence of glucose, SDZ affected soil respiration as well the bacterial community structure: additional bands appeared and some bands already visible at the beginning of incubations increased in intensity. A clear relationship between SDZ concentrations and changes in DGGE patterns became visible. During 48 days of incubation, changes in DGGE patterns were minimal in microcosms with 50 mg SDZ/kg soil indicating an inhibition of strains, which were capable of growing on glucose in the presence of lower SDZ concentrations. Only a few soil bacterial isolates were weakly inhibited by SDZ in agar diffusion disk test. Contrastingly, CTC significantly inhibited growth of 12 soil bacterial isolates in disk tests, but no effects on soil respiration and bacterial community structure could be observed. In the presence of the soil matrix the growth inhibitory potential of CTC decreased due to adsorption or complexation. This was confirmed in growth inhibition experiments with soil suspensions and time-dependent sampling.

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Contents

List of abbreviations used in the text

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionisation
ASE	Accelerated solvent extraction
ATP	Adenosintriphosphat
$CaCl_2$	Calciumchloride
CE	Capillary electrophoresis
CO_2	Carbondioxid
Conc.	concentrated
CTC	Chlortetracycline
CV	Coefficient of variation
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC_{50}	Toxic effect data
ED_{50}	Effective dose values
EDTA	Ethylendiamintetraessigsäure
EEC	European Economic Council
E_H	Evenness
EMEA	European Medicine Evaluation Agency
ESI	Electro spray ionisation
FEDESA	European Federation of Animal Health
G	Glucose
GC	Gas chromatography
GC-clamp	40-45 bases GC-rich sequence
GLP	Good laboratory praxis
H'	Diversity Index
HCl	Hydrochloric acid
HCOOH	Formic acid
HPLC	High performance liquid chromatography
i.d.	Diameter
ISTD	Internal standard

LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave solvent extraction
MeOH	Methanol
MIC	Minimal inhibitory concentration
MIT	Microbial Inhibition Test
MS	Mass spectrometry
MV	Mean value
NaOH	Sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
OTC	Oxytetracycline
PABA	Para-amino benzoic acid
PAH	Palycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PLE	Pressurised liquid extraction
RISA	Ribosomal intergenic spacer-analysis
RNA	Ribonucleic acid
RP	Reversed phase
S	richness
SD	Standard deviation
SDM	Sulfadimidine
SDZ	Sulfadiazine
SIR	Substrate-induced respiration
SPE	Solid-phase-extraction
SWE	Superheated water extraction
TAE	Trisacetic-EDTA-buffer
TCY	Tetracycline
TGGE	Temperature gradient gel electrophoresis
THFA	Tetrahydrofolic acid
V	Volt
WHC	Waterholding capacity
WHO	World Health Organization

1. General Introduction

1.1. Background

Among the veterinary medicines used in intensive animal production more than 70% of the administered pharmaceuticals are antibiotic agents. In 1999, the total amount of antibiotics consumed in the EU and Switzerland amounted to 5000 tons while 3500 tons are used for therapeutic purpose and the remaining 1500 tons are used as feed additives for growth promotion [87]. Minor amounts of administered antibiotics are retained in the medicated animals and most of the agents are quantitatively excreted as the parent compound and as metabolites of which the acetylated form is temporarily inactivated [161]. The animal excrements are either directly released into the environment by grazing animals or indirectly by spreading animal excrements as fertilizer onto agricultural soils.

The actual intention of using antibiotics is to inhibit bacterial growth or to kill bacteria, and thus there is a serious concern about qualitative and quantitative effects on resident microbial soil populations, possibly leading to disturbances in soil functioning.

Drugs, contrary to heavy metals and pesticides, were rarely viewed as potential environmental pollutants although developed with the intention of performing a biological effect and thus they could have the potential to disrupt the natural microbial populations [162].

Systematic investigations of environmental behaviour of antibiotics and our knowledge about existing contaminations in soil are not sufficient and only few studies deal with this problem. Drugs in the environment did not capture the attention of the scientific or popular press until the last couple of years, with some significant papers presented by Halling-Sørensen et al. [161], Hirsch et al. [94], and Ternes [159]. First in the new millennium more studies dealt with this problem [1, 81, 29, 127, 134, 136, 160, 185].

Besides chemical analysis of soils contaminated with antibiotics, ecotoxicological tests with a focus on the effects on microbial soil populations are necessary.

As a first step in an environmental risk assessment it is necessary to elucidate the concentration and the stability of the drug [23]. Several chemical methods have been described for the analysis of antibiotics: high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) [22, 61-67]. Among the various methods liquid chromatography mass spectrometry (LCMS) or even tandem mass spectrometry MS/MS seems to be the method of choice for estimating the low concentration of antibiotics in water and soil. The preferred extraction methods are liquid-liquid extraction followed by a sample clean up procedure. In a few cases,

pressurised liquid extraction (PLE), known as accelerated solvent extraction (ASE) was applied [10, 25, 27].

To analyse the structure and diversity of bacterial communities in soil several methods have been developed. Agar-plating of soil suspensions is insufficient for estimating biodiversity, because less than 1% of the total bacterial population has been found to be culturable on standard media. Isolated bacteria may account for only a minor proportion of the total bacterial diversity in soil [30]. Recently, molecular techniques have been applied for assessing biodiversity. Denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer-analysis (RISA), amplified ribosomal DNA restriction analysis (ARDRA) or sequencing are typical examples for molecular techniques in ecological microbiology [179, 211, 213, 221, 224]. A DGGE analysis of PCR-amplified 16S rDNA fragments is a relatively fast and simple method and has been used for several population structure analyses in soils [212, 213, 223, 231, 233]. For measuring the microbial activity soil respiration is widely used. Soil respiration and kinetics have been applied in this work also to assess the effects of xenobiotics on soil microorganisms [135, 202, 203].

1.2 Selection of substances investigated in this thesis

From the quantitative high usage antibiotic groups, tetracyclines and sulfonamides, two important representative compounds [138, 141, 170, 241] were selected: sulfadiazine (SDZ) and chlortetracycline (CTC) as chlortetracycline hydrochloride. In the current work SDS was used in chemical and microbiological experiments and CTC in biological experiments in order to include another common used antibiotic (see Consumption and use of antibiotics). Sulfonamides and tetracyclines are frequently found in waste- and surface-water and pig manure. Both are measured in concentration ranges able to effective inhibition or killing of bacteria [131, 142, 170].

In the following sections details and mechanisms of inhibition of sulfonamides and tetracyclines are described.

1.3 Scope of this thesis

Only a few studies investigated the effect of tetracyclines or sulfonamides on the soil microflora, especially the effect on microbial functions.

The goal of this study was to determine if and how the veterinary medicines sulfadiazine and tetracycline in its pure form influence the bacterial population in soil. The procedure involved various amounts of sulfadiazine and chlortetracycline and two different kinds of soils. The antibacterial effects of these antibiotics were investigated with classical biological methods like plate counts and respirometry and were combined with modern molecular biological methods like polymerase chain reaction (PCR) and DGGE. The resistance of culturable soil bacteria against sulfadiazine and chlortetracycline was determined with the agar diffusion test method. Additionally, the growth inhibitory potentials of CTC in water-extractable soil solutions were tested with sensitive soil bacterial isolates in agar diffusion disk tests.

All experiments (see materials and methods/design of the microcosm experiments) were carried out with microcosms to follow the changes in the population structure and microbial activity under the influence of the selected antibiotics.

In addition, an analytical method was established for the simultaneous measurement of SDZ and its metabolite acetyl-sulfadiazine (acetyl-SDZ) in soil. Experiments were carried out to optimise and validate the chemical method. Methods published on extraction, chromatographic separation and detection of SDZ unfortunately describe only extraction solvents for mixtures of antibiotics and not the optimum conditions for sulfadiazine solely. This was the reason for optimising of the solvent mixture.

The poor information on suitable storage procedures was the reason for studying the influence of storage conditions on the recovery of SDZ in this work.

The main aim of the present work was to combine biological and chemical investigations to measure the effects of the important antibiotic substance sulfadiazine in soil. In addition the reaction of the soil microbial population to the supplementation of chlortetracycline was studied.

2. The state of the art and basics

2.1 Antibiotics in the environment

2.1.1 Definitions, classification and background

Antibiotics are natural metabolic products of fungi, actinomycetes and bacteria that kill or inhibit the growth of microorganisms. Antibiotic production is particularly associated with soil microorganisms and in the natural environment it is thought to provide a selective advantage for organisms in their competition for space and nutrients. Although the majority of antibacterial and antifungal agents in clinical use today are derived from natural products of fermentation, most of them are then chemically modified to improve their antibacterial or pharmacologic properties. However, some agents are totally synthetic (e.g. sulphonamides, quinolones). Therefore the term "antibacterial" or "antimicrobial" agent is often used in preference to "antibiotic" [100].

The earlier used classification of antibacterial agents in synthetically gained chemotherapeutics and antibiotics, i.e. antimicrobial active substance of biological derivation, is nowadays extensively abandoned. In the meantime, antibiotics traditionally gained from microorganisms are even produced synthetically [101].

The spectrum of efficacy of an antibiotic substance defines against which pathogen the substance is effective, in concentrations that can be achieved against infections in the human being. Substances with a narrow activity spectrum e.g. penicillin V is only affective against some Gram-positive bacteria as *Streptococcus*, *Neisseria*, *Spirochetes* and a few others. Antibacterial agents with activity against a multiplicity of diverse bacteria are called broad spectrum antibiotics (tetracyclines, sulfonamides).

There are three ways of classifying antibacterial agents:

- According to whether they are bactericidal or bacteriostatic.
- By target site.
- By chemical structure.

Due to antibacterial effect of chemotherapeutic drugs it is possible to differentiate two different types of effects: bacteriostatic and bactericidal. Bacteriostatic agents inhibit growth of bacteria whereas bactericidal agents kill bacteria. Bacteriostatic agents are successful in the treatment of infections because they prevent the growth of the bacterial population.

Also a convenient way of classifying antibacterials is on the basis of their site of action.

The four main target sites for antibacterial action are:

- Cell wall synthesis.
- Protein synthesis.
- Nucleic acid synthesis.
- Cell membrane function.

A classification based alone on chemical structures is not of practical use because of the diversity. However a combination of target site and chemical structure provides a useful working classification. Classification of the antibacterial agents with some examples according to various textbooks [100, 101, 105]:

- Inhibitors of Cell Wall Synthesis: Beta-lactams, Glycopeptides, Cycloserine, Bacitracin.
- Inhibitors of Protein Synthesis: Aminoglycosides, Tetracyclines, Chloramphenicol, Macrolides, Lincosamides.
- Inhibitors of Nucleic Acid Synthesis:
    - Inhibitors of synthesis of precursors: Sulfonamides and Trimethoprim.
    - Inhibitors of DNA replication (Quinolones) and RNA polymerase (Rafamycins).

2.1.2 Application of antibiotics

Antibiotics are widely used in human and veterinary medicine to prevent or to treat microbial infections as well as in livestock production to promote the growth of animals. All antibiotics used in veterinary medicine are the same or closely related to antibacterials used in human medicine [93].
In human medicine antibacterials are mostly used therapeutically against different bacterial diseases or as chemical prophylaxis (infection prophylaxis for travellers in malaria regions or complications prophylaxis in operations).
In veterinary practice antibiotics are used to treat disease and protect livestock's health but also are used precautionary as feed additives. The exact purposes with the appropriate definitions according to the National Committee for Clinical Laboratory Standards (NCCLS) are presented below.

Therapeutic purpose

Therapy is defined as the administration of an antimicrobial to an animal, or group of animals, which exhibit frank clinical disease.
In that case the antibiotics should be exactly suitable (antibacterial spectrum as narrow as possible)

or in case of doubt an antibiogram should be done. Intermediate broad spectrum antibiotics as tetracyclines or sulfonamides can be used momentary.

Prophylactic purpose

Prevention/prophylaxis is defined as the administration of an antimicrobial to exposed healthy animals considered to be at risk, but before expected onset of disease and for which no aetiological agent has yet been cultured.

This is typical at the beginning of pig fattening, to control bacterial infections of fish or to prevent recurrent urinary- infections with *E. coli* (sulfonamides-trimethoprim).

Growth promoters

Growth promotion is defined as the administration of an antimicrobial to growing animals, usually as a feed additive, over a period of time, resulting in improved physiological performance of the animals.

This use of antibiotics for animal growth promotion has been controversial because of the potential transfer of antibiotic resistance. Also the food consumers have a demand for antibiotic free food.

2.1.3 Consumption and use of antibiotics

The available database on antibiotics used in husbandry in European countries is poor and incomplete because only few European Union (EU) member states (the Scandinavian countries and the Netherlands) have already started to collect those data. FEDESA, representing the most important companies in the veterinary pharmaceutical industry, has provided some data on the quantities of antibiotics on request of the EU Commission. Furthermore, information on the European use of antibiotics for animal husbandry has been compiled recently in reports on the situation of antimicrobial resistance prepared by the Scientific Steering Committee of DG XXIV of the European Commission and by the Committee of Veterinary Medicinal Products of the European Medicine Evaluation Agency (EMEA) [138]. In 1996, about 10,200 tons of antibiotics were used by EU countries of which 50% were applied in veterinary medicine and as growth promoters in animals [148]. In 1999, 13,288 tons of antibiotics were used in the EU and in Switzerland, of which 65% were used in humans, 29% in the veterinary medicine and 6% as growth promoters [152]. The EU Commission Press Release Food Law News reported similar data in 2002. In some estimates of antimicrobial use in veterinary medicinal products, farm animals consumed 4,700 tons (35%) of all antibiotics administered in the EU, largely for therapeutic purposes (29%), whereas humans consumed 8,500 tons (65%) [92].

The most important groups of the predominant therapeutically used pharmaceuticals are tetracyclines (chlortetracycline (CTC) and tetracycline (TCY)), with 52%, followed by sulfonamides (sulfadiazine (SDZ) and sulfadimidin (SDM) with 19% [141].

Tetracyclines and sulfonamides are broadband antibiotics and therefore they are widely used in livestock farming not only in European countries [69, 132]. Accordingly Kumar et al. [124] reported that tetracyclines (oxytetracycline (OTC) and CTC) were the most common antibiotics present in swine, beef, and turkey manures.

Sulfonamides are among the most used chemotherapeutics in veterinary practice, because of their low cost, their broad spectrum of activity in preventing or treating bacterial infections, and their effectiveness as growth promoters [72]. According to Ungemach [138] sulfonamides are the fifth most widely used group of veterinary antibiotics within the EU, accounting for 2% of sales in 1997. Rassow and Schaper [168] reported that in the Weser-Ems region, Germany, the common antimicrobial agents are tetracyclines and sulfonamides. Also Thiele-Bruhn et al. [170] gave details on the administrated antibiotics in Mecklenburg-Western Pomerania, Germany, for the period from October 2000 until September 2001.

The antibiotics tetracycline and sulfonamides were the most important groups prescribed with the main substances chlortetracycline and sulfadiazine. Details are shown in the figure 1.

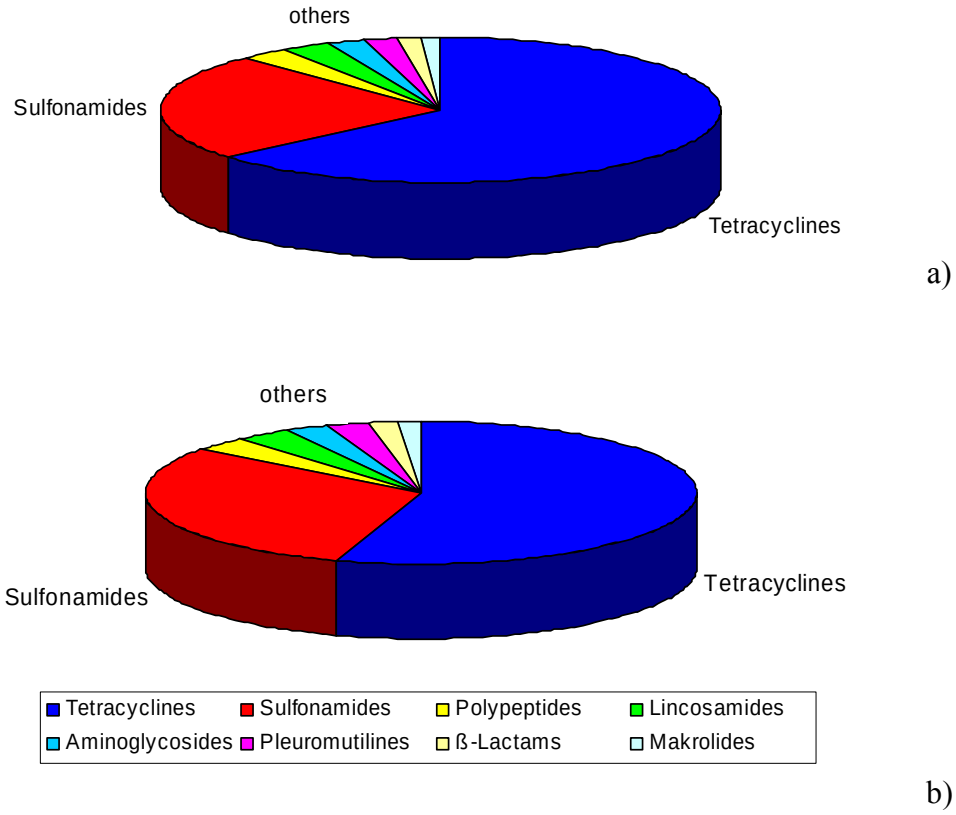


Figure 1.

Administrated antibiotics in Mecklenburg-Western Pomerania, Germany, for the period from October 2000 until September 2001 via feed additives: a) husbandry, b) piggeries according to Thiele-Bruhn [170].

2.1.4 Input and exposure of antibiotics into the environment

After administration of antibiotics to humans or animals and passing through the organism, a significant amount is excreted through urine or faeces. However, the majority of the used antibiotics leave the organism as a mixture of the parent compounds and metabolites. For example CTC is excreted in 70% unchanged [94] and the parent sulfonamides are excreted between 40% and 90% from the treated organism [169].

Most medical substances are metabolised to phase I or phase II metabolites before being retrieved from the body. Phase I reactions (usually consist of oxidation, reduction or hydrolysis) cause the change of the structure of the pharmaceutical and the products are often more reactive and sometimes more toxic than the parent drug. Phase II reactions (involve conjugation with glucoronic

acid, sulphuric acid, acetic acid, amino acid etc.) are coupling reactions and the products are more water soluble and mostly biological inactive. In some cases the phase II products can be transformed by hydrolysis in to the parent drug [101]. Berger et al. [95] showed that N-4-sulfadimidine (phase II metabolite) was converted to the parent drug - sulfadimidine in samples of liquid manure. On the basis of these facts, not only the parent compound can be a risk for the environment, but also its metabolites.

These drugs and their metabolites can enter the environment via several exposure routes (figure 2).

Exposure

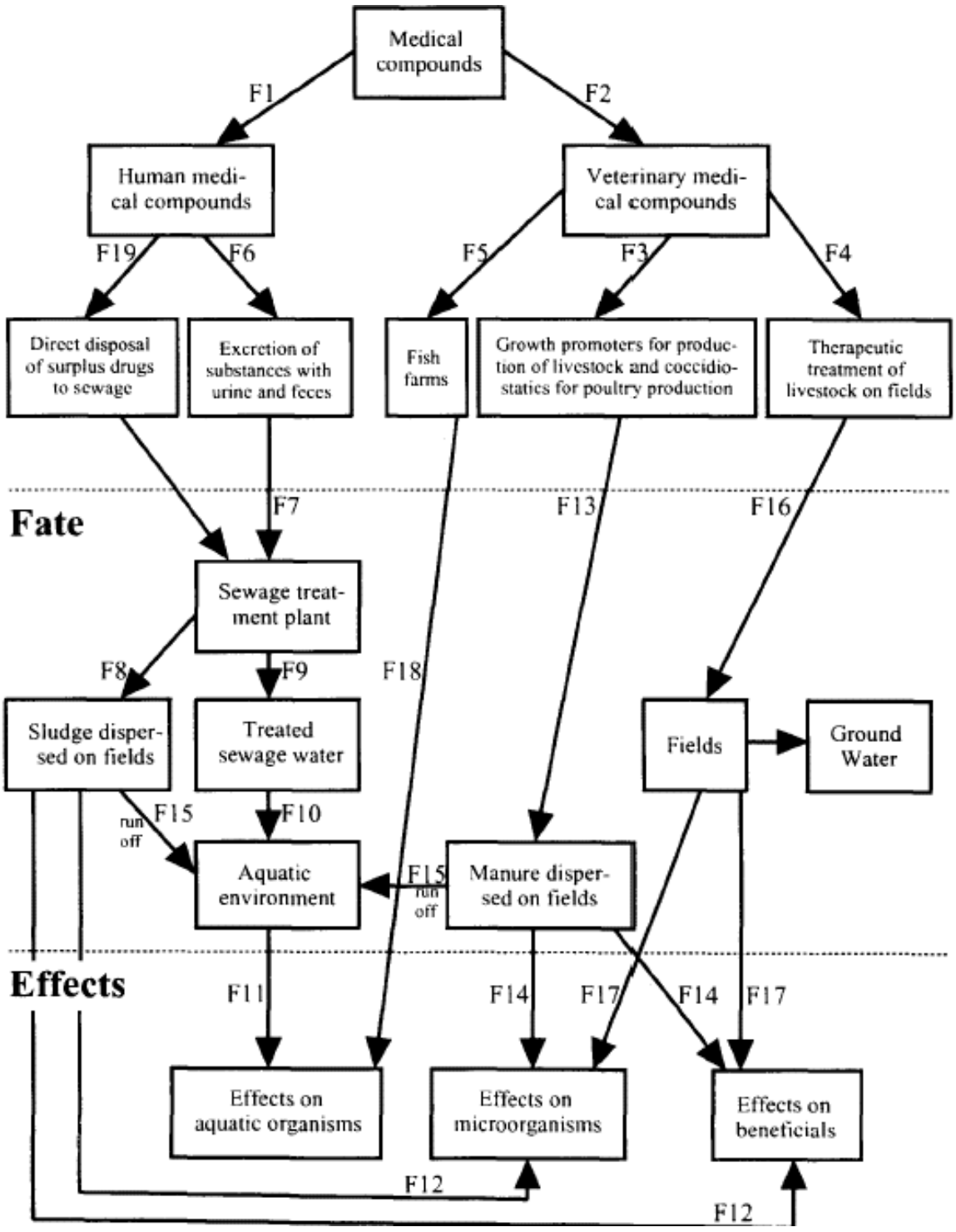


Figure 2.

Anticipated exposure routes of both veterinary and human medicinal substances in the environment according to Halling-Sørensen [161].

Human medical substances will enter the sewer system with the urine and faeces and attend the sewage treatment plant. If the drugs are not completely mineralized, they are released into surface water or absorpted by sludge and, if the sludge is used to fertilize arable land, they may enter the topsoil of fields [91]. In the case of veterinary medicines, the excrements are either directly released into the environment by grazing animals or indirectly by spreading manure onto agricultural soils. A direct release of contaminated excrements can result in a high local concentration of the specific pharmaceutical and may affect soil organisms.

Antibiotics are also extensively used in aquaculture as feed additives in fish farms. The main groups of pharmaceuticals used in aquaculture are tetracyclines, sulfonamides and also chloramphenicol, which is forbidden for veterinary use in the EU since 1995 [94].

In addition soils are a natural source of antibiotics. Soils are a habitat and source of indigenous, antibiotics producing microorganisms. Among numerous other soil microorganisms, 30 to 50% of *actinomycetes* isolated from soil are able to synthesize antibiotics [173]. Such antibiotics, biosynthesized *in situ*, are found especially in the soil rhizosphere with concentrations of up to 5 µg/g [174, 175].

2.1.4.1 Fate and occurrence

Pharmaceutical compounds like antibiotics, from human and agricultural sources, have been detected in soils, sediments, surface waters and ground waters in many countries. Some of the antibiotics are hydrophilic and increased mobility may lead to ground water contamination [27] as found by Velagaleti [125] and Ternes [159].

Heberer [165] reported, that in some investigations carried out in Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, The Netherlands, and the US, more than 80 different pharmaceuticals and several drug metabolites have been detected up to µg/l-level in the aquatic environment, mostly due to the discharge of effluents of sewage treatment plants.

The Federal Environmental Agency in Austria reported in 2002 on contaminated waste water from treatment-plants with pharmaceuticals e.g. sulfamethoxazole [130]. Sulfonamides were found in six of fifty-one samples of drinking water in Bavaria [143]. Hirsch et al. [94] found trimethoprim and a sulfonamide in a sewage treatment plant and in surface water and two sulfonamides (sulfamethoxazole 0.47 µg/L and sulfamethazin 0.16 µg/L) in groundwater.

Sulfamethoxazole and sulfamethazine have also been detected in the US and Germany in a few other groundwater samples [15, 96, and 98] and Holm et al. [99] reported on the presence of different sulfonamides in high concentrations in groundwater samples in Denmark. Tetracycline drugs (chlortetracycline, oxytetracycline and tetracycline) were detected by Lindsey et al. [96] and Kolpin

et al. [97] in US surface water samples. However, tetracyclines could not be detected in the soil- and ground water [1, 69, and 96].

Several investigations report on findings of antibiotics in sediments from fish farms [166, 172].

In spite of the numerous findings of pharmaceutical compounds and their metabolites only relatively few studies exists on the effect of the antibiotics on the environment including uptake by plants from manure-amended soils. Kumar et al. [124] reported on the occurrence of antibiotics in plants after fertilisation of the soil with contaminated manure. The authors found that *Zea mays*, *Allium cepa*, and *Brassica oleracea* adsorbed increasing amounts of chlortetracycline with increasing amounts of antibiotic present in the manure. Also Migliore et al. [163] described effects of available residues of sulfadimethoxine on *Panicum miliaceum*, *Pisum sativum* and *Zea mays.*

Little is known about the occurrence and fate of antibiotics in soil. Only few investigations have reported findings of medical substances in other field samples than sediment or treated waste water samples.

Some antibiotics have been detected in soil in the range of several hundred µg/kg, but the margin of the residual concentrations range from a few µg up to g/kg.

Hamscher et al. [1] found tetracycline in concentrations between 10 and 100 µg/kg in several soils after liquid manure fertilisation while Höper et al. [69] reported on concentrations of different antibiotics e.g. TCY, CTC and SDM below 20 µg/kg soil in pig manure treated field soils. CTC concentrations up to 249 µg/kg were measured in soil, and in the manure crust even up till 1435 µg/kg.

Hamscher et al. [1] found 86.2 µg/kg (0-10 cm), 198.7 µg/kg (10-20 cm), and 171.7 µg/kg (20-30 cm) of TCY in soil samples and 4.6-7.3 µg/kg CTC (all three sublayers) after fertilization with liquid manure.

Warman and Thomas [126] determined CTC in soil amended with poultry manure. They demonstrated that drug metabolites excreted by medicated livestock are decomposed by bacterial action in the liquid manure and reconverted into the active drug.

2.1.4.2 Assessment of antibiotics

Literature on the fate of pharmaceuticals in soil is still insufficient; however, research and publications on this topic have increased in the last few years.

Hartig et al. [15] described sulfonamides as potential organic micropollutants in water. Samuelsen et al. [60] reported that no degradation of OTC, SDZ and other antibiotics in marine aquaculture sediment took place over a period of 180 days. Beside this, recent studies concerning other

pharmaceutical residues in the aquatic environment have clearly shown that elimination in municipal sewage treatment plants is often incomplete [94, 165].

Persistence of antibiotics in soils depends on many factors including soil type, climate, and class of antibiotics. The fate of antibiotics partly depends on degradation. The degradation of xenobiotics in soils is mainly driven by microbial processes and numerous antibiotics are susceptible to enzymatic transformation reactions [81]. However, the degradation of antibiotics is hampered by fixation to the soil matrix. Already persisting antibiotics were determined in soils [170]. The pharmaceuticals may accumulate in soil [83, 164] and influence soil organisms [151].

Höper et al. [69] classified CTC as persistent because the substance accumulated in the soil under specific conditions. Also Hamscher et al. [1] found that tetracyclines not only occurred in significant amounts in soil after repeated fertilizations with liquid manure but also persisted and accumulated in the environment.

Kay et al. [122] and Blackwell et al. [239] investigated the fate of veterinary antibiotics e.g. OTC on agricultural clay soils. He found that the presence of slurry in combination with highly sorptive antibiotica, such as OTC, will not increase their mobility due to facilitated transport. OTC residues (0.2 µg/kg) from the experimental soil columns were only detected in one single sample collected from the 0-5 cm layer of one soil core.

CTC has been shown to persist in soil; however, this was dependent on temperature [1].

Ungemach et al. [93] pointed out that tetracyclines may have a potential risk and that investigations on the environmental effects of these antibiotics are necessary.

This shows that current knowledge and evaluation of fate, occurrence, assessment and potential risk of tetracyclines and especially sulfonamides in the environment is insufficient and more investigations are needed.

2.1.5 Resistance of bacteria to antibiotics

In medical science a resistant organism is defined as an organism that will not be inhibited or killed by an antibacterial agent at concentrations of the drug achievable in the body after normal dosage [100]. Two types of resistances are to divide: the primary resistance and secondary resistance. Primary resistant or born resistant means that some species are innately resistant to some families of antibiotics either because they lack a susceptible target or because they are impermeable to the antibacterial agent e.g. all strains of *Pseudomonas aeruginosa* against benzylpenicillin or some *E. coli* strains against tetracyclines [100, 101].

Secondary resistance or acquired resistance means that a spontaneous resistance occurs first during the antibiotic therapies. This resistance can be divided in one-step- and multi-step resistance. One-step resistance occurs quickly after the beginning of a therapy. Multi-step resistance occurs first after repeated therapy with the same agent and is developed slowly and step by step.

In parallel with the rapid development of a wide range of antibacterial agents since the 1940s, bacteria have proved to be extremely adept at developing resistance to each new agent that comes along. The rapidly increasing incidence of resistance associated with a decreasing in the discovery of novel antibacterial agents to combat resistant strains is now recognized worldwide as a serious threat against the treatment of life-threatening infections [100].

An important question in the recent time is if the antibiotics used in agriculture have a great impact on the environment or can contribute to antibiotic resistance in pathogenic bacteria of humans. Many scientists hotly debate and try to answer this question. Smith et al. [146] carried out model studies and suggested that transmission of antibiotic-resistant bacteria from agriculture can have a bigger impact on human populations than hospital transmission and that the agricultural antibiotic use can have important quantitative effects on the spread of resistances in the community.

Antibacterial resistance is a serious threat to the efficacy of antibacterial substances. Antibiotics must have various effects, because different kinds of bacteria with diverse features are the target organism of antibiotics. As antibiotics are nature's own weapons for maintenance of all microbial ecosystems, the resistance is a natural part of the regulatory factors in any ecosystem and genes coding for resistance have existed as long as microbes. The increased use of antibiotics during the last five decades has caused a genetic selection of more harmful bacteria [129]. Although antibiotic concentrations in most soils are not at therapeutic levels which cause inhibitory effects on a bacterial population, they may still influence the selection of antibiotic resistant bacteria in the environment. Resistance can be transferred to other bacteria living in other environments such as ground water or drinking water. In general, knowledge of sub-inhibitory concentrations and their effects on environmental bacteria is poor, especially with respect to resistance [160, 191]. This development of resistance can be favoured by the use of antibiotics in concentrations below therapeutic levels or may be selected by antibiotic substances in hospital effluent, municipal sewage, aeration tanks, and the anaerobic digestion process of sewage treatment plants or in soil. Furthermore, resistant bacteria are excreted and discharged into sewage or soil and other environmental compartments [160] and the resulting antibiotic residues and resistant microorganisms can affect the natural soil microbial community and soil functions and may even harm animals and humans via the food chain [215, 216]. The resistance problem in the environmental compartments such as waste water, surface water, ground water, sediments and soil is described by Kümmerer [191]. The author concluded that the

increasing antibiotic resistance is seen as an ecological problem. Antibiotic resistance in sediment bacteria are often found in locations with fish farms as antibiotics are commonly used therapeutically in fish farming. Samuelsen et al. [166] reported findings of sediment bacteria resistant to various antibiotics used as feed additives in fish farms.

2.1.5.1. Antibiotics as the growth promoters

Since more than 50 years antibiotics in the low dosage have been used as growth promoters as feed additive of the feed animals [147]. This procedure based on the detection that some antibiotic substances cause significant better feed utilisation, accelerate the weight gain and reduce the frequency case of illnesses' in the fattening farm. It is supposed that the mechanism of the growth promoting effect is associated with the inhibition of the gut flora of the animals by the antibiotics, but the exact mechanism has not been clearly elucidated [153].

The concentrations of antibiotics in the feed for therapy and prophylaxis are usually higher than the concentrations of antibiotics for growth promotion. The risk of underdosing might favour the selection of bacterial resistance. In 1997, the WHO concluded that the use of antibiotics in any ecosystem may cause selection of resistant bacteria and that low dose and long-term treatment with antibacterials exert a higher pressure on selection of bacterial resistance than full-dose therapy [158]. Several studies dealt with this problem and described the growth promoting role in resistance development [146, 147, 154, 155, 156, 157].

In the US and several other countries, classes of antibiotics active against Gram-negative bacteria, such as tetracyclines, have been used for growth promotion for decades and are still being used today [154]. In the US tetracyclines belong to the most used antimicrobial growth promoters.

Routine use of antimicrobials in food producing-animals for growth promotion constitutes a serious public health problem, especially in the case where the same classes of antimicrobials are used in humans [154]. Hence, in some countries, for instance Denmark, the farmers took a step in response to consumers concerns and voluntarily discontinued the use of all antimicrobial growth promoters [154]. In Sweden the use of them is banned since 1986 and in Switzerland since 1999. According to the EU Regulation 1831/2003, using of the antibiotic growth promoters in feed since 2006 is also forbidden.

2.1.6 Ecotoxicology of antibiotics

Antibiotics are biologically active molecules. The intention of their use is to inhibit bacterial growth or to kill bacteria. As this use can lead to resistance of bacteria there is a serious concern about qualitative and quantitative effects on resident microbial soil populations, possibly leading to disturbances in soil functioning. Effective inhibition or killing of bacteria in therapeutic application of antibiotics is normally achieved by 1-25 mg/l [133]. Unfortunately, very little is known about the ecotoxicology of antibiotics.

Bacterial toxicity tests mainly use a single species and other matrices as soil. The growth inhibition test with *Pseudomonas putida* and the bioluminescence test with *Vibrio fischeri* are among these [142]. Toxicological studies investigate the effects of antibiotics on aquatic organisms as *Daphia magna*, a micro-algae, an aquatic weed or on soil fauna etc. (see Table 1) but little is known about effects on total microflora in soil [69] and on the impact of antibiotics on environmental bacteria [167, 134]. Most of the toxicity tests presented in the literature are performed as acute toxicity tests. Halling-Sørensen et al. [161] suggested that, due to the fact that at least some of the antibiotics exposed to the environment are found to be rather persistent, it would be more relevant to perform life cycle test on organisms representing different trophic layers in order to identify the hazard of the substance in question rather than to perform acute toxicity tests.

Table 1.

Selected data on the toxicology of antimicrobial substances in environmental compartments.

