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Accelerated microbial degradation of the veterinary antibiotic sulfamethazine in agricultural soil

Natalie Seyfried

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Vorsitzender: Prof. Dr. Johannes Kollmann

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1. Prof. Dr. Jean Charles Munch
2. Prof. Dr. Siegfried Scherer

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Abstract

Veterinary antibiotics like sulfamethazine are administered to farming animals in large amounts worldwide and contaminate agricultural soils through manure fertilisation.

Once the drugs reach the soil ecosystem they can affect it by changing the pattern and functions of the soil microbial communities for example by stimulating antibiotic resistant organisms. Another critical point is the accumulation of antibiotics in soil, and their transport to other environmental compartments. To reduce or avoid these risks, soil remediation approaches need to be developed which eliminate antibiotics effectively and permanently from soils, without further soil damages.

In this thesis different strategies were applied, to develop an approach for decontamination of soils from sulfamethazine, whereat a major difficulty was the fact that the substance the microorganisms are supposed to degrade has been developed to hamper their growth.

The first challenge was the selection of an antibiotic that was

- I. frequently used in veterinary medicine
- II. posing an environmental risk due to its accumulation in soils
- III. not naturally degraded by soil microorganisms
- IV. not adsorbing to soils too strongly, instead it should be bioavailable

and which was thereby suitable for soil incubation experiments.

To select the appropriate substance, three different antibiotics were tested for their sorption characteristics in agricultural soil samples (Tetracycline, Tetracycline*HCl and Sulfamethazine). Sulfamethazine exhibited the highest solubility in the soil pore water, and therefore the highest bioavailability. Thus, this substance was selected for further degradation experiments.

Sulfamethazine was purchased with a ^{14}C-labelled aromatic ring for improved tracking of residues in soils as well as in the atmosphere.

The second task was to select a soil suitable for soil inoculation experiments. This soil should not adsorb the antibiotic too strongly to support the bioavailability and provide a large sulfamethazine fraction available for microbial biodegradation in the soil pore water.

To investigate sorption characteristics of different soils, three soils were selected, differing in their properties like pH and soil type. These three soils were spiked with sulfamethazine and

over two weeks, the amount which was dissolved in the soil pore water was determined by extraction. The soil exhibiting the biggest portion of dissolved sulfamethazine in the pore water, standing for bioavailable sulfamethazine, was selected for all following experiments (sandy loam).

Thereupon, the effects of a single sulfamethazine application on the microbial biomass in this soil were determined. After an incubation time of up to three weeks, soil samples were chloroform fumigated and extracted.

The third step was originally to adapt a soil with its microbiota to the degradation of sulfamethazine by different methods and thereupon, to isolate a sulfamethazine degrading bacterium.

To enhance this adaptation process, different soil experiments were conducted. Soil samples were

   I.   incubated at different soil moisture regimes and different sulfamethazine concentrations.

  II.   incubated at different temperatures, also with different sulfamethazine concentrations.

 III.   spiked with sulfamethazine several times.

As this adaptation process needs more time than is available for a PhD-work, an existing sulfamethazine degrading strain and its microbial community was obtained from a Canadian soil, adapted to degradation within 10 years of sulfamethazine application.

Finally, soil incubation experiments were performed with sandy loam soil samples, spiked with sulfamethazine, at environmentally relevant concentrations of 1 µg g^{-1}. The isolated strain and the soil-borne microbial community (also including this strain) were introduced to soil samples separately. The soil was decontaminated successfully with the most promising variant being the inoculation with the whole microbial community established on carrier material. In this way, 57% of the applied sulfamethazine was degraded within 46 days, whereas sulfamethazine degradation by the control soil samples without microbes was negligible (0.7%). The degrading bacteria were still able to mineralise sulfamethazine successfully after 112 days, showing the stability and sustainability of this inoculation approach.

In the cases in which sulfamethazine was degraded in high quantities, the large fractions of non-extractable ^{14}C-residues in soil samples were presumably of biogenic origin, consisting of biomolecules containing fixed ^{14}C. Since these biomolecules (for example sugars or amino acids) are harmless to the environment, the risk of persisting non-extractable sulfamethazine residues in soils is decreased, when high mineralization is performed.

This soil remediation approach, presented here for a bacteriostatic substance, was already successfully applied to other soil samples, even in outdoor lysimeter experiments. Other organic compounds (pesticides) were used in other soil materials, indicating a high potential to effective remediate soils also from many other contaminants while conserving soil functionality, particularly for food production.

Zusammenfassung

Veterinärantibiotika wie Sulfamethazin werden in der Tierhaltung in großen Mengen eingesetzt, wodurch sie schließlich über die Ausbringung der Gülle als Düngemittel Ackerland kontaminieren.

Geraten die Medikamente einmal in das Ökosystem Boden, können sie es beeinflussen durch Veränderung der Zusammensetzung und der Funktionen der mikrobiellen Gemeinschaft sowie durch Anreicherung antibiotika-resistenter Bodenorganismen. Ein weiterer kritischer Punkt ist die Anreicherung von Antibiotika in Böden und deren Verlagerung in andere Umweltkompartimente. Um diese Risiken zu reduzieren oder zu verhindern müssen Ansätze zum Bodenschutz entwickelt werden, die die ausgebrachten Antibiotika effektiv und dauerhaft von Böden eliminieren.

In dieser Promotionsarbeit wurden verschiedene Maßnahmen zur Bodendekontamination von Sulfamethazin erprobt. Die größte Herausforderung dabei war, dass diese von Mikroorganismen abzubauende Substanz entwickelt wurde, um deren Wachstum einzudämmen.

Die erste Aufgabe war es, ein passendes Antibiotikum auszuwählen, das

  I.   in der Tiermedizin häufig verwendet wird
 II.   aufgrund seiner Anreicherung im Boden ein Umweltrisiko darstellt
III.   nicht natürlicherweise von Bodenorganismen abgebaut wird
 IV.   nicht zu stark am Boden adsorbiert wird und somit bioverfügbar ist

und aufgrund dieser Eigenschaften für Bodeninkubationsexperimente geeignet ist.

Um die geeignete Substanz auszuwählen, wurden drei verschiedene Antibiotika auf ihre Sorptionseigenschaften in Proben eines landwirtschaftlichen Bodens getestet (Tetrazyklin, Tetrazyklin*HCl und Sulfamethazin). Sulfamethazin zeigte die größte Löslichkeit im Porenwasser und damit die höchste Bioverfügbarkeit. Daher wurde diese Substanz für die weiteren Abbau-Versuche ausgewählt.

Das Sulfamethazin wurde mit einem ^{14}C-markierten aromatischen Ring bezogen, um dessen Abbauprodukte und Rückstände in Bodenproben sowie in der Atmosphäre gezielt verfolgen zu können.

Die zweite Aufgabe war die Auswahl eines für Inokulationsexperimente geeigneten Bodens. Dieser Boden sollte das Antibiotikum Sulfamethazin nicht zu stark adsorbieren, um es im Porenwasser für den mikrobiellen Abbau zur Verfügung zu stellen. Um die Sorptionseigenschaften verschiedener Böden zu untersuchen, wurden drei Böden ausgewählt, die sich in ihren Eigenschaften wie pH Wert und Bodenart unterschieden. Diesen drei Böden wurde Sulfamethazin zugegeben und über einen Zeitraum von zwei Wochen wurde die im Porenwasser gelöste Menge mittels Extraktion bestimmt. Der Boden mit dem größten Anteil an gelöstem Sulfamethazin im Porenwasser, das für bioverfügbares Sulfamethazin steht, wurde für alle weiteren Experimente ausgewählt (sandiger Lehmboden).

Daraufhin wurden die Auswirkungen einer einmaligen Sulfamethazin-applikation auf die mikrobielle Biomasse dieses Bodens ermittelt. Die Bodenproben wurden nach einer bis zu dreiwöchigen Inkubationszeit mit Chloroform begast und anschließend extrahiert.

Ursprünglich sollte der dritte Schritt darin bestehen, über verschiedene Methoden einen Boden, mitsamt dessen Mikrobiota, an den Abbau des Antibiotikums zu adaptieren, und ein Sulfamethazin-abbauendes Bakterium zu isolieren. Um diesen Adaptionsprozess zu beschleunigen wurden verschiedene Bodenexperimente durchgeführt. Die Bodenproben wurden

   I.   bei unterschiedlichem Wassergehalt und unterschiedlichen Sulfamethazin Konzentrationen inkubiert.
   II.   bei unterschiedlichen Temperaturen inkubiert, ebenfalls mit unterschiedlichen Konzentrationen des Antibiotikums.
   III.   mehrfach mit Sulfamethazin behandelt.

Da dieser Adaptionsprozess mehr Zeit benötigt als eine übliche Promotionsdauer, wurde ein bereits bekanntes Sulfamethazin abbauendes Bakterium aus einem 10 Jahre lang adaptierten Kanadischen Boden bezogen.

Schlussendlich wurden Inkubationsexperimente mit Proben eines sandigen Lehmbodens durchgeführt, die in einer umweltrelevanten Konzentration von 1 µg g^{-1} mit Sulfamethazin versetzt wurden. Dabei wurden der isolierte Einzelstamm und die bodenbürtige mikrobielle Gemeinschaft separat in die Bodenproben eingebracht. Diese Bodenproben wurden erfolgreich dekontaminiert wobei die vielversprechendste Variante die Bodeninokulation war, mit der gesamten bakteriellen Gemeinschaft die auf einem Trägermaterial eingebracht worden war. Auf diese Weise wurden 57% des applizierten Sulfamethazins innerhalb von 46 Tagen

abgebaut, während der Sulfamethazin Abbau in den Kontroll-Bodenproben ohne zugesetzte Bakterien vernachlässigbar gering war (0.7% des applizierten Sulfamethazins). Die abbauenden Bakterien waren nach 112 Tagen noch aktiv und mineralisierten das Sulfamethazin erfolgreich, wodurch die Stabilität und Nachhaltigkeit der Inokulations-Methode gezeigt werden konnte.

Da das Sulfamethazin in großen Mengen abgebaut wurde, waren die erhöhten Anteile an nicht-extrahierbaren ^{14}C-Rückständen in den Bodenproben wahrscheinlich biogenen Ursprungs, bestehend aus Biomolekülen, die das fixierte ^{14}C enthielten. Da solche Biomoleküle (wie z.B. Zucker oder Aminosäuren) generell harmlos für die Umwelt sind, ist das Risiko persistenter nicht-extrahierbarer Sulfamethazin-Rückstände in Böden verringert, bei hohen Abbauleistungen.

Dieser Bodensanierungsansatz, der hier für eine bakteriostatische Substanz präsentiert wurde, wurde bereits erfolgreich auf andere Bodenproben und auch in Freiland-Lysimeter-Anlagen mit anderen organischen Substanzen (Pestizide) in anderem Bodenmaterial angewendet. Dies zeigt das hohe Potential, Böden auch von vielen anderen Kontaminanten zu befreien, unter Erhalt der Bodenfunktionen, insbesondere der Gewinnung von Lebensmitteln.

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1.   Introduction

Soil remediation approaches are to be developed to decontaminate soils from veterinary antibiotics like sulfamethazine (SMZ), as husbandry leads to the increased use of these compounds. They enter the ecosystem soil with slurries, may affect soil microbial functioning and disseminate resistance genes in soil biota. The necessary preliminaries for conducting this study were

first, the selection of an antibiotic, which is frequently used in veterinary medicine and suitable for soil incubation experiments, which can be ^{14}C labelled for better tracking of residues in soil;

second, selection of a soil which only moderately adsorbs the antibiotic, so it can be available for microbial degradation;

third, a degrading microorganism to mineralise the antibiotic in the soil.

This introduction provides the background information to these points and to the principle necessity of a soil conserving remediation approach.

1.1   Veterinary antibiotics

Antibiotics are frequently used in human medicine as well as in veterinary medicine to prevent and cure infectious diseases. Since Alexander Fleming discovered antibiotic activity through a *Penicillium* fungus in 1929, many other antibiotics were isolated from fungi or bacteria. These antibiotics are secondary metabolites naturally produced in soils by mould fungi like *Penicillium chrysogenum* (penicillin) or *Acremonium chrysogenum* (cephalosporin) and bacteria like *Bacillus* species as well as *Streptomyces* species (Munk 2000). As secondary metabolites, these antibiotics are not essential for the growth of the microorganisms, and are therefore produced in stationary phase (fig. 1). Instead, they allow the producing organisms to have survival benefits in the biodiverse soil habitat.
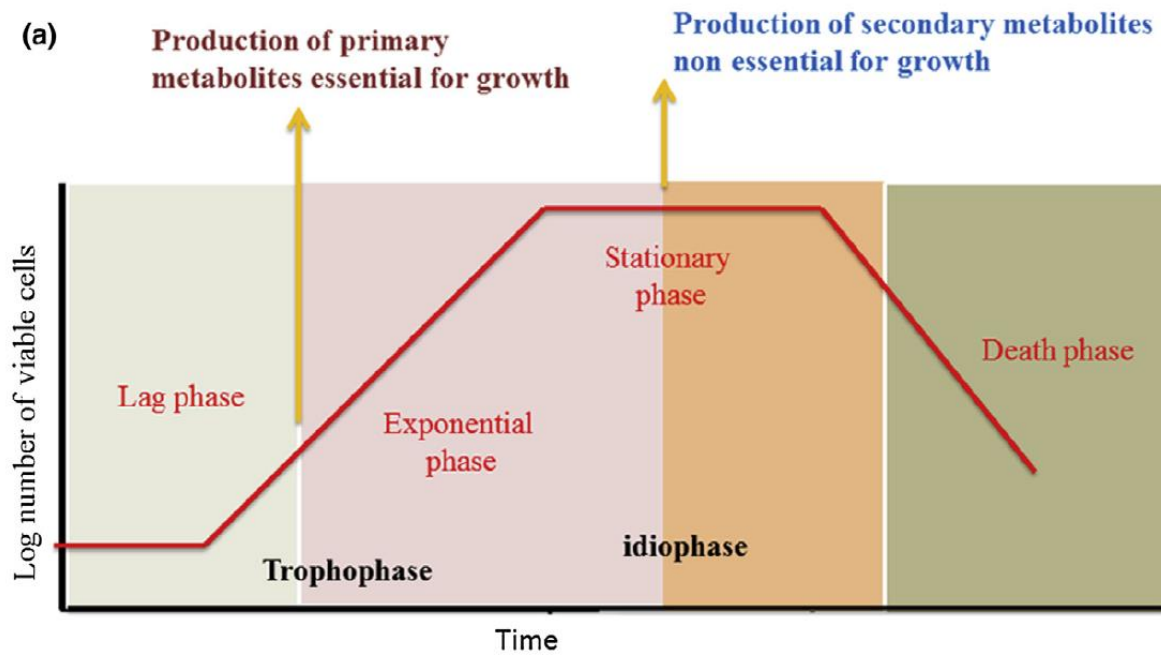
Fig. 1. Schematic representation of a microbial growth curve; the time of the antibiotic production (secondary metabolite) is labelled. From Gokulan et al. (2014).

Besides the natural antibiotics, many semi-synthetic or fully synthetic antibiotics are produced which effect different types of microbes. Generally, an antibiotic can be bacteriostatic or bactericidal. The bactericidal substances kill the bacteria within some hours while the bacteriostatic antibiotics limit their growth via different mechanisms. As the frequent usage of antibiotics in human as well as in veterinary medicine has led to resistant microorganisms, it is necessary to continuously develop or isolate new antibiotic substances. When patients already possess resistant microbes making the common antibiotics non-effective, they cannot be used for curing their infections anymore. In this case some special so called "reserve antibiotics" are applied. These reserve antibiotics should actually only be used in these severe cases. With this background it is obvious, how dangerous a misuse of these reserve antibiotics in animal husbandry is. But also the common antibiotics should only be used for animals which have infections and a medication should be avoided whenever it is possible. In Europe, the usage of antibiotics as growth promoters was therefore prohibited in 2006 (European Community 2003). The global average use of antibiotics was estimated by Van Boeckel et al. (2015) to be 63.151 ± 1.560 tons in 2010, with an expected drastically increase over the next 20 years. An overview of the antibiotics used in six countries is provided by Kim et al. (2011), presented in table 1. For the usage in Germany see 1.1.1.

Table 1. Usage of the three most frequently used veterinary antibiotics in six countries. The numbers in parenthesis indicate the percentage of the substance compared to the total usage of veterinary antibiotics in that country. From Kim et al. (2011).

Country	Amount used (tonne)			
	Total	Tetracyclines	Sulfonamides	Macrolides
Denmark	112	30 (27[a])	13 (12)	22 (20)
Korea	1,595	723 (45)	237 (15)	59 (4)
Norway	6	0.3(5)	1.5 (25)	–
Sweden	16.4	1.6 (10)	2.5 (15)	1.0 (6)
UK	390	240 (61)	74 (19)	37 (9)
USA	11,148	3,230 (29)	–	–

1.1.1   Main groups of veterinary antibiotics used in Germany

The main groups of veterinary antibiotics used in Germany are penicillin, tetracyclines, sulphonamides, macrolides and polypeptide antibiotics. Data from 2014 show that 1.238 t of antibiotics have been sold to veterinary surgeons in Germany, with sulphonamides contributing 121 t (Germap 2015). A ranking of antibiotics sold in Germany in 2014 is shown in table 2.

Table 2. Antibiotics sold to veterinary surgeons in Germany in 2014, modified from Germap (2015).

Antibiotic group	Sold to vets in 2014 (t)
Penicillin	450
Tetracyclines	342
Sulphonamides	**121**
Macrolides	109
Polypeptide antibiotics	107
Aminoglycosides	38
Antifolates	19
Lincosamides	15
Pleuromutilines	13
Fluoroquinolones	12.3
Amphenicoles	5.3
Cephalosporins 3rd generation	2.3
Cephalosporins 1st generation	2.1
Cephalosporins 4th generation	1.4
Sum	1 238

The antibiotic investigated in this study, sulfamethazine, is an antibiotic from the group of sulphonamides (third rank in Germany, marked blue in table 2), which are synthetically produced antibiotics with bacteriostatic effect.

The first sulphonamide was developed in 1935 by Gerhard Domagk (Frey and Löscher 2010). Sulphonamides block the dihydropteroate synthase via competitive inhibition to the 4-aminobenzoic acid, so the dihydropteroate synthase can no longer produce folic acid, which is an important component for the synthesis of DNA, RNA and proteins (Löscher et al. 2014). The location of the interference is shown in fig. 2. In this way, the cell growth is hampered and the immune system of the treated animal eliminates the pathogenic cells (Löscher et al. 2014). Sulphonamides are effective against gram-negative as well as gram-positive bacteria, including many pathogens, like *E. coli*, species of *Klebsiella*, *Pneumococcus*, *Salmonella*, *Shigella*, *Staphylococcus* and *Streptococcus*. However, several resistances have been detected in *E. coli*, isolated from calves, pigs, dogs and cats (Löscher et al. 2014). The microbes can develop resistances by producing a higher amount of para-aminobenzoic acid to lower the competitive effect of the sulphonamides, or by adapting the dihydropteroate synthase. These developments are intensified, when the antibiotic is not administered long enough, or the dose of the sulphonamide was too low (Löscher et al. 2014). In agricultural farming, SMZ is mainly used in cattle, pig, sheep and goat husbandry (Löscher et al. 2014).
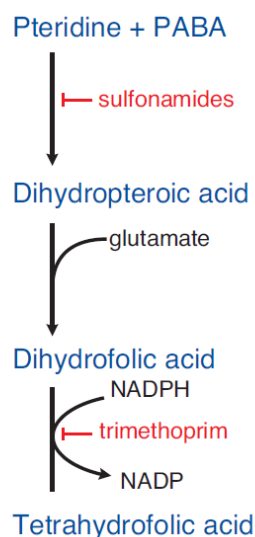
Fig. 2. Sulphonamides block the synthesis of folic acid by competitive inhibition of dihydropteroate synthase, so the para-aminobenzoic acid (PABA) cannot bind to the active centre of that particular enzyme and dihydropteroic acid is not formed. From Goodman and Gilman (2011).

Due to the fact that sulphonamides block the folic acid synthesis, only microbes that depend on the synthesis of folic acid are sensitive; organisms that take up folic acid from their environment are insensitive (Goodman and Gilman 2011). Mammalian cells are not able to synthesise folic acid are therefore also not affected by the sulphonamide drugs (Goodman and Gilman 2011) and thus have a natural resistance to sulphonamides (Löscher et al. 2014).

1.1.2   Main entrance of antibiotics to soils and environment

When administered to animals (or humans) antibiotics are required to reach their site of action in a suitable quantity. As parts of the drug may be altered or eliminated by the metabolism of the treated animal, it is necessary to administer sufficient amounts to ensure that the required quantity reaches its site of action. Consequently, portions of these drugs will be excreted by medically treated animals.