Substance	**Matrix**	**Effects**	**Effects on**	**Reference/Year**
Oxytetracycline, Tylosine	Soil	Toxic effects EC_{10} and EC_{50}	Earthworms, springtails and enchytraeids	[151] 2000
Sulfadimidin, sulfathiazol	Manure	Resistance	*E. coli*, streptococci	[131] 1988
Sulfadiazine, Tetracycline, Oxytetracycline and other	Water	Toxicity (acute and chronic tests)	Freshwater crustacean *Daphnia-magna*	[144] 2000
Chlortetracycline and other	Purified water	Toxic effect EC_{50}	Freshwater algal species (cyanobacteria and green algae)	[145] 2000

The toxic effect data (EC_{50}) of antibacterial agents on various aquatic species found in the literature show values in the range of mg/l. However, it is difficult to draw conclusions about effects in other habitats as water. For example, it was shown that inhibitory effects of tetracyclines on activated sludge bacteria occurred already at significantly lower contents. The EC_{50} for CTC was 0.03 mg/l, for OTC 0.14 mg/l and for TC 0.3 mg/l [134]. Another example is the minimal concentration of CTC for inhibition of microbes being between 0.01 and 0.5 mg/l [147, 148]. In soil however, much higher EC_{50} values were calculated for OTC, tylosin and sulfachloropyridazine of 50, 30 and 75 mg/kg dry soil, respectively [135]. Also average effective dose values (ED_{50}) of 47.6 mg/kg soil for SDZ and 25.4 mg/kg soil for CTC found by Thiele-Bruhn [132] were in the same range. In another recent paper, Thiele-Bruhn and Beck [136] report on ED_{10} values ranging from 0.003-7.35 mg/kg soil for sulfapyridine and OTC, depending on the antibiotic compound and its soil adsorption. In spite of this, Warman et al. [126] observed no influence on soil respiration even at high concentrations of CTC (50 mg/kg soil) in an investigation on ecotoxicity on soil microorganisms and neither Hund-Rinke et al. [127] did find an influence of tetracycline on soil respiratory activity with concentrations up to 50 mg/kg soil.

Thiele-Bruhn and Beck [136] considered that the effective doses and concentrations of antibiotics are lower than those reported from other organic pollutants, which documents the ecotoxic relevance of the antibiotics. In their experiments the microbial inhibition varied among antibiotics and soils and was influenced by the susceptibility and activity of the microbial community in the soil, the soil sorptive properties, and the intrinsic toxicity of the antibiotics. Consequently the effects of different antibiotics in different soils are depending on various factors and difficult to predict. Presently, there is a basic lack of information concerning the effects of sulfadiazine on soil microorganisms, their function, diversity or resistance. While some studies described the effects on selected bacteria [128] only a few investigations have shown that antibiotics inhibit soil microorganisms [139, 140].

2.1.7 Legal Regulations for veterinary pharmaceuticals

Since 1992 the assessment of environmental effects of veterinary products is required prior to the marketing of new pharmaceutical products. According to the relevant directives (the Directive 81/852/European Economic Council (EEC) and 92/18/EEC are today replaced by Directive 2001/82/EC) [199]. These directives have been implemented in the national legislations of the Member States. Comparable with industrial chemicals and biocides it is necessary to explore the

properties of these substances, the exposure routes, the environmental fate and ecotoxicity to assess the risk.

Thus, the environmental risk assessment consists of two phases. The first phase assesses the exposure of the drug to the environment. In the second phase, information about the physical/chemical, pharmacological and/or toxicological properties are obtained and assessed in relation to the extent of the environment exposure. All tests have to be performed under GLP quality standard. It is described as a practical guidance by the Committee for Veterinary Medicinal Products [225].

There are no regulations or requirements concerning the environmental properties or potential effects of existing "old" pharmaceuticals but also they can directly affect microorganisms and assessing their functionality. The "old" pharmaceuticals are defined as those pharmaceuticals registered between 1978 and 1998. From this group for example tetracyclines and sulfonamides may have a potential risk and investigations on the environmental effects of these antibiotics are therefore necessary.

2.1.8 Characterisation of selected antibiotics

2.1.8.1 Sulfonamides

In 1932, Gerhard Domagk discovered the antibacterial effect of Sulfachrysoidin, one azo dye called Prontosil rubrum, on *Streptococcus* bacteria [102]. In 1935, the parent compound sulphanilamide became the first clinically effective antibacterial agent. The p-amino group is essential for activity, but modifications at the sulfonic acid side chain have produced many related agents.

Sulfonamides have a selective toxicity on bacteria as they act in competition with para-amino benzoic acid (PABA) for the active site of dihydropteroate synthetase, an enzyme that catalyzes an essential reaction in the synthetic pathway of tetrahydrofolic acid (THFA), which is required for the synthesis of purines and pyrimidines and therefore for nucleic acid synthesis (see figure 3).

The selective toxicity depends on the fact that many bacteria synthesize THFA, whereas human cells lack this capacity and depend on an exogenous supply of folic acid. Bacteria that can use preformed folic acid are similarly unaffected by sulfonamides.

Sulfonamides have an effect against some Gram-positive and Gram-negative bacteria and the activity spectrum is very broad. They are very efficient against *Streptococci*, *Pneumococci*, *Actinomyzetae*, *Nocardieae*, *Chlamydia*, *E. coli*, *Salmonella*, *Shigella*. In addition sulfonamides have also an affect on some Protozoa e.g. *Toxoplasma gondii* or Plasmodia.

To this class of potentiated sulfonamides that frequently are applied in livestock husbandry belongs Sulfadiazine (N-[2-pyrimidinyl]-sulfanilamide) [16]. Sulfadiazine (SDZ) is used for skin infections or as a general antibiotic when the actual identity of the infecting organism is not known.

The structural formula of sulfadiazine is shown in figure 4.

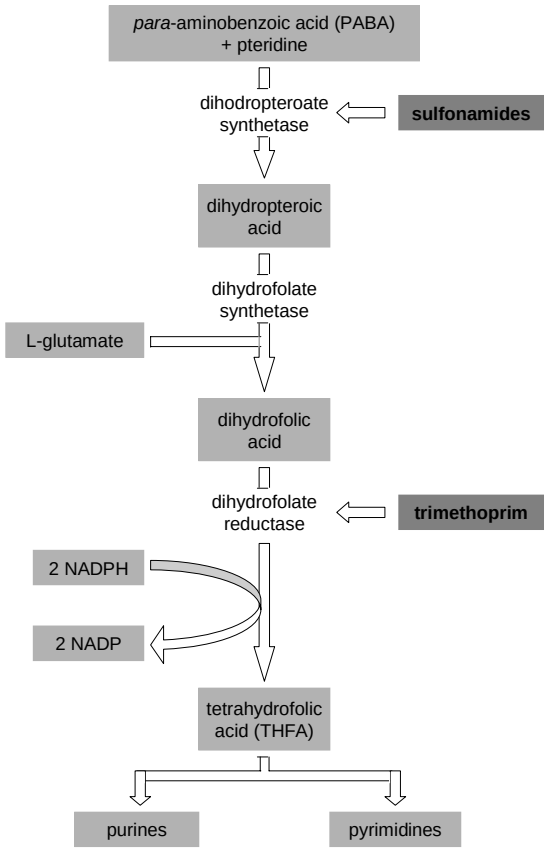


Figure 3.

Sulfonamides and trimethoprim inhibit in series the steps in the synthesis of tetrahydrofolic acid by interacting with key enzymes in the pathway according to Mims [100].

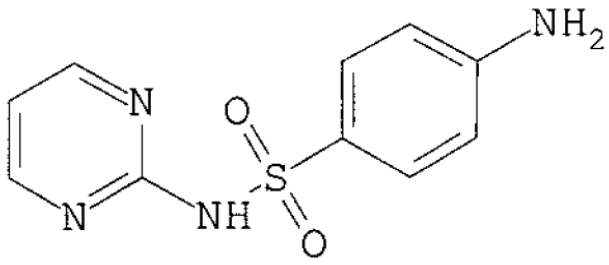


Figure 4.

Chemical structure of sulfadiazine.

The sulfonamides are metabolised in the human body as a detoxification step through N-4-acetylation or oxidation. The acetyl metabolites are not bacteriostatic, besides, they are better soluble and less toxic as the parent compound [101].

After excretion metabolites can even be transformed back in to the original active drug [94]. Berger et al. [95] reported that N-4-acetylated sulfamethazine was converted to sulfamethazine in liquid manure.

Beside acetyl-sulfadiazine (acetyl-SDZ) other sulfadiazine metabolites are known. In the urine from rhesus monkeys treated with SDZ, the presence of five metabolites of SDZ was revealed: N4-acetyl-sulfadiazine, 4-OH-sulfadiazine, 5-OH-sulfadiazine, 5-OH-glucuronide sulfadiazine and 5-OH-sulfate sulfadiazine. The last three 5-OH metabolites have not been found in humans [116]. Very little is known on the occurrence and fate of SDZ metabolites in soil or manure. Haller et al. [14] could quantify SDZ and the metabolite acetyl-SDZ in animal manure. Pfeifer et al. [80] examined the structural characterization of sulfadiazine metabolites in pig manure and found in addition to acetyl-SDZ 4-hydroxysulfadiazine as the major metabolites and a third unknown minor metabolite with the mass 295 m/z.

In a study on sorption and transport of SDZ Wehrhan [232] found acetyl-SDZ and hydroxyl-SDZ, together with a third unknown metabolite after contact of SDZ to soil.

2.1.8.2 Tetracyclines

Tetracycline was first synthesized by Lloyd Conover working for Pfizer Inc. and was patented in 1955. Within three years it became the best selling antibiotic in the U.S.A. Tetracyclines are a family of large cyclic-structures that have several sites for possible chemical substitutions. Tetracyclines

inhibit protein synthesis by preventing aminoacyl transfer RNA from entering the acceptor sites on the ribosome.

Tetracyclines are easily distributed in the body and penetrate host cells to inhibit intercellular bacteria.

They are antibiotics with a broad antibacterial spectrum and bacteriostatic activity, and have a good activity against acute diseases caused by Gram-positive and Gram-negative bacteria. In veterinary medicine they are routinely used for prevention and control of disease of food-producing animals [107, 109]. Among others they are used in the treatment of infections caused by mycoplasmas, chlamydia by some respiratory infections, urinary infections or systemic infections [100, 104, 105]. Administration of these antibiotics results in healthier and faster-growing animals.

Chlortetracycline (CTC) is the oldest member of the tetracycline group or antibiotics. It is obtained through anaerobic fermentation of *Streptomyces aureofaciens* and is prepared industrially by bulk fermentation [109]. The chemistry of tetracyclines is complex. The structural formula of CTC is shown in figure 5.

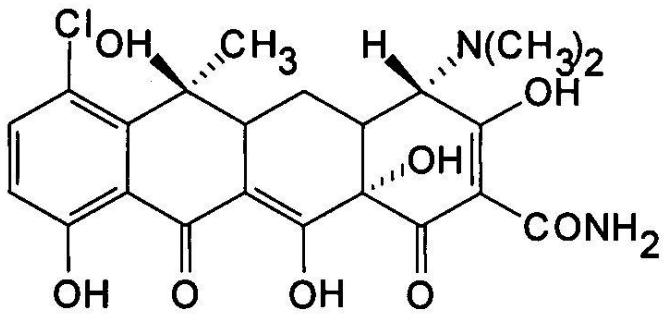


Figure 5.

Chemical structure of chlortetracycline.

Tetracyclines are well known to form chelate complexes with metal ions and to bind to proteins and silanol groups [1]. They build willingly complexes with Mg^{2+} und Ca^{2+} ions. Especially stable are trivalent aluminium- and iron-complexes [164]. Sithole et al. [119] suggested that tetracycline interaction with soil organic matter occurs as a result of binding to divalent cations, ion exchange interactions and hydrogen bonding between acidic groups in humic acids and polar groups on tetracycline. Tolls [164] described that sorption of tetracycline appears to be strongly related to the particle size of the solids, which in turn is related to the specific surface. Jones et al. [118] showed cation exchange with clay minerals, and surface complexation to soil iron and aluminium oxides. Hamscher et al. [1] have shown that tetracyclines are sorbed and accumulated in agricultural soils

amended with antibiotic-contaminated manure. Höper et al. [69] also noted that tetracyclines are very sorptiv. Those different effects reduce the antibacterial effect of tetracycline. Lunestad et al. [120] presented the reduction in the antibacterial effect of oxytetracycline in sea water by the formation of complexes with magnesium and calcium.
Also for CTC many metabolites are known. Most investigations and effects of CTC in environmental samples are restricted to the parent substance and not to the metabolites.

2.2 Soil microbiology and analysis of microbial community

Soil microbiology is defined as a discipline that describes the fate, activity, number and interactions of microorganisms present in soil and how they are affected by their environment [178]. Soil microorganisms can be involved in plant-pathogenic reactions, as well as in biological transformations of xenobiotic compounds added to soil. Soil also represents a natural reservoir of genetic information and soil organisms, many of which are unculturable and hence unknown.
Different methods are known to assess soil microbial populations (communities). In Domsch et al. [180] 15 of these methods for soil microbial population and biomass studies were compared including direct observations, physiological methods like total microbial, bacterial and fungal biomasses, O_2 uptake, soil enzyme analyses and ATP-analyses. The authors concluded that such methods are of limited use in general soil ecosystem studies.
Additional methods for the estimation of microbial activities, as for example dimethyl sulphoxide reduction (DMSO), soil respiration or aerobic biodegradation of ^{14}C-labelled organic matter in soils are described in Alef [90].
In recent years new techniques have become available for the measurement of bacterial diversity and community structure. The molecular methods based on recovery of community DNA from soil offers a great potential for the investigation of the nonculturable part of complex microbial communities [183].
A number of recent publication analysed and described molecular biological methods for the determination of changes in the microbial community in environmental samples [111, 208, 209, 210, 211, 212, 213], other compare the diversity of the cultivable bacteria with the diversity of the total bacterial population [200, 211, 214]. Only few studies consider the use of both bulk community reactions and microbial community structure analysis to examine the effects of some antibiotic: tylosine [181] and TCY [127]. Up to now only a few studies are concerned with the influence of antibiotics on soil microbial community using molecular biological methods [136, 181, 183, 184]. The antibiotic substances sulfapyridine and OTC exerted a selective pressure on soil microbial

community in form of a shift from soil bacteria to soil fungi [136], the addition of tylosin to soil led to a permanent change in the bacterial community structure [183], and addition of sulfachloropyridazine resulted in small changes in the community level profile [184]. In contrast, the addition of TCY had no influence on the composition of phospholipids fatty acids [127]. Mueller et al. [181] investigated the effect of antibiotic tylosin on soil bacterial community with denaturing gradient gel electrophoresis (DGGE) analysis. A small difference in the diversity of 16 S rDNA of the samples compared to the control soil was found. The author concluded that there is no method currently available for exploring the whole bacterial community in one analysis, but that a combination of methods is necessary to obtain a detailed view of its structure and diversity.

The objective of current study was to investigate the impact of SDZ and CTC on the bacterial community in soil and to compare these effects to those obtained with the measurements of microbial respiratory activity. Changes in the bacterial community structure were visualized by changes in patterns of DGGE.

2.2.1 Molecular biological methods

In the past, studies on the diversity of natural microbial populations have been restricted to culturable microorganisms. However, for at least half a century it has been known that the major part of the structurally intact bacterial cells in soil appears to lack the capacity to grow on standard laboratory media [178]. Therefore, the understanding and knowledge of the dynamics of natural microbial communities have remained limited because only a minor fraction of all cells in natural ecosystems is accessible to cultivation techniques. Only recently methods for direct extraction of nucleic acids from different environmental samples became available, allowing a cultivation-independent analysis of microbial communities. Nowadays, the isolation and analysis of DNA is a powerful approach with in soil and environmental microbiology.

The 16S ribosomal DNA (rDNA) exists in all bacteria and contain the highly and less conserved primary and higher order structure elements. The more conserved regions occur in all organisms and report on earlier events during evolution and the less (variable) positions carry the information from lower levels of phylogenetic relationships [185, 218]. For this reason one of the most commonly applied molecular techniques in environmental microbiology during recent years has been the phylogenetic analysis of 16S rDNA gene sequences amplified by PCR from DNA or RNA extracted from samples of soil, water, or sediments [219]. The rDNA genes can be PCR-amplified directly from community DNA using so-called universal 16S rDNA primers [219, 220]. To determine the

community structure of microbial communities among the sequences of DNA fragments denaturing gradient gel electrophoresis (DGGE) [221], temperature gradient gel electrophoresis (TGGE) [179, 211], ribosomal intergenic spacer-analysis (RISA) [224], and different other approaches can be used. An overview of these approaches has been given by Pedrós-Alió [227].

A DGGE analysis of PCR-amplified 16S rDNA fragments is a relative fast and simple method for microbial ecological studies to obtain the genetic "fingerprints" of the microbiological population. This analysis provides a direct outline of the community composition in both a quantitative and semi-quantitative way. This makes it an excellent approach not only for the analysis of the genetic diversity of mixed microbial communities, but also in order to study population dynamics [226]. This makes the technique a powerful tool for monitoring community behaviour after environmental changes in mixed bacterial populations over time [223].

2.2.1.1 Structural analysis of soil microbial communities by denaturing gradient gel electrophoresis (DGGE)

Gradient gel electrophoresis was originally developed and used in medical research to detect point mutations. Since Muyzer et al. [221] applied this method to environmental microorganisms, analyses of microbial communities using DGGE have become increasingly popular.

DGGE is based on the electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels. DNA fragments of the same length but of different sequences can be separated according to their melting properties. DNA is electrophoresed through a linearly increasing gradient of denaturants; the fragments remain double-stranded until they reach the conditions that cause melting of the lower temperature melting domains. Branching of the molecule caused by partial melting of the regions with low melting temperature sharply decreases the mobility of the DNA fragments in the gel. A GC-clamp (40-45 bases GC-rich sequence) attached to the 5'end of the forward primer prevents the complete melting of the PCR product. With DGGE, double-stranded DNA is separated in a linearly increasing denaturing gradient of urea and formamide at elevated temperature. The banding pattern visible after staining reflects the different melting behaviour of the DNA sequences amplified from mixed communities. For the optimal separation of the different products different condition how temperature, time, gradient conditions should be adjusted [179].

2.2.1.2 Diversity Index

The results of the differences in the bacterial community after the addition of a bacterial with and without antibiotics were evaluated by determining the Shannon-Wiener index, which is calculated as

$$H' = -\sum_{i=1}^{s} p_i \ln p$$

Where p_i is the proportion of the total intensity accounted by the *i*th band and ln is the natural logarithm. Evenness *(E)* is calculated as E=*H'*/ln *S*, where *S* is the richness or total number of bands.

Among a variety of different diversity indices, the Shannon index is one of the most widely used, although it has to be taken into account, that this index requires clearly defined species and a distinct identification of individuals, requirements that are not met when dealing with bacteria. Estimation of the Shannon index provides composite values for the number and distribution of morphotypes, DGGE bands, and substrates utilized that represent different aspects of bacterial diversity.

2.2.2 Soil respiration

Soil respiration is one of the oldest and still the most frequently used parameter for quantifying microbial activities in soils [90]. A bacterial population, also complex, can be tested e.g. in respiration inhibition test [142].

The basal respiration is defined as the respiration without the addition of an organic substrate to soil. Substrate-induced respiration (SIR) is the soil respiration measured in the presence of an added substrate such as glucose, amino acids, etc. [90]. The activity of the soil bacteria can be estimated by measuring the CO_2 production and/or O_2 consumption with different techniques as e.g. incubation of soils in jars, adsorption the CO_2 in NaOH and titration by HCl or estimation of O_2 uptake in e.g. an apparatus called “Sapromat”. Some additional methods are described by Alef [90].

Respiration methods are used to measure microbial activity in soil, studying different effects of biological processes in soils, often used for assessment of the side effects of chemicals such as pesticides and heavy metals [90, 202, and 203].

However, some papers describe the use of respirometry to study the effects of antibiotics. In a lysimeter study, Hund-Rinke et al. [127] investigated the potential effects of tetracycline on the soil microflora after application of manure containing tetracycline.

The suitability of the respiration method to study effects of antibiotics was tested by Vaclavik et al. [135] who described this method as simple, fast, cost-effective and characterized it as suitable and stable for use.

2.2.3 Growth inhibition

Originally the Microbial Inhibition Test (MIT) was used for the detection of bacterial sensitivity. MIT was developed already 1952 for milk (*B. subtilis*). It based on the detection of growth inhibition of various sensitive bacterial strains and in the clinical medicine based on microbial agar diffusion tests. The basic microbial inhibition assay involves a standard culture of a test organism, usually *Bacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus cereus* and others. These are seeded in an agar or liquid growth medium which is then inoculated with milk or urine for example and incubated for periods of up to several hours. Samples can be applied directly to the medium or on a filter paper disk impregnated with liquid sample. During incubation the liquid diffuses into the medium and if the sample contains sufficient concentrations of inhibitory substances the growth of the indicator organism will be reduced or inhibited. Depending on the format of the test the presence of an inhibitory substance is indicated by zones of growth inhibition [176].

For environmental samples the antibiotic resistance can be determined using the agar diffusion test as described by Smalla, Niemi, Kresken and Schwartz [192, 193, 195, 196], minimal inhibitory concentration (MIC) test described by Stock and Wiedemann [194] and by Kresken and Hafner [195] or with a microdilution test system used by Kresken and Hafner [195].

Only a few studies described the effects of sulfonamides or tetracyclines in the environment with the growth inhibition test, for example Samuelsen et al. [60] found no change in the antibacterial activity of SDZ-treated sediments after 6 months but they activity of OTC was lost already after 1 month.

Hund-Rinke et al. [127] investigated the influence of TCY in soil. They studied the function and structure of the biocoenosis as well as the distribution of resistance genes. The results showed that the establishment of resistance genes was independent from the addition of tetracycline to the soil and no effects in the growth inhibition test could be revealed.

2.3 Chemical analysis of sulfonamides and tetracyclines in environmental samples

The determination of traces of organic substances in environmental samples usually requires following steps: extraction or enrichment of the substance, clean-up, chromatographic separation and detection. From the many techniques known, the choice of a method is dependent on the matrix and physicochemical properties of a substance.

Many publications describe methods for the determination of sulfonamides in non-environmental samples [18, 22, 53-59] in particular in food or feed. For environmental samples water and waste

water are the principal matrices. However, during the last years methods for the determination of antibiotics in soil and manure gain in importance (see table 4 in section “chromatography and detection”).

The first application of LC-MS for the analysis of sulfonamides dates from 1982 and reports on the detection of residues in urine und plasma from racehorses as described by Niessen [68] in his review on the analysis of antibiotic and antibacterial compounds. For environmental samples LC-MS was not used for analysis of antibiotics. According to Höper [69] no selective and sensitive analytical method for determination of antibiotics residues in complex matrices such as manure, soil or seepage existed before 1999. First protocols for the application of LCMS for antibiotic analysis in environmental matrices were developed for the determination of tetracycline by the Hamscher group [1].

Based on available information in the literature a variety of methods has been proposed for the analysis of tetracycline in biological matrices [106]. Also in the food- or feed samples the HPLC methodology has been applied increasingly. However, the poor recoveries necessitate an improvement of existing methods. Recently Hamscher et al. [1, 186] described an improved method for the determination of persistent tetracycline residues in soil. In 2005 a few reports on the quantification of tetracycline in soil were published [122, 123]. The obtained recoveries in these studies varied between 33.0 – 75.9% and were concentration and soil dependent. The chemistry of tetracycline is very complex, due to the fact that the molecule is capable of a wide range of chemical reactions.

Because a methodology for the determination of tetracycline was not available at the start of the present study the analysis of CTC was set aside.

The following sections will treat in more details the extraction and chromatographic separation as well as the detection of sulfonamides known from literature and points out the open questions which were the basis for the analytical experiments in this thesis.

2.3.1 **Extraction**

2.3.1.1 Basics of extraction

Environmental samples are mostly not suitable for direct analysis. They are either too diluted (water) or too complex (soil, manure). For this reason sample preparation e.g. extraction is a very important step.

A number of techniques have been developed to improve some important criteria such as automatisation of the extraction technique, reducing the sample preparation time, reducing the solvent consumption and cutting the costs. Poole and Poole [48] described such techniques e.g., MAE (microwave solvent extraction), PLE (pressurised liquid extraction) or SFE (supercritical fluid extraction). PLE-based methods have the advantage over MAE that no additional filtration step is required. As soil is a complex matrix, exhaustive extraction is important to obtain high recovery rates.

The clean-up steps were simplified, because of the cleaner extracts provided by the modern extraction techniques. The high specificity of LC-MS/MS makes it possible to minimise clean-up procedures, and even a new trend is noticeable: no sample clean-up at all, possibly achieved by dilution [14, 51, 60, 65].

For the extraction of sulfonamides from environmental samples conventional liquid extraction is often used [14, 16, 17, 21, 23]. However, in the last years the new extraction technique PLE was increasingly used [3, 10, 25, 27, 75]. No reports could be found on the use of MAE as another modern and possibly advantageous extraction technique.

Some publications compared modern extraction techniques with classical methods, e.g. Soxhlet [32, 38, 43, 47, 48, 50, 52] and comparable efficiencies with acceptable reproducibility have been reported.

Poole and Poole [48] wrote about the modern extractions techniques: “The initial capital costs are high compared with conventional solvent extraction approaches, and although this will be paid back over time through a higher level of automation and sample throughput, for some the ‘sticker shock’ factor has resulted in a conservative wait-and-see attitude…In any case, hot solvent extraction in the form of MASE, ASE or SFE…are about establish themselves as the main tools for extracting solid environmental samples as we move into the next millennium”.

For those reasons PLE and MAE and not the traditional sample preparation techniques were used and compared in the present work. The extraction solvent must be able to solubilise the analytes from the matrix and minimise the co-extraction of other matrix components. The compatibility with the later treatment steps of such analytical technique is necessary [39].

Mixtures of low- and high-polar solvents generally provide more efficient extractions than single solvents if analytes with a wide range of polarities are extracted [39].

The choice of the solvent is an important step in the extraction procedure and dependent on the analyte or analyte-mix. With more than one analyte a compromise has to be found. The best solvent for sulfadiazine is not the most suitable one for the whole sulfonamides group. The most common extraction solvent described in the literature for sulfonamides group is ethyl acetate and ethyl acetate with additives [14, 17, 16, 23]. Other authors prefer methanol/water [10, 25], methanol and citric acid [3] or acetonitrile [21]. For the multi-residue method the most efficient extraction solvent mixture was water/methanol (50:50, v/v). Pure solvents are used very seldom.

The liquid-liquid extraction is realized mostly at room temperature. With PLE and MAE a high temperature increases the rate and extent of extraction. The reason is an increase in analyte solubility and a weakening of analyte-matrix interactions. In addition it allows fast and more complete extractions with a smaller solvent volume. The most common extraction temperature using PLE is 100 °C [10, 25] or 80 °C [27].

2.3.1.2 Extraction with superheated water (SWE)

In 2002 Smith [78] reported in a review on the extractions with superheated water. Water as a non-toxic and inexpensive solvent could be the new choice in the extraction procedure. He showed the application of SWE for the determination of alkylbenzenes, PAHs and PCBs in environmental samples with extraction temperatures between 50°C to 450°C, but mostly at about 200 °C. So far, there have been no reports of the applications of SWE in pharmaceutical analysis. The hot-water extraction procedure may be the method of choice for the liberation and quantification of extractable and probably bioavailable SDZ from soil. In the current work a few SWE were used to get an idea on the extraction power.

2.3.1.3 Pressurised liquid extraction (PLE)

Pressurised liquid extraction is also called accelerated solvent extraction (ASE). Until 2000, the most frequently used term was ASE. The Dionex Corporation (Sunnyvale, CA, USA) developed and commercialised the technique by introducing an extraction unit, ASE™ 200, that is used by many laboratories world-wide. The American Chemical Society has introduced the abbreviation PFE (pressurised fluid extraction) in their journals. PFE is also used by the US Environmental Protection Agency in their EPA Method 3545 [38]. Another term for PLE is PSE (pressurised solvent extraction). Most of the applications reported in the literature were performed with a Dionex ASE 200, until now the only commercially available PLE system [37].

Originally, the use of PLE was mainly focused on the extraction of environmental pollutants present in soil matrices, sediments, and sewage sludge [37, 38, 78]. Nowadays the ASE 200 is used not only in environmental analysis, but also in food analysis and synthetic material analysis and is a good alternative to the classical extraction techniques [40].

PLE is a technique with short extraction times and low solvent consumption and is totally automated. With this system, a solid sample is enclosed in a sample steel cartridge with a stainless steel frit at the outlet that is filled with an extraction fluid and used to statically extract the sample under elevated temperature and pressure conditions for short time periods. Compressed gas is used to purge the sample extract from the cell into a collection vessel.

With the ASE 200 system pressures up to 200 bar and temperatures up to 200°C can be applied and up to 24 samples can be extracted automatically.

PLE is also described in the literature as a good extraction tool for pharmaceuticals in soil by Schlüsener et al. [27], Christian et al. [10], and Jacobsen et at. [3] or for other substances in environmental matrices by Luque-Garcia et al. [28], Goebel et al. [25] and Otaka et al. [77].

2.3.1.4 Microwave solvent extraction (MAE)

Microwave solvent extraction is also known as Microwave-assisted solvent extraction (MASE).

The first applications of MAE were performed using domestic microwave ovens in the late 1980s, later several articles described applications where commercial extractors were used. The first application was related to the determination of PAHs and PCBs from soils and sediments. Since then, several other compounds have been extracted efficiently.

A lot of studies used MAE as an extraction technique in environmental and non environmental samples [32, 33, 34, 35, 36]. MAE has been successfully applied to the simultaneous extraction of toxic organic contaminants from different solid matrices, such as PAHs, PCBs, phenols and pesticides [34].

Table 2.

MAE as an extractions method in different compartments.

Matrix	Extracted substance	Reference/Year
Rubber	Plasticizer from polyethylene and polystyrene, softeners from PVC and other additives	[35] 2000
Soil	Carbamate pesticides	[34] 2003
Soil	Phenols	[32] 1998
TCMP Tongmaichongji	Bioactive constituents (danshensu, puerarin, ferulic acid)	[33] 2004
Soil, sediments, mussels	PAHs, PCBs, triazine herbicides	[36] 2000
Soil	Organ chlorine pesticides (BHC, DDE, Dildrin)	[43] 2004
Soil	Chlorophenols	[44] 2002
Fly Ash and Sea Sediments	Polychlorinated Biphenyls and Polychlorinated Dibenzo-p-Dioxins	[45] 2003
Soils, clays, sediments, sludges, solid wastes	Chlorinated pesticides, PAHs, PCBs, Chlorinated herbicides, Phenols, Organ phosphorus pesticides and chlorinated herbicides, Dioxins and furans	[46] 2000
Sediments	Short-chain chlorinated alkanes	[52] 2004

PVC polyvinyl chlorine; PAH polycyclic aromatic hydrocarbons; PCB polychlorinated biphenyl;
TCMP traditional Chinese medicinal preparation; BHC 1,2,3,4,5,6-hexachloro cyclohexane;
DDE 1,1-dichloro-2,2-bis(p-chloro phenyl)ethane)

None of them was used for extraction of pharmaceuticals. The numerous applications of MAE for the determination of different substances in environmental matrices are given in table 2.

MAE consists of heating a sample, mostly in liquid organic solvents with microwave energy. Microwave energy is a non-ionizing radiation that causes molecular motion by migration of ions and rotation of dipoles. The effect of microwave energy is strongly dependent on the nature of the solvent and the matrix. Camel [47] described the details of the principle of MAE, the effects of microwaves and the main applications of MAE to environmental samples.

MAE is a technique similar as PLE with short extraction times and low solvent consumption.

Although this technique is older than PLE, only a few studies compared the performances of PLE and MAE. In terms of extraction efficiency, both techniques were comparable for several pollutants from real contaminated soils [47]. Frost et al. [121] reported that PLE is less matrix-dependent than MAE. Camel [47] reported that MAE gives extraction efficiencies comparable to SFE and PLE, with similar extraction times.

2.3.2 Storage of samples and extracts

The effects of storage of environmental samples on the determination of antibiotics are only sporadically described in the literature. For example manure samples were both frozen and stored at -20 °C [14] or directly stored in the refrigerator at 4 °C [23]. Goebel et al. [25] freeze-dried sludge samples and stored them at -25 °C until analysis and Samuelsen et al. [60] kept their marine sediment samples until analysis at -20 °C.

Hamscher et al. [17] and Schlüsener et al. [27] stored their soil samples in the dark at 4°C while in the work of Christian et al. [10] the soil samples were frozen without a temperature declaration. Little information is also found in the literature on the influence of storage of the extracts from the environmental samples, e.g. Sorensen et al. [21] reported, that SDZ extracts from marine sediment were stable for at least 14 days when stored at 5-7 °C and Hirsch et al. [4] stored the water extracts after enrichment at -20 °C.

2.3.3 Chromatography and Detection

The chromatographic separation of different substances can be done essentially with the help of HPLC (high performance liquid chromatography), GC (gas chromatography) or CE (capillary electrophoresis). Other techniques like thin layer chromatography is not very often used in the environmental analysis. In contrast to the very few methods published on the determination of sulfonamides in environmental matrices many methods have been described for the determination of residues of sulfonamides in animal tissues, feed and food (table 3). A review of several analytical strategies for the screening of veterinary drugs in edible products was presented by Aerts et al. [71] and Gentili [72] presented 11 current LC-ESI-MS-MS (electro spray ionisation ESI) methods from different authors for determining sulfonamides residues in different food matrices. In these methods various extraction and clean-up techniques are utilized. The most widely used technique for quantitative analysis is high performance liquid chromatography combined with mass spectrometric [18, 22, 53-59] or UV detection [22, 64- 66, 79].

Also GC-MS or GC-MS/MS after derivatisation were used for the determination of sulfonamides [61-63]. The chromatographic techniques allow quantitative multi-analyte determinations and compound identification. In spite of the high resolving power of gas chromatography, liquid chromatography is the method of choice for determination of antibiotics, which are rather polar (like sulfadiazine) and sometimes heat sensitive.

Instead of HPLC, some authors also used capillary electrophoresis coupled with MS [67].

Thanks to improvements in analytical chemistry, many pharmaceutical compounds are more easily detected in the surface-water and waste-water environmental compartments [74].

The determination of pharmaceuticals in solid environmental matrices is not easy because these substances can be non-accessible due to sorption or biodegradation and in addition the very low concentrations make the detection difficult.

In the last few years the LC-MS/MS technique became the method of choice for determination of pharmaceuticals in environmental matrices [3, 10, 14, 17, 21, 23, 25, 75]. HPLC-UV was used sometimes [15, 16, 79] and GC/MS was used for groundwater and sludge samples [24, 75]. A review on the environmental determination of pharmaceutical compounds is given by Beausse [74].

For the separation of the sulfonamides with HPLC, the RP (reversed phase mode) was used. In all methods described in table 4 RP-C18 columns were used. The column length, diameter (i.d.) and particle size are variable. In environmental analysis most columns used were 150-250 mm in length and 2 mm i.d. with a particle size from 3-5 µm, except for some that had a length of 100 or 125 mm. In modern MS analysis the trend goes to the short columns with 100-125 mm in length and a particle size of 3 µm [84].

Analytical techniques and chromatographic properties for the determination of sulfonamides in environmental matrices are outlined in table 4.

Table 3.

LCMS/MS methods for detection of sulfadiazine in biological matrices.

	Matrix	API tandem MS-technique	Mobile phase	Recovery (%)	LOD/LOQ µg/kg	Reference Year
SDZ	Pig's kidney	ESI (+)- QqQ*	MeOH 7%, ACN 8%, 0.01 mol/L ammonium acetate in water 85%	71-77	Not reported	[53] 1994
SDZ	Milk	ESI (+)- QqQ	Water und ACN with 0.1% formic acid; gradient elution	72-96	LOD: 0.1-1	[54]1996
SDZ	Liver and kidney tissues	ESI (+)- QqQ	MeOH/ ACN/0.05M formic acid (10/15/75)	71-98	LOD: 30	[55]2000
SDZ	Kidney tissues	ESI (+)- QqQ	A: Water and 0.3% formic acid B: acetonitrile and 0.3% formic acid gradient elution	78-82	LOD: 5-14	[56]2000
SDZ	Milk	ESI (+)- QqQ	A: acetonitrile B: 10 mM ammonium acetate (pH 3.5): gradient elution	69-87	LOQ: 5-10	[57] 2001
SDZ	Honey	ESI (+)- QqQ	A: 0.3% formic acid and 5% acetonitrile in water B: 0.3% formic acid in pure acetonitrile: gradient elution	44-73	LODs dependent on the different kind of honeys	[58] 2002
SDZ	Eggs	ESI (+)- tandem ion trap MS	A: 0.1% formic acid in water B: methanol: gradient elution	83	LOC: 5-10	[59] 2002
SDZ and other	Bovine kidney	ESI (+)- QqQ	A: water with 0.3% formic acid B: acetonitrile with 0.3% formic acid: gradient elution	80,82-85,14 concentration dependent	LOQ: 27	[18] 2000
SDZ and TMP	Animal feed	1) HPLC-UV 2) LCMSMS APCI (+)- QqQ)	2) A: 1% acetic acid in water B: MeOH; isocratic elution 70%A/30%B	1) 30.6 2) 20.4	1) LOD 1160 2) LOD 52	[22] 2002

*QqQ triple quadrupole

API atmospheric pressure chemical ionisation; ESI electro spray ionisation

LOD limit of detection; LOQ limit of quantification

ACN acetonitrile; MeOH methanol

Table 4.
Analytical methods for detection of sulfonamides in environmental matrices. (Abbreviations see table 3).