Veterinary antibiotics enter the soils through manure application from treated animals or directly by excretion to the soil surface from treated grazing animals (Jørgensen and Halling-Sørensen 2000; Winckler and Grafe 2001). Once introduced to soil, these antibiotics can contaminate other environmental compartments and affect terrestrial as well as aquatic organisms (Fig. 3), especially in the case of high solubility and low sorption to the soil matrix.
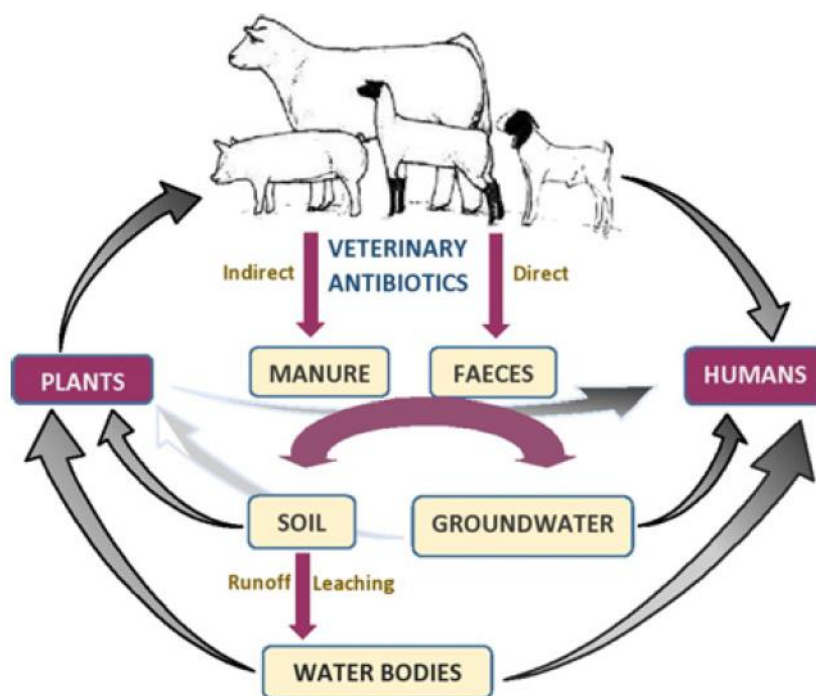
Fig. 3. Veterinary antibiotics distribution pathways in the environment. From Tasho and Cho (2016).

Different antibiotics that have been excreted from treated animals were recovered in the environment. Hamscher et al. (2002) detected minimum inhibitory concentrations of tetracycline and chlortetracycline in soil after manure application. Christian et al. (2003) identified many antibiotics from different groups (β-lactams, fluoroquinolones, sulphonamides, macrolides and lincosamides) in water samples from 16 river banks in Germany. Yan et al. (2013) screened the surface water of the Yangtze estuary for 20 different antibiotics over time, where chloramphenicol, sulphonamides, and macrolides were present in all samples, reflecting the widespread distribution of veterinary antibiotics in nature.

1.1.3   Degradation of antibiotics in soils

Several different studies investigating antibiotic degradation from different groups in diverse test matrices are summarised by Boxall et al. (2004). Tetracycline degradation with half-lives of 4.5 to 106 days was investigated in soils, sediments and aerated as well as non-aerated manure from pig and poultry farming (Lai et al. 1995; Kühne et al. 2000; Winckler and Grafe 2001).

The metabolism of the sulphonamide antibiotic used in this work, sulfamethazine, was studied previously by Berger et al. (1986). Sulfamethazine was administered to pigs via their food in common concentrations for prophylactical treatment and was degraded to 50% within seven days in liquid manure. The most common sulfamethazine-metabolite is described in 1.1.4. Several different possible transformation variants are shown in fig. 4a.
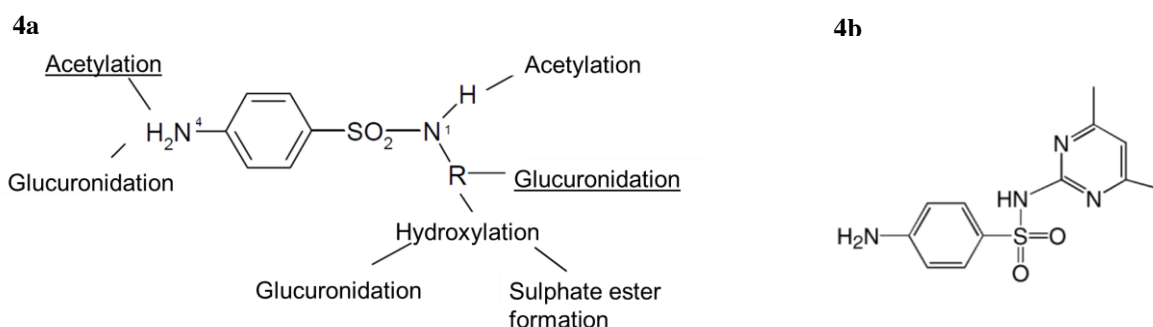
Fig. 4.
4a. Possible starting points for sulphonamide metabolism with main pathways underlined. From Forth 1996, modified by Harms (2006).
4b. Chemical formula of Sulfamethazine.

1.1.4   Occurrence and behaviour of sulfamethazine in soils

The specific veterinary antibiotic from the group of sulphonamides, used in this work radiolabelled and non-labelled, is sulfamethazine (4-Amino-N-(4,6-dimethyl-2-pyrimidinyl) benzenesulphonamide; CAS number: 57-68-1, SMZ). It is a bacteriostatic veterinary sulphonamide antibiotic, effective against gram-negative and gram-positive bacteria, used against infections of the respiratory tract, mainly in swine farming (Haller et al. 2002; Iglesias et al. 2012). One metabolite is N-4-acetyl-sulfamethazine (CAS number: 100-90-3); it was detected in different studies (Haller et al. 2002; Harms 2006). The chemical structure of SMZ and this metabolite are shown in fig. 5.
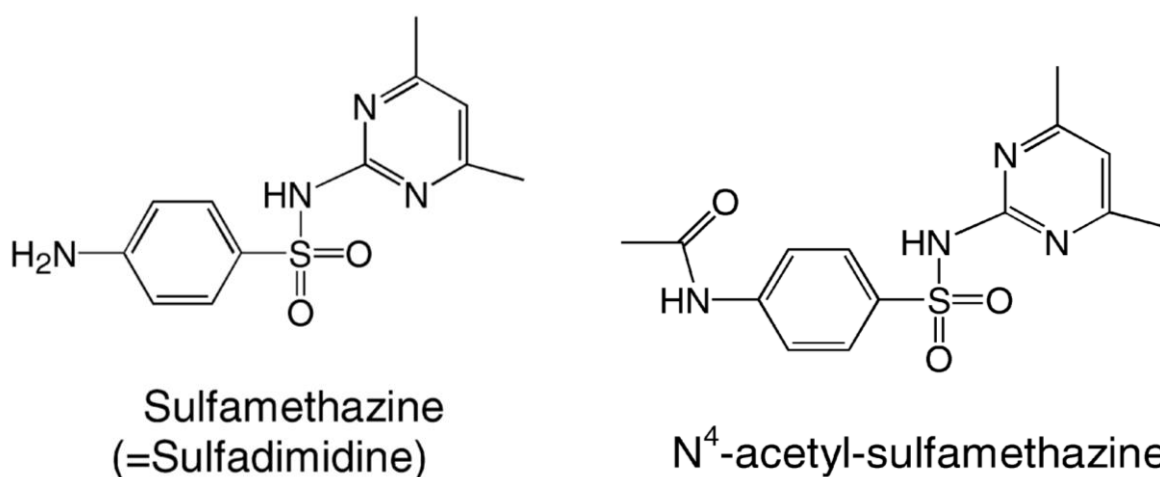
Fig. 5. Chemical structure of sulfamethazine and the metabolite N-4-acetyl-sulfamethazine. From Haller et al. (2002).

After administration to animals, SMZ was recovered in manure, in soils and in other environmental compartments. When investigating cattle treated by intravenous administration, Bevill et al. (1977) discovered 18% of the unchanged SMZ in the urine. Additionally, they presented three metabolites that were formed and eliminated by renal excretion, whereof one was probably the acetylated form of SMZ, the N4-Acetyl-Sulfamethazine.

Berger et al. (1986) investigated the urine and faeces of SMZ treated pigs whereby they discovered, that 46% of the administered SMZ was excreted, of which about 50% were the N-4-Acetyl-SMZ. They furthermore stated that the N-4-Acetyl-SMZ was cleaved and hence

transformed back to the active parent compound during the storage of manure. In this way the amount of the active compound can be increased by transformation of the metabolite back to the parent compound. Haller et al. (2002) investigated SMZ treated pigs and calves of six farms, where they detected the antibiotic in their manure in the range of mg kg^{-1}. When bringing out the manure of the SMZ treated animals, the risk of contaminating other environmental compartments is increased (Jørgensen and Halling-Sørensen 2000; Winckler and Grafe 2001). Christian et al. (2003) even detected SMZ seven months after liquid manure application in soil samples (15 µg kg^{-1}) and also in surface waters (7 ng L^{-1}). These results demonstrate the persistence of SMZ in the environment.

1.2   Importance of soils

The constantly increasing world population makes the challenge of food production, and therefore the need of arable soils, more and more complex (Lal 2013). After a rapid increase between 1980 and 2015 the world population forecast by the United Nations states that the population will grow from 7.3 billion in July 2015, to 9.7 billion in the year 2050 (United Nations 2015). Both, the estimated growth from 1980 to 2015 and the predicted growth until 2050 are presented in Fig. 6a and b.

a

Estimated population growth, 1980-2015
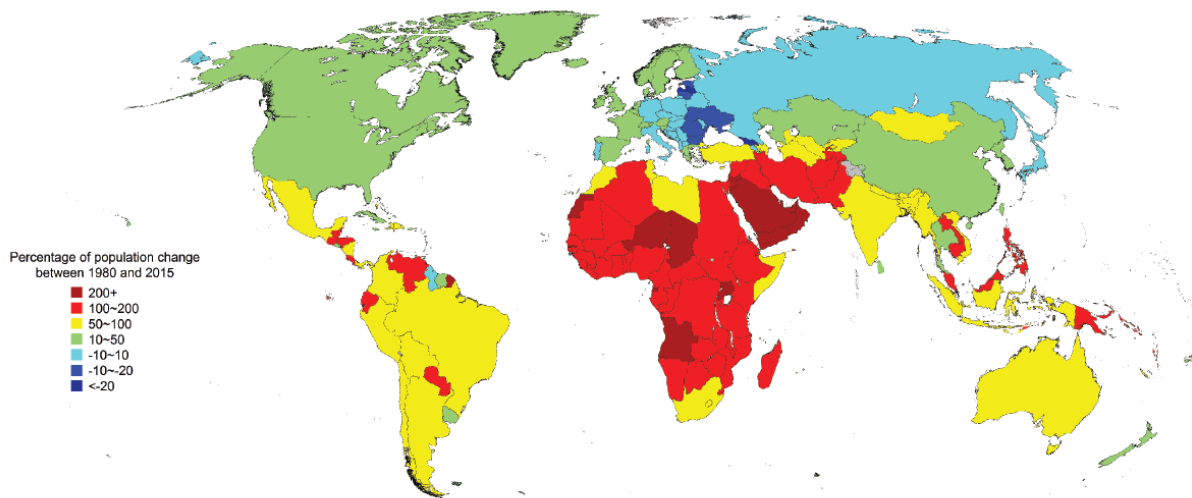
Percentage of population change
between 1980 and 2015
- ■ 200+
- ■ 100~200
- ■ 50~100
- ■ 10~50
- ■ -10~10
- ■ -10~-20
- ■ <-20