	Matrix	**Analytical technique**	**Mobile phase for HPLC**	**Extraction**	**SDZ Recovery (%)**	**LOD/LOQ µg/kg**	**Reference**
SDZ and TMP	Marine sediment	LCMS/APCI (+)-MS	Acetate buffer and 10% ACN; isocraically	Liquid-liquid extraction with ACN	72	For SDZ LOD 4	[21] 2002
SDZ, AcSDZ and other	Manure	LCMS/APCI (+)-MS	A: 5% ACN in water with 0.1% HCOOH and 1mM ammonium acetate B: 0.1% HCOOH in ACN gradient elution	Liquid-liquid extraction with ethyl acetate	80.5	LOQ 5	[23] 2003
SDZ and other	Groundwater	LCMS/ESI-QqQ	A: 20mM ammonium acetate in water B: 20mM ammonium acetate in ACN/MeOH 2:1 gradient elution	SPE	Tap water: 25 Surface water: 14	LOD (ng/l) 9.1	[24] 2001
SDZ and other	Agricultural soils	LCMS/MS ESI (+) QqQ	Methanol with formic acid and water	Citric acid and Methanol PLE	48.5-85.8 soil and concentration dependent	LOQ: 1-10	[3] 2004
SDZ and other	Animal manure	LCMS/ESI (+) and ESI (-)	A: water with 1mM ammonium acetate and acetic acid and 10% ACN B: CAN Gradient elution	Liquid-liquid extraction with KOH and ethyl acetate	47-80 pH dependent	LOQ: >100	[14] 2002
SDZ, AcSDZ	Environmental water samples	LCMS/MS	Not reported	Online SPE	SPE recovery: 91	LOQ in nanopure water: 10 ng/l	[19] 2003
SDZ and other	Soil and manure	LCMS/MS Ion trap with ESI (+)	A: 0.5% formic acid in water with 1mM ammonium acetate B: acetonitrile Gradient elution	Liquid-liquid extraction with 1mM citrate buffer and ethyl acetate	Soil: 41.8 Manure: 89.9	LOQ Soil: 5 manure: 50	[17] 2005
SDZ	Soil	LCMS ESI (+)	A: 0.05 M ammonium acetate with 0.05% acetic acid B: acetonitrile Gradient elution	Liquid-liquid extraction with acetate buffer and ethyl acetate	85	LOD: 20	[16] 2005
SDZ and other	Municipal waste water	LCMS/MS ESI (+) Quattro LC (quadrupole-hexapole-quadrupole)	A: acetonitrile-water 3:97 with 1% formid acid B: acetonitrile-water 75:25 with 1% formid acid Gradient elution	Enrichment with SPE	SDZ: 49-94	0.8 µg/l	[15] 1999
SDZ and other	Sewage sludge	ESI (+)- QqQ	1) MeOH/Water containing 1% formic acid; gradient elution 2) (A) Water,10% ACN, ammonium acetate and (B) 80% ACN and 20% A; gradient elution	PLE Water/MeOH 50:50	1) For SDZ 63 2) For SDZ 83	For SDZ LOQ: 4	[25] 2005
SDZ and other	Swine manure			PLE mixture of 80% methanol with 0.2 mol L^{-1} citric acid (pH 3)	Concentrations dependent 59–73%		[70] 2006
SDZ and Metabolites	Pig manure	Turbo ionspray (+)-QqQ	A: 5% ACN in water with 0.1% HCOOH and 1mM ammonium acetate B: 0.1% HCOOH in ACN gradient elution	Liquid-liquid extraction with ethyl-acetate	Not reported	Not reported	[80] 2005

2.3.3.1 Mass spectrometry

Combined liquid chromatography-mass spectrometry (LC-MS) was originally developed as an alternative to gas chromatography-mass spectrometry (GC-MS). The field of applications of LC-MS mainly regards the determination of thermo labile compounds. The development of this technology started in the early 1970s. The theoretical and historical considerations are described in the encyclopaedia of analytical science [85] as well as in textbooks on MS and LC-MS [86, 87].

The combination of liquid chromatography and tandem mass spectrometry allows unequivocal identification of traces of antibiotics and antibacterial agents in complex biological matrices [72]. The power of a mass spectrometer as chromatographic detector lies in its capacity to determine, by means of the molecular weight, the precursor ion and its products of fragmentation. The combination of LC with mass spectrometry allows, in addition a more definitive identification than with LC only, namely the quantitative determination of compounds that, because they belong to the same class, could not be fully resolved with chromatographic methods. The European Commission states in the Decision 2002/657/CE [73] that "methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods".

The high selectivity and sensitivity of MS/MS allows analysis of more complex matrices and simplification of the sample preparation.

The summary of MS and MS/MS methods with HPLC-coupling for the analysis of sulfonamides in environmental and non environmental samples is presented in tables 3 and 4.

Ionisation/Ion sources

During the last 30 years different ionisation techniques were developed and used: moving-belt interface, thermospray interface, and particle beam interface. Nowadays the atmospheric pressure ionisation (API) with atmospheric pressure chemical ionisation (APCI), atmospheric pressure photo ionisation (APPI) and electro spray ionisation (ESI) are successful in use.

With API ionisation the ions are created and/or evaporated at atmospheric pressure outside the mass spectrometer (analyser) in the laboratory atmosphere in the ion source. The effluent from the HPLC is sprayed into the ion source [82].

Short characteristic of API sources:

ESI: the ionisation occurs in the liquid phase (in the spray) where ions might already be present or are created by application of a high electrical potential (3-5 kV) to the sprayer tip. Evaporation of the liquid is assisted by pneumatic pressure and by heat.

APCI: the first step is the heat-assisted evaporation of the solvent followed by a cascade of ionization reactions in the gas phase (vapour).
APPI: like APCI but photo ionisation is induced by means of a xenon lamp (10 eV).

Table 5.

Comparison of electrospray (ESI) with atmospheric pressure chemical ionisation (APCI.)

ESI	**APCI**
• Ions formed by solution chemistry	• Ions formed by gas phase chemistry
• Good for thermally labile analytes	• Good for volatile/thermally stable analytes
• Good for polar analytes	• Good for non-polar analytes
• Good for large molecules	• Good for small molecules

The ESI and APCI are the most successful ionisation techniques. With these sources it is possible to ionize the whole range of compounds from apolar to polar. ESI and APCI interfaces are the sources of choice to promote the ionisation of antibiotics; they complement each other well with regards to polarity and molecular mass of analytes [72].

Mass analyzer

Depending on the requirements (quantitative analysis, qualitative screening, structure determination) different mass analyzers are in use.
The quadrupole (single Quad), quadrupole ion trap (IT) and time of flight (TOF) are the analysers used most for the LC coupling, either alone or combined to give tandem mass spectrometers as the triple quadropole (QqQ or Q_1 Q_2 Q_3) and hybrid instruments, such as the quadrupole/time of flight (QqTOF), the quadrupole ion trap/time of flight (QITTOF) and the quadrupole/linear ion trap (QqQ_{LIT}).
The quadrupole has been the most widely used instrument for low resolving power applications for nearly 30 years. In terms of sensitivity and linear range, QqQ currently offers the best available performance for quantitative determination. For further improvement of MS-MS methods, the QqTOF mass spectrometer and the QqQ_{LIT} mass analyzer might be very promising approaches [72].

2.3.3.2 Quantification and selection of internal standards

The quantification of the analytes is often very problematic due to matrix effects. Components from the complex matrices can co-elute with an analyte and compete for ionization with analyte molecules.

The quantification of an analyte can be done in different ways, e.g. external standardization, standard addition (add a defined amount of the same substance) or internal standard addition. The simplest method of choice is the external standardization (calibration curve). In this method the occurrence of matrix effects is not compensated. Compensation may be achieved with the use of an internal standard (ISTD). However, it is only effective if the ISTD is subject to the same type and extent of matrix effect as the analyte. The ISTD should co-elute with the analyte. The best ISTD for LC-MS is the stable isotopically labelled analyte or a chemically very close homolog of the analyte which co-elutes with the analyte. Also standard addition is a possible quantification tool.

In the literature on determination of SDZ in environmental matrices some authors used external calibration methods [17, 23] others described the internal standard method as the preferred method [14, 15, 19, 20, 25]. The different calibration methods and standards used in the different matrices are shown in more detail in table 6.

Table 6.

Calibration methods for sulfonamides in environmental matrices.

Matrix	**Calibration method**	**References/Year**
Sewage sludge	Internal calibration curve with d4SMX as surrogate standard for all sulfonamides	[25] 2005
Soil and manure	External standard calibration	[17] 2005
Soil	Internal standard calibration with chloridazone	[20] 2005
Municipal waste water	Internal standard calibration with sulphaphenazole	[15] 1999
Manure	External standard calibration	[23] 2003
Animal manure	Internal standard calibration with sulfamethazine-phenyl-$^{13}C_6$ for all sulfonamides	[14] 2002
Environmental water	Internal standard calibration with isotope labelled sulfonamides for almost each analyte (sulfonamides)	[19] 2003

3. Materials and Methods

3.1 Microbiological methods

3.1.1 Design of the microcosm experiments

Microcosms were spiked with two different pharmaceuticals. In experiment I the influence of sulfadiazine (SDZ) and in experiment II the influence of chlortetracycline (CTC) as chlortetracycline hydrochloride on the orthic luvisol soil (see section "Characterising the types of soil used in the microcosm experiments") bacterial community structure and respiration was studied. Parallel to experiment I an experiment Ia with the gleyic cambisol was started. Both experiments (I and Ia) were used for the chemical analysis of sulfadiazine (and acetyl-sulfadiazine) during the incubation.

Experiment I and Ia with sulfadiazine (SDZ)

- Soil with annealed soil as control
- Soil with annealed soil and glucose (5 g/kg soil)
- Soil with annealed soil + 1 mg/kg SDZ
- Soil with annealed soil + 10 mg/kg SDZ
- Soil with annealed soil + 50 mg/kg SDZ
- Soil with annealed soil and glucose + 1 mg/kg SDZ
- Soil with annealed soil and glucose + 10 mg/kg SDZ
- Soil with annealed soil and glucose + 50 mg/kg SDZ

Experiment II with chlortetracycline (CTC)

- Soil with annealed soil as control
- Soil with annealed soil and glucose (5 g/kg soil)
- Soil with annealed soil + 1 mg/kg CTC
- Soil with annealed soil + 10 mg/kg CTC
- Soil with annealed soil + 50 mg/kg CTC
- Soil with annealed soil and glucose + 1 mg/kg CTC
- Soil with annealed soil and glucose + 10 mg/kg CTC
- Soil with annealed soil and glucose + 50 mg/kg CTC

Samples for microbial and chemical analysis were taken over a period of 64 days (some chemical analysis over 106 days).

3.1.1.1 Characterising the types of soil used in the microcosm experiments

The microcosm-experiments were carried out using two types of soil, a loamy sand and a sandy loam. The sandy loam is a typical orthic luvisol from the experimental test-site „Im Brühl", Jülich/Merzenhausen in Northrhine-Westfalia (NRW), Germany.

The loamy sand is a gleyic cambisol from the test-site Kaldenkirchen-Hülst, NRW, Germany.

Both soils were used as natural soils, as annealed soil (heat treated at 600 °C for 48 hours) or after sterilisation with gamma radiation (14 h with 2.5 kGy/h or a total dosis of 35 kGy).

The results of the physicochemical characterisation of both soils [41, 42] done by the Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Bonn and the Institut für Bodenkunde der Rheinischen Friedrich-Wilhelms-Universität Bonn are shown in table 7.

Table 7.

Physico-chemical characterisation on the soils from the two test-sites (means of two parallel measurements).

	Orthic luvisol	**Gleyic cambisol**
pH ($CaCl_2$)	7.0	5.35
WHC_{max}[%]	45.8	27.42
C [%]	0.92	0.99
Total N [%]	0.081	0.06
Sand, total [%]	3.3	73.3
Sand, coarse [%]	0.5	1.8
Sand, middle [%]	1.0	25.2
Sand, fine [%]	1.8	46.3
Silt , total [%]	80.6	23.1
Silt, coarse [%]	56.5	17.9
Silt, middle [%]	19.9	3.6
Silt, fine [%]	4.2	1.7
Clay [%]	16.1	3.6

3.1.1.2 Field sampling and microcosms experiments

10 soil cores (Ø 30 mm) were taken randomly from the soil surface to 30 cm depth. The upper 5 cm were discarded and the remaining cores were mixed and sieved (< 2 mm).

The microcosms consisted of 221 g of moist soil (200 g dry weight) in sterilised Erlenmeyer flasks. Antibiotics and glucose as additional carbon source (5 g/kg) were mixed with 10 g annealed soil. The annealed soil was blended with the moist soil to obtain homogeneously distributed final concentrations 1, 10 and 50 mg/kg soil (dw) of antibiotic.

Soil moisture was adjusted to 40% of the maximum WHC with sterilised water.

All microcosms (Erlenmeyer flasks) were plugged with sterilised cellulose, covered with aluminium foil and incubated at 20 °C in the dark. Soil moisture was regularly checked by weighing and deionised water was added if necessary.

Soil samples were taken from the microcosms at different times (for example 1, 7, 14, 48 days) in order to obtain a representative sample from the microcosms at each sampling.

5 samples of soil were taken randomly from different sites of the microcosm to obtain a sample of approximately 10 g and mixed well. An aliquot of 500 g was taken for DNA extraction and 5 g for the chemical analysis. The chemical- and DNA extraction from this soil was taken either within the same day or the samples were collected and stored at – 20 °C until further processing. All microcosm experiments were done in duplicate.

3.1.2 Determination of water content in the soil samples

The determination of water content in the soil samples was carried out gravimetrically following the method of Forster [90].

About 10 g of field-moist soil was dried overnight at 105 °C in an open container and cooled down for 1 hour in an exsiccator before weighing.

3.1.3 Isolation of bacteria

For the extraction of bacteria from soil 0.5 g sieved soil was suspended in 1.7 ml sterile Ringer solution (1/4 strength), and ground for 1 min. in a Mikro-Dismembrator S (B. Braun Biotech International, Melsungen, Germany) at 2500 rev/min. After centrifugation (2 min, 500g), the supernatant was diluted in decadal steps and plated onto Mueller-Hinton agar. Morphologically

different colonies were subcultured in 10^{-1} Standard 1 nutrient broth and plated again. Isolates obtained this way were considered as pure cultures when PCR products (universal primers see below) had only one band in DGGE. The bacterial isolates were tested for susceptibility against SDZ and CTC in agar diffusion disc tests.

3.1.4 Agar diffusion disc test

The agar diffusion test was carried out according to the German DIN 58940 standard [197]. In this test, small filter paper disks (Ø 6 mm) were impregnated with antibiotic solution (10 µl each, in duplicating concentrations from 0.25 to 32 ml/l and for SDZ additionally 64 ml/l). The soil bacteria isolates were spread on Mueller-Hinton agar. The disks were placed onto agar surfaces containing a lawn of the test organisms. After 48 h of incubation at room temperature the diameters of the inhibition zones were measured, including the diameter of the disk. Strains were considered susceptible when growth inhibition zones were >10 mm at the highest concentration (32 mg/l). Smaller zones (<10 mm) indicated a weak inhibition. If no inhibition zone was detectable, strains were considered to be resistant.

3.1.4.1 Bioavailability of CTC in soil slurry

Due to negative respiratory activity of CTC (no changes) in soil but good results in the growth inhibition test (concentration dependant growth inhibition) the bioavailability of CTC in soil slurries was tested.

In a separate experiment, the bioavailable fractions of CTC were determined using aqueous supernatants obtained from soil slurries of different concentrations (2, 10, 100 g soil in 100 ml milli Q-water) and after different incubation intervals (1, 2, 5, 10 min). The soil slurries were stirred and CTC was added to give final concentrations of 50 mg/l. Samples were taken after the respective time intervals and centrifuged (1 min, 10000g). The growth inhibition potentials of the supernatants were tested with the most CTC-sensitive soil bacterial isolate selected from the disc diffusion assays. The antimicrobial effective concentrations of CTC in the supernatants were calculated from a calibration curve obtained with standard CTC solutions (0.25-32 mg/l). The experiment was performed in duplicate.

3.1.5 Poured plate method

Soil bacteria were extracted from 0.5 g sieved soil as described above. After centrifugation (2 min, 500 g), the supernatant was diluted in decadal steps. 1 ml of the soil bacteria solution obtained this way was pipetted into a Petri dish with agar (Mueller-Hinton agar) containing antibiotic substances. The dishes were incubated at room temperature. The colonies having grown on the plates with different antibiotics concentrations were counted and photographed with the control plate (without antibiotics) after 48 h.

The agar solution was prepared according to the manufacturer's instructions. To the agar being still liquid at 40-45 °C the SDZ or CTC solutions were added. The final antibiotic concentrations were 3.2; 6.4; 16; and 32 mg/l SDZ or CTC. Every step was done under a clean bench to avoid contamination.

3.1.6 Soil respiration

In parallel to the soil microcosms, the soil respiratory activity was determined using a "Sapromat" (Voith, Heidenheim, Germany). Concentration of chemical amendments (SDZ, CTC and glucose) and incubation conditions were identical to those described in section microcosms design except a smaller amount of soil (75 g) was used.

The estimation is based on the measurement of the O_2 uptake during the incubation of soil in a closed system. The O_2 is delivered in the system electrochemically. A "Sapromat" consists of a regulated temperature water bath, containing the measuring units and an instrument for recording the results. A measuring unit consists of a reaction vessel (A) with a CO_2 absorber, an O_2 producer and a pressure meter (C). The produced CO_2 will be adsorbed by a NaOH solution. O_2 uptake during the soil respiration causes an underpressure. This is indicated by a pressure meter, which regulates the electrolytic oxygen production as well as the display and graphic recording (D) of the results. The O_2 uptake is shown on the display in milligrams of O_2 [90]. The figure 6 shows the schematics of a "Sapromat". The measurements were performed at 20 °C.

The microbial activity of CTC treated soil was measured for a period of 16 days. For SDZ treated soil the period was longer and accounted 40 days. The data obtained from the measurement equipment were calculated as the cumulative O_2 consumption (mg O_2/100 g soil). Experiments were done in duplicate.

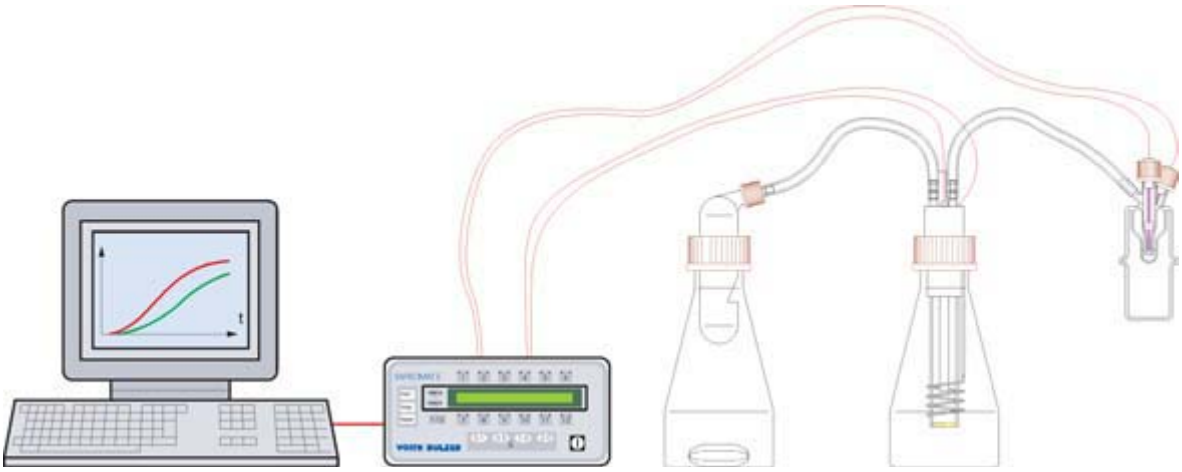


Figure 6.

Estimation of soil respiration in a "Sapromat® E" according H + P Labortechnik GmbH.

3.1.7 Molecular biological methods

To assess the response of soil bacterial community structure to the two antibiotics used, the molecular biological technique PCR-DGGE was applied.

The samples were obtained from the microcosm experiments with orthic luvisol and gleyic cambisol soil. The microcosms were prepared separately on the same day and from the same batch of soil as used for the respiration measurements. All incubations were done in duplicate and these duplicates gave identical banding patterns as shown exemplarily (figure 35) for samples taken after 6 weeks of incubation.

In this section the DNA extraction and purification, the polymerase chain reaction (PCR) and the denaturing gradient gel electrophoresis (DGGE) are described.

3.1.7.1 Total community DNA extraction and purification

DNA of the total community was extracted from 500 mg aliquots of sieved soil (<2 mm) using "Fast DNA SPIN KIT for soil, BIO 101" (QBiogene, Carlsbad, CA, USA). The extracted DNA was purified by using "Wizard® DNA Clean-Up System" (Promega, Madison, WI, USA). Extraction and purification protocols were according to the manufacturer's instructions.

3.1.7.2 Polymerase chain reaction (PCR)

The segment of eubacterial 16S rDNA from nucleotide 968 to 1401 (*Escherichia coli* numbering [222]) was amplified from soil DNA using an eubacterial universal primer set
U-968-GC and L-1401, described by Nübel et al. [107] for the detection of individual strains of *Paenibacillus polymyxa* and for the detection of soil bacteria by Ferris and Heuer [110, 111]. The GC clamp prevents complete melting of DNA during electrophoresis in the denaturing gradient polyacrylamide gels [112].

Used primers:

U968GC:

5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3'

L1401:

5'-CGG TGT GTA CAA GAC CC-3'

PCR was performed with the following approach:

Primer U968 10 pmol/ml	2 µl	MWG Biotech, Germany
Primer L1401	2 µl	MWG Biotech, Germany
20 mM dNTP mix	2 µl	ABgene, Surrey, UK
Reactionbuffer*	5 µl	ABgene, Surrey, UK
25mM $MgCl_2$	3 µl	ABgene, Surrey, UK
DMSO	1.5 µl	Sigma, Germany
TermoStart Taq Polymerase	0.25 µl	ABgene, Surrey, UK
DNA extract	1 µl	
Water (sterile, pure 18,2 MΩcm)	ad 50 µl	Milli-Q® Synthesis A10, Millipore

*Reaction buffer:

750 mM Tris-HCl, pH 8.8 at 25 °C, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween)

The temperature program was as follows:

	First denaturation step	10 min	95 °C
35 cycles:	denaturation	1 min	95 °C
	annealing	1 min	54 °C
	elongation	1 min	72 °C
	Final elongation step	10 min	72 °C

3.1.7.3 Agarose gel electrophoresis

The success of the PCR was tested by the addition of one positive control (pure DNA from *E. coli*) and one negative control (sterile, pure water) in every amplification. The quality of amplification products was checked with agarose gel electrophoresis (1.2% (w/v)). The wells were loaded with 5 µl PCR products and 1 µl loading dye (50 mM EDTA, 15% (w/v) Ficoll 400, 0.25% (w/v) Bromphenolblue, 0.25% (w/v) Xylencyanol, pH 8) and the electrophoresis was carried out in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) at 80 mA for 1 h. After electrophoresis the agarose gel was stained with ethidium bromide and documented using PDQuest (BioRad Laboratories, Hercules, USA). The length of the PCR products was checked with special molecular weight marker.

3.1.7.4 Denaturing gradient gel electrophoresis (DGGE)

DGGE of the PCR products was performed using a DCode system (BioRad Laboratories, Hercules, USA). The 6% (w/v) polyacrylamide gels (acrylamide-bis (37.5:1) were made with denaturing gradients ranging from 30% to 70% (100% denaturant contained 7M urea and 40% formamide; table 8). Gels were polymerised to a sheet of PAG film. Polymerisation of the denaturing gel was induced by adding 1 µl TEMED and 1 mg ammonium persulphate. The polymerisated polyacrylamide gels were placed in the 60 °C warm tank containing 1 x TAE buffer. The wells were loaded with PCR products and loading dye (0.25 ml Bromphenolblue, 0.25 ml Xylencyanol, 7 ml Glycerin, 2.5 ml pure water) and the electrophoresis was carried out at 100 V and 16 h. The running conditions were taken from the work of Bulawa [114] and Liebich [115].

The advantage of this system is that two gels can be run in parallel with a good reproducibility between different runs.

Table 8.

Composition of acrylamide solutions.

Acrylamide solutions		**0% denaturant**	**100% denaturant**
Acrylamide	ready-to-use solution 40% (37.35:1) for electrophoreses; Merck KGaA, Germany	15% (v/v)	15% (v/v)
TAE (50x)- buffer	Bio-Rad Laboratories Central Europe	2% (v/v)	2% (v/v)
Formamide	deionised min. 99.5%; Carl Roth GmbH & Co, Germany		40% (v/v)
Urea	min.99.5%, p.a.; Carl Roth Gmbh & Co., Germany		42% (v/v)

3.1.7.5 Silver staining

After DGGE the gels were stained with silver nitrate. During the staining the gels were completely submerged and carefully horizontally shaken. The silver staining protocol shows the individual steps (table 9).

Table 9.

Silver staining protocol.

Fixation solution	Ethanol	10% (v/v)	3 x 3 min.
	Acetic acid	0.5% (v/v)	
Staining solution	Silver nitrate	0.1% (v/v)	10 min, followed with careful rinse with water
Developing solution*	Sodium borohydride	0.01% (w/v)	10-20 min. develop
	Formaldehyde	0.15% (w/v)	
	NaOH	1.5% (w/v)	
Stop solution	Sodium carbonate	0.75% (w/v)	5-10 min.

*freshly prepared

After silver staining the gels were scanned (DUOScan f 40, Agfa) and analysed using image analysis GelCompar ® II (Applied Maths, Sint-Martens-Latem, Belgium) or Quantity One 4.3.1. (BioRad, Hercules, USA). By careful inspection of the lane intensity curves in combination with enlarged images of the lanes, bands were detected and quantified. Background intensity was subtracted. The position and peak intensity of each band relative to the cumulative intensity value of all bands in the

given lane was recorded and only bands which accounted for more than 1% of the total lane intensity were considered. For the calculation of similarity and cluster analysis of the DGGE patterns the Dice correlation matrix and unweighted pair group method using arithmetic averages were used.
To analyse the effects of the different amendments of antibiotics on the bacterial diversity the Shannon-Wiener index was used.

3.1.8 Sterilisation of soil

A sterile product is one that is free of viable microorganisms. The sterility of individual items within a batch of sterilized products cannot be guaranteed in the absolute sense.
Through sterilisation of soil the microbial activity should be completely inhibited. Several methods for the sterilization are presented by Alef [90]:

- Sterilization by autoclaving
- Soil fumigation with chloroform
- Irradiation of soils with gamma irradiation
- Inhibition of microbial activity by azide, cyanide and toluene.

By autoclaving and fumigation of soil the soil structure is destroyed. The toxicity of cyanide and azide is a big disadvantage of the last method. The main advantages of irradiation are that no disturbance of soils occurs during this process and no toxic substances are used. Alef recommends a dose of 2.5 Mrad for soils samples.
Trevors [205] described numerous methods for soil sterilization and inhibition of microbial activity in detail. He reported that sometimes even a higher dose as 2.5 Mrad is used for sterilisation of soil, for example in Canada, where 4 Mrad was used.

3.1.8.1 Gamma irradiation and sterility testing

According to ISO 11137:1995(E) [88] radiation is a physical process, involving the exposure of the subject to ionizing radiation. The subject is exposed to gamma rays from cobalt 60
(^{60}Co) radionuclides or coesium 137 (^{137}Cs) radionuclides, or to an electron or x-ray beam from an electron beam generator. When properly applied, radiation sterilization is a safe and reliable process.
According to DIN EN 552 medicinal products are treated with 25 kilo Gray and according to ISO 11137 [88] the sterility should be tested microbiologically. Soybean Casein Digest Broth, with an incubation temperature of 30 ± 2 °C and an incubation period of 14 days, is generally recommended for sterility test when a single medium is used.

In accordance to ISO 11137 [88] method using bioburden information exist. This method of choosing a sterilization dose is based upon experimental verification that the response to radiation of the microflora in the subject is higher than that of the microbial population standard resistance. Also food products can be treated with gamma-radiation, for reason of preservation or in order to kill pathogenic microorganisms as for example: salmonella.

At an irradiation dose of 10 kilo Gray all Gram negative bacteria and fungi, and most Gram positive bacteria, but not spores and virus will be affected [89].

In the current work gamma irradiated soil was used as control soil for studies on degradation of SDZ. The sterilisation of the soil samples was done by the gamma irradiation. The soil samples in the PE-bottles were irradiated with 35 kilo Gray for 24 hours at maximal 45 °C at the Research Reactor Division of the Research Center Jülich.

The sterility test of the soil samples was tested according to the sterility test DAB 10 V.2.1.1 [206] using soy peptone-caseine peptone-medium (CSB).

3.2 Analytical methods

3.2.1 Selection of solvents for the extraction of antibiotics from soil

The following solvents were considered as appropriate and the extraction potential of different extraction solvents was tested: methanol/water, acetonitrile/water and acetone/ acetonitrile/water in different proportions.

Together with the solvent selection, the extraction temperature was investigated; of special interest was a comparison between 80 °C and 100 °C.

For the extraction of SDZ from soil a solvent mixture had to be chosen, which was suitable for both PLE and MAE.

Portions of 5 g soil (moisture 12% of the maximum WHC) were spiked with SDZ (concentration of SDZ was 3.33 mg/kg) and extracted with: Acetonitril/Water 50:50 (v/v); Acetonitril/Water 80:20 (v/v); Methanol/Water 50:50 (v/v); Methanol/Water 80:20 (v/v); Acetonitril/Aceton/Water 45:45:10 (v/v) at 80 and 100 °C.

All sample extractions were carried out five fold as described below and were analysed with LCMS/MS.

3.2.2 Soil extraction

3.2.2.1 Soil extraction with ASE

The extraction of SDZ by PLE was performed using an ASE 200 system from Dionex (Dionex, Idstein, Germany). The system was operated with 24 pressure resistant steel extraction cells with a volume of 11 ml and lined with cellulose-fibre filters from Macherey & Nagel (MN GF-2; Ø 19 mm, Düren, Germany). Soil samples (orthic luvisol soil) of about 5 g from the microcosms were filled into the extraction cells between the cellulose filter and layers of diatomaceous earth (Bulk Isolute ® Sorbent; Isolute® HM-N; Separtis GmbH, Germany), spiked with SDZ (final concentration 3.33 mg SDZ/kg soil) and extracted with the respective extraction solvent. The extracts were flushed into the sample collection vial. The conditions for the ASE are given in table 10.

Table 10.

Example of ASE extraction conditions (for 100 °C and ACN/Water 80:20 (v/v)).

Extraction solvent	ACN/Water 80:20 (v/v)
Temperature	100 °C
Pressure	1500 psi (10 MPa)
Heat-up time	5 min
Static extraction time	8 min
Static cycles	2
Cell flushing	50% cell volume
Purge time	60 sec

PLE extracts (approximately 20 ml) were flushed into the scale bottle and diluted with 5% acetonitrile in water with 0.1% formic acid (HCOOH) (v/v) to 25 ml. After mixing an aliquot for the liquid chromatography mass spectroscopy (LCMS/MS) measurement was filled in the vial.

3.2.2.2 Soil Extraction with MAE

For the extraction of SDZ with MAE (close microwave digestion system Ethos SEL, Milestone Inc., Shelton, CT, USA) pressure resistant glass in Teflon (PTFE) extraction vessels lined with cellulose-fibre filters were used. This instrument is able to extract 6 samples concurrently. Homogeneous mixing of the sample is achieved by a special built-in magnetic stirring.

About 5 g of the orthic luvisol soil samples from the microcosms were filled into the glass cells on a layer of diatomaceous earth and the cellulose filter, spiked with SDZ (final concentration 3.33 mg

SDZ/kg soil) and 5 ml ACN/ water 80/20 was added. The glass cell was transferred into a teflon jar filled with 5 ml ACN/ water 80/20 and closed. The extraction was carried out 10 min. static (after the heat-up period) at 100 °C and the current intensity was automatically adapted.

In the current work a system with 6 vessels and fibre optic temperature control up to 300 °C was used. Associated EasyControl Software (Milestone Inc., Shelton, CT, USA) worked with sophisticated process control algorithms; the Ethos MAE can thus precisely follow the temperature curve (temperature increase, static temperature and time) by continuously modulating the microwave power for precise extraction (± 1°C). Declarations of pressure or power were not required.

After the extraction procedure the extract was sucked out the teflon jar. A twofold extraction as with PLE was not possible because of clogging in the glass cell. Analysis of the extracts was as described for PLE-extracts.

At the start of this work, only the microwave extraction was available at the institute. The optimisation of the extraction method with PLE was carried out at the Institute of Plant Nutrition (Dr. R.J. Schneider), University of Bonn.

3.2.3 Chromatography and Detection

The measurement of soil extracts was carried out using liquid chromatography separation coupled with mass spectrometry detection HPLC-MS/MS.

The method of Pfeifer et al. [4] was utilized and optimised. The separations according to Pfeifer et al. were performed using a Luna RP18 column (2mm i.d., length 150 mm, particle size 3 µm) and a SavetyGuard (Phenomenex, Torrance, CA, USA) at 30°C. The flow rate was 0.3 ml/min. The HPLC gradient was produced by using two mobile phases: phase A consisted of 5% acetonitrile in water (v/v) with 0.1% HCOOH (v/v) and 1 mM ammonium acetate and phase B consisted of 0.1% HCOOH (v/v) in pure acetonitrile. Chromatographic separation was achieved with the following gradient: 0 min 5% B, 1.5 min 20% B, 5.5 min 40% B, 6 min 100% B, 8.5 min 100% B, 9 min 5% B, 16 min 5% B. Ten µl of each sample were injected. For the mass spectrometry an APCI 2 source was used.

With this method a wide tailing occurred. The abandonment of 1 mM ammonium acetate in the phase A combined with a lower flow rate and a longer run time solved this problem.

The separations were done using the same C18 (2) column LUNA (Phenomenex, Aschaffenburg, Germany) as described above by Pfeifer et al. [4] with a security guard column C18, length 4 mm and 4 mm i.d. (Merck, Darmstadt, Germany).

The HPLC system (AGILENT HP 1100, Binary gradient pump version 1.3 (Agilent, CA, USA) consisted of an autosampler CTC Analytics HTC PAL, Version 1.3.1 (Chromtec Düsseldorf, Germany), a column oven AGILENT and degasser Chromtech (Düsseldorf, Germany). The massspectrometric detection was done with a triple quadropole mass spectrometer (TSQ Quantum 1.0 SR1; Thermo Electron, San Jose, USA) (figure 7).

Except for the comparison of the ionisation modes (APCI and ESI), the normal ionisation source used was ESI operated in a positive ion mode (ESI +).

The purity of nitrogen for MS operation was 99.5%. As collisions gas argon 5.0 (Messer, Griesheim, Germany) was used.

The software for the LCMS/MS system was X-Calibure Version 1.3 (Thermo Electron, San Jose, USA). The separation and detection conditions are given in tables 11 and 12.

Table 11.

Separation conditions of the HPLC method.

Eluent	A: 5% acetonitrile in water with 0.1% HCOOH (v/v) B: acetonitrile 100% with 0.1% HCOOH (v/v)		
Gradient	Time	Eluent A [%]	Eluent B [%]
	0	95	5
	3	95	5
	8	75	25
	11	45	55
	13	5	95
	18	5	95
	21	95	5
	33	95	5
Flow rate	0.2 ml/min		
Column temperature	25 °C		
Injectionvolume	5 µl injection loop		

Table 12.

ESI settings.

Parameter	Setting
Spray voltage	4100 V
Sheath gas pressure	45 arbitary units
Aux gas pressure	10 arbitary units
Capilary temperature	160 °C
Capilary offset	35 V
Scan Width	0.2 m/z
Scan Time	0.4 s
Collision Gas Pressure	1.5 mTorr
Tube Lens Offset	138 V
Mode	SRM

Figure 7.

The Triple Quadropol LC-MS.

3.2.3.1 Qualitative and quantitative analysis

The identification of SDZ and acetyl-SDZ was made by comparing retention times and substance specific mass spectra using liquid chromatography separation coupled with mass spectrometry detection HPLC-MS/MS (triple quad instrument); (figure 7).

The quantification of SDZ and acetyl-SDZ was done by comparing the intensity of the characteristic fragmentation pathways of SDZ and acetyl-SDZ.

SDZ Parent Mass (H+) 251.1
Product Mass 156 (22 CE) 108 (32 CE) 92 (34 CE)
D4-SDZ Parent Mass 255.1
Product Mass 160 (22 CE) 112 (34 CE) 96 (34 CE)
Acetyl-SDZ Parent Mass 293.3
Product Mass 198 (22 CE) 134 (30 CE) 108 (36 CE)
(CE= Collisonenergy)

The retention times of SDZ and acetyl-SDZ and the product ion spectra of the substances are shown in figure 8.

For the quantification of SDZ an isotope labelled sulfadiazine (SDZ - D4) was added as internal standard. Due to the identical behaviour of analytes and internal standards during measurement (figure 9), problems with ion suppression effects in the MS were eliminated.

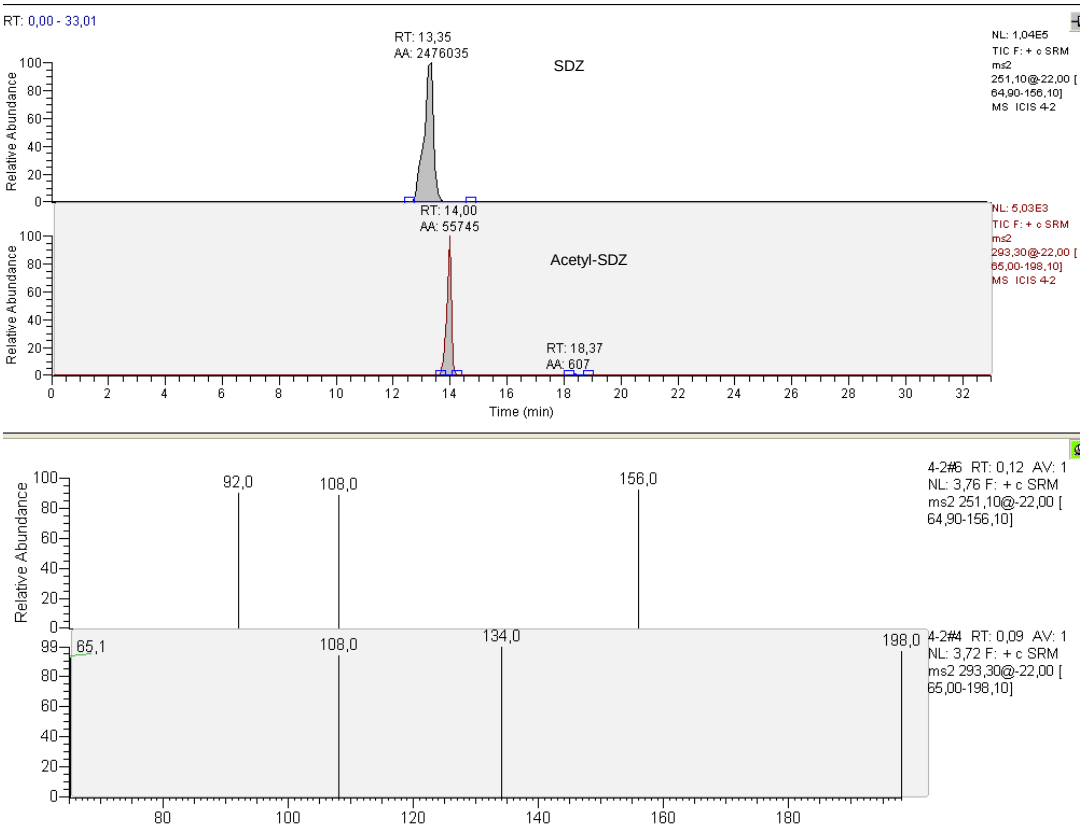


Figure 8.

LC-MS/MS chromatogram and product ion spectra of SDZ and acetyl-SDZ.

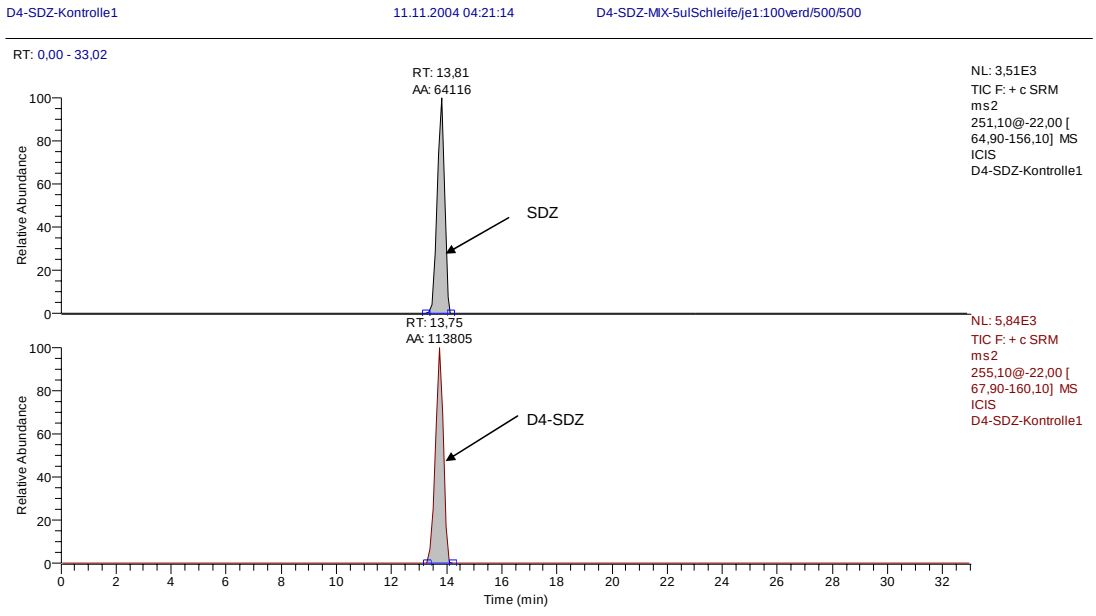


Figure 9.

Chromatograms of pure SDZ and the internal standard D4-SDZ .

The internal standard was added after completing extraction procedures, but prior to LC-MS analysis. Samples were fortified with the internal standard at a concentration of approximately 0.02 to 0.2 µg/ml. The peak areas from SDZ and SDZ-D4 were proportional and SDZ concentrations were calculated. All calculations were done using the following equations:

- The correction factor (CF) was calculated using the integrated peak areas and the known concentrations of the both substances:

$$CF = \frac{Conc.(SDZ) \bullet Peakarea(SDZ - D4)}{Conc.(SDZ - D4) \bullet Peakarea(SDZ)}$$

- The concentration (M) of the unknown amount of SDZ in the samples was calculated using the known amount of added SDZ-D4 and the integrated peak areas of SDZ and SDZ-D4:

$$M = \frac{Amount(SDZ - D4) \bullet Peakarea(SDZ) \bullet CF}{Peakarea(SDZ - D4)}$$

3.2.3.2 Selection of ionisation technique (ESI or APCI)

The accuracy and stability of the measurements were compared using both ionisation sources. This was done by measuring the analyte repeatedly.

A standard solution of SDZ in ACN with a concentration of 0.65 ng/ml was injected up to 20 times. Results should also give limits on handling and maintenance of the ionisation sources.

3.2.4 Stability of SDZ in soil

Soil samples of 5 g orthic luvisol soil (dry: moisture 12% of the maximum WHC and moist: 40% of the maximum WHC) were spiked with SDZ (concentration of SDZ was 3.33 mg/kg) and extracted with PLE and MAE (conditions see above) and analysed with LCMS/MS.

3.2.5 Storage of samples

Soil samples were stored at –18 °C up to the maximum of two months.
Extracts of soil samples were stored for 96 days at + 4 °C in a refrigerator and at – 18 °C and – 80 °C in a deep freezer.

3.2.6 Measurements of SDZ and acetyl-SDZ in microcosms

All samples from the microcosm experiments (see: Materials and methods/Design of the microcosm experiments/microcosm design) were periodically sampled and analysed in duplicate.
In experiment I the samples were taken after 1, 7, 10, 17, 27, 37 and 64 days. In the repetition of the experiment I the intervals were: 1, 7, 14, 21, 30, 64 and 106.
The microcosms were filled with SDZ-free orthic luvisol soil.

3.2.7 Statistical analysis and validation

Validation is the procedure which is used to prove that a test method consistently yields what is expected. In order to get appropriate results, adequate accuracy and precision during validation are required.
According to the requirements of the validation of an analytical method the following validation characteristics were measured:

- Precision and linearity
- Recovery
- Limit of Detection and Limit of Quantification (LOD, LOQ)

Outlier values were analyzed with the outlier test according to Grubbs.
ANOVA was used to test statistical significance P.
LOD and LOQ were calculated with B.E.N. Version 1.0 for Microsoft Excel according to DIN 32645 [9].
Graphical illustrations of the data were done with Origin 7G (OriginLab Corporation, Northampton, USA).

Coefficient of variation (CV)

For the precision of the method the coefficient of variation was analysed.

The coefficient of variation was calculated with Excel according to the following equation:

$$CV = \frac{s}{\bar{x}} \times 100 \qquad (1)$$

Outlier test according to Grubbs

Significant differences were calculated according to the following equation:

$$PW = \frac{\left| x^* - \bar{x} \right|}{s} \qquad (2)$$

Decision:

	PW ≤ rM (n, P = 90%)	random difference
rM (f, P = 90%) <	PW ≤ rM (n, P = 95%)	probable difference
	PW > rM (n, P = 95%)	significant difference

Precision and linearity

Precision describes the degree to which data generated from replicated or repetitive measurements differ from one another.

The bigger the relative standard deviation the poorer is the precision of the measurement. These figures give a good indication of how stable the actual measurement system is, what the condition of the equipment is and how well selected the possible integration parameters are [5].

Which precision is acceptable depends on the complexity of the method, the matrix and the concentration of the analytes. In quality control of pharmaceutical products the precision of the method (CV) should be within 1 or 2% deviation. However, in the environmental analysis a practical limit (CV) of 10 to 15% is quite acceptable [12 and 13].

Instrumental precision of the measurement with LCMS/MS

[1] x^*: Data point; $\bar{x}$: Arithmetic mean; s : Standard deviation

[2] *PW*: Examinationquantity; *rM*: Grubbs-table value

The instrumental precision of the measurements was assessed using SDZ standards in pure acetonitrile (0.375, 0.75, 7.5, and 25 ng/ml) and an average of six independent injections of each concentration.

Influence of the matrix on the precision of the method

SDZ free soil samples of 5 g were extracted and the extracts enriched with 25, 7.5, 0.75, and 0.375 ng/ml SDZ. For the precision assessment an average of six injections of each concentration was used.

Linearity

Quantitation requires that one knows how the response measured depends on the analyte concentration. This knowledge is obtained using external or internal standardisation and formulated into a mathematical expression. The calibration equation reads as follows:

$$y = bx + a \qquad (3)$$

For the proof of linear results of measurements, the samples used for the assessment of precision were used and the calculation conducted with ORIGIN 7 G.

Recovery in soil

The recovery of analytes is influenced by such factors as concentration of the analytes, sample matrix, and time of storage. Because recovery often varies with concentration, the spiked and the analyte concentrations should be as close as found in practical samples. Matrix effects can cause wide variability in recoveries, especially with organic compounds (e.g. in soil). Therefore, to be valid, recoveries of a spiked standard must be determined in the same matrix as the sample.

According to the Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry [6] recovery is derived from the measurement of „spiked blanks“. These may be controls or simulated field samples containing a variety of known added concentrations of the analyte. The recovery is calculated according to the equation:

$$\%\ recovery = \frac{C(found)}{C(added)} \times 100$$

C (found) is based on the net analyte signal for the “spiked” blank.

[3] y: measured response (peak area); x: concentration; b: slope of calibration curve = sensitivity; a: intercept

All experiments on recovery were carried out fourfold with orthic luvisol and gleyic cambisol soil from Merzenhausen and Kaldenkirchen. Those soils were previously checked on the occurrence of sulfadiazine residues.

As a negative control the orthic luvisol soil was used, as no sulfadiazine residues were detectable. For recovery SDZ was spiked in three concentrations: 1 mg/kg, 10 mg/kg and 50 mg/kg soil. Prior to spiking the SDZ to natural moist soil, an amount of SDZ standard solution dissolved in acetonitrile was mixed with annealed soil and after evaporation of the acetonitrile. The whole soil sample was than homogenised and immediately extracted. The extracts were prepared as described before and measured with LCMS/MS.

Limit of Detection (LOD) and Limit of Quantification (LOQ).

LOD and LOQ were examined according to Principles of Environmental Analysis [7].

The limit of detection is defined as the lowest concentration level that can be determined to be statistically different from the blank.

The limit of quantification is defined as the level above which quantitative results may be obtained with a specified degree of confidence. Confidence in the apparent analyte concentration increases as the analyte signal increases above the LOD.

LOD- and LOQ-values were determined for SDZ-solutions without soil and after the extraction of soil containing SDZ to get these values for the entire method [8].

Therefore separate calibration curves in the range of (0.001- 1000 ng/g) were made according to DIN 32645 [9].

4. Results and discussion

4.1 Optimisation of the Method for the Measurement and the Recovery of Sulfadiazine from soil

4.1.1 Optimisation of extraction solvent and temperature

For the recovery of SDZ from soil different mixtures of extraction solvents and extraction temperature were tested with both, Microwave solvent extraction (MAE) and Pressurised liquid extraction (PLE). The results are presented in table 13 and figure 10.

Table 13.

The influence of variation of the extraction solvent mixtures, extraction temperature and extraction device on recovery of spiked orthic luvisol soil. The recovery is given as mean with standard deviation(s) and coefficient of variation (CV) (n=3).

	Extraction	PLE			MAE		
	temperature (°C)	recovery (%)	s	CV	recovery (%)	s	CV
A/A/W 45/45/10	100	107.88	15.91	14.75	95.05	2.23	2.35
A/A/W 45/45/10	80	92.52	4.74	5.12	90.92	2.94	3.23
Me/W 80:20	100	91.81	10.84	11.81	98.28	11.8	12.01
Me/W 80:20	80	95.17	1.11	1.17	96.69	0.52	0.54
Me/W 50:50	100	80.38	4.38	5.45	95.26	4.47	4.69
Me/W 50:50	80	85.39	8.11	9.50	85.9	12.88	14.99
A/W 80:20	100	82.35	1.54	1.87	94.32	1.63	1.73
A/W 80:20	80	86.64	7.1	8.19	92.43	7.86	8.50
A/W 50:50	100	89.34	7.19	8.05	100.51	4.14	4.12
A/W 50:50	80	83.92	2.92	3.48	85.6	3.66	4.28

A/A/W: Aceton/Acetonitril/ Water

Me/W: Methanol/Water

A/W: Acetonitril/Water

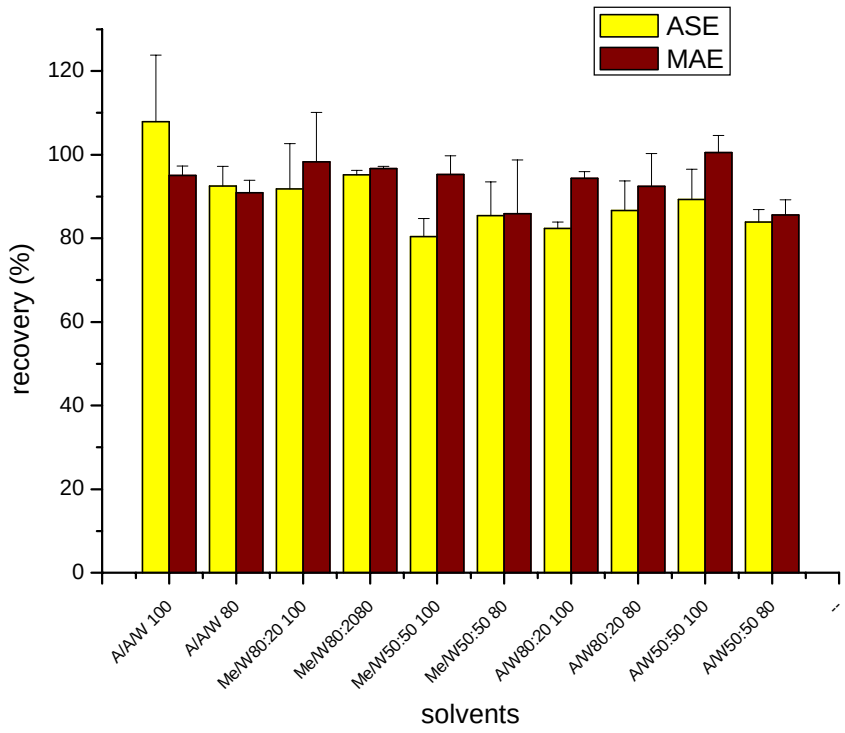


Figure 10.

The influence of a variety of the extraction solvent mixtures, extraction temperatures and extraction device on recovery of SDZ spiked orthic luvisol soil. The recovery is given with standard deviation (n=3), with error bars indicating standard error of difference.

A variety of solvents and solvent mixtures were tested to find the most efficient extraction medium for sulfadiazine. Each recovery value reached more than 80%. MAE recovery was between 85.6% and 100.51% with variable CV. However, two mixtures with CV > 10% were dropped; but the 7 mixtures left were all strong and suitable extraction solvents. The selection for an extraction solvent equally suitable for both MAE and PLE, e.g. high recovery and high precision resulted in the choice of two solvents: methanol/water 80:20 (v/v) mixture at 80 °C and acetonitrile/water 80:20 (v/v) mixture at 100 °C extraction temperature. The applicable solvent mixtures do not correspond exactly to the until now known and used extraction methods (due to amount of examined substances). Extraction mixtures are described mostly for the extraction of many antibiotics and not for the optimum extraction of only one antibiotic. Although the recovery with acetonitrile/water decreases as with methanol/water, the acetonitrile/water mixture was selected because no further sample preparation for LCMS/MS was necessary.

With methanol/water mixtures evaporation of the solvent would have been required followed by

resolution in the eluent acetonitrile/water used in HPLC. Losses would have been possible and thus the acetonitrile/water mixture was a good compromise.

4.1.2 Comparison of extraction methods

The soil extraction methods MAE and PLE were compared concerning recoveries, reproducibility, amount of used solvent and time necessary for sample preparation and extraction.

The recovery using MAE was 92.43% (CV 8.5%) and using PLE 82.7% (CV 1.73%), however PLE showed a better reproducibility. As for environmental samples a precision of 10% is demanded [12, 13] the precision and recovery of both methods are acceptable. The same amount of solvent is required for the extraction in both methods. A problem with MAE however is the amount of soil. It is not possible, to take more as 5 g soil for extraction, because of clogging in the suction equipment and problems of extract filtration. If bigger amounts of soil need to be extracted, because of an expected low concentration of the analyte, the PLE has an advantage over MAE.
The MAE allows only one extraction procedure in contrast to the PLE, where up to three extraction steps are possible. The expenditure of time for the preparation of the extraction is the same for both methods, however MAE is more time consuming in total, because of the fact that the extracts have to be filtered. The PLE delivers ready filtered extracts. In addition PLE runs automatically.
Concluding remarks: Both methods are equally suitable techniques for extracting SDZ from soil and they are rather environmental-friendly due to a low solvent consumption. With MAE the filtration of the extract is necessary, resulting in an increasing preparation time. The PLE is faster and more cost-effective (no sample cleaning after extraction) due to reduced sample preparation time. The sample preparation scheme used is simple and easier to perform than the methods previously reported [23, 27].
In any case, hot solvent extraction in the form of MAE or PLE can be the main tool for extracting solid samples [48] and is a good and fast alternative to conventional extraction techniques. PLE was already used in the antibiotic extraction from soil by Jacobsen et al. and Stoob et al. [3, 237]. Probably the recovery for one of the extraction techniques (either for MAE or for PLE) can be increased by further optimisation of the extraction solvents.

4.1.3 Choice of the LCMS/MS ionisation source

To test both ionization sources a standard solution containing 0.65 mg SDZ/ml was injected 20 times.

The comparison of APCI and ESI showed that the APCI ionization source was instable especially after the heating up period. Maintenance and waiting time after cleaning were not very favourably for ESI. With APCI the relative standard deviation of the peak area obtained was 21.6%, while with ESI only 6.1%. Thus, results with ESI were much better which is probably also the reason why ESI is recommended in the literature (table 3 and 4). Jacobsen et al. and Stoob et al. [3, 237] also used ESI ionization method for LCMS/MS after extraction of sulfonamides from agricultural soil using PLE.

4.1.4 Stability cheque of stored extracts

The results of storage stability measured over a period of 96 days with LCMS/MS immediately after extraction and on day 21, 76, 85 and 96 are displayed in the figures 11, 12 and 13.

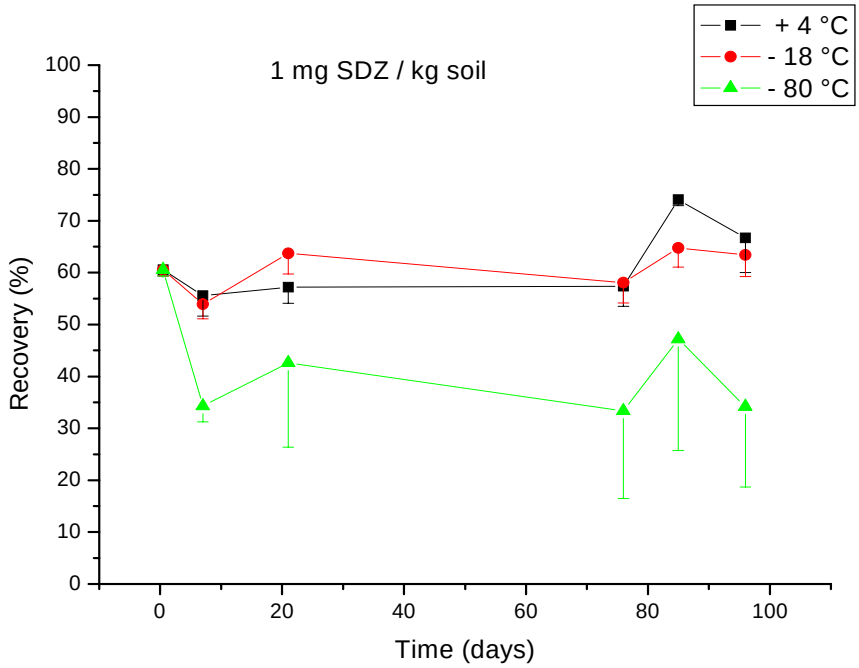


Figure 11.

Recovery of SDZ (1 mg/kg soil) after extraction and storage at 3 different temperatures. The recovery is given with standard deviation (n=3), with error bars indicating standard error of mean.

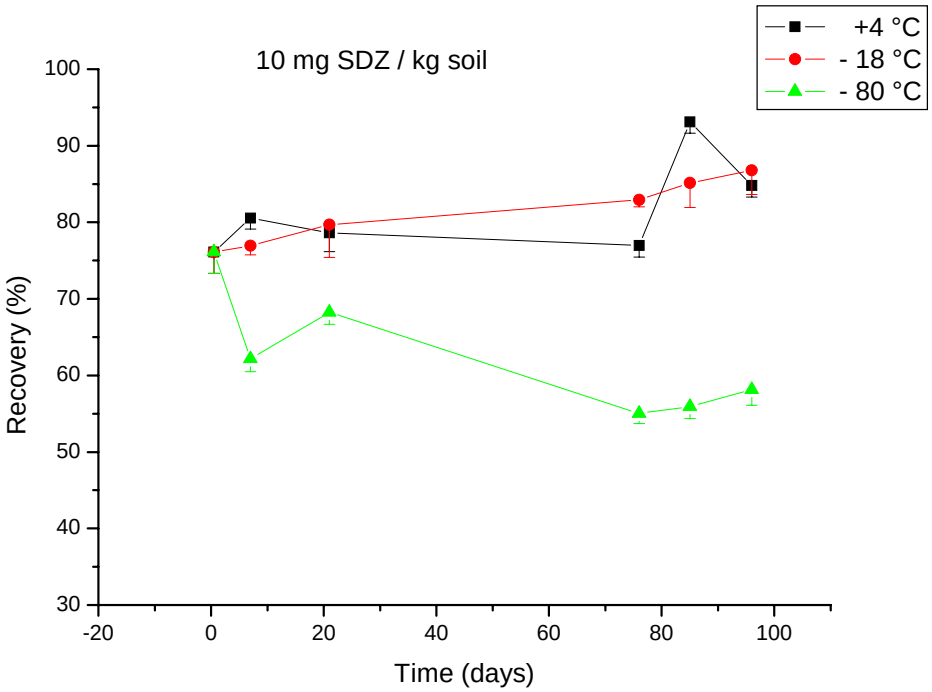


Figure 12.

Recovery of SDZ (10 mg/kg soil) after extraction and storage at 3 different temperatures. The recovery is given with standard deviation (n=3), with error bars indicating standard error of mean.

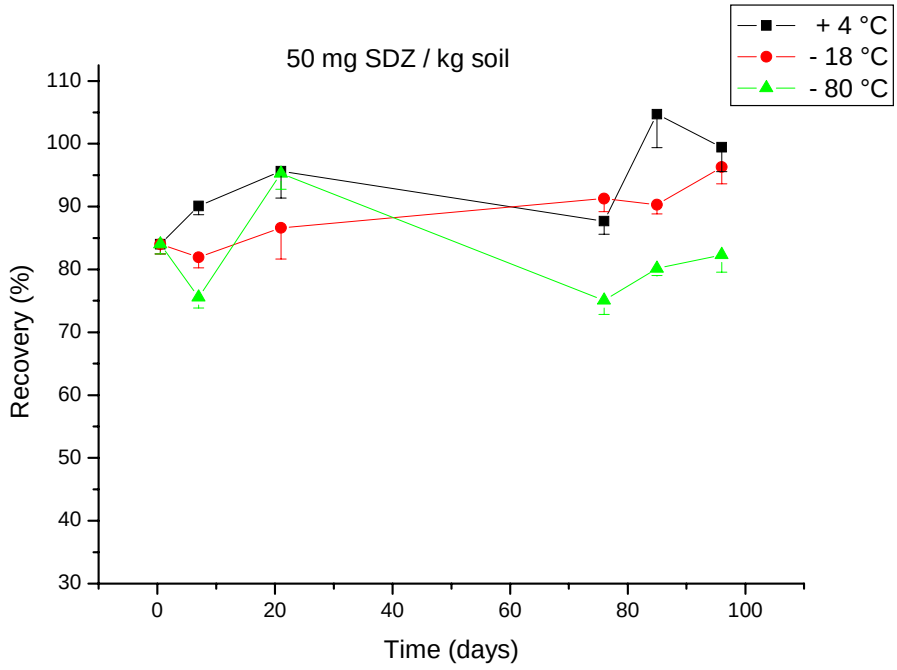


Figure 13.

Recovery of SDZ (50 mg/kg soil) after extraction and storage at 3 different temperatures. The recovery is given with standard deviation (n=3), with error bars indicating standard error of mean.

With 1 mg SDZ/kg soil the mean values of SDZ concentration at the storage temperatures of +4 and –18 °C were not significantly different while storage at –80 °C yielded significantly lower values. The relative standard deviation of the mean values was rather high.

The tendencies of the obtained results with 10 mg SDZ/kg soil are the same as with 1 mg/kg soil, but with a lower standard deviation at –80 °C. The mean values at 50 mg/kg soil did not differ significantly at all storage temperatures (table 14).

Table 14.

Differences in significance of different SDZ concentrations. Different letters indicate significant difference (P<0.05).

	+ 4 °C	**- 18 °C**	**- 80 °C**
1 mg/kg SDZ	a	a	b
10 mg/kg SDZ	a	a	b
50 mg/kg SDZ	a	a	a

As a preliminary result it is not recommended to store extract samples at –80 °C, if concentrations of SDZ in soil of 10 mg and below are expected.

Because SDZ concentrations as high as 50 mg/kg soil are not to be expected in environmental samples, the results of this experiment shows that storage of extracts at either + 4 °C or – 18 °C is advisable, if storage can not be avoided. Such an experiment was not described in the literature until now and thus not comparable with other findings. In most works the final extracts are applied directly to the analysis and not stored.

4.1.5 Durability of the analyte in frozen soil samples

The durability of SDZ in frozen samples at -18 °C for 70 days is shown in table 15.

Table 15.

SDZ concentrations measured directly after addition and after 70 days of storage at -18 °C.

	Fresh			after 70 days		
SDZ conc. [mg/kg soil]	**MV**	**SD**	**CV**	**MV**	**SD**	**CV**
50	82.50	2.47	2.99	82.49	6.24	6.71
10	83.12	1.54	1.85	80.71	7.65	9.48
1	87.02	2.95	3.39	53.72	3.60	7.56

MV: mean value % of added conc.

70 days of storage did not led to significant losses of SDZ in soils with either 10 or 50 mg SDZ/kg soil. In the sample with 1 mg SDZ/kg soil one third was lost after 70 days storage. However it is not clear, if the low end concentration measured was due to deep freezing and storage as such or due to soil adsorption during deep freezing and thawing.

In summary it can be said that there was no decrease of concentration due to deep freezing and storage at the higher concentrations of SDZ tested. Stoob et al. [237] described a usual practise to freeze and store the samples at -20 °C directly after collection. The storage stability was confirmed by the extraction of the reference soil sample 1 year after the first extraction resulting in almost identical values with maximal deviations of ± 15%. Also Accinelli et al. [238] stored the samples at -20 °C until they were analyzed.

The storage of samples at -20 °C seems to be a good compromise if direct extraction is not practicable. Soils with an expected concentration of 1 mg SDZ/kg soil and below should be extracted as soon as possible or supplementary monitoring with low SDZ concentration should be done.

4.1.6 Method validation

Precision and linearity

The results of the LCMS/MS precision measurement, determined by repeated injections of standard SDZ solutions, are shown in table 16.

Table 16.

Precision of LCMS/MS measurement (coefficient of variation; n=6).

Spiked amount of SDZ	25 ng/ml	7.5 ng/ml	0.75 ng/ml	0.375 ng/ml
CV [%]	2.76	2.83	2.79	2.66

The CVs at all measured concentrations were between 2.6% and 2.8% which is satisfactory. However, the results allow no conclusions on matrix dependent precision of the method.

Influence of the soil matrix on precision

The influence of the soil matrix on precision, tested by spiking different concentrations of SDZ to soil extracts, is presented in table 17.

Table 17.

Precision including matrix effects (coefficient of variation; n=5)

Added Sulfadiazin	22.5 ng/ml	6.75 ng/ml	0.675 ng/ml	0.337 ng/ml
CV [%]	2.71	4.08	3.37	6.27

The precision of the total method including soil matrix effects was between 2.7% and 6.3%. These values are somewhat higher than those obtained without matrix effects. Matrix effects were more pronounced in lower concentrations.

Linear calibrations curve was obtained with SDZ standard solutions and a regression coefficient (R) in the range of 0.99997 and a linearity range of 0.337-22.5 ng/ml.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD for SDZ in pure ACN was 43.67 pg/ml, the LOQ was 148.54 pg/ml (correlation coefficient: 0.999997; CV: 0.83%). These values show the high sensitivity of LCMS/MS measurements, proving that they are a good tool for the detection of low concentrations in environmental analytics. For validation in general however, these values are not very relevant because the sensitivity of the whole method, including extraction losses and matrix effects is crucial for LOD and LOQ estimations.

LOD and LOQ of the total method

The limit of detection for the orthic luvisol soil was 7.38 µg/kg and the limit of quantification 24.92 µg/kg (correlation coefficient: 0.999966; CV: 2.49%).

For the gleyic cambisol soil the limit of detection was 28.69 µg/kg and the limit of quantification was 24.92 µg/kg (correlation coefficient: 0.999966; CV: 2.49%).

Concluding remarks: A sensitive and selective quantification of the investigated antibacterial agent SDZ in soil was achieved in the present study by using LCMS/MS and ESI as an ionisation source. The SDZ amount applied was in the range of 1 to 50 mg/kg soil and concentration could be measured down to 30 µg/kg. The demonstrated LOQ and LOD were higher than the limits obtained by Jacobson et al. [3] probably due to the missing SPE clean-up step. The obtained LOD for orthic luvisol is comparable with results obtained by Stoob et al. [237] who also did not use a cleanup step. PLE followed by a SPE clean-up can improve the limits and achieves an excellent applicability for environmental samples in very low residue concentrations but was not necessary in this work, when high concentrations of SDZ were applied.

4.1.7 Recovery of SDZ in soil samples

As presented in table 18 the recoveries of spiked SDZ were between 82.5% and 87%. In addition to the recovery standard deviation (SD) was calculated as well as the coefficient of variation (CV).

Table 18.

Recovery of SDZ from orthic luvisol soil (n=6).

	Recovery		
	1 mg/kg	**10 mg/kg**	**50 mg/kg**
Recovery (%) meanvalue	87.02	83.12	82.50
SD	2.95	1.54	2.47
CV (%)	3.39	1.85	2.99

The mean recovery values for the three concentrations differ significantly (P = 0.00919). Nonetheless the obtained recovery values as well as the low CV and SD are acceptable for environmental analysis.

Since the recovery depended on both, matrix and contact time, and therefore could not be estimated for every single sample, no corrections were made for possible incomplete extractions in the microcosm experiments.

In conclusion, the response of SDZ residues detected in the LCMS/MS method was evaluated for linearity, and the LOD and LOQ for the instrument and LOD and LOQ for the whole method were determined. The validation data obtained in our recovery studies show that this method is suitable for the analysis of SDZ in soil. It is accurate, rapid and sensitive. No problems occurred with contamination of the LCMS/MS instrument or carry over from run to run. Due to lack of clean-up and possible matrix effects, the quantification was assured by a corresponding isotope labelled internal standard. The presented recoveries are satisfactory and comparable with Jacobsen et al. and Pfeifer et al. [3, 23], whose recovery was at the level of approximately 85% for SDZ. The validation shows that the soil extraction method used and described in this study is applicable for the compound analysed.

All in all, a good precision was obtained for the whole procedure including the following steps: sampling, spiking, extraction, spiking an internal standard and quantification by LCMS/MS.