Data source: World Population Prospects: The 2015 Revision
The boundaries and names shown and the designations used on this map do not imply official endorsement or acceptance by the United Nations.
Dotted line represents approximately the Line of Control in Jammu and Kashmir agreed upon by India and Pakistan. The final status of Jammu
and Kashmir has not yet been agreed upon by the parties.
Final boundary between the Republic of Sudan and the Republic of South Sudan has not yet been determined.

b

Projected population growth, 2015-2050
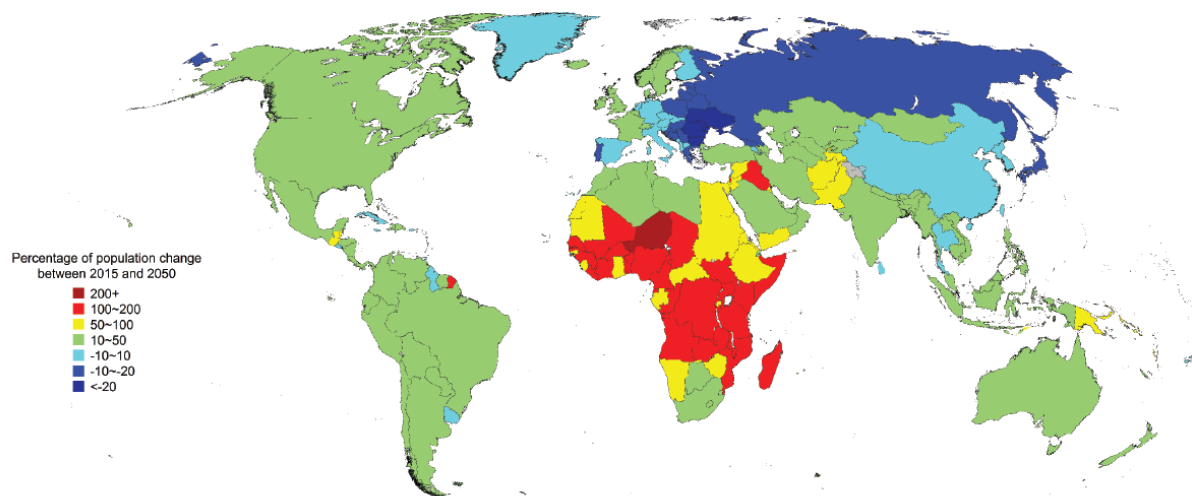
Percentage of population change
between 2015 and 2050
- ■ 200+
- ■ 100~200
- ■ 50~100
- ■ 10~50
- ■ -10~10
- ■ -10~-20
- ■ <-20

Data source: World Population Prospects: The 2015 Revision
The boundaries and names shown and the designations used on this map do not imply official endorsement or acceptance by the United Nations.
Dotted line represents approximately the Line of Control in Jammu and Kashmir agreed upon by India and Pakistan. The final status of Jammu
and Kashmir has not yet been agreed upon by the parties.
Final boundary between the Republic of Sudan and the Republic of South Sudan has not yet been determined.

Fig. 6 a and b. The dark red area symbolises the countries with an increase of more than 200% in the respective time span, red symbolises a growth between 100 and 200%. The variants of blue symbolise countries that lose 10 to 20 percent of their population in the respective time span. From United Nations, Department of Economic and Social Affairs, Population Division (2015), World Population Prospects: The 2015 Revision, World Population 2015 Wallchart. ST/ESA/SER.A/378.

However, conserving arable land as a finite resource to produce food for the next generations of humankind is not the only reason, why we have to preserve our soils. Besides food production, there are many other essential soil functions that we need for our daily life and to ensure basic life conditions on earth by buffering climate parameters and global geochemical cycles. The European Commission defined six main soil functions to be:

- Biomass production through agriculture and forestry
- Filtering (groundwater), buffering function and location for transformation processes
- Representing a big percentage of the worlds biological habitats and gene reservoirs
- Territorial base for technical, industrial and socio-economic structures (Industrial areas, buildings, areas for sports and recreation and others)
- A source of raw materials (gravel, sand, water and others)
- A geogenic and cultural heritage, forming the landscape, containing palaeontological and archaeological treasures

Main soil functions (such as ecological functions like biomass, gene reservoir and filtration functions; technical soil functions, soil use for forestry and non-ecological functions like raw materials, infrastructure, basis for human activities and cultural heritage) are also presented in detail in Blum (2005). More soil functions are displayed in fig. 7.
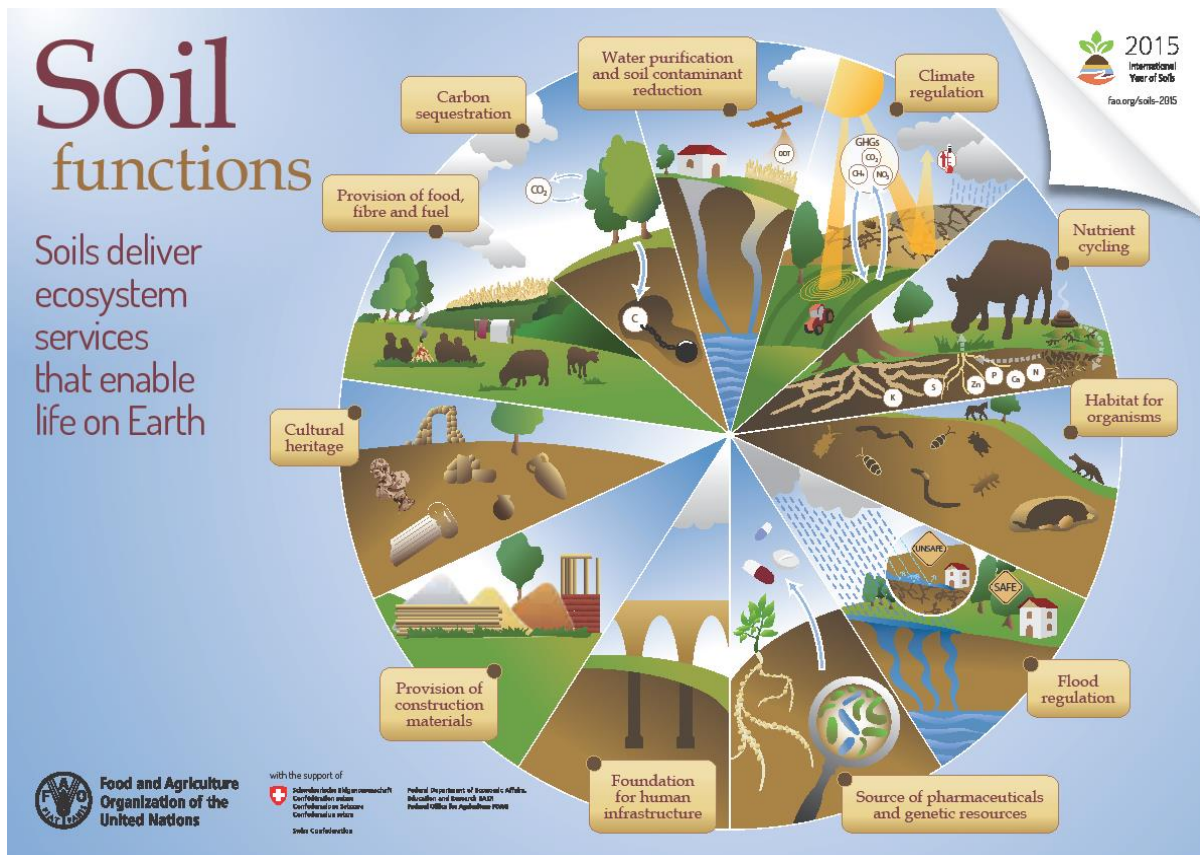
Fig. 7. Visual presentation of the different soil functions. Food and Agriculture Organization of the United Nations (FAO), FAO Information material for the International Year of Soils (IYS 2015), Office for Corporate Communication, 2015.

Despite these numerous essential soil functions for global ecological conditions and human survival, soils are destroyed and endangered by different factors every day.

1.2.1   Endangered soils

There are many aspects, in which the imprudent use and loss of arable land affect the population leading to many difficulties we have to face, when trying to use soils more sustainable. Lal (2013) listed these crucial points in which we need to preserve soil regarding the food production, and he also provided a solution in form of "sustainable intensification". The main problems endangering soils are soil degradation, loss of soil organic matter, extreme weather events, soil erosion, soil compaction, acidification, soil contaminations and reduction of soil fertility (Blum 2013; Lal 2013).

Soil is lost due to erosion by wind and water often in combination with miss management practices, soil sealing, industrial contamination and depletion, whereby we lose huge areas of

soil daily. In Germany we lose about 120 ha per day only due to soil sealing for buildings and infrastructure (Montanarella 1999). Once the topsoil is gone, it takes 1000 – 4000 years to produce 10 cm of agricultural soil (Montanarella 1999). Therefore it is important to safe, protect and responsibly treat the remaining intact soils that we have as well as remediate the contaminated ones in a mild conserving way.