4.1.8 Extraction with hot and superheated water

As routine method of the samples extraction acetonitrile (ACN)/water was used. It is not certain if this solvent extraction delivers the amount of analyte, that would be bioavailable to the bacteria. In the literature the possible bioavailable fraction of organic compounds is discussed in particular for pesticides like atrazine, phenanthrene, isoproturon, dicamba or carbaryl [187, 188, 189, 190], suggesting that extraction with calcium chloride ($CaCl_2$) or hot water delivers the bioavailable fraction, while extraction with methanol would give the potential bioavailable fraction [187, 188, 190]. Therefore we compared the hot water extraction with the ACN/water extraction (table 19 and 20).

Table 19.

Recovery of SDZ of the extraction with 100 °C water (due to n=2, the statistical error was set as the deviation of the mean from maximum and minimum value).

	Recovery		
	1 mg/kg	**10 mg/kg**	**50 mg/kg**
Mean recovery (%)	71.67	96.16	91.54
SD	5.51	9.9	1.23
CV (%)	7.69	10.29	1.34

Table 20.

Recovery of SDZ of the extraction with 200 °C water (due to n=2, the statistical error was set as the deviation of the mean from maximum and minimum value).

	Recovery		
	1 mg/kg	**10 mg/kg**	**50 mg/kg**
Mean recovery (%)	72.88	85.7	93.26
SD	5.3	2.32	2.29
CV (%)	7.27	2.71	2.46

The extraction of SDZ with hot water resulted in an extremely high recovery of the soils spiked with 10 and 50 mg/kg, suggesting a high bioavailability of SDZ in soil. The influence of the extraction temperature (100 or 200 °C) seems to be irrelevant. Due to a lack of literature data on hot water extraction of antibiotics, there was no possibility to compare this result with literature data except perhaps for the study of Stoob et al. [237] who extracted sulfonamides from soil with a mixture of buffered water and acetonitrile (85:15) at 100 and 200 °C. His results showed a five-fold increase in soil in extraction efficiency at 200 °C compared to 100 °C for aged residues. The tests with the addition of ACN revealed slightly higher extraction efficiencies compared to pure water. The authors concluded that water is the solvent of choice for sulfonamides because of the polarity of the sulfonamides and the thermal stability of the sulfonamides makes it possible to use such high temperatures for extraction. The presented results are similar to those obtained in this study, but additional experiments should be carried out for confirmation.

Water used as extraction medium in PLE is not only friendly to the environment, but also very easy to couple with the eluents used in HPLC. Problems with evaporation of the extraction solvent and renewed dissolving are avoided in this case. The extraction power of water should not be disregarded but more appreciated than in past studies. One possibility could be to repeat the chemical analysis of the extracted material and to analyse bioavailability in parallel.

4.1.9 Stability of SDZ in soil

4.1.9.1 Influence of contact time on recovery

The results of the stability of SDZ in dry soil followed over 140 hours are shown in figure 14.

The amount of SDZ recoveries varied between 3.17 mg/kg after 2.5 h and 2.86 mg/kg after 140 h. The latter amount was still 86% of the spiked amount of SDZ. The extractable fraction of SDZ decreased from initially 95% to 86% slowly and without sudden decrease.

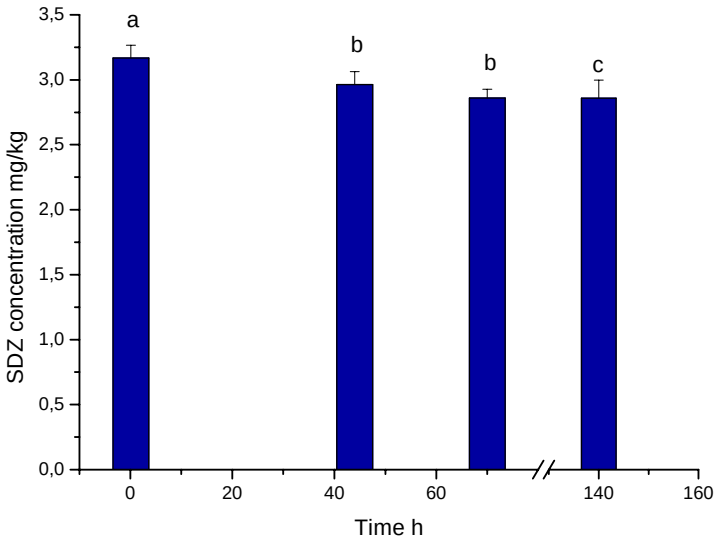


Figure 14.

Measured SDZ concentrations over a period of 140 h in dry soil. SDZ amount spiked was 3.33 mg/kg. Bars with different letters differ significantly (P<0.005). The recovery is given with standard deviation (n=3), with error bars indicating standard error of difference.

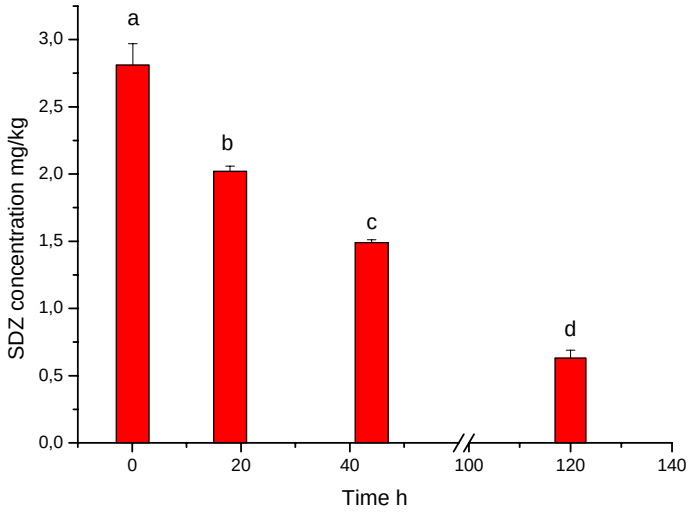


Figure 15.

Measured SDZ concentrations over a period of 120 h in moist soil. SDZ amount spiked was 3.33 mg/kg. Bars with different letters differ significantly (P<0.05). The recovery is given with standard deviation (n=3), with error bars indicating standard error of difference.

The results of the influence of the contact time on the recovery of SDZ were at first in contradiction with those of Wehrhan [113] and Kreuzig [228] who found that the non extractable fraction increases with incubation time. Also Hamscher [17] reported that the recovery of SDZ from spiked soil samples decreased time dependent from 74% to 18% for samples extracted 5 minutes or 7 days after spiking. The negligible losses of the extractable SDZ in our experiment were not consistent with the present knowledge. The explanation for the obtained results was the absence of soil moisture.
The experiment was repeated with moist orthic luvisol soil (40% of the maximum WHC) and the results are presented in figure 15.

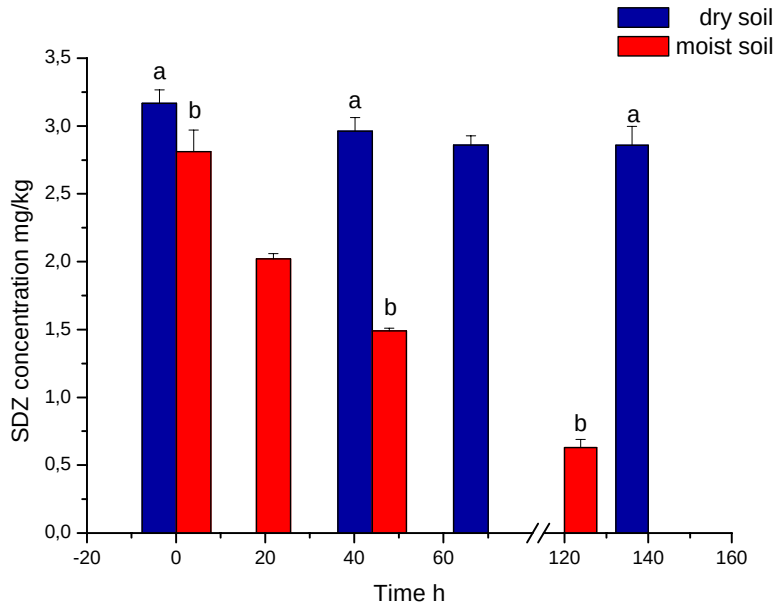


Figure 16.

Comparison of SDZ concentrations over a period of 140 h in dry and moist soil, SDZ amount spiked 33.3 mg/kg. Bars with different letters differ significantly (P<0.05). The recovery is given with standard deviation (n=3), with error bars indicating standard error of difference.

The amount of SDZ recovered in the moist soil, declined during 120 h from 2.81 mg/kg to 0.63 mg/kg or 18.9% of the start concentration (figure 15).
The concentration of SDZ in both experiments (dry and moist soil) decreased with increasing contact time (figure 16). In the case of the moist soil, the decrease was very rapid, while in dry soil the concentration was still high. In the moist soil, after about 2 days the concentration was below half of the spiked amount and after 5 days about 20% of the start concentration was reached. These results were consistent with other reports, which indicated that about 50% of SDZ was not detectable

anymore after three days of incubation [228] or by Stoob et al. [237] about 50% after 6 days. Wehrhan [232] observed that the extractable fraction of ^{14}C-SDZ residues decreased from initially 70% to approximately 20% of the applied radioactivity within 20 days and only about 30% was detectable already after 5 days.

This observed dissipation of SDZ in orthic luvisol soil and thus the low extraction efficiency for aged SDZ residues can be a result of different processes like interaction with soil constituents through sorption or reaction with humic substances, or due to biological processes in the soil [237]. Also transformation into metabolic products is thinkable. Processes like mineralisation are of minor importance as shown by Wehrhan [232] and Kreuzig et al. [228], who observed that mineralisation of ^{14}C-SDZ plays no role as potential sink of SDZ in moist soil under the prevailing laboratory conditions.

The experimental results indicated that the sample matrix and moisture has a significant effect on the recovery of the target analyte. The extraction efficiency decreased significantly with increasing contact time between SDZ and the soil.

4.1.9.2 Recovery and aging of SDZ in sterilized soil

To evaluate the influence of microbial activity on the process of declining recovery of SDZ in time, two different soils were sterilised and spiked with SDZ and compared with non-sterilised moist soils (figure 17).

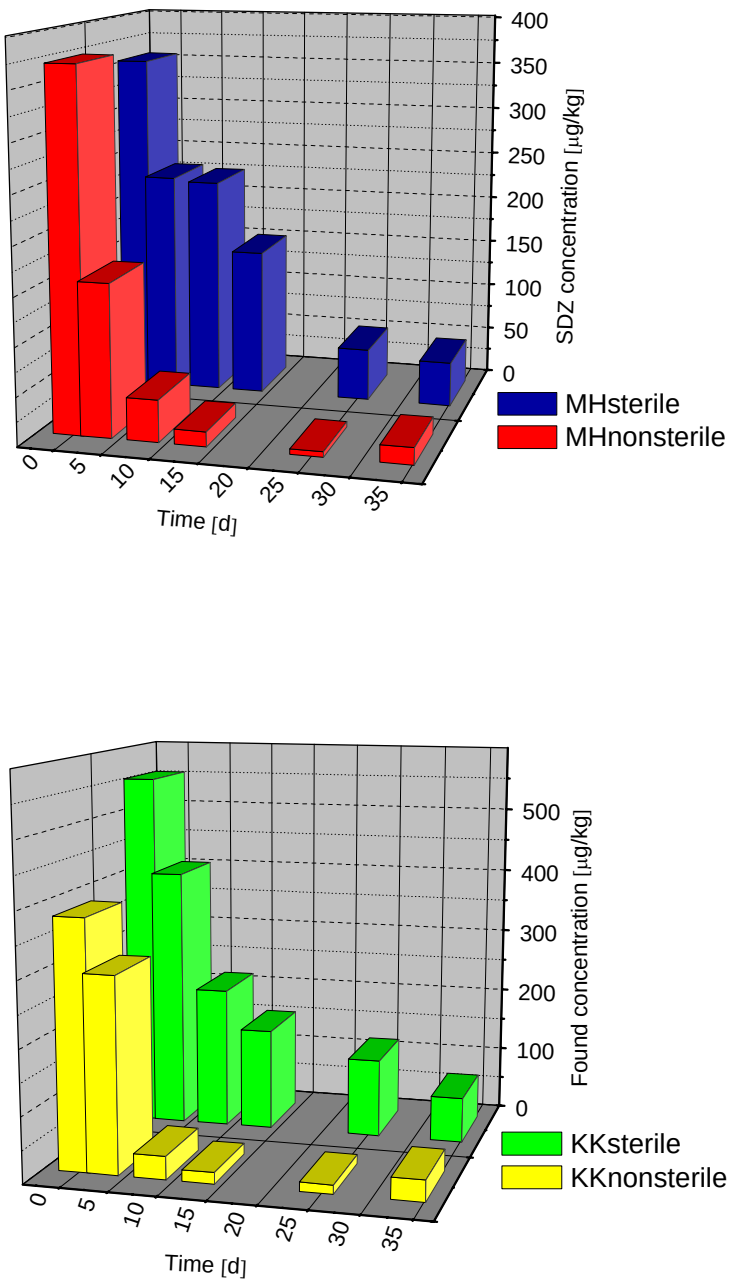


Figure17.

SDZ recovery over a period of 35 days in sterilised and non-sterilised MH(Merzenhausen, orthic luvisol) and KK (Kaldenkirchen, gleyic cambisol) soil. SDZ concentration spiked to the soils was 833 µg/kg. (n=6).

In both soils and variants (sterilised and none sterilised) the extracted concentrations of SDZ declined with time, although with some delay in the sterilised soils. After 2 days 51 or 45.9% (respectively for MH and KK) of the initial amount of SDZ could be extracted from the non

sterilised soil, for the sterilised soil these values were 68% , 42.8% respectively. After a period of 36 days the recovery was below 10% and the differences between the sterilised and non sterilised soils were significant (table 22).

Acetyl-SDZ could not be detected in any of the extracts.

Table 22.

Differences of significance in SDZ concentrations of sterile and non sterile soils measured over 36 hours. Different letters mean, that the data differ significantly (P<0.05).

days	MH non sterile	MH sterile	KK non sterile	KK sterile
2	a	b	ab	c
5	a	b	c	d
10	a	b	a	b
15	a	b	a	b
27	a	b	c	d
36	a	b	c	d

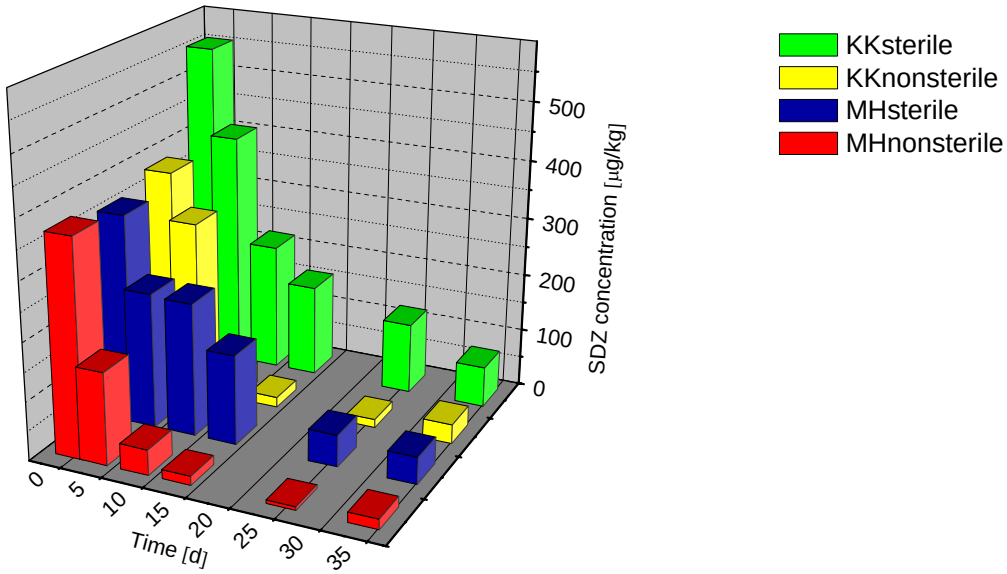


Figure 18.

Combination of differences between sterile and non-sterilised MH (Merzenhausen, orthic luvisol) and KK (Kaldenkirchen, gleyic cambisol) soil. SDZ concentration spiked to the soils was 833 µg/kg. (n=6).

In sterilised as well as in non-sterilised soils the concentration of SDZ decreased with increasing contact time. The extractability in the sterilised gleyic cambisol soil was much higher at the beginning of the experiment than in the other soil. After 10 and 15 days these differences were still significant. The initial concentration of SDZ was in the sterilised soils decreased 75% after 10 days and in the non-sterilised soils significantly to below 90%. After 36 days contact time the differences had disappeared and all the recoveries were in the range from 2 to 5%. The decreasing recoveries from soil with increasing contact time were already discussed in the previous subchapter. However, these results suggest that microbial degradation was not the predominant mechanism in the dissipation of the SDZ in both soils, although the influence of biological processes in the soil can not be fully excluded. These results were consistent with the findings of Stoob et al. [237].

In contrast Accinelli et al. [238] found that the half-life for SDZ was significantly longer in autoclaved soils than in non-autoclaved soil and that microbial degradation was the predominant mechanism in the dissipation of the sulfonamides in soil. Because the differences in the degradation rates in his experiment between the two soils were consistent, it is possible that not only biological degradation but also the chemical reactions were responsible for the dissipation process.

When degradation of the antibiotic would be mainly due to microbial processes, this could have been clarified with the use of sterile soils. Due to the lack of microorganisms, the substances can not be degraded microbial and hence sterile soils should give a higher recovery. The fact, that a continuous decrease of SDZ is measured, could either depend on an irreversible binding of SDZ to the soil matrix, or be the result of a transformation in different known (acetyl-SDZ) and unknown metabolites.

Evaluating the results, in principle more SDZ could be extracted from sterilised soil compared to non-sterilised soils during the first days of storage. This trend decreases with time, and becomes minimal after 27 days.

4.2 Concentration effects on microbial population using microcosms experiments

4.2.1 Results of chemical soil analyses from the microcosm experiments

The soil samples from Merzenhausen (orthic luvisol soil) that were used for the microcosms can be regarded as uncontaminated with antibiotics. No manure has been applied during the last 10 years and SDZ measurements with LCMS/MS were below LOD. In contrast the soil from Kaldenkirchen (gleyic cambisol soil) had been manured and a SDZ contamination slightly above the LOD could be measured. Additionally, the orthic luvisol soil had a greater capacity for the dissipation of SDZ than the gleyic cambisol soil.

The analyses were difficult due to the high LOD. The detected amounts of SDZ were not exceeding 28 µg/kg and therefore close or below LOD. Under consideration of the stability it is supposed, that the values were significantly higher directly after manuring. The question is if the detected concentration is equivalent to the extractable amounts (10%) or if the natural dissipation in open land differs from laboratory conditions and thereby influences the SDZ recovery of the applied manure.

In two microcosm experiments the fate of SDZ was followed by sampling in regular intervals over 64 and 106 days. The results were checked by one way ANOVA to guarantee comparability. The differences in results of both series of all three concentrations of SDZ are not significant (1 mg/kg: $P = 0.70203$; 10 mg/kg: $P = 0.90817$; 50 mg/kg: $P = 0.88519$).

For all three concentrations of SDZ, the greatest decrease took place within the first 24 hours after antibiotic application (figures 19 - 22, 24, 25).

In the microcosms with 1 mg/kg SDZ added only 0.5 mg/kg soil could be extracted after one day. Thereafter the concentrations of SDZ decreased after 64 days to 0.03 and 0 mg/kg (below LOD), respectively. After 106 days the SDZ concentration was below LOD.

Due to a lack of other reference material and the fact that not all of the metabolites of SDZ found in soil are identified, the soil samples of the microcosms were only tested for the presence of acetyl-SDZ. In all samples from the 1 mg/kg microcosm series no acetyl-SDZ was detectable.

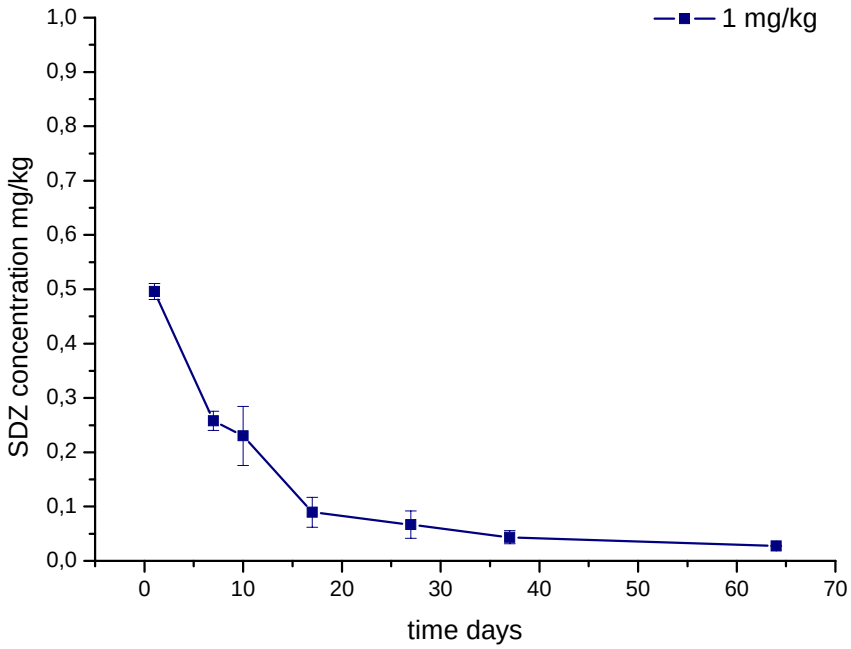


Figure 19.

Changes in extractable SDZ concentrations over 64 days in microcosms with 1 mg/kg soil added (n=4)

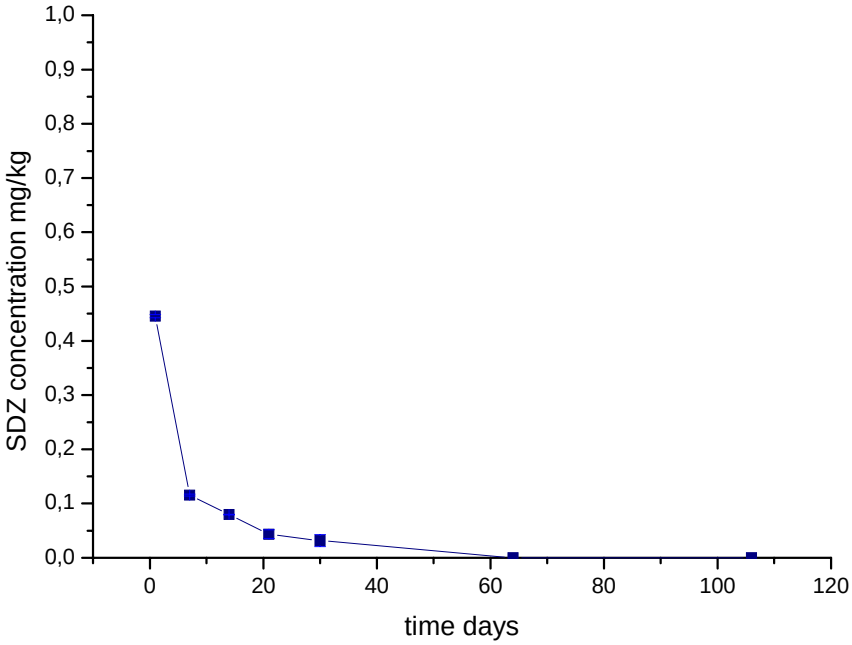


Figure 20.

Changes in extractable SDZ concentrations over 106 days in microcosms with 1mg/kg soil added (n=4).

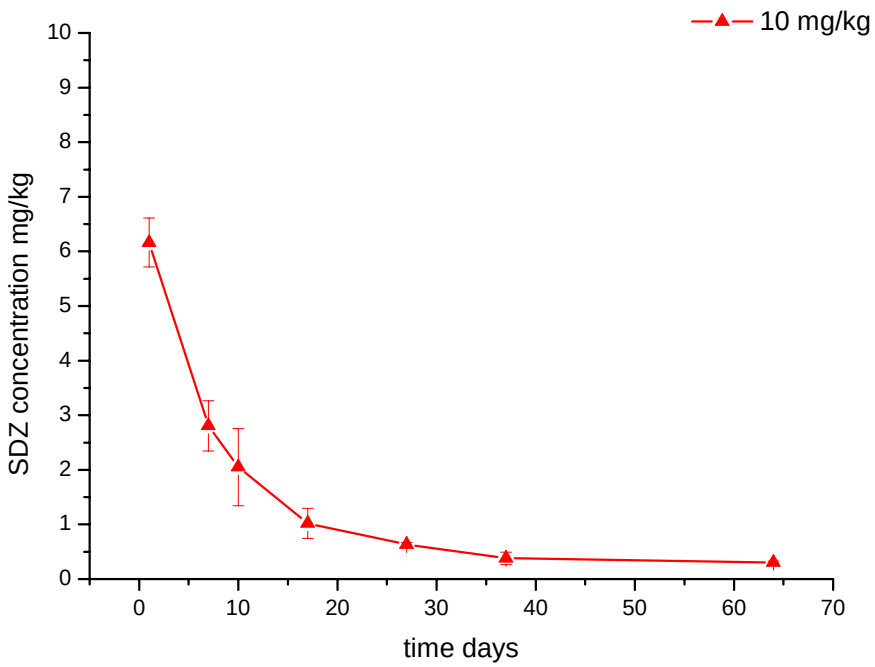


Figure 21.

Changes in extractable SDZ concentrations over 64 days in microcosms with 10 mg/kg soil added (n=4).

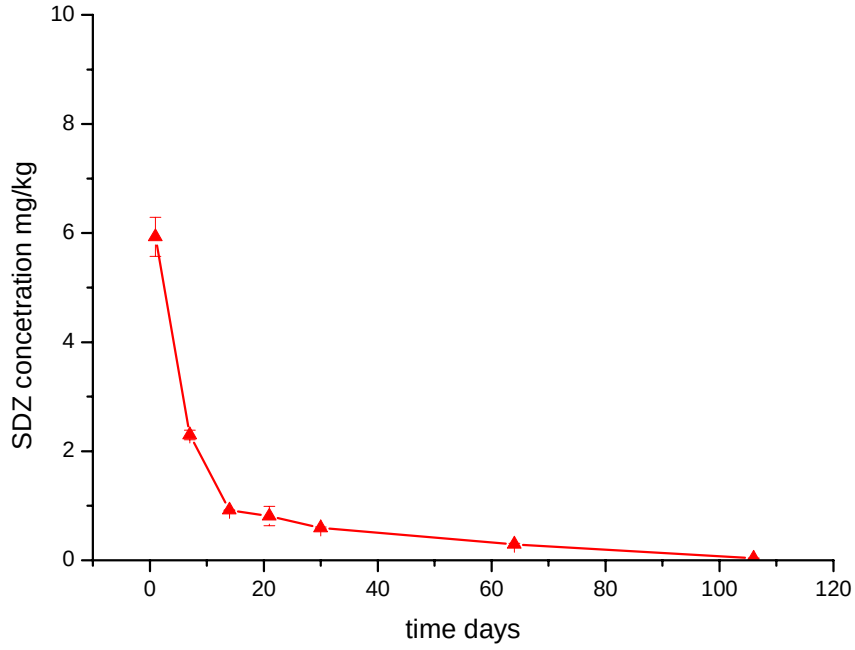


Figure 22.

Changes in extractable SDZ concentrations over 106 days in microcosms with 10 mg/kg soil added (n=4).

In the microcosms with SDZ concentrations of 10 mg/kg soil the extractable concentration of SDZ was 6 mg/kg soil after one day, which means a reduction of 40%. After 64 days the SDZ concentration was 0.3 mg/kg in both series and after 106 days only 0.03 mg/kg could be measured which is a loss of almost 100%. In a few samples the metabolite acetyl-SDZ could be detected (figure 23).

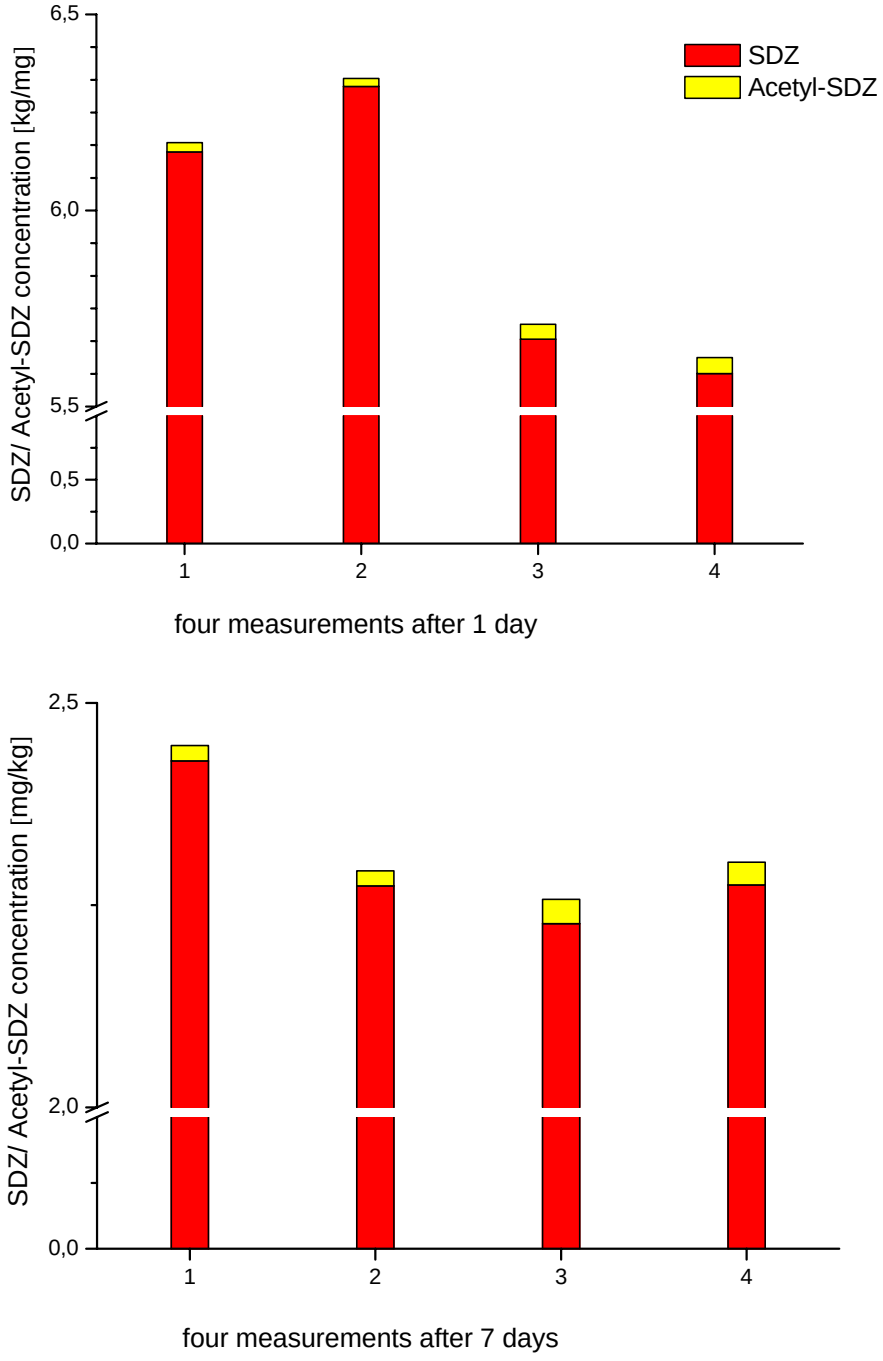


Figure 23.

Concentrations of SDZ and acetyl-SDZ in spiked soil (10 mg/kg) after 1 and 7 days.

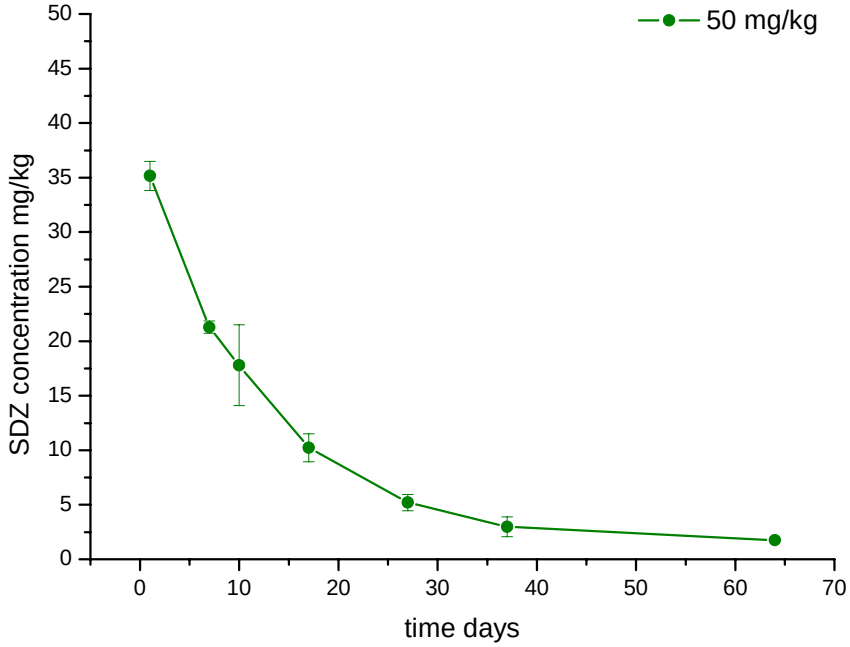


Figure 24.

Changes in extractable SDZ concentrations over 64 days in microcosms with 50 mg/kg soil added (n=4).

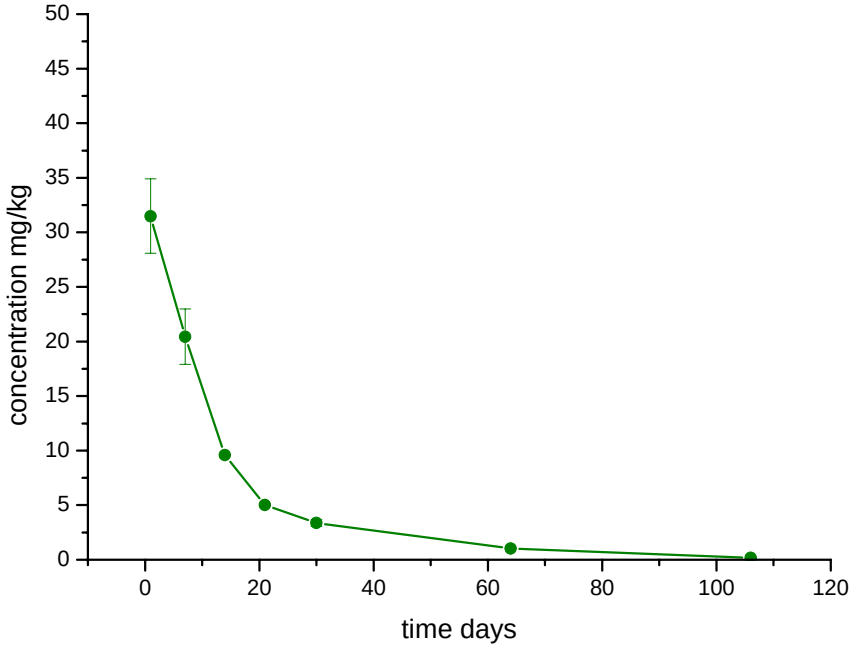


Figure 25.

Changes in extractable SDZ concentrations over 106 days in microcosms with 50 mg/kg soil added (n=4).

The results give the opportunity to follow the dissipation kinetics of SDZ in soil in the microcosms experiment.

A first order kinetic model described as: $\ln c = \ln (c^0 * e^{-kt})$, where t is the time, c the found concentration and k the rate was fitted to the data.

The results are shown in figures 26, 27 and 28 and indicated that dissipation rate changes with concentration. First order kinetics only satisfactorily described the measurements with a coefficient of variation equal to 85% for the case of 10 mg/kg 80% and 92% for the case with an initial concentration of 50 mg/kg. For the case with an initial concentration of 1 mg /kg (figure 26) the coefficient of determination was only 48%. This indicates that a one rate process is not sufficient to describe the measurements and improved model description with two or three different dissipation rates might provide better results. Wehrhan described [232] that the long-term adsorption kinetic indicates a time-dependent, non-linear sorption behaviour of SDZ and used a complex sorption model involving non-linear equilibrium and rate-limited reversible or irreversible sorption as flexible tool to describe all experimental data with one set of parameters.

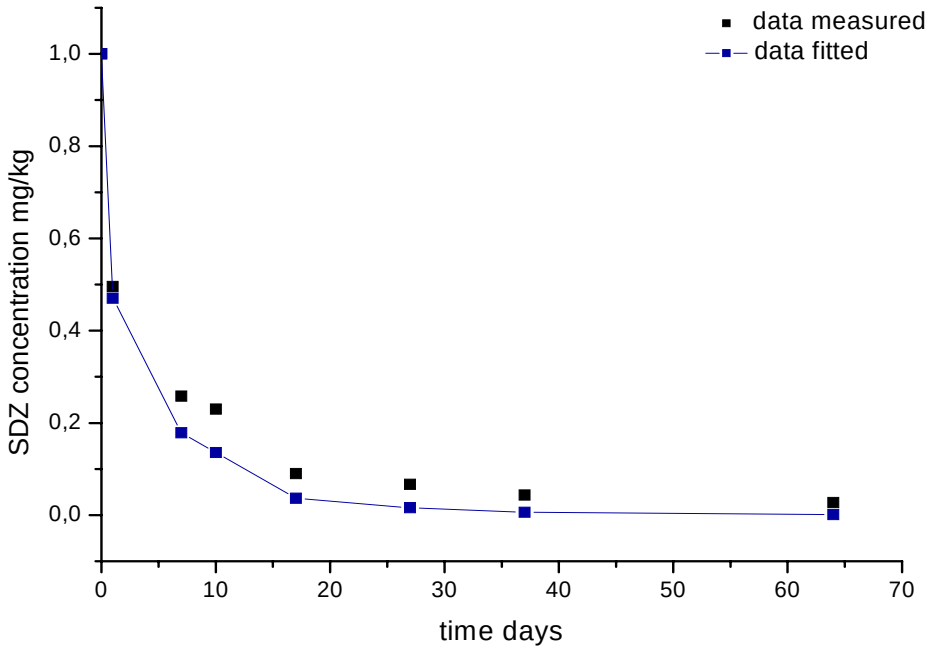


Figure 26.

Dissipation of SDZ over 64 days in microcosms with 1 mg/kg soil added.

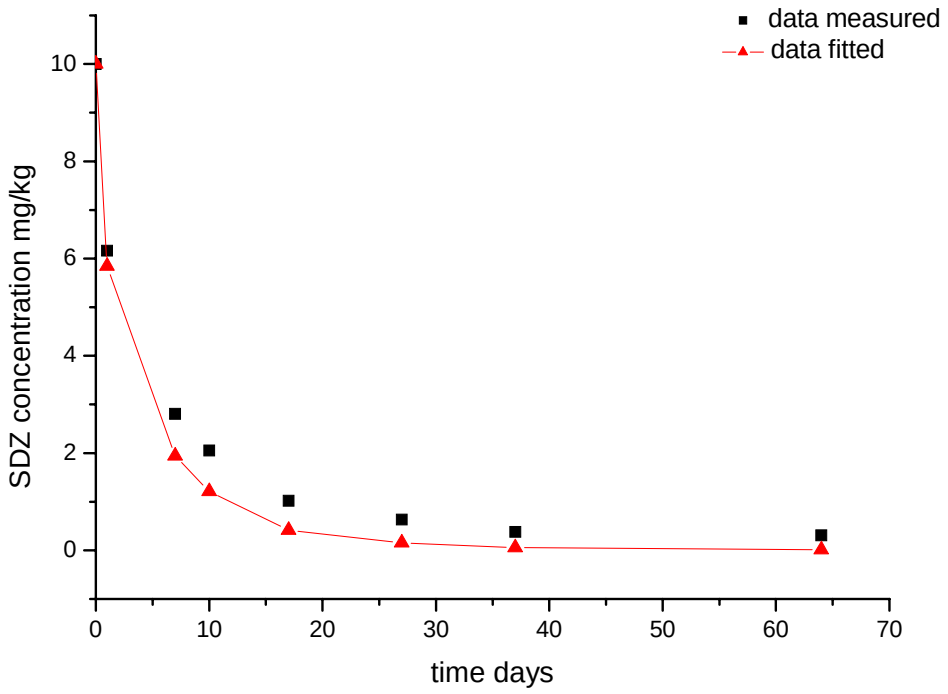


Figure 27.

Dissipation of SDZ over 64 days in microcosms with 10 mg/kg soil added.

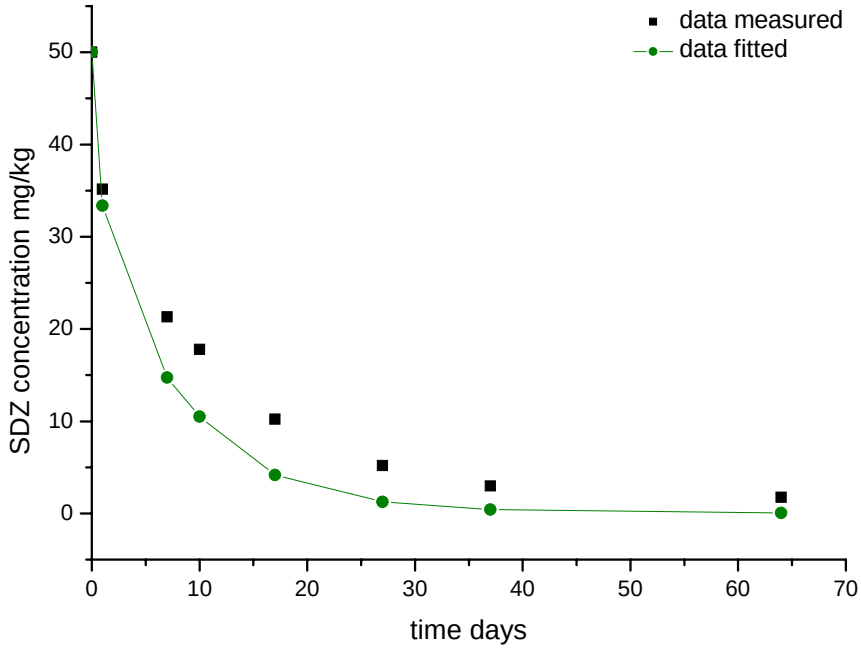


Figure 28.

Dissipation of SDZ over 64 days in microcosms with 50 mg/kg soil added.

Acetyl-SDZ could be detected after 1 and 7 days in low concentrations of 0.02-0.04 mg/kg. After 10 days the metabolite concentration was below LOD. In the microcosms with 50 mg SDZ/kg soil 31-35 mg of SDZ could be extracted after one day (35% of the added SDZ is dissipated). After a continuous decrease the concentration was 1-1.8 mg/kg after 64 days. Acetyl-SDZ was measured only in a few samples in low concentrations between day 1 and 37. The measured concentrations of acetyl-SDZ were in the range of 0.01-0.8 mg/kg, which is equal to maximally 3.7% of the amount of SDZ measured (figure 29).

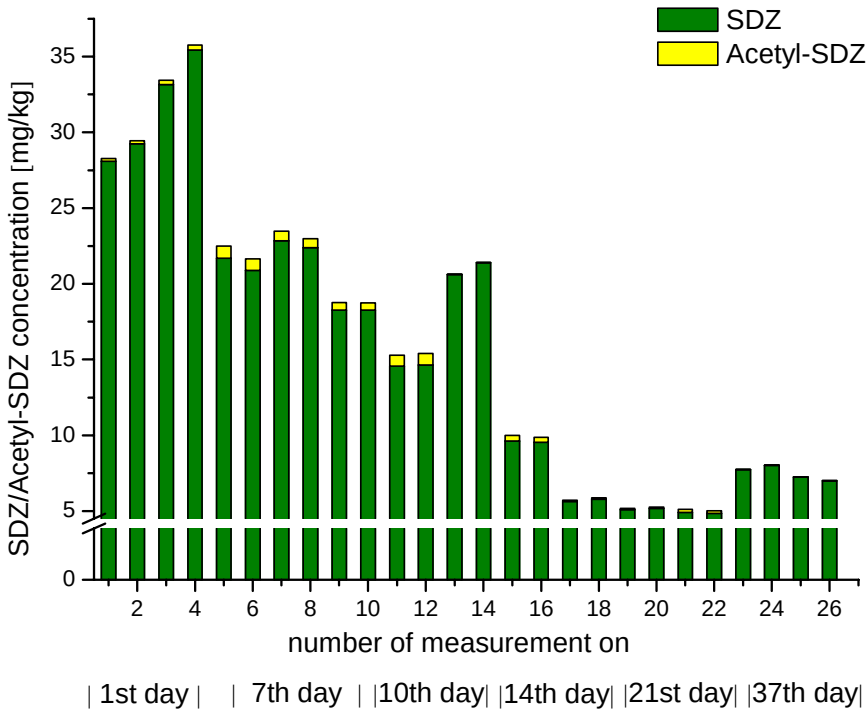


Figure 29.

Concentrations of SDZ and acetyl-SDZ in spiked soil (50 SDZ mg/kg) after 1, 7, 10, 14, 21 and 37 days.

Summarising, both series (over 64 and 106 days) showed an almost identical dissipation of SDZ over time at all three concentrations. The amount of detectable acetyl-SDZ was maximally 3.7% of the measured SDZ-concentration and dependent on the concentration of SDZ in the soil. The degradation test showed a steady dissipation of SDZ over time independent from the concentration and could be due to an irreversible adsorption of either the parent compound or the transformation metabolites to the soil compounds. This present study showed that the dissipation of SDZ is

dependent on the contact time with the soil. This is in agreement with other authors [16, 17, 136, 232, 237]. Stoob et al. [237] observed that already after 90 minutes contact time of SDZ with soil the efficiency of the extraction decreased. After 6 days the recovery obtained about 55% and after 17 days only 30%.

Also Thiele-Bruhn and Beck [136] observed that the detectable concentration of sulfapyridine strongly declined with time and reached non-detectable concentrations within 14 days. Wehrhan [232] measured decrease in the concentration of total ^{14}C (soil was spiked with ^{14}C-SDZ) with increasing time (50% of ^{14}C was not extractable after 3 days and about 90% after 28 days). The fraction of SDZ-metabolites increased during the course of her experiments. Wehrhan's experiments hint towards a fast transformation of SDZ in the soil environment and showed that the particle surfaces of the soil material seem to be essential for the transformation processes. Heise et al. [235] reported about a laboratory test on the bioavailability of ^{14}C-sulfamethoxazole where 93% ± 7% became non-extractable fraction in the test period of 14 days. Also the analysed extractability of ^{14}C-SDZ was similar. The authors explained that the rapid disappearance of SDZ is mainly attributed to the formation of non-extractable residues, indicating the high affinity to the soil matrix. Also Kreuzig et al. [228] report on 93% of non-extractable residues of SDZ within 3 days and explained it as indication of rapid formation of non extractable SDZ residues in soil.

In contrast the concentration of the metabolite acetyl-SDZ measured by other authors [232, 237] was much higher than in this study. Stoob et al. [237] showed that the amount of acetyl-conjugates showed constant responses in the dissipation over several weeks (contact time 60 days) and accumulated for about 15% at the end of the experiment. In this study, a decrease of the acetyl-SDZ concentration in time was also obtained but the extracted concentrations were lower. A possible explanation could be that the PLE solvent is not optimal for extracting acetyl-SDZ from soil and higher extraction efficiency may be achieved by using another solvent or solvent-mixtures. However, for SDZ, satisfactory recoveries were obtained.

In conclusion, in agreement with other authors [16, 17, 136, 232, 237] it is assumed that SDZ as an example for sulfonamides dissipates with increased contact time in moist soil and the extractability of SDZ decrease very fast. The disappearance is due to chemical reactions, which led to the formation of non-extractable SDZ or of transformation products like acetyl-SDZ and partially as a result of biological processes in the soil. Probably non extractable sulfonamides may persist for a long time in the soil and may accumulate. The availability of the non-extractable sulfonamides for microorganisms, however, is unknown.

4.3. Effects of SDZ and CTC on microbial populations using microcosms experiments

4.3.1. Soil respiration

The results of the microbial activity for soil amended with SDZ are presented in figures 30-34 and those for soil amended with CTC in figures 35 and 36.

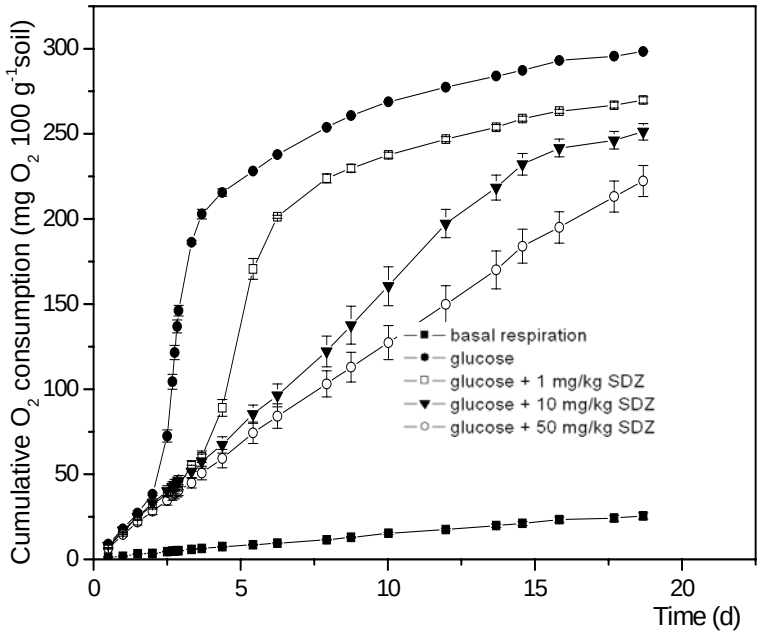


Figure 30.

Cumulative oxygen consumption of SDZ-amended soil with and without glucose. Error bars indicate standard error of mean.

Figure 30 shows the data obtained from soil respiration measurements in presence of glucose and SDZ and glucose. Without an additional carbon source the data did not differ from the soil basal respiration (without glucose and SDZ: 1.4 mg O_2/100 g soil pro day).

For all concentrations of SDZ (0 - 50 mg/kg soil) oxygen consumption increased immediately after the addition of glucose. During the first 36 hours, the oxygen consumption rates yielded identical values of 16 mg O_2/100 g soil. A cut-out of the first period of the time is presented in the figure 31.

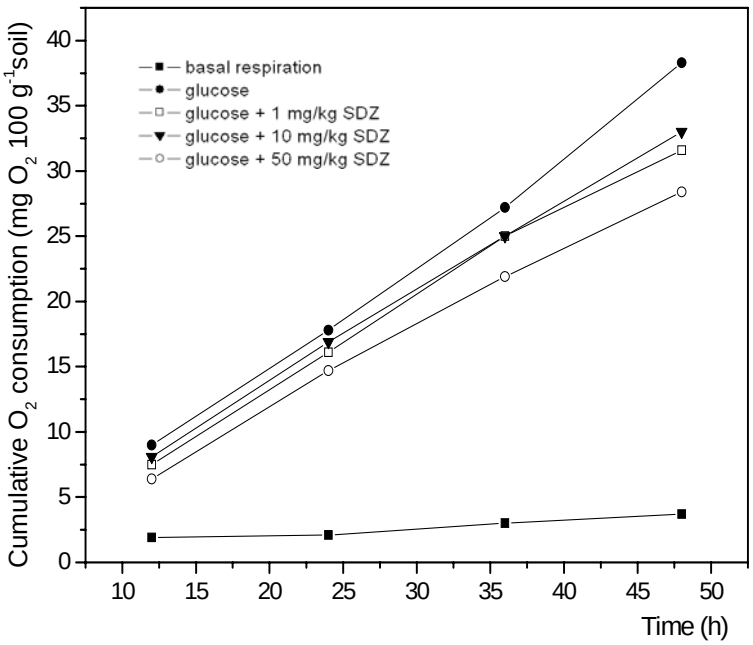


Figure 31.

Cumulative oxygen consumption of SDZ-amended soil with and without glucose.

After the first 36 hours, the kinetics of cumulative oxygen consumption displayed an increasing inhibition with increasing concentrations of SDZ. Glucose without SDZ induced the earliest increase in oxygen consumption followed by the incubations with glucose and 1 mg SDZ/kg soil and glucose and 10 mg SDZ/kg soil corresponding to the particular concentrations.

The first order derivation of the cumulative oxygen consumptions (see figure 32) accentuates the onset of growth in the incubations with 1 mg SDZ/kg soil and glucose, and a weak increase with 10 mg SDZ/kg soil and glucose. In the presence of 50 mg SDZ/kg soil and glucose, the oxygen consumption proceeded more or less at a constant or somewhat declining rate. This experiment was than continued by a repeated addition of glucose (5 mg glucose/kg soil) on day 19 (figure 33).

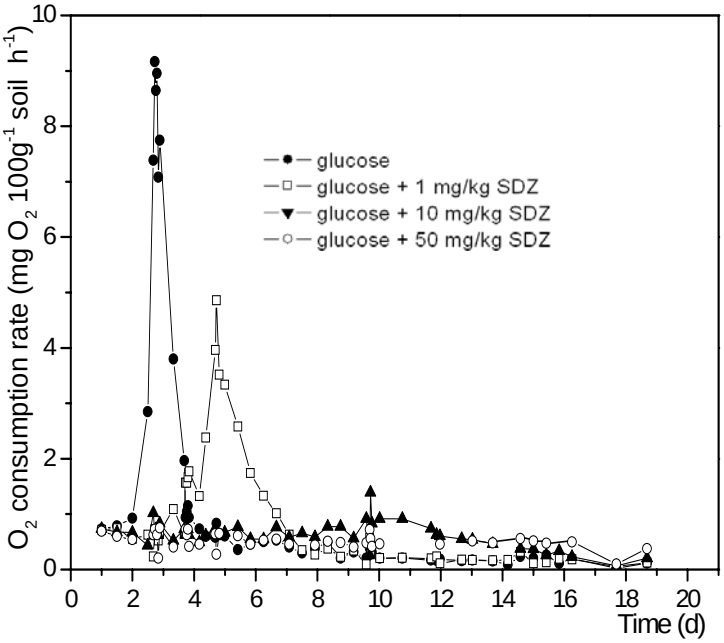


Figure 32.

First order derivation of the cumulative oxygen consumption.

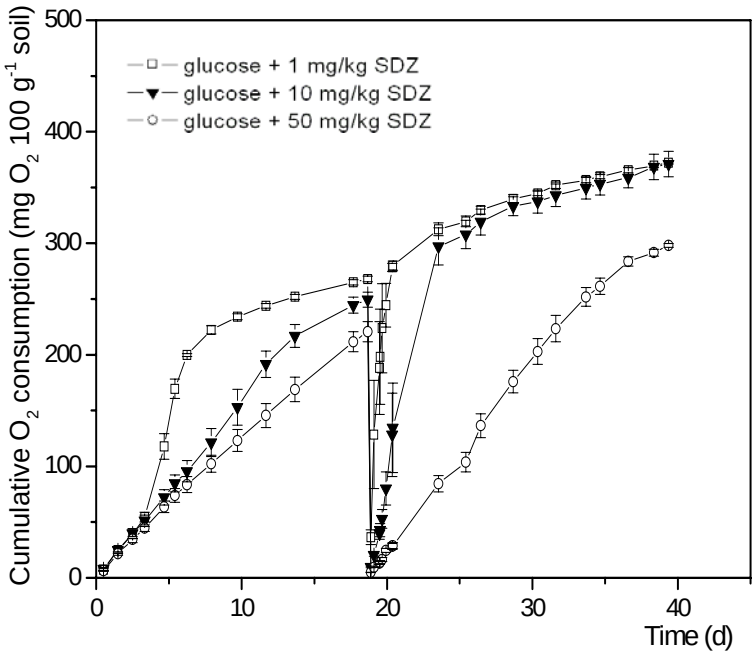


Figure 33.

Cumulative oxygen consumption of soil amended with glucose and SDZ and repeated addition of glucose but without SDZ on day 19. Error bars indicate standard error of mean.

In the setups that had been spiked with 1 and 10 mg SDZ/kg soil, oxygen consumption started immediately without a detectable lag phase. The setup with initially 50 mg SDZ/kg soil continued to respire at a constant rate, similar to the rate observed in the first 19 days. The results are presented in the figure 33.

The experiment with SDZ but without glucose addition was also continued by the addition of glucose on day 19th resulting in SDZ concentration-dependent oxygen-consumption rates without lag phases. The results are presented in figure 34.

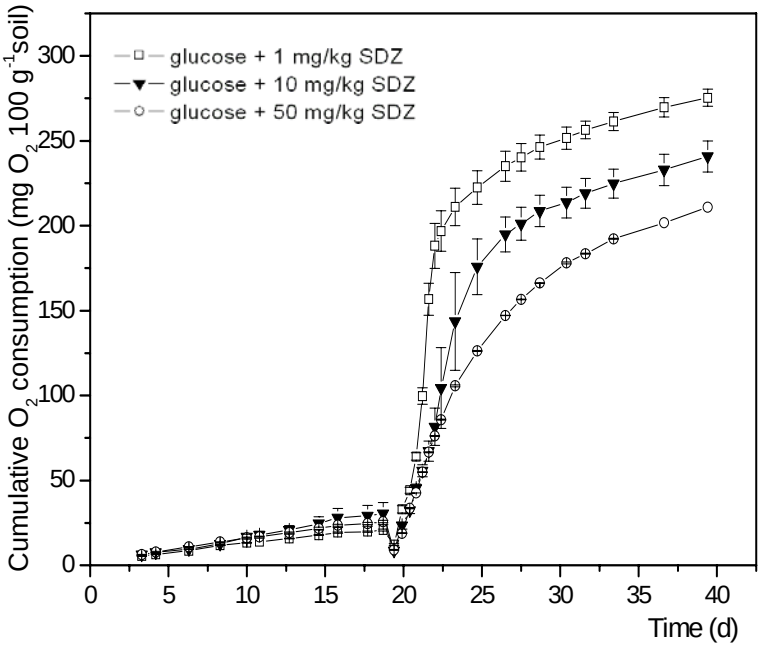


Figure 34.

Cumulative oxygen consumption of soil amended with SDZ only and subsequent addition of glucose without SDZ on day 19. Error bars indicate standard error of mean.

Analogue to the previous experiments the influence of the presence of CTC in soil was also investigated.

Similar to the experiment with SDZ, the soil respiration measurements with added CTC but without an external carbon source gave no detectable differences compared to the soil basal respiration. In contrast to the previous experiment the kinetics of the oxygen consumption were not affected, when soil with 50 mg CTC/kg was treated with glucose.

The results are presented in figure 35.

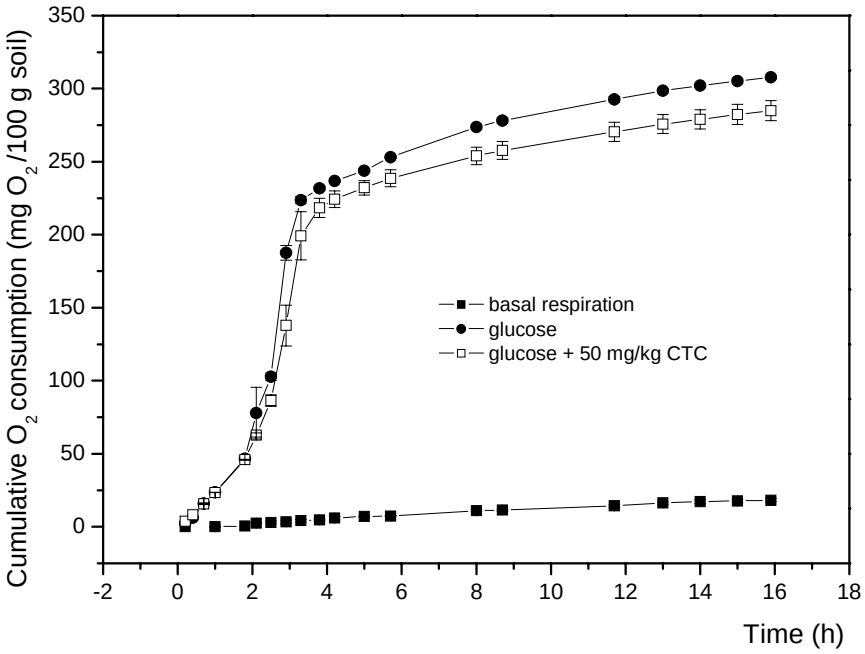


Figure 35.

Cumulative oxygen consumption of glucose-amended soil with CTC and without CTC. Error bars indicate standard error of mean.

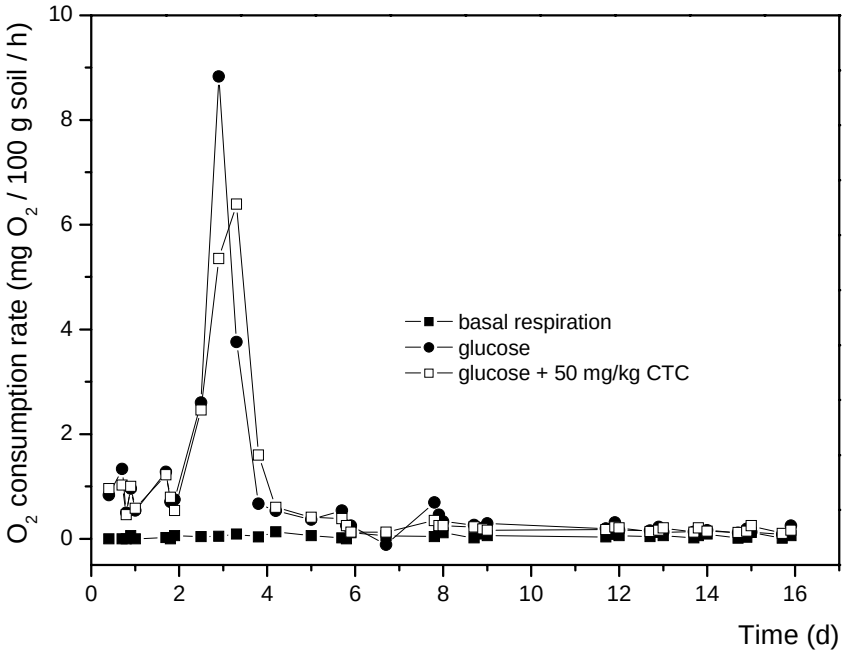


Figure 36.

Influence of 50 mg CTC/kg soil on oxygen consumption rates. First-order derivation.

The first-order derivation in the figure 36 shows that the maximum peak for the glucose supplemented soil was similar to CTC/glucose amended soil. The rates are indicative of exponential microbial growth and thus CTC in a dose as high as 50 mg/kg soil has no inhibition effect on the growth of the soil bacteria.

To study the effects of SDZ and CTC on soil microbial activity, the respiratory activity in soil microcosms was measured. Neither with SDZ (1, 10, 50 mg/kg soil), nor with CTC (50 mg/kg soil) a suppression or a stimulation of respiration was detected during 20 days of incubation. This is in accordance with the results of Hund-Rinke et al. [127] who described slightly reduced mean respiration activities first at the highest tetracycline concentration (500 mg/kg) they used over the whole incubation period of 16 weeks but in contrast to the findings of Vaclavik et al. [135] who found a 1.3-1.7 times increase in respiration above background respiration with different tetracyclines, including CTC, and sulfonamides, sulfachloropyridazine, at initial concentrations of 60 and 600 mg/kg soil. Samuelsen et al. [60] tested the stability of the antibacterial agents e.g. SDZ and no decrease in the sediment concentration was found for SDZ. According to the author these effects of tetracyclines and sulfonamides on the activity and biodiversity of soil microorganisms were expected [132]. He investigated the microbial activity after treatment with different antibiotics e.g. chlortetracycline and sulfadizine in different agricultural soils with the iron (III) reduction test. In aqueous solution tetracycline has a significant antimicrobial effect, however in soil, measurable effects are observed at considerably higher concentrations [127].

Due to the absence of a lag phase and the fact that the antibiotics themselves could be quantitatively re-extracted at the end of the incubation, it can be concluded that the antibiotics did not serve as substrates, but the reason for the increased respiration remained unclear. For SDZ, it is rather unlikely, that a substantial degradation resulting in an increased respiration would have occurred. From the parallel chemical experiment (measurement of SDZ concentration in microcosms) it is clear, that within the first 7 days the extractable SDZ concentration in soil decreased rapidly leading to 20% of the initial concentration at day 7 and to less than 5% of the initial concentration after 40 days in the present study. Kreuzig et al. [228] measured a microbial degradation of ^{14}C-labelled SDZ to CO_2 of less than 1% of the initial concentration of 250 µg/kg soil within 28 days and a rapid transfer to non-extractable residues.

In these experiments, CTC had probably no effect on respiration due to the strong inactivation (binding to Calcium) in the presence of the used orthic luvisol soil.

With glucose-induced respiration (SIR) concentrations of SDZ and CTC up to 50 mg/kg soil showed no differences in respiration rates during the first 6-8 hours in comparison to the addition of glucose

alone. This is not a surprising result, because the antibiotic activity of SDZ and CTC is based on the mechanism that the inhibition time should be long enough to enable microorganisms to grow. For that reason, Thiele-Bruhn and Beck [136] extended the incubation time in their experiments up to 48 hours. Accordingly, the respiratory activity in this experiment showed a clear delay of 36 hours in the onset of exponentially increasing oxygen consumption.

4.3.2. PCR DGGE

DGGE profiles of amplified 16S rDNA fragments from DNA extracted from the bacterial fractions in soil were very complex. The profiles from both soils (orthic luvisol and gleyic cambisol) are shown in the figure 35.

All incubations were done in duplicate and these duplicates gave identical banding patterns as shown exemplarily (figure 37 and 6.1 appendix) for samples taken after 3 or 6 weeks of incubation.

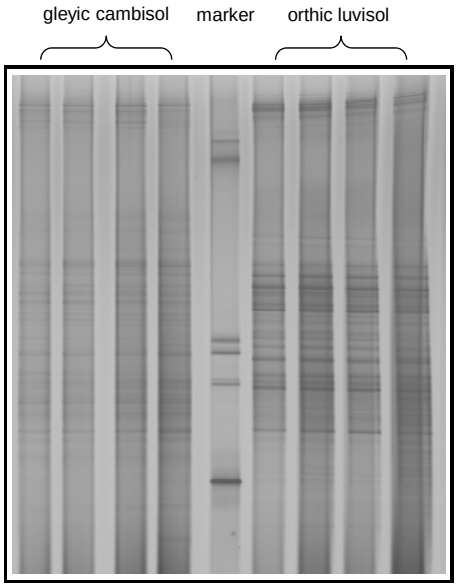


Figure 37.

DGGE patterns of PCR amplified 16S rDNA fragments from soil bacterial communities in orthic luvisol and gleyic cambisol soil.

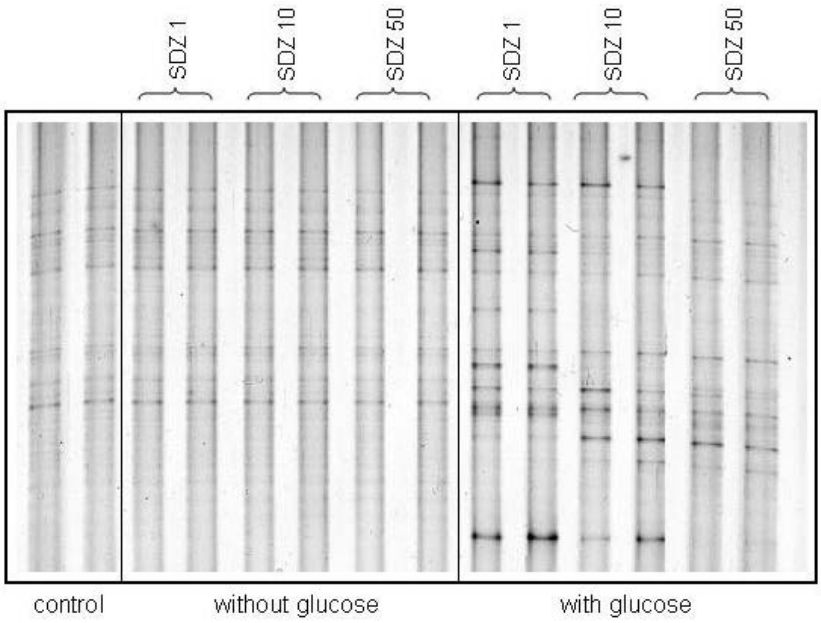


Figure 38.

DGGE patterns of individual microcosms from duplicate runs after 6 weeks of incubation (control: without glucose and SDZ; SDZ 1, 10, 50 mg/kg soil with and without glucose). Orthic luvisol soil.

All incubations with SDZ but without glucose gave identical banding patterns at any date of sampling and no differences were visible compared to the control soil.

However, incubations with glucose only compared to incubations with glucose and SDZ caused significant differences between the different setups. The DGGE patterns are presented in figure 38 and figure 6.2 in appendix.

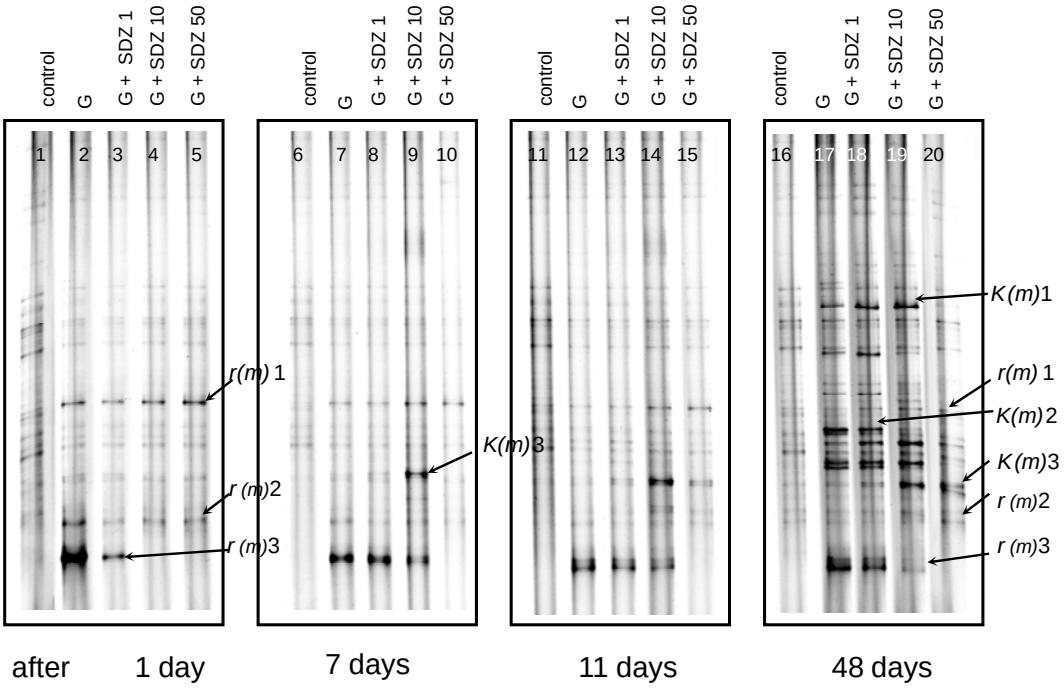


Figure 37.

DGGE patterns of soil samples taken from the microcosms after the respective incubation time periods (control: without glucose and SDZ; G: with glucose;

G + SDZ: with glucose and SDZ and the respective concentrations of 1, 10, 50 mg SDZ/kg soil). r and K denotation refer to different assumptive strategists as discussed in the text; Orthic luvisol soil.