Most of the soil threats are man-made. The human impact on soils is summarized in fig. 8 (Blum 2013).
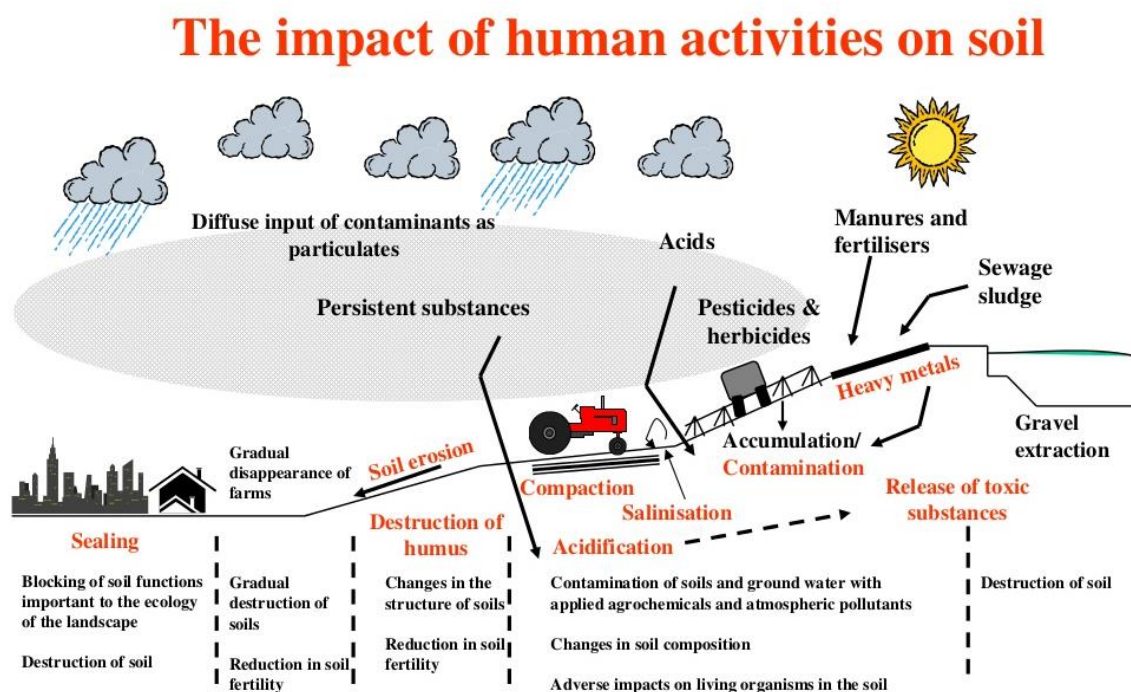
Fig. 8. Different human impacts on soils are shown in this graph, by Winfried E. H. Blum, presented during the Regional Conference on the Asian Soil Partnership, Nanjing, China, 2012. ©FAO: http://www.fao.org

One important soil threat is the contamination with many different chemicals produced by humans, such as industrial, medical or agricultural products, oil, heavy metals, polycyclic aromatic compounds from combustions and other organic pollutants. These xenobiotics enter the soils directly or indirectly via application to plants (pesticides); via excretion in the manure of animals (veterinary medicals), deposition from the atmosphere, waste water or via accidents on industrial production sites (organic compounds, heavy metals, polycyclic

aromatic hydrocarbon), at neglected deposits (oil) or at waste disposal sites (organic compounds, heavy metals). From soils, these contaminations can reach other environmental compartments, where they can have different effects on plants, animals, water bodies and also on our health. To prevent these unfavourable effects of polluted soils, we should decimate the usage of these chemicals, but also decontaminate the soils that have been and continue to be polluted.

1.2.2   Soil remediation techniques

For soil remediation in Germany and some other countries, different methods are applied (LUBW 1993):

Soil flushing technique: A gas or a liquid is flushed through the soil and then pumped out to wash off the contaminations. Soil structure can be impaired by this method.

Solvent extraction: The soil is flushed with solvents for decontamination. These solvents can be aggressive and have negative effects on the soil ecology.

Thermal treatment: In a pyrolysis treatment, the soil is anaerobically heated to evaporate or destroy the contaminant. Another thermal treatment is to combust the soil, under oxygenation. The exhausted air must be filtered and the soils can be partly or completely destroyed by these treatments.

Biological treatments: Soil can be decontaminated with microbes, if the contaminant is biodegradable and via uptake by plants in case of heavy metals.

1.2.3   Soil remediation with microbial communities

In Germany there are some methods for microbial soil decontamination in use, manly targeted on environmental pollutants like old, neglected waste deposits and contaminations after accidents. In preliminary laboratory experiments, the degradability of the environmental pollutant is tested, and if microbial degradation is possible, it is preferred over thermal treatment. Microbial degradation preserves the soil fabric, soil flora and fauna and shows a better ecological balance as well as balance of energy (Sutter 1996).

For microbial soil remediation, contaminated soil can be treated *in situ* which means the soil stays where it is for the decontamination process, whereas in the *ex situ* methods, soil can be taken out and treated on-site or transported to another handling site (Sutter 1996).

Most common are the on-site treatments, where soil is taken out for remediation and then treated *ex situ* on-site without any transportation. Manly, the soil conditions are improved for microbial degradation by adding microbes, trapping the exhaust air and cleaning it with biological filters and activated-carbon filters (Sutter 1996).

Second common is the *in situ* remediation, where the ground water can be pumped out, filtered on-site, oxygenated and enriched with nutrients before being led back to the soil. Another *in situ* method is pumping air out of the soil and filtrate it with biological filters. These methods can have a very long duration (Sutter 1996).

A rarely used *ex situ* method is removing soil before decontaminating it in bioreactors, where microbes are added and nutrients, oxygen, temperature, as well as other parameters can be regulated in closed systems (Sutter 1996).

As these methods are developed for heavy contaminations like waste deposits or chemical accidents, veterinary contaminants and farming products are barely considered in these decontamination methods. The preservation of soil quality is not as important as eliminating the contaminations, often only applied to prevent leaching towards water bodies.

The advantages of soil remediation using degrading microbial strains over using *in situ* or *ex situ* flushing, extraction or combustion methods are distinct. The soil does not have to be removed, the soil structure is not destroyed, the soil is not treated with aggressive chemicals and agricultural soils can be used for food production again, after decontamination with a gentle microbial degradation approach.

Many studies have been conducted to remediate soil from organic pollutants using the help of microorganisms for degradation. After testing single strains or a selection of competent strains for their ability to degrade pollutants, awareness of the advantages of using microbial communities for soil remediation, has arisen and studies with and without carrier material for soil inoculation with microbial communities were undertaken (Dejonghe et al. 2001; Owsianiak et al. 2010; Li et al. 2016). A disadvantage can be a longer time span for decontamination.

The remediation approach applied here comprises the above mentioned advantages and is a very successful approach for degrading the tested substance SMZ.

1.3   Objective of this work

Based on the lack of applicable opportunities to remediate soils from antibiotics, the aim of this study was to provide an applicable, effective approach for soil decontamination leaving the agricultural soils in unchanged quality, so they can still be used for farming and food production. To achieve this goal, different strategies were operated.

1. Different antibiotics were tested in different soils for their adsorption behaviour and bioavailability to select the suitable antibiotic plus soil for inoculation experiments.

2. To obtain a microorganism that is able to degrade the selected antibiotic, several different soil incubation methods with different SMZ concentrations, application frequencies, and different soil moisture and soil temperatures were tested.

3. The microbial community was isolated from a soil, adapted to SMZ mineralisation by long time SMZ application in field conditions.

4. Soil inoculation experiments were conducted with this adapted microbial community. The mineralisation capability of the microbial community was compared to the isolated SMZ degrading strain *Microbacterium* sp. C448 from the same soil. The strain was obtained from the Canadian working group which investigated this soil.

5. The microbial community was further investigated for its composition and the single strain was tested for additional antibiotic resistances.

The soil inoculation approach used here has been investigated in previous studies with other chemicals and soils. It was successfully applied for accelerating the mineralisation of the herbicide isoproturon (Li et al. 2016) in the laboratory and in outdoor lysimeter experiments (Grundmann et al. 2007). It has been verified for the chemical 1,2,4-trichlorobenzene, using a different microbial community and a different soil (Wang et al. 2010; Wang et al. 2013).

In this work, I used a soil-borne microbial community, transferred to sandy loam soil samples to mineralise the veterinary antibiotic SMZ. The SMZ molecule was radiolabelled to observe its degradation in soil and trace its extractable and non-extractable residues.

2.   Materials and Methods

The aim of the study was to decontaminate soil samples from SMZ and thereby verify a remediation approach, which has been used for other organic substances before.

The first step was to identify a veterinary antibiotic suitable for degradation experiments, frequently used, persistent and with detectable residues in soils. In the second step a soil for the inoculation experiments was selected out of three soils differing in their properties, by investigating the antibiotic sorption and desorption behaviour in these soils. The third step was to enrich a soil-borne microbial community, able to degrade the selected antibiotic, from an *in vitro* antibiotically treated soil, and to use this microbial community for soil inoculation experiments with the investigated remediation approach. As a soil-borne, SMZ degrading strain could not be enriched by conducting laboratory soil adaptation experiments, we received the SMZ-degrading single strain *Microbacterium* sp. strain C448 from Canadian scientists that isolated this strain (Topp et al. 2013). The soil this strain originated from had been exposed to an annual dose of 1 mg kg^{-1} for six years and 10 mg kg^{-1} SMZ for additional four years and thus, adapted to SMZ degradation. This microbial strain as well as a soil aliquot from this adapted soil was provided. From this adapted soil aliquot, we enriched the whole adapted soil-borne microbial community, including this key degrading strain. Soil inoculation experiments in this work were conducted using the isolated strain originating from the Canadian soil and using the microbial community, enriched by us from a sample of the same Canadian soil.

2.1   Chemicals

Uniformly ^{14}C-ring-labelled sulfamethazine (4-amino-N-(4,6-dimethylpyrimidin-2-yl) ben-zenesulfonamide, CAS number: 57-68-1, SMZ) was purchased from Campro Scientific GmbH (Veenendaal, The Netherlands) with a specific radioactivity of 673.4 MBq mmol^{-1}, and a radiochemical purity of > 98%. Non-labelled SMZ, Tetracycline Hydrochloride (TC*HCl) and Tetracycline (TC) were obtained from Sigma Aldrich (Taufkirchen, Germany). For the experiments, aqueous ^{14}C-labelled and non-labelled SMZ solutions were blended to produce the respective ^{14}C-SMZ application standards (spec. radioactivity ranging from 80 to 500 MBq mmol^{-1}). Carbo-Sorb® E and the scintillation cocktails Permafluor® E+, Ultima

GoldTM XR and Ultima-FloTM were purchased from PerkinElmer (Waltham, USA). All other chemicals were obtained in analytical grade from Merck (Darmstadt, Germany).

2.2   HPLC analysis parameters

Antibiotics in the extracted soil pore water (2.4.1 and 2.4.2) were analysed by HPLC using the components and gradient program presented in table 3 and 4. Acetonitrile and water were used as mobile phases, while the water was blended with 0.600 g L^{-1} of CH_3COOH to make the mobile phase acidic for better separation of peaks.

Table 3. HPLC components used for detection of antibiotics in the soil pore water.