Figure 37 (and 6.3 in appendix) shows the differences in the microbiological community during the incubation time of 48 days of the treated soil with different concentrations of SDZ.

After day one, a few apparently new or more intensive bands (r(m)1, r(m)2, r(m)3 in lane 2) appeared in the soil samples amended with glucose (figure 37). Bands r(m)1 and r(m)2 became clearly visible in all glucose and SDZ treatments. Band r(m)3 the most intense one after glucose addition, also appeared in the samples with glucose and 1 mg SDZ/kg soil after day one. From day 7 onwards this band became also visible in the treatment with glucose and 10 mg SDZ/kg soil but not in soils with glucose and 50 mg SDZ/kg soil. Other bands (K(m)1-K(m)3), prominent by band intensities, appeared in the course of the incubation. Although visible in the DGGE patterns from the control, the glucose and all glucose and SDZ-treated samples, K(m)1 and K(m)2 appeared as more intensive bands in the treatments with glucose, glucose and 1 mg and 10 mg SDZ/kg soil (except K(m)2) after 48 days of incubation. K(m)3 became more prominent in the treatment with glucose and 10 mg SDZ/kg soil and later an also in the treatment with glucose and 50 mg SDZ/kg soil.

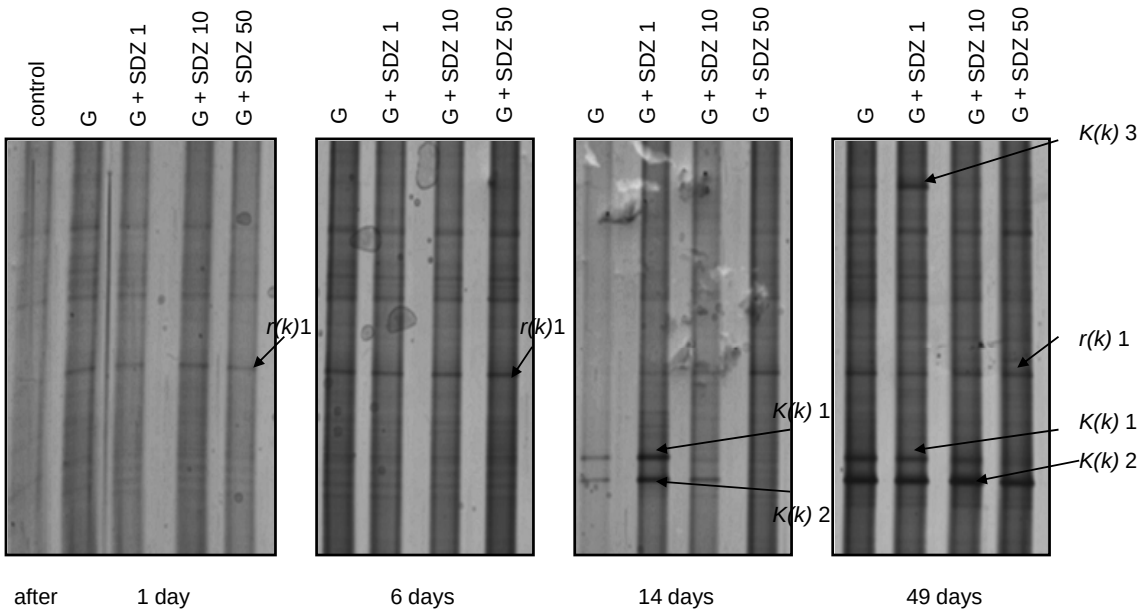


Figure 40.

DGGE patterns of soil samples taken from the microcosms after the respective incubation time periods (control: without glucose and SDZ; G: with glucose;

G + SDZ: with glucose and SDZ and the respective concentrations of 1, 10, 50 mg SDZ/kg soil). r and K denotation refer to different assumptive strategists as discussed in the text; gleyic cambisol soil.

In the experiment with gleyic cambisol soil after one day only one more intensive band (r(k)1) appeared in the sample treated with glucose (figures 40 and 6.4 in appendix). This band was visible in all samples with glucose and glucose with SDZ during the whole experiment. From day 14 on other more intensive bands appeared (K(k)1 and K(k)2) and after 49 day K(k)2 showed very intensive bands in all glucose- treated samples. K(k)1 appeared very clearly in the bands treated with glucose and glucose with 1 mg SDZ/kg soil. In the sample with glucose and glucose with 10 mg SDZ/kg soil the intensity of K(k)1 (after 14 days) was weaker and in the sample with glucose and 50 mg SDZ/kg soil very week. The intensity of K(k)2 is time dependent and the same for every concentration and achieved after 49 days the maximum. Similar to the band pattern of the orthic luvisol soil, at the end of the incubation time other band (K(k)3) appeared very prominent by band intensity, most prominent in the treatment with glucose and 1 mg SDZ/kg soil.

At least the orthic luvisol soil the appearance of new bands and bands with stronger intensities in the DGGE banding patterns shows a dependency on SDZ concentrations and correlates with the onset of exponentially increasing oxygen consumption. The correlation between bacterial community shifts in

DGGE and oxygen consumption kinetics indicates a significant role for bacteria in SDZ-dependent glucose utilization. However, it must be considered that fungi could also have contributed to the glucose mineralization and might be the reason for the weak increase in *r*-strategists in the gleyic cambisol soil. DGGE bands, which responded positively in the initial stages of the experiment, were designated as "*r*" to indicate that these sequences could belong to *r*-strategists which respond rapidly to input of nutrients. It is remarkable, that only relatively few bands appeared or became stronger in intensity after the addition of glucose (Figure 39). In orthic luvisol soil the number of these strategists was higher then in gleyic cambisol soil. Using the SIR method with glucose as substrate, Stenström et al. [201] often found that *r*-organisms constitute a minority of the biomass in arable soils. Combining these findings, one may conclude that *r*-organisms in arable soils are both low in biomass and numbers of species.

For the orthic luvisol soil DGGE bands responded positively after consumption of the primary substrate, glucose (K(m)1-K(m)3, day 11 and 48 resp. 14 and 49), may be due to *K* strategists (specialists in utilizing resources when they are becoming scarce in the later course of a succession). K(m)3 and partly K(k)3 did not respond to glucose at all but increased in intensity in the presence of 10 mg SDZ/kg soil in orthic luvisol soil and in gleyic cambisol soil in the presence of 1 mg SDZ/kg soil. An intensive band first appeared with 10 mg SDZ/kg soil (orthic luvisol) at day 7 and remained clearly visible until day 48. The possible explanation from Zielezny et al. [233] was that K(m)3 had an advantage in competition for metabolits, rather than the primary substrate glucose in the presence of 10 mg, and to some extent of 50 mg SDZ/kg soil.

Both soils showed a similar behaviour of the *K*-strategist microorganisms. The difference was the smaller fraction of the *r* -strategists. Thus, the explanation of this behaviour could be the slightly different substrate availability and the presence of fungi in the gleyic cambisol soil which was not considered in this work.

The influence of different soil treatments on the bacterial diversity based on DGGE bands and intensities is exemplary shown for the orthic luvisol soil in table 23.

Table 23.

Diversity as indicated by Shannon-Wiener index (H'), richness (S) and evenness (E_H) of all samples estimated by the DGGE band patterns. Orthic luvisol soil.

Time	Treatment	Shannon-Wiener Index (*H'*)	Richness (*S*)	Evenness (E_H)
1 day	Control	2.77	18	0.96
	Glucose (G)	2.29	16	0.81
	G + SDZ 1	2.45	15	0.88
	G + SDZ 10	2.45	15	0.91
	G + SDZ 50	2.38	15	0.88
7 days	Control	2.72	17	0.96
	Glucose (G)	2.40	16	0.87
	G + SDZ 1	2.05	17	0.72
	G + SDZ 10	2.05	16	0.74
	G + SDZ 50	2.59	15	0.95
11 days	Control	2.68	19	0.91
	Glucose (G)	2.57	21	0.82
	G + SDZ 1	2.25	19	0.79
	G + SDZ 10	2.15	19	0.77
	G + SDZ 50	2.68	18	0.93
48 days	Control	2.82	19	0.96
	Glucose (G)	3.05	27	0.87
	G + SDZ 1	3.09	27	0.87
	G + SDZ 10	3.09	28	0.86
	G + SDZ 50	2.88	20	0.96

Species richness (*S*) was similar in all treatments and at all sampling days, except for the increase in *S* in the 48-day samples for glucose, glucose and SDZ 1 and glucose and SDZ 10 treatments. The diversity (Shannon-Wiener index, *H'*) decreased after the addition of glucose and glucose with SDZ for the first three sampling times. After longer incubation of 48 days the highest values for *H'* were obtained for glucose, glucose and SDZ 1 and 10 mg. Evenness (E_H) scored the highest values for the controls and the treatment with glucose and 50 mg SDZ.

The addition of glucose + 1 mg SDZ and glucose + 10 mg SDZ had a strong influence on the diversity index (*H'*) and evenness (E_H). Both values declined in the beginning, however, after 48 days of incubation *H'* and E_H reached high values and richness (*S*) increased. The addition 50 mg SDZ/kg soil together with glucose suppressed the influence of glucose on *H'*, *S*, and E_H for at least

up to 48 days. From these data the conclusion can be drawn that glucose addition leads to an increase in diversity after 7 weeks and this increase is not very much influenced be the addition of up to 10 mg SDZ/kg soil. However, this interpretation should be handled with care because the calculation of Shannon index requires clearly defined species and a distinct identification of individuals, requirements that are not met when dealing with bacteria [181]. Whether the bands in DGGE represent the most abundant species, the most easily extractable or PCR amplifiable species, or a combination of all these groups is uncertain [181]. Additionally, only a part of the whole soil bacterial community is covered because data processing abolishes DGGE bands with intensities below 1% of total band intensities in a given line.

The cluster analysis of DGGE patterns from the microcosms study after 48 and 49 days of incubation with glucose and SDZ of the orthic luvisol and the gleyic cambisol soil respectively are shown in figures 41 and 42.

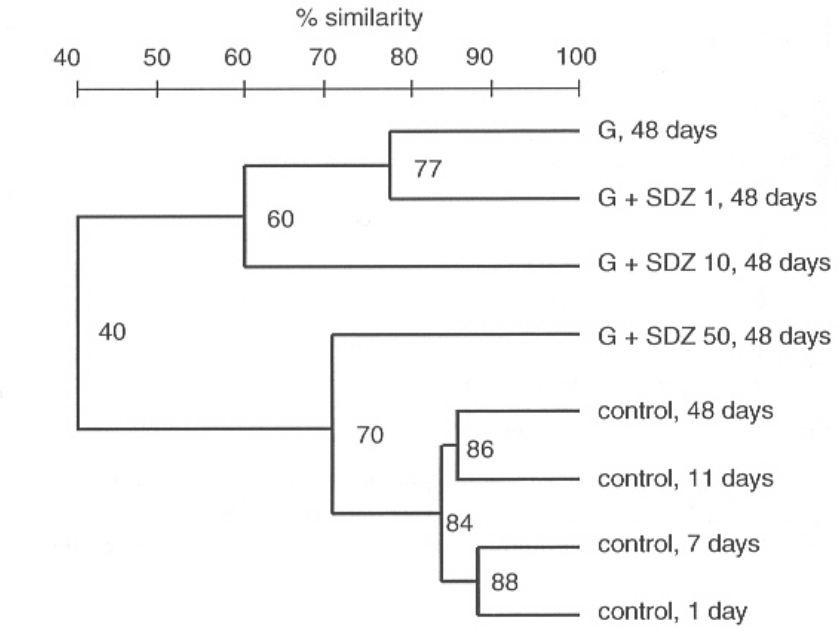


Figure 41.

Cluster analysis of DGGE patterns from orthic luvisol soil generated from all samples taken after 48 days of incubation and all controls using Dice correlation matrix and UPGMA.

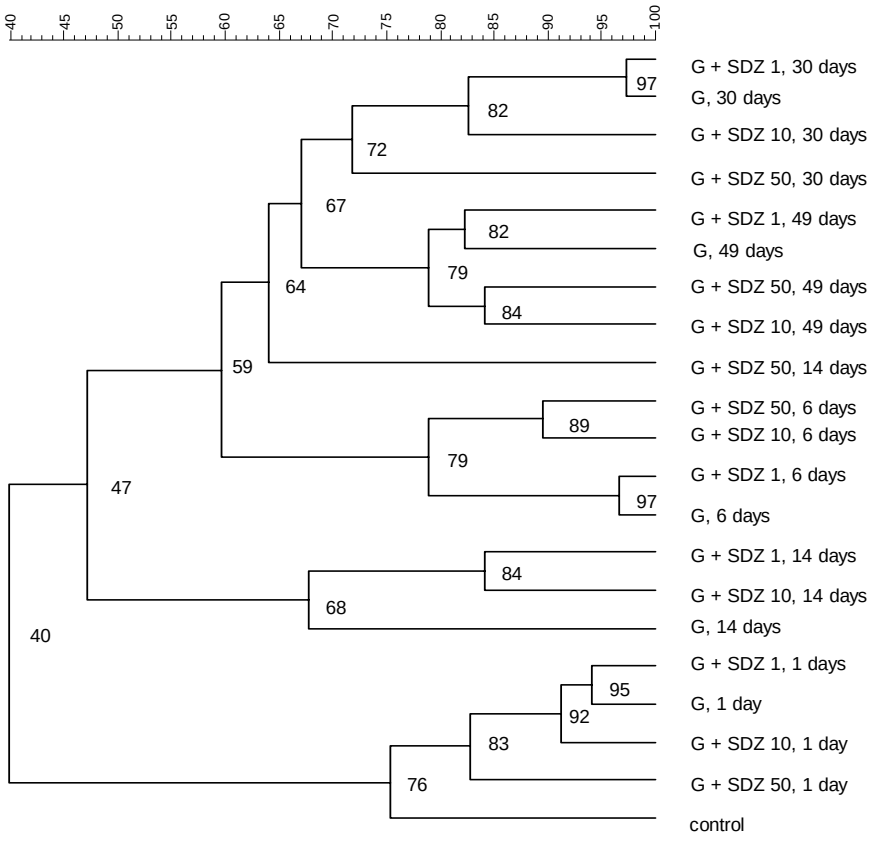


Figure 42.

Cluster analysis of DGGE patterns from gleyic cambisol soil treated with SDZ and generated from all 49 days samples and all controls using Dice correlation matrix and UPGMA.

Overall changes of the DGGE patterns were summarized by constructing a phylogenetic tree of lane similarities for the controls and the DGGE patterns after 48 (49) days. The highest conformity existed between the soils amended with glucose only and with glucose + 1 mg SDZ/kg soil. This indicates only a marginal effect of SDZ in the lowest concentration. Pronounced differences in band pattern intensities were found between the soils treated with glucose only and soil samples with glucose + 10 mg/kg soil. In the treatment with 50 mg/kg soil oxygen consumption continued at a constant rate, showing the bacteriostatic effect of SDZ at this concentration. This effect was confirmed by the DGGE banding pattern. Glucose and 50 mg SDZ/kg soil gave only a weak intensification of bands at the beginning of the incubation and after 48 (49) days the intensity pattern was in highest concordance with the control (without glucose and SDZ).

As described for SDZ the impact of CTC on the eubacterial community structure in both soils was examined by PCR-DGGE analysis.

All incubations done in duplicate gave identical banding patterns. Figure 43 shows exemplarily the similarity for samples taken after 7 days of incubation of the orthic luvisol soil.

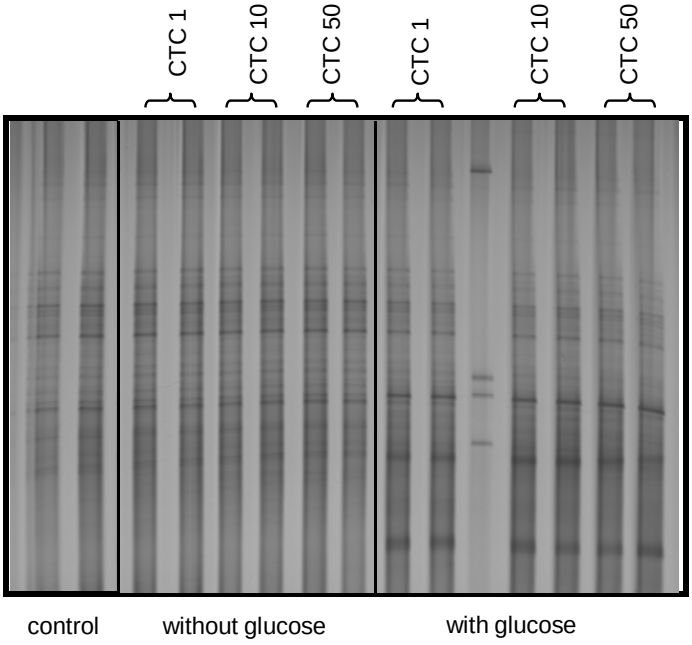


Figure 43.

DGGE patterns of individual orthic luvisol soil microcosms from duplicate runs after 7 days of incubation (control: without glucose and CTC).

The DGGE analysis of soil samples treated with CTC in the three different concentrations (1, 10, 50 mg CTC/kg soil) revealed that none of the concentrations of CTC tested showed significant differences in neither the orthic luvisol (figure 44) nor the gleyic cambisol soil (figure 6.5 in appendix).

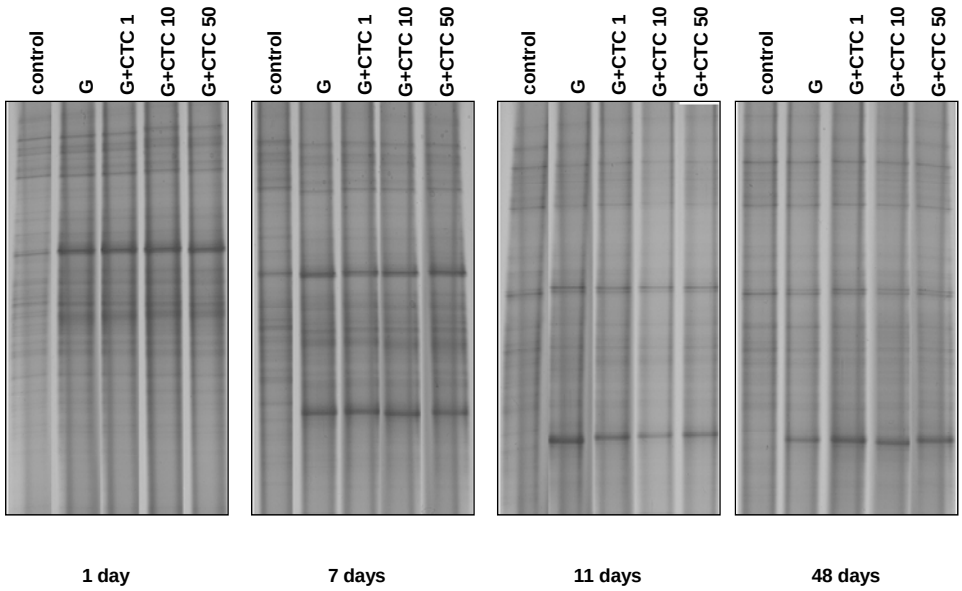


Figure 44.

DGGE patterns of soil samples taken from the microcosms after the respective incubation time periods (control: without glucose and CTC; G: with glucose; G + CTC: with glucose and the respective concentrations of 1, 10, 50 mg CTC/kg soil). Orthic luvisol soil.

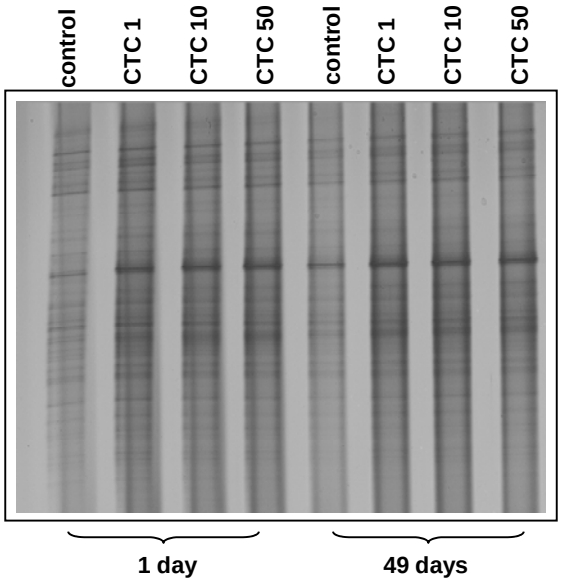


Figure 45.

DGGE patterns of runs without glucose after 1 and 49 days in orthic luvisol soil.

Similar to the results obtained with SDZ all incubations with CTC (without glucose) gave identical banding patterns at any date of sampling and no differences were visible compared to the control soils (figures 43 and 45).

However, the incubations with glucose (alone and with CTC) showed significant differences to the control soils. In the DGGE profiles a band became more intensive (r(m)1) after 1 day incubation. More clearly are the marked differences after 7 days. Band r(m)2 became clearly visible in all glucose + CTC treatments. In the treatments with glucose and CTC (concentration independent) from day 7 to 49 this two additional bands were intensive. No new bands became visible after a longer period of contact of glucose and CTC with the soil (figure 44). In contrast to the effects of SDZ no K strategists were detected.

The analysis of the DGGE patterns showed that the microbial communities of the CTC (only) treated and non treated soil were similar. As expected the clustering of the DGGE profiles revealed that all DGGE patterns had a relatively high level of similarity (> 80%) to each other for samples taken during the time of 49 days. The results of the DGGE analysis (appendix figure 6.5) of these bands and their tentative phylogenetic affiliation are shown in figure 46.

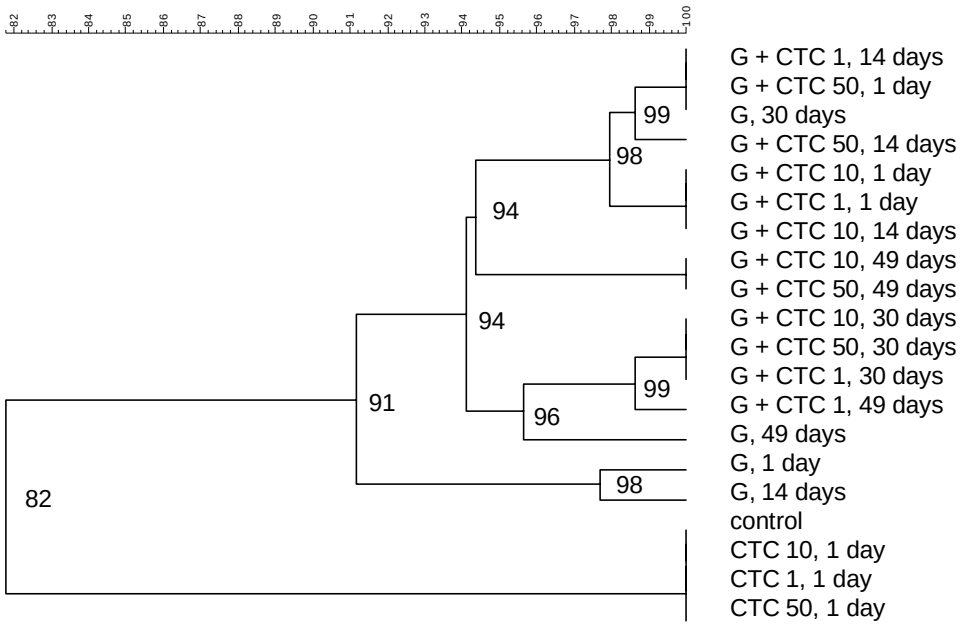


Figure 46.

Cluster analysis of DGGE patterns from gleyic cambisol soil treated with CTC and generated from all 49 days samples and all controls using Dice correlation matrix and UPGMA.

In the cluster analysis of the CTC treated soil (gleyic cambisol) it can be recognized that there is a high level of genetic homogeneity between the samples within two groups: soil without and with glucose. The soil samples belonging to the group with glucose and CTC (different concentrations) have barely genetic variation (matching 91%). Also in the orthic luvisol soil a very high conformity (>90%) existed between the patterns (figures 6.6 and 6.7 in appendix). These results indicate that soil bacteria were not affected by the added CTC. Halling-Sørensen et al. [123] measured the concentration of CTC in manured soil and observed a dissipation of CTC during a few weeks. The authors suggest a strong sorption of CTC to the organic fraction of the soils and in addition that a stronger sorption through increased residence time has possibly sequestered the compound within the matrix and may have rendered CTC non-extractable with time. In this experiment neither the respiratory activity nor the microbial population analysis gave suppression or showed influence of CTC on the soil microbial community probably due to strong sorption.
To find explanations for the absence of inhibitory effects of CTC in the soil the results of an experiment with soil slurries will be presented in the next subsection.

4.3.3 Bacteriological examination with the poured plate technique

Poured plates were used to follow the influence of antibiotic concentrations on culturable soil bacteria. The supernatant of soil slurries after centrifugation and diluted 4-fold were spread on the Müller-Hinton-Agar containing the antibiotic concentrations.
The results are shown in figures 47 and 48.

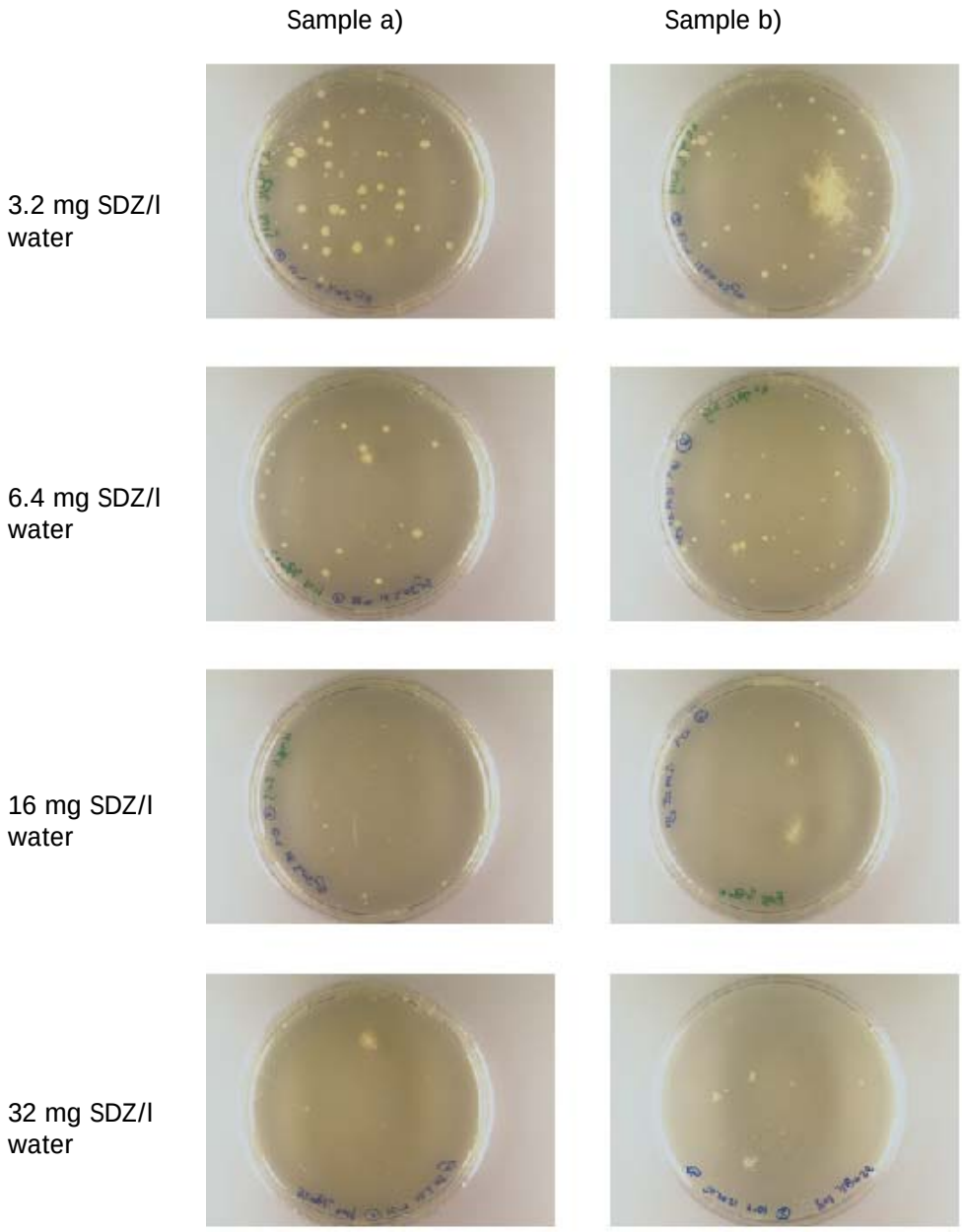


Figure 47.

Bacterial growth on agar plates of centrifuged supernatant from soil slurries after addition of different SDZ concentrations (3.2, 6.4, 16 and 32 mg SDZ/l water) to the agar broth.

With 3.2 mg SDZ/l 59/44 colonies could be counted and with 32 mg SDZ/l only 15/17 colonies grow (figure 51). Bacterial growth was clearly inhibited in dependence of the SDZ concentration which was in contrast to the inhibition with CTC. With the lowest concentration of 3.2 mg CTC/l the colony number obtained was 95/98 and with 32 mg CTC/l 117/102 were counted (figure 48).

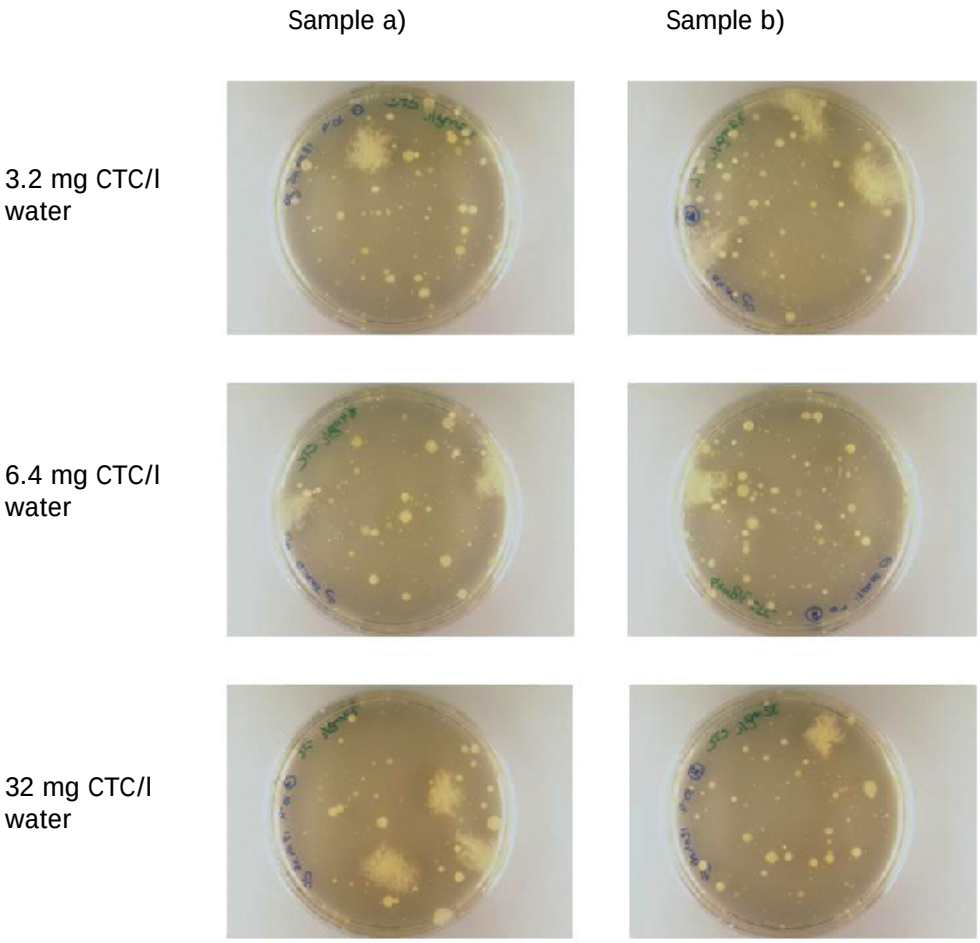


Figure 48.

Bacterial growth on agar plates of centrifuged supernatant from soil slurries after addition of different CTC concentrations (3.2, 6.4 and 32 mg CTC/l water) to the agar broth.

The results suggest that culturable soil bacteria are sensitive to SDZ and CTC shows no inhibitory activity at all. The obtained results are in accordance with the respirometric and DGGE results presented above.

4.3.4 Bacterial examination with the agar diffusion disk test

Sensitivity tests (agar diffusion disk test) of 47 soil bacterial isolates showed that the growth of only a small number of isolates was inhibited by SDZ or CTC. In presence of SDZ, 5 out of 47 strains tested were weakly inhibited but with a poor correlation to SDZ concentrations.

CTC inhibited the growth of 12 strains (including the 5 strains sensitive to SDZ) and the extent of growth inhibition was clearly correlated to CTC concentrations (figure 49).

An Example of absent inhibition by SDZ but a good, concentration dependent inhibition of CTC of one of the 47 strain is illustrated in figure 50.

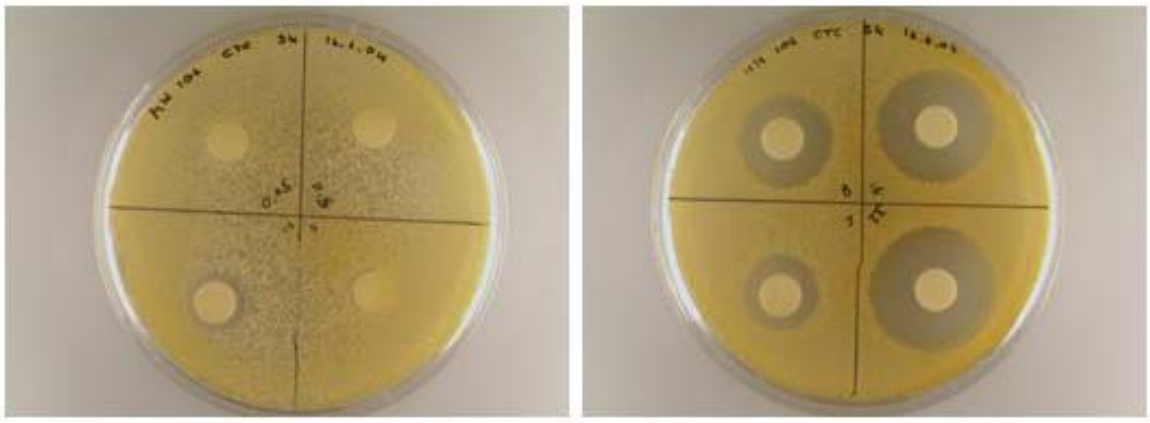


Figure 49.
Concentration dependent growth inhibition of a soil bacteria strain by CTC. (On the left: above 0.25 and 0.5/below 2 and 1; on the right: above 8 and 16/ below 4 and 32).

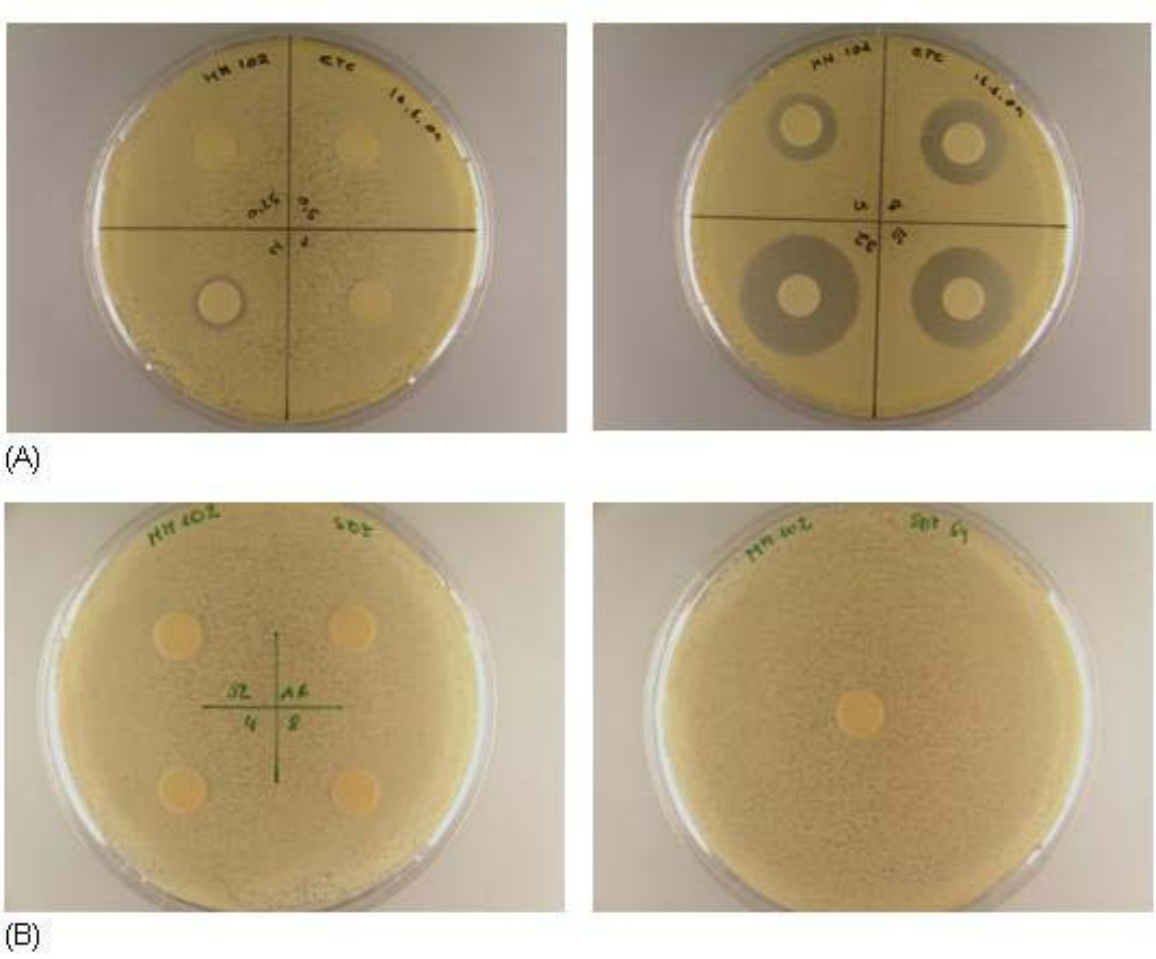


Figure 50.

Concentration dependent growth inhibition of a soil strain by SDZ and CTC. A) Inhibition by CTC. (On the left: above 0.25 and 0.5/below 2 and 1; on the right: above 4 and 8/below 32 and 16). B) No inhibition by SDZ. (On the left: above 32 and 16/below 4 and 8; on the right: 64).

Since these results were apparently not in agreement with the findings from the soil microcosms, the impact of the soil matrix on the growth inhibitory potential of CTC was tested in the supernatants of soil slurries as described in materials and methods. In supernatants from batches with the highest soil amendments, no growth inhibitory effects could be detected even after the shortest incubation time interval of 1 minute. The decrease in bioavailability of CTC in soil slurries is presented in figure 51.

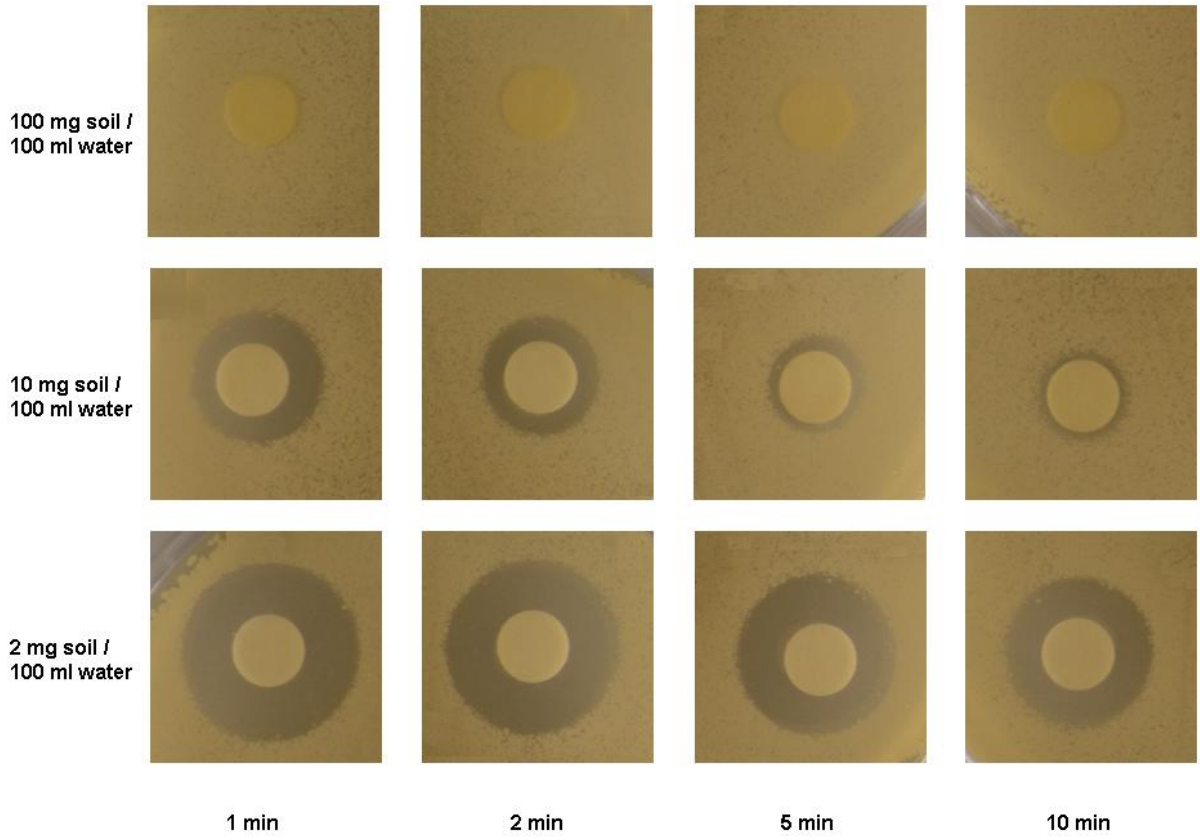


Figure 51.

Agar diffusion test of centrifuged supernatant from soil slurries in different concentrations (100, 10, and 2 g soil per 100 ml water) and at different time intervals (1, 2, 5, 10 minute) after addition of CTC (50 mg/l).

A time dependent decrease in growth inhibition was detectable in slurries diluted 10 fold. Even in 50-fold diluted soil slurries, an influence of the soil matrix on the antimicrobial effectiveness of CTC in the supernatants over time was detected. The strong matrix effect of soil is consistent with the results from the microcosm experiments and respirometric measurements insofar as there were no effects detectable in presence of CTC.

The bioavailable concentrations of CTC were calculated from calibration curves obtained with the same soil bacterial isolate and are presented in the figure 52.

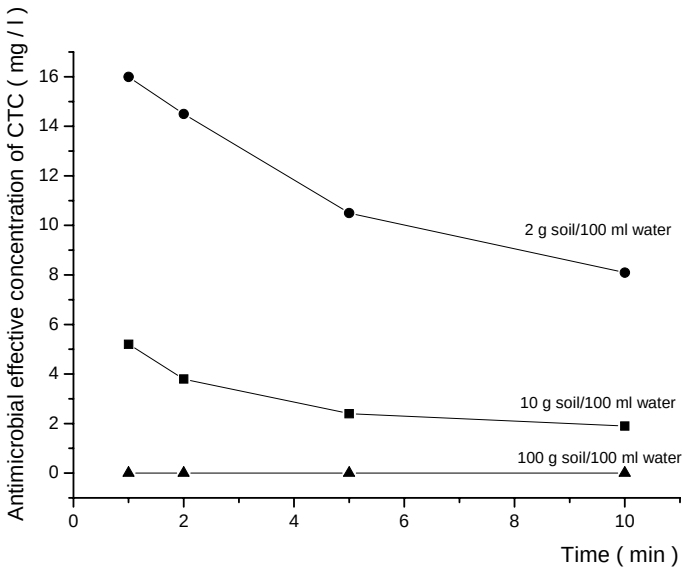


Figure 52.

Attenuation of bioavailable CTC concentratios as a function of soil/water ratio and contact time. Bioavailable concentrations were calculated from calibration curves obtained with the same soil bacterial isolate.

Considering the results obtained with soil bacterial isolates in the agar diffusion disk test, such a dramatic inhibition potential of SDZ as seen in soil respiration was not expected since only 5 strains out of 47 isolates were weakly inhibited by SDZ. However, cultured bacteria may not be representative for the soil bacterial population. Felske et al. [229] demonstrated that the predominant bacteria as identified by direct DNA/RNA extraction from soil were not correlated with the bacteria found by cultivation.

In the agar diffusion disk test with CTC, 12 out of 47 soil bacterial isolates were growth inhibited. With CTC, contrary to SDZ, neither an influence on soil respiration (figure 35) nor on the bacterial community structure with the DGGE method (figure 44) was detected. This was probably due to inactivation of CTC on contact with soil. In the experiments with orthic luvisol soil, the impact of the soil matrix on the growth inhibitory effect of CTC was clearly demonstrated (figures 48 and 49). Even diluted soil slurries had the potential to mask the antimicrobial effect of CTC during a contact time of less than 1 min. The mechanism of inactivation of CTC is possibly due to a strong chelation with calcium and magnesium ions [120, 230]. In addition to chelation, adsorption to soil [81] is also an important factor for the mitigation of antibacterial effects of tetracyclines. These findings are in agreement with those of Hund-Rinke [127] who concluded that tetracyclines are not likely to cause undesired effects on the soil microflora. However, reports from other authors reveal that tetracyclines

are well able to affect microbial populations in soils [135, 136]. These controversial results accentuate the requirement for further studies concerning the impact of antimicrobial substances in the environment.

The major processes leading to a reduction in antimicrobial activity of antibiotics after addition to soil are inactivation by chemical or biological modifications of the parent compound, chelation with polyvalent cations, biodegradation and adsorption to soil components. In batch equilibrium studies with 10 mg SDZ/kg soil Thiele-Bruhn and Aust [79] found a non-desorbable fraction of 24% after 1 day. The non extractable fraction increased with incubation time [228]. After 28 days incubation time they found 90% of the applied radioactivity in the non-extractable fraction. Heise et al. [235] described the rapid formation of non-extractable residues of SDZ in manured soil, 95% of ^{14}C –SDZ were transferred into the non-extractable fractions. The results obtained in this thesis are in agreement with those from Thiele Bruhn and Aust [79] and Heise et al. [235]. The non-extractable residues after one day of the contact time of SDZ with soil achieved 50% to 70% (concentration dependent) and after 30 days more than 90% of the amount of SDZ applied. The high affinity of sulfonamides to the soil matrix resulting in a low bioavailability of the SDZ to microorganisms was clearly shown by Heise et al. [235]. However, strong sorption and consequently reduced bioavailability can only partly explain the results obtained in the respirometric experiments where glucose was added repeatedly (figure 33) or after 19 days SDZ contact with soil (figure 34). However, the nature of non-extractable sulfonamides residues and their accumulation potential in soil is unknown.

Mineralization kinetics of glucose added to soil 19 days after initial amendment with 10 and 50 mg SDZ/kg soil (with or without glucose) were still inhibited compared to the 1 mg/kg treatment. Assuming that a fraction of SDZ had been adsorbed (about 90% of SDZ was not extractable) after 19 days, the concentration would still be high enough to have antimicrobial effects. Addition of SDZ together with a carbon source (glucose) had a more pronounced and longer lasting impact on microbial response to a second addition of glucose compared to pre-incubation with SDZ alone. Although kinetics were inhibited, repeated applications of glucose, with the exception of the glucose + 50 mg SDZ/kg soil treatment, mineralized more rapidly than in the first application. In addition to the possibility of adsorption, another explanation could be that the contact time of 3 weeks induced tolerance of the soil microbial biomass to the antibiotic, as has been shown for other sulfonamide antibiotics [184].

5. Conclusions and outlook

In this study the influence of the veterinary antibiotics sulfadiazine and tetracycline on soil bacteria and the quantification of the extractable concentration of SDZ were investigated.

It was demonstrated that LCMS/MS combined with PLE extraction and no further sample clean up, is an effective and time saving method for the detection of SDZ in environmental samples like soil. To my knowledge acetyl-SDZ was determined for the first time after spiking soil with non labelled SDZ. A comparison of the extraction methods (microwave solvent extraction MAE and pressurised liquid extraction PLE) revealed that both methods are suitable to obtain good recovery rates for SDZ. The soil extraction procedure was optimised for SDZ only and probably higher recoveries would have been achieved for acetyl-SDZ by modifying the method (solvent and temperature choice) in respect to this compound. Acetyl-SDZ was measured in small amounts in soil after spiking the soil with SDZ. Thus it is not only generated in animals as a detoxification reaction.

Dissipation of SDZ was not observed in dry soil. After wetting the soil leading to a condition which is normal for natural conditions and especially after manuring SDZ dissipated rather fast. The dissipation in soil could not be described by first order kinetics.

An explanation would be that the dissipation of SDZ over time exhibits at least two rates due to a fast reversible and an irreversible adsorption of either the parent compound or transformation products to the soil compounds.

The comparison of sterilised and non-sterilised soil showed that microbial transformation or degradation of SDZ in soil can not be excluded. After 104 days it was still possible to measure SDZ residues with the microwave solvent extraction), PLE (pressurised liquid extraction applied extraction method. However, the nature of the non-extractable sulfonamides residues and their accumulation potential in soil is unknown [235] and deserve further research.

The influence of SDZ on the microbial communities was different in the different soils and effects were only measured in the presence of an assimilable carbon source. The results indicate that SDZ was only slightly (first at concentrations higher than 20 mg/ L, which is considerably above predicted soil concentrations of sulfonamides) active against the cultured bacteria, isolated from the soil . This was confirmed by the results obtained with DGGE assuming, that the extracted soil bacteria are representative for the soil bacterial population. CTC in soil showed no antimicrobial effect with and without an additional carbon source which can be explained by a strong sorption to the soil resulting in a low bioavailability.

On the basis of the obtained results it is not very likely that SDZ and CTC potentially present in soil will have any direct effect on soil functions (except perhaps on nitrifiers [240], which was not chequed in this work) or microorganisms. However, Chander et al. [234] state that even if antibiotics are tightly sorbed to soil, they can still play a role in the emergence of antibiotic resistant bacteria in the environment and Hamscher et al. [1] and Thiele-Bruhn and Beck [136] concluded that although extractable concentrations of antibiotics in field soils are generally low, initial concentrations immediately after addition can be high enough to affect soil microorganisms. The potential risk of sulfadiazine for the environment is still unclear.

Further research in the assessment of antimicrobial effects of veterinary antibiotics on soil bacterial populations and the fate of veterinary antibiotics should focus on:

- Further characterisation of SDZ, acetyl-SDZ and other still unknown metabolites (as e.g. described by Wehrhan [232]) for example with hot water extraction, which could be a measure of bioavailability of these compounds.
- Affinity of ^{14}C-SDZ residues (metabolites) to the soil matrix and the formation of metabolites in sterilised and non-sterilised soils.
- In contrast to the single application of antibiotics in the current study design, an increase in the effective concentration can be expected by repeated manuring, due to accumulation of SDZ and CTC in soil.
- Microbial measurements should be carried out in the field to study the influence of manure as natural nutrient and carbon source together with antibiotics on soil microorganisms.

6. Appendix

List of used chemicals

- 20 mM dNTP mix; ABgene, Surrey, UK
- 25mM $MgCl_2$; ABgene, Surrey, UK
- 50x TAE-buffer; Bio-Rad Laboratories Central Europe
- Acetic acid 99-100% for synthesis; Merck KGaA, Germany
- Acetone 98% p.a.; Merck KGaA, Germany
- Acetonitrile 99,9% for HPLC; Biosolve, Valkenswaard, NL
- Acrylamide–bis ready-to-use solution 40% (37.35:1) for electrophoreses; Merck KGaA, Germany
- Ammonium acetate 98%; KMF, St. Augustin, Germany
- Ammonium-persulphate min. 98%, p.a. ACS; Carl Roth GmbH & Co., Germany
- Bulk Isolute® Sorbent; Isolute® HM-N; Separtis GmbH, Germany
- Certified ™ Molecular Biology Agarose; Bio-Rad Laboratories Central Europe
- Chlortetracycline hydrochloride from *Streptomyces aureofaciens* for fluorescence; Vetranal®, standard for analytical purposes (84.4%); Riedel-de Haen®;Seelze, Germany
- Copper(II)-sulfate pentahydrate; Merck KGaA, Germany
- D(+)-Glucose monohydrate for biochemistry; Merck KGaA, Germany
- Dimethylsulfoxide for molekcular biology ≥ 99.5%, p.a.; Carl Roth GmbH & Co., Germany
- DMSO; Sigma, Germany
- Ethanol absolute for synthesis; Merck KGaA, Germany
- Ethidium bromide solution 1%; Carl Roth GmbH & Co., Germany
- EZ Load ™ Precision Molecular Mass Rulers (EZ Precision Molecular Mass Standard) 50 µg/ml; Bio-Rad Laboratories Central Europe
- FastDNA® Spin® Kit (for soil); BIO 101 Systems; Q-BIOgene
- Formaldehyde solution 37%, stabilised for synthesis; Merck KGaA, Germany
- Formamid deionised min. 99.5%; Carl Roth GmbH & Co, Germany
- Formic acid 98% for HPLC; Romil, UK
- Methanol 99,9% for HPLC; Merck KGaA, Germany
- Mueller- Hinton- Agar; Merck KGaA, Germany
- PAG film: GelBond, Amersham Biosciences, Buckinghamshire, UK

- Primer L1401; 10 pmol/ml; MWG Biotech, Germany
- Primer U968 10 pmol/ml; MWG Biotech, Germany
- R2A Agar; Oxoid LTD, England
- Reaction buffer; ABgene, Surrey, UK
- Ringer-tablets for preparation of Ringer solution; Merck KGaA, Germany
- Silvernitrate p.a.; Merck KGaA, Germany
- Sodium hydroxide, pellets pure; Merck KGaA, Germany
- Sodium borohydride, granulate for synthesis; Merck KGaA, Germany
- Sodium carbonate waterfree p.a.; Merck KGaA, Germany
- Standard I- Nutrient broth for microbiology; Merck KGaA, Germany
- Sulfadiazine Vetranal®; min 99.9% for HPLC; Riedel-de Haen®;Seelze, Germany
- Sulfadiazine; min. 99.0%; Sigma- Aldrich Chemie GmbH, Germany
- TEMED p.a. 99% for electrophoreses; Carl Roth GmbH & Co., Germany
- TermoStart Taq Polymerase; ABgene, Surrey, UK
- Urea, min. 99.5%, p.a.; Carl Roth GmbH & Co., Germany
- Water, pure 18,2 MΩcm; Milli-Q® Synthesis A10, Millipore, Germany
- Wizard® DNA Clean-Up System; Promega, USA

Figures

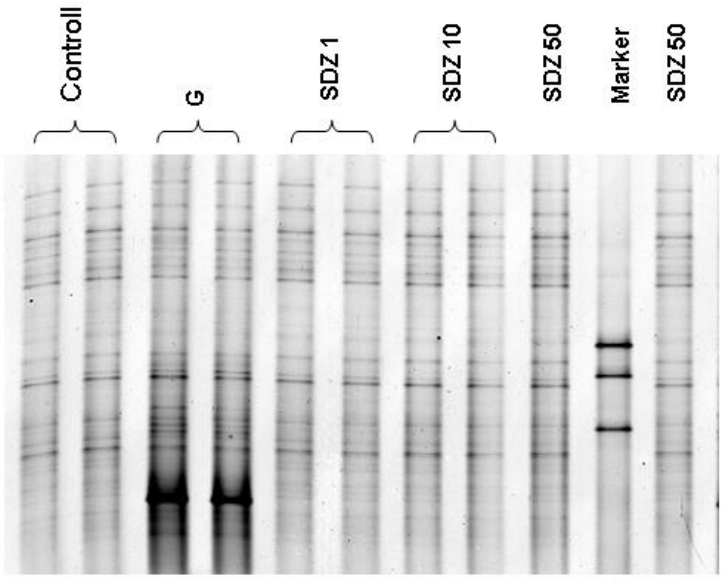
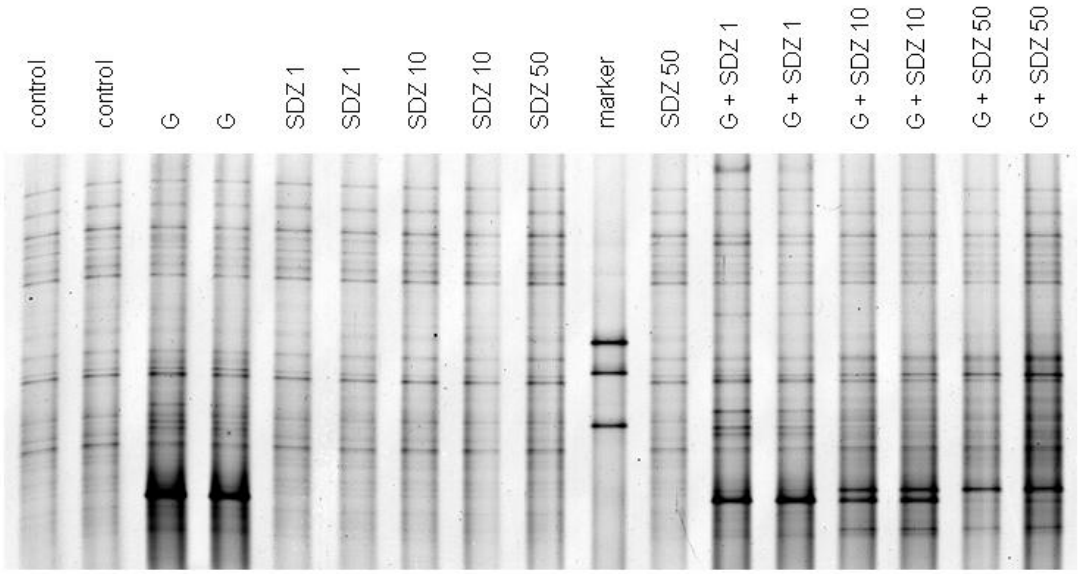


Figure 6.1
DGGE patterns of duplicates taken from orthic luvisol soil microcosms after 21 days.

(A)



(B)

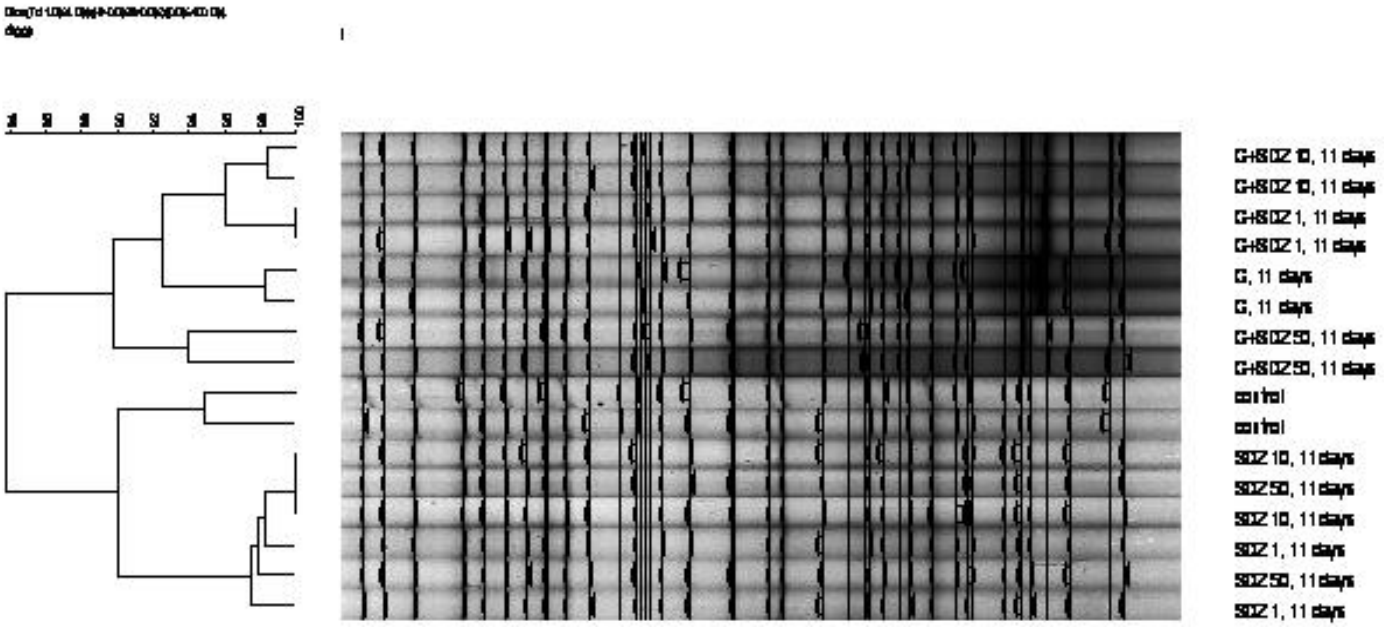


Figure 6.2
(A) DGGE patterns of orthic luvisol soil samples after 11 days incubation with SDZ and with and without Glucose. (B) Digital analysis of the DGGE with Gel Compar Software; Dice correlation.

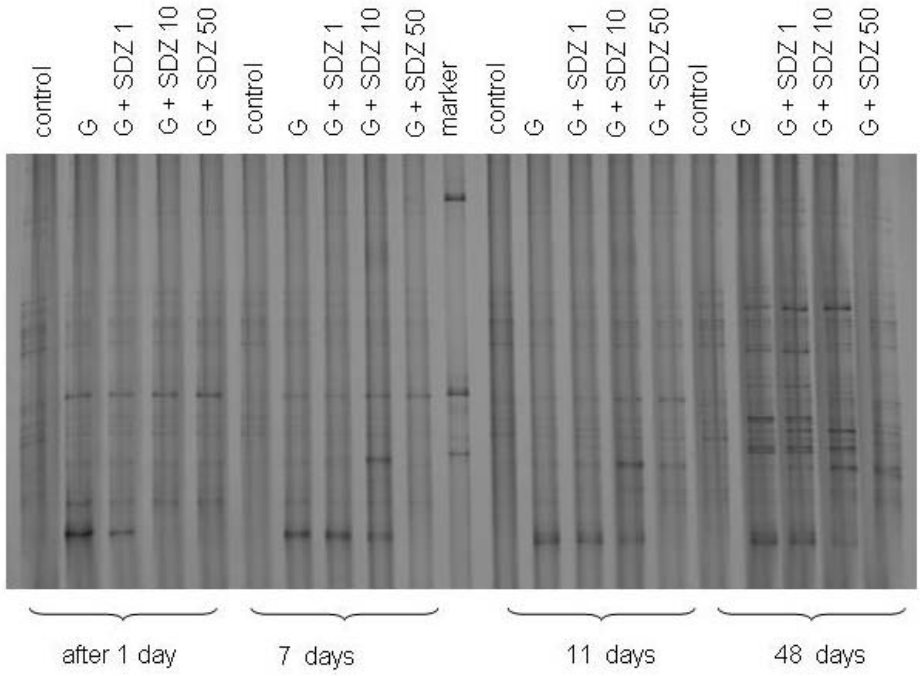


Figure 6.3
DGGE patterns of orthic luvisol soil samples taken from the microcosms after the respective incubation time periods after treatment with SDZ in different concentrations.

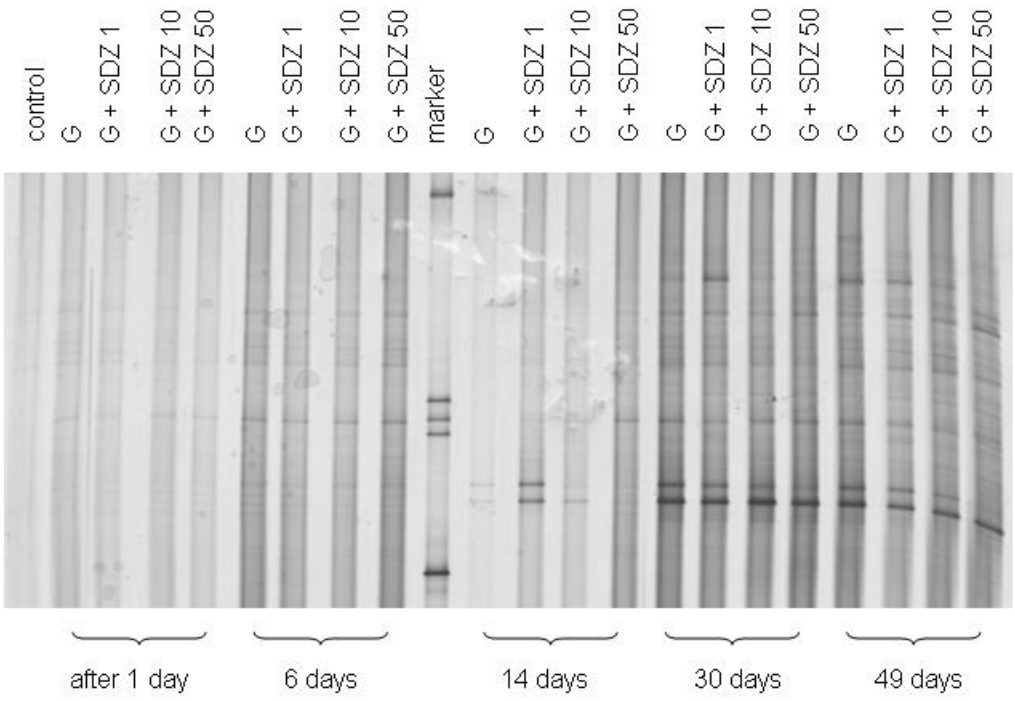


Figure 6.4
DGGE patterns of gleyic cambisol soil samples taken from the microcosms after the respective incubation time periods after treatment with SDZ in different concentrations.

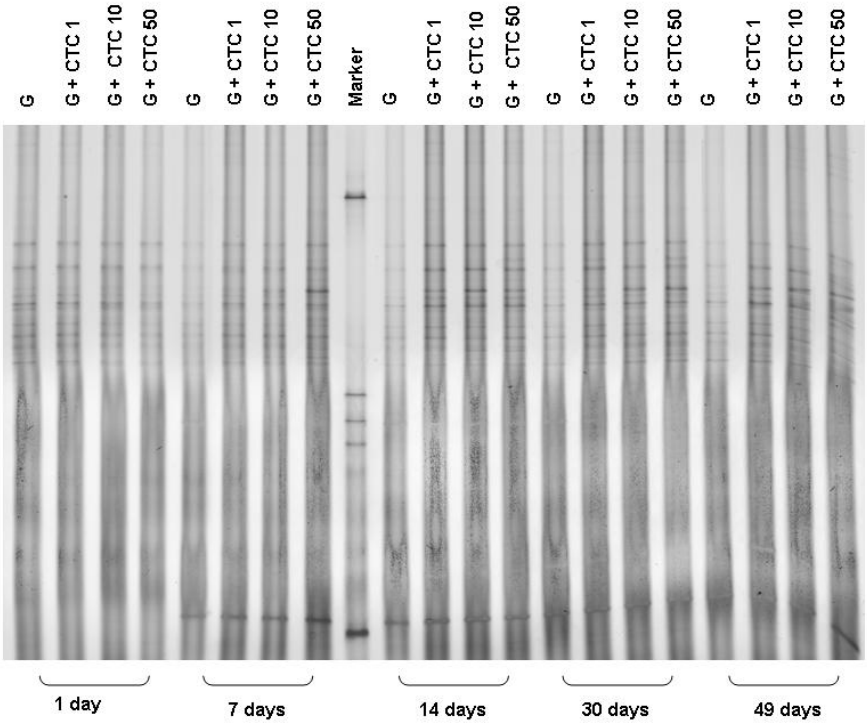


Figure 6.5
DGGE patterns of soil samples taken from the gleyic cambisol soil microcosms after treatment with CTC (the incubation periods of 49 days).

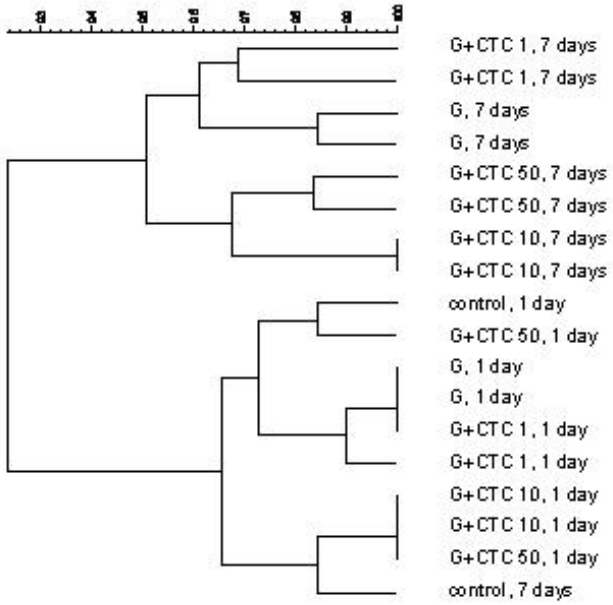


Figure 6.6

Cluster analysis of microbial community profiles obtained with PCR-DGGE after 1 and 7 days after CTC treatment. Orthic luvisol soil.

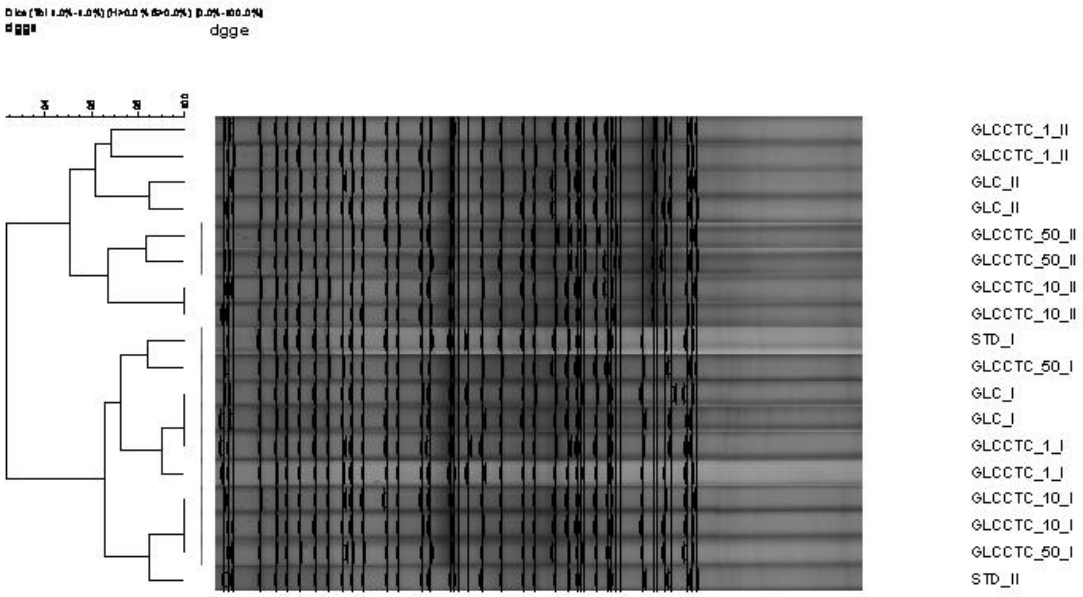


Figure 6.7.

DGGE patterns of orthic luvisol soil samples after 1 and 7 days incubation with CTC with and without glucose. Digital analysis of the DGGE with Gel Compar Software: Dice correlation.

7. Literature

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