HPLC component	Type and producer
UV/VIS-Detector	D-7000 (Merck Hitachi, Darmstadt, Germany)
Pump	L-7100 (Merck Hitachi, Darmstadt, Germany)
Autosampler	L-7200 (Merck Hitachi, Darmstadt, Germany)
Pre-column and column	LiChrospher 100 RP-18 (Merck, Darmstadt, Germany)
Mobile phases	Acetonitrile, H_2O with acetic acid
Flow rate	0.8 ml min^{-1}
Injection volume	20 µl

Table 4. HPLC gradient program used for detection of antibiotics in the soil pore water. Acidic acid was added to the water phase.

Time (min)	Acetonitrile	H_2O (acidic)
0	25	75
12	60	40
17	60	40
20	25	75
30	25	75

2.3   Soils

All soil materials were sampled from agricultural fields (0-10 cm depth), air dried, sieved (< 2 mm), and stored at -20 °C. Physical and chemical soil properties and taxonomy are presented in table 5.

Table 5. Soil parameters of all utilized soil materials.

Site name	Scheyern 1	Scheyern 2	Neumarkt	Hohenwart	Feldkrichen
Soil type	aric anthrosol	mollic gleysol	haplic arenosol	aric anthrosol	calcaric regosol
Classification[1]	sandy loam	silty loam	sandy soil	loamy sand	loamy clay
Clay (%)	20	22	4	13	33
Silt (%)	40	60	8	19	34
Sand (%)	40	18	88	68	33
pH CaCl$_2$	7.8	5.4	5.8	6.7	7.2
TOC	1.5	1.5	1.0	1.0	2.7
Total N	0.2	0.2	0.1	0.1	0.3
Latitude (°)	48.493	48.500	49.250	48.600	48.150
Longitude (°)	11.432	11.467	11.483	11.383	11.733
Altitude (m)	497	478	467	392	521
Water content at pF 2.18 (% w/w)	27.38	29.57	10.79	16.14	32.8
SMZ in soil pore water after 7 days (%)[2]	83.90	51.55	25.55	not tested	not tested

[1]Classifications are consistently used in this work, when referring to different soil material.
[2]See fig. 15

Prior to each experiment, the soils were thawed and stored at 4°C for one week, then moistened to a water tension of -15 kPa (pF 2.18) and equilibrated at room temperature (20 ±1 °C) for another week. Thus, the indigenous soil microorganisms were activated at soil typical conditions. All experiments with constant soil moisture were conducted at pF 2.18, a water tension where soil microbes show the best performance in degrading organic compounds (Schroll et al. 2006). In consideration of this specific water tension, the water content of the sandy loam soil samples was adjusted to 27.4% (w/w), the silty loam to 29.6% the sandy soil to 10.8%, and the loamy sand to 16.1%.

Loamy sand soil material was used for experiments to stimulate the indigenous soil bacteria to degrade SMZ by suppling it in different concentrations and frequencies. This soil was never treated with antibiotics in the last 10 years before sampling. It was managed with a barley, maize and wheat crop rotation. Finally the field was laid fallow for three years.

The results of the *in situ* bioavailability experiment of SMZ in three soils (sandy loam, silty loam and sandy soil, table 5), led to the decision to use soil material originating from the sandy loam soil for all further soil inoculation experiments. This soil was sampled on the agricultural research farm Scheyern, Germany. It was managed by organic farming with a crop rotation consisting of potatoes, winter wheat, sunflower, winter rye and grass-clover-alfalfa as intertillage and cover crop.

Loamy clay soil material was only used for one soil adaptation scenario (table 7) in 5 g aliquots (three replicates), to introduce the soil microbes from this soil into loamy sand soil samples, to increase the probability for adaptation of soil microorganisms to the degradation of SMZ.

2.4   Bioavailability of antibiotics in soil samples

First, three antibiotics were tested for their sorption behaviour in soil samples, whereupon one antibiotic (SMZ) was selected for all further experiments. After selection of this antibiotic, samples from three different soil types were tested for their SMZ sorption behaviour. Furthermore, the general effect of SMZ application to the indigenous soil microorganisms was investigated.

2.4.1   *In situ* bioavailability and desorption of three antibiotics from soil

The appropriate antibiotic for prospective soil inoculation experiments was selected by investigating three antibiotics, regarding their *in situ* bioavailability and desorption in soil for a period of 14 days (TC, TC*HCl and SMZ, no radioactive labelling was used in this experiment). To investigate the sorption kinetics and the bioavailability of the antibiotics in soil for 14 days, their residues in the soil pore water were extracted according to Folberth et al. (2009a) and analysed by HPLC.

The loamy sand soil (35 g dry weight equivalent per replicate) was equilibrated as described in 2.3, spiked with aqueous antibiotic solution (5 µg g^{-1}), and with aqueous NaN$_3$ solution

(100 µg g^{-1}) to prevent any microbial degradation during the experiment period and incubated in 250 ml amber bottles in the dark (3 replicates for each antibiotic). For spiking the soil samples, 1000 µl of the aqueous application standard of the different antibiotics was applied dropwise to a 5 g oven dried (105 °C, 24 h) soil aliquot and carefully mixed. The spiked aliquot was transferred to fresh, equilibrated soil (30 g, dry weight) in the amber bottle and thoroughly stirred with a spatula. The soil was adjusted to a density of 1.3 g cm^{-3} and a soil water tension of -15 kPa. The incubators were closed with parafilm and stored in a desiccator containing some water at the bottom. Every other day of incubation, the soil samples were extracted to determine the residues of the antibiotics in the soil pore water, regarded as the current *in situ* bioavailability of these antibiotics (Folberth et al. 2009b). For extraction, the incubated soil samples were centrifuged for 90 min at 9000 rpm and 20 °C (centrifuge: Sorvall RC 6+, rotor: F14-6x250y; both Thermo Scientific, Braunschweig, Germany). After the centrifugation an aliquot of 20 µl of the soil pore water was injected into the HPLC device for residue detection. Soil samples were transferred back into the soil incubators and rewetted with the equal volume of distilled water (Elix, Millipore, Eschborn, Germany) that has been removed during the extraction process. Samples were incubated for two more days before the next extraction (six extractions for TC and TC*HCl, eight extractions for SMZ).

From these extracted amounts of antibiotics, dissolved in the soil pore water, the fraction which was desorbed from soil was calculated for each centrifugation step. The soil pore water could not be extracted totally, so the antibiotics that remained in the non-extracted soil pore water after the first extraction were subtracted from the amount recovered in the second extraction step. In this way only the amount that was actually desorbed since the last extraction was taken into account.

2.4.2   *In situ* bioavailability of sulfamethazine in different soils

After the suitable antibiotic was selected it was purchased in a radiolabelled form for best tracking of SMZ residues and all following experiments were conducted with this ^{14}C-labelled SMZ. In order to select a suitable soil for upcoming experiments, material from three soils was investigated in view of the *in situ* bioavailable SMZ fraction in their soil pore water.

The three soils (sandy loam, silty loam and sandy soil, 35 g dry weight equivalent) were spiked with aqueous ^{14}C-SMZ (1 µg g^{-1}) and incubated in 250 ml amber bottles (30 replicates for each soil, NaN$_3$ was not used here). The equilibration of these three soils, the SMZ-

spiking, the adjustment of the water content and the pore water extraction was conducted similarly to the sample treatment described in 2.4.1.

The experiment lasted for 21 days and 3 of the 30 replicates were extracted via centrifugation on day 1, 2, 3, 4, 7, 8, 9, 10, 14, and 21.

The ^{14}C-SMZ fractions in the soil pore water were quantified in a liquid scintillation counter (Tricarb 2800TR, PerkinElmer, Waltham, USA). Therefore, two aliquots of 1 ml of the soil pore water were each mixed with 4 ml Ultima Gold XR in a scintillation vial (PerkinElmer, Waltham, USA) and the ^{14}C-radioactivity was measured in this liquid scintillation counter.

To exclude microbial SMZ degradation in these sorption experiments, 30 aliquots of the sandy loam soil were sterilised by gamma radiation. Afterwards, the SMZ sorption experiment was repeated exactly in the same way as the non-sterilised soil samples, using ^{14}C-SMZ, incubating for 21 days and extracting the soil samples via centrifugation. The SMZ sorption to the sterile and the non-sterile sandy loam soil samples was analysed comparatively. Sterilisation was conducted in a closed unit irradiation chamber (Gammacell 220, Atomic Energy of Canada Limited, nuclide: [60]Co, 9 Gy/min) for 72 h.

2.4.3   Effect of SMZ application on microbial biomass in the sandy loam soil

The general effect of a single SMZ application on the soil microorganisms was investigated by analysing microbial carbon in SMZ treated and untreated soil samples using chloroform fumigation extraction. In the broader sense, the extraction was conducted according to the DIN ISO 14240-2 for fumigation extraction, with some changes. Six soil replicates (180 g dry weight equivalent per replicate) were equilibrated as described in 2.3; three of them were spiked with non-labelled SMZ (1 µg g^{-1}). All replicates were incubated in 500 ml amber bottles in the dark, closed with a rubber stopper. Six aliquots (5 g wet weight) of each soil incubator were sampled for microbial biomass analysis by chloroform fumigation on day one, day six and day twenty. Three of these aliquots were fumigated and three were not. The chloroform fumigation procedure with ethanol free chloroform was conducted in a desiccator for 24 hours, as described in the DIN ISO 14240-2. Both, the non-fumigated and the fumigated samples were extracted with 20 ml of a 0.01 M $CaCl_2$ solution by shaking for 45 min in an overhead shaker (Reax 2, Heilolph, Schwabach, Germany). Afterwards, samples were filtrated through a cellulose filter (240 mm, Whatman, from Sigma Aldrich, Taufkirchen, Germany) and immediately frozen at -20 °C until analysis.

The organic content was measured using a TOC/TNb (total organic carbon and total bound nitrogen) analyser (Dimatoc 2000, Dimatec, Essen, Germany) and the difference between the fumigated and the non-fumigated samples was calculated. The concentration of microbial carbon in soil was calculated using this equation:

$$c_{mic}[\mu g\ g^{-1}] = \frac{TOC_{fum}[\mu g\ ml^{-1}]*(20\ ml+ soil\ water[ml])}{soil\ weight\ [g\ oven\ dry\ matter]*0.45} - \frac{TOC_{nfum}[\mu g\ ml^{-1}]*(20\ ml+ soil\ water[ml])}{soil\ weight\ [g\ oven\ dry\ matter]*0.45}$$

C_{mic} = microbial carbon in the sample

TOC_{fum} = organic carbon in the fumigated sample

TOC_{nfum} = organic carbon in the non-fumigated sample

20 ml of the 0.01 M CaCl was used

0.45 = k_{ec}, a factor to consider the extractable amount of microbial carbon after fumigation

2.5   Stimulation of soil microorganisms to degrade SMZ

To stimulate the indigenous soil microorganisms to degrade SMZ and to accelerate this adaptation process, different experimental setups were run. The loamy sand soil was selected for adaptation experiments due to its sandy structure which supposedly does not adsorb SMZ too strongly. Furthermore, former experiments conducted in free air lysimeters, showed that this soil adapted to the degradation of other chemicals (pesticides) quickly. To possibly enhance this adaptation process the SMZ application rate, the SMZ concentrations as well as the soil moisture and temperature were varied. Per replicate 35 g soil (dry weight) were equilibrated as described in 2.3, non-labelled SMZ was applied in different concentrations to the soil and the soil samples were incubated in the dark (soil density of 1.3 g cm^{-3} and soil water tension pF 2.18 for experiments with constant water content). At the beginning of these experiments, all variants were set up with non-labelled SMZ. After an incubation-adaptation time of 224 days, ^{14}C-labelled SMZ was applied to the soil incubators to investigate whether any degrading microbes have grown in the soil and ^{14}C-CO_2 was formed. Additionally, new radioactive incubation variants were started (table 6).

2.5.1   Experimental setup of soil samples with constant moisture

For all experiments with constant soil moisture (pF 2.18), the soil was incubated in 250 ml amber bottles in the dark, closed with a rubber stopper. The rubber stopper was pierced by a hollow needle for air exchange to guarantee constant oxygen supply for the soil microbes. A schematic soil incubator is shown in fig. 9. On top of the needle a syringe filled with soda lime pellets was connected for absorbing atmospheric CO_2. Thus, no atmospheric CO_2 could enter the incubator during the air exchange through the needle.

At the bottom of the rubber stopper a 25 ml plastic beaker was attached and filled with 9 ml of a 0.1 N NaOH solution to trap the ^{14}C-CO_2 originating from ^{14}C-SMZ, mineralised in the soil. The soda-lime pellets in the syringe above the needle, absorbing the atmospheric CO_2, made sure that the liquid NaOH in the cup inside the incubator was solely absorbing the CO_2 from the soil respiration, not from outside the incubator. Three times per week, the NaOH solution in the plastic cup was exchanged. An aliquot of 2 ml was mixed with 3 ml Ultima Flo and the ^{14}C-CO_2 was measured in the liquid scintillation counter. To keep the soil moisture continuously at pF 2.18, it was gravimetrically surveyed weekly and if necessary, evaporated water was refilled with distilled water.

Glass wool

Soda lime pellets

Rubber stopper

Hollow needle

0.1 N NaOH

Soil sample
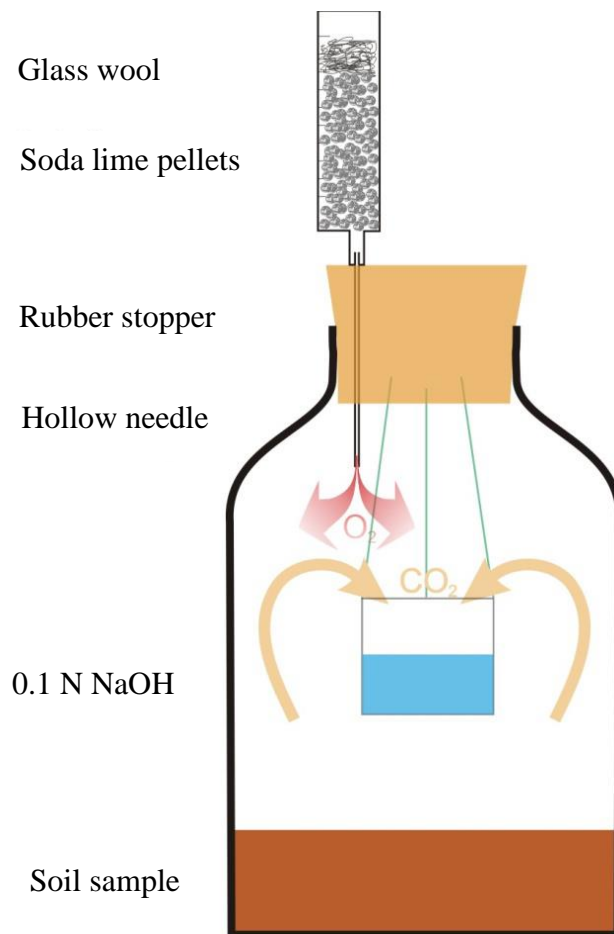
Fig. 9. Soil incubator for constant soil moisture with NaOH filled cup for ^{14}C-CO_2 trapping used for soil inoculation experiments where CO_2 was quantified, with constant soil moisture (Four soil incubation variants (2.8) and three incubation phases of MCCP (2.9)), modified from Kiesel (2014).

Table 6. Different "adaptation" treatments with pre-exposure to non-labelled SMZ. The numbers in brackets behind the sample ID are the numbers of replicates.

Sample ID	Pre-exposition to non-labelled SMZ					Sample ID	1st application of ^{14}C-SMZ				Sample ID	2nd application of ^{14}C-SMZ		
CM1 (20)[1]	20 °C	100 µg g^{-1}	1 application	224d	→	CM1-1 (5)[1]	20 °C	5 µg g^{-1}	46 d	→	CM1-2 (2)[1]	20°C	100 µg g^{-1}	160 d
CM2 (20)[1]	20 °C	5 µg g^{-1}	1 application	224d	→	CM2-1 (5)[1]	20 °C	5 µg g^{-1}	46 d	→	CM2-2 (2)[1]	20°C	10 µg g^{-1}	160 d
CM3 (20)[1,2]	20 °C	5 µg g^{-1}	4 applications	224d	→	CM3-1 (5)[1]	20 °C	5 µg g^{-1}	46 d	→	CM3-2 (2)[1]	20°C	10 µg g^{-1}	160 d[3]
FM1 (20)[4]	20 °C	5 µg g^{-1}	1 application	224d	→	FM1-1 (5)[4]	20 °C	5 µg g^{-1}	46 d	→	end			
						CM4 (5)[1,5]	20 °C	5 µg g^{-1}	46 d	→	end			

[1] CM = constant soil moisture of -15 kPa

[2] These replicates received 3 further applications of non-labelled SMZ á 5 µg g^{-1}; incubation time after each re-application: 28-91 days

[3] These replicates received 8 further applications of ^{14}C-SMZ á 5 µg g^{-1} within the 160 days of incubation

[4] FM = fluctuating soil moisture (between -15 kPa and -1500 kPa, pF 2.18-4.18)

[5] CM4 = with the first radioactive application on all samples, theses 5 replicates were started with fresh soil, to serve as ^{14}C-control.

Table 7. Different "adaptation" treatments without pre-exposition to non-labelled SMZ. The figures in brackets behind the sample ID are the numbers of replicates.

Sample ID	Single application of ^{14}C-SMZ		
CM4 (3)[1]	30 °C	10 µg g^{-1}	160 d
CM5 (3)[1]	30 °C	100 µg g^{-1}	160 d
FM1 (5)[2]	20 °C	10 µg g^{-1}	160 d
FM2 (4)[2][4]	20 °C	100 µg g^{-1}	41 d
SI(3)[3]	20 °C	100 µg g^{-1}	209 d
Control 10 (4)[1]	20 °C	10 µg g^{-1}	160 d
Control 100 (4)[1]	20 °C	100 µg g^{-1}	160 d

[1]) CM = constant soil moisture of -15 kPa

[2] FM = fluctuating soil moisture between -15 kPa and -1500 kPa, pF 2.18-4.18

[3] Soil inoculum = 5 g dw fresh soil loamy clay, 30 g dw loamy sand

[4] These replicates received 8 further applications of ^{14}C-SMZ á 5 µg g^{-1}; incubation time after each reapplication: 10-21 days

Table 6 and 7 present the experimental design of all variants for enhancing adaptation in the loamy sand soil, with and without pre-exposition to inactive SMZ. Three times 20 samples were pre-exposed to inactive SMZ, receiving different treatments (table 6). The replicates of CM1 received a single high dose of SMZ (100 µg g^{-1}), CM2 with 5 µg g^{-1} served as control and the CM3 replicates received four non-labelled SMZ applications over time. Incubation of these samples lasted for 224 days. The experiment started with non-labelled SMZ, so there was no need for NaOH in the cups attached to the rubber stopper in this period. The water content was constantly regulated to pF 2.18. After 224 days, ^{14}C-SMZ was applied to identify possible SMZ degradation. For this purpose, five replicates of each treatment were selected to be continued, the other samples were discarded. The five replicates each, received a 5 µg g^{-1} dose of ^{14}C-SMZ and were incubated for another 46 days. The cups were filled with NaOH for ^{14}C-CO_2-trapping, which was exchanged and measured in the scintillation counter 3 times per week, to detect possible ^{14}C-CO_2-evolution, indicating SMZ mineralisation.

As no noteworthy mineralisation was identified after 46 days, two replicates of the five replicates of each treatment, received a second dose of ^{14}C-SMZ. The two replicates of CM1 (table 6) which received a high dose of inactive SMZ in the first application, now also received a high dose of ^{14}C-SMZ (100 µg g^{-1}). The CM2 replicates received 10 µg g^{-1}, and the CM3 samples received 10 µg g^{-1} plus 8 further applications of 5 µg g^{-1} ^{14}C-SMZ. Incubation after this SMZ application lasted for 160 days.

After such a long incubation period, new incubators with freshly equilibrated, non-adapted soil were started to make sure, nutrients in soil are fully available for indigenous, possibly SMZ degrading microorganisms and ^{14}C-SMZ was applied to all new variants. Therefore 10 µg g^{-1} and 100 µg g^{-1} were applied to three replicates each, with constant soil moisture and incubated at 30 °C (CM4, CM5, table 7). Five samples with fluctuating soil moisture received 10 µg g^{-1} and four samples with fluctuating soil moisture received 100 µg g^{-1} SMZ. Four replicates with constant soil moisture, incubated at 20 °C received a low dose of SMZ (10 µg g^{-1}, "Control 10") and four received a high dose (100 µg g^{-1}, "Control 100"). Those samples served as control samples for the high and the low dose SMZ treatments. The incubation times of all radioactive treatments are shown in table 7.

2.5.2   Experimental setup of soil samples with fluctuating moisture

This system was used for soil samples with fluctuating soil moisture, a scenario to accelerate the natural adaptation process of the soil microorganisms to SMZ degradation. It is schematically shown in fig. 10.
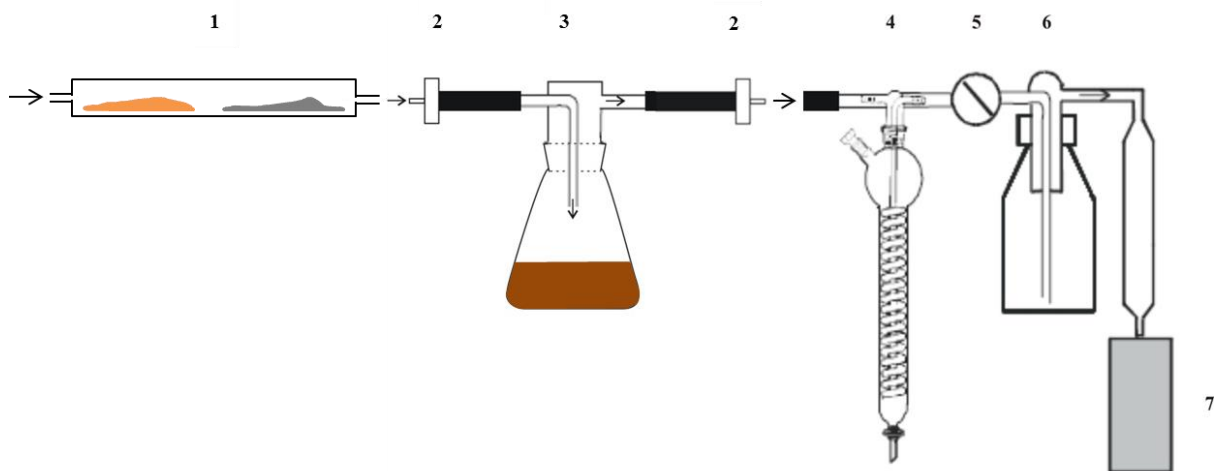
Fig. 10. Laboratory system used for aeration of liquid cultures and particular soil samples.
1: Plastic tube containing silica gel pellets (orange) and soda lime pellets (grey) 2: Sterile membrane filters, 3: Erlenmeyer flask containing soil samples, 4: Wash bottles for CO_2 trapping (2 of them were used in a row for each sample), 5: Vent for airflow regulation 6: Wulff bottle, 7: pump, → Direction of air flow. Modified after Lehr et al. (1996) by Kiesel (2014) and by me.

In this setup, the vacuum pump (no. 7) was continuously drawing air from the atmosphere through the soil incubators passing the ^{14}C-CO_2 trapping system.

The airflow entered the system through a plastic pipe (no. 1) and was constantly dried by silica gel pellets (orange), while CO_2 from the atmosphere was absorbed by soda lime pellets

(grey, Merck, Darmstadt, Germany). Thereupon, the air passed a membrane filter (0.20 µm, Sartorius, Göttingen, Germany, no. 2) and was drawn into 100 ml Erlenmeyer flasks containing the soil samples (no. 3). After passing another membrane filter, the air from the samples, containing the ^{14}C-CO_2 was led through the wash bottles (no. 4) which were filled with a 0.1 N NaOH-solution for collecting CO_2 and finally through a Wulff bottle (no. 6) into the pump (no. 7). Number 5 represents the vent to adjust the airflow. The NaOH-solution was exchanged three times per week to quantify the trapped ^{14}C-CO_2 by taking an aliquot of 2 ml, as described in 2.5.1.

The aeration continuously dried the soil samples in the Erlenmeyer flasks. Once per week, all soil samples were rewetted. The evaporated amount of water was measured gravimetrically and the missing volume was refilled dropwise on the soil samples with distilled water. Thus, the soil moisture was fluctuating between pF 4.18 and pF 2.18.

2.6   Analysis of the SMZ degrading bacteria

To gain more precise information about the SMZ degrading strain and the associated microbial community, further analyses were conducted. The morphology of the single strain *Microbacterium* sp. C448, obtained from Canada, was investigated by electron microscopy and additional resistances of this strain to other antibiotics were tested on agar plates. A growth curve was established to reveal the growth characteristics of the strain *Microbacterium* sp. C448. The DNA of the microbial community, enriched from the same Canadian soil the degrading strain was isolated from, was analysed to characterize the different bacterial members potentially associated to the degrading strain.

2.6.1   Morphology of *Microbacterium* sp. C448

The shape and size of the SMZ degrading strain was investigated in the electron microscope. Ten microliter of the cells of *Microbacterium* sp. C448 in liquid culture were fixed on a silicon chip with 10 µl of a glutaraldehyde solution (2.5%), washed in an alcohol dilution series and finally three times in pure ethanol (100%) for 15 minutes, before being critical point dried in a BAL-TEC CPD 030 (Liechtenstein). After being brought to the specimen stub, the silicon chip holding the sample was vapour coated with platinum. The samples were subsequently examined in a Hitachi S-5200 field emission scanning electron-microscope (FESEM).

2.6.2   Growth curve of *Microbacterium* sp. C448

The strain *Microbacterium* sp. C448 was grown in liquid culture in a test tube for seven days and the optical density was measured. The liquid culture consisted of LB medium (10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) and 250 µl bacterial suspension inoculum, added to 100 ml LB medium. The liquid culture was incubated at 37 °C on an orbital shaker.

2.6.3   Additional resistances of *Microbacterium* sp. C448

Microbacterium sp. C448 was tested for further resistances to other antibiotics. It was grown in a LB liquid culture, for 3 days before LB-agar plates were prepared and 100 µl of the liquid medium, containing the single strain, were distributed on the plates with a Drigalski spatula. Two different antibiotic test rings were placed on one agar plate, respectively. These paper rings each contained eight different antibiotics, adjacent on the rings. Agar plates with the single strain and the test rings were incubated at 37 °C for 7 days before the sensitivity of the strain to the different substances was evaluated by the growth pattern of the bacteria.

2.6.4   DNA analysis of the microbial community

For the DNA extraction, the microbial community was enriched on carrier particles in liquid cultures (mineral salt medium) with non-labelled SMZ (10 mg L^{-1}). The mineralisation was measured in parallel liquid cultures via ^{14}C-SMZ (10 mg L^{-1}) and ^{14}C-CO_2, to survey the sample handling. The DNA was extracted from the carrier particles using the fastDNA^{TM} SPIN Kit for Soil and the corresponding FastPrep$^{®}$ Instrument for cell lysis in the samples. After the DNA was extracted, the sample preparation for the illumina sequencing was conducted according to the illumina sequencing standard protocol. The base pairs number 8 to 343 were sequenced using paired end sequencing with following primers:

Primer F: S-D-Bact-0008-a-S-16

Primer R: S-D-Bact-0343-a-A-15

Most sequences were analysed to genus level, while some were not.

2.7   Preparation of bacteria for soil inoculation

2.7.1   Cultivation of the degrading strain *Microbacterium* sp. C448

This single strain, used for soil inoculation experiments (results in chapter 3.7) was received on agar plates from the Canadian group and cultivated in liquid cultures prior to soil inoculation as described in 2.7.4.

2.7.2   Enrichment and cultivation of SMZ-degrading bacteria

For enrichment of the microbial community associated with the key degrading strain *Microbacterium* sp. C448, 2 g soil material (< 2 mm, wet weight) from the adapted Canadian soil was added to 19 ml culture medium in 100 ml Erlenmeyer flasks (Duran, Mainz, Germany). This mineral salt medium was prepared according to Topp et al. (2013) with the exception that vitamins (biotin and thiamine) and trisodium citrate were not included in the medium. The soil-mineral salt medium mixture was amended with 1 ml of an aqueous solution of ^{14}C-SMZ to reach a final concentration of 10 mg L^{-1} and a total volume of 20 ml. To establish the microbial community on the carrier material, 40 sterilized Seramis® carrier particles (2-4 mm sized clay particles, containing Kaolinite, Illite and Quartz as main minerals, total N: 3-8 mg L^{-1}, P_2O_5: 5–10 mg L^{-1}; K_2O: 100–120 mg L^{-1}, pH in H_2O approximately 7, expanded for high pore volume (> 80%), dried and fired, produced by Seramis GmbH, Mogendorf, Germany) were added to this liquid culture containing the 2 g of soil. To avoid microbial contamination of these liquid cultures, membrane filters (0.20 µm, Sartorius, Göttingen, Germany) were installed at the air inlet and outlet of each Erlenmeyer flask. The liquid cultures were incubated on a rotary shaker at 75 rpm in the dark at 20 °C. The experimental setup is presented in fig. 11.

Three times per week the ^{14}C-CO_2, evolved from the mineralisation of ^{14}C-SMZ, was quantified according to the procedure described in section 2.7.3. After 6-9 days of incubation the **M**icrobial **C**ommunity, established on **C**arrier **P**articles (MCCP) was transferred to fresh ^{14}C-SMZ spiked liquid medium. In total ten subsequent transfers were carried out.
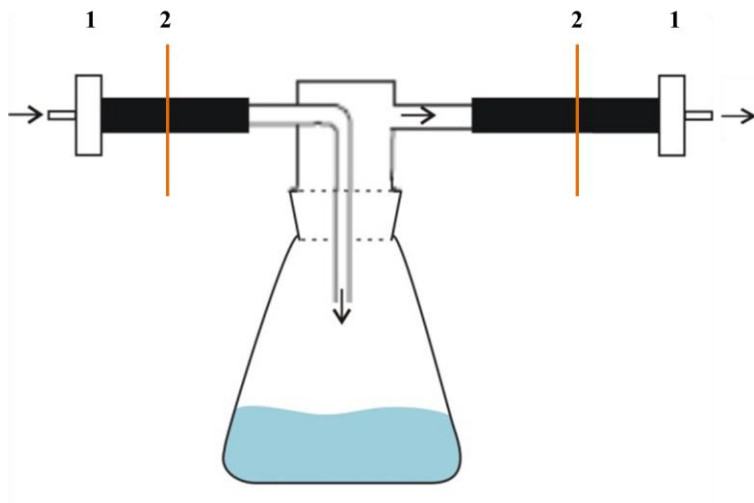
Fig. 11. Erlenmeyer flask containing mineral salt medium. This system was used to cultivate the Microbial Community (MC), as well as the Isolated Strain (IS), in Liquid Culture (LC) or established on Carrier Particles (CP). For aeration in order to trap the ^{14}C-CO$_2$, this incubator was connected to the pump exactly like the aerated soil samples shown in fig. 10, the clamps were opened and air was pumped through the flask for one hour.
1: Membrane filters at air outlet and inlet. 2: Clamps to close incubator, while incubating in the dark.

2.7.3   Analysis of ^{14}CO$_2$ in liquid cultures

The aeration system described in 2.7.2 was used to measure the ^{14}C-CO$_2$ generated in all liquid cultures. Therefore the incubator containing the liquid cultures shown in fig. 11 was connected to a vacuum pump and wash bottles as shown in fig. 10 three times per week.

The liquid cultures were enriched in Erlenmeyer flasks and incubated on an orbital shaker (75 rpm) in the dark. They were connected to the system and aerated (1.3 l h^{-1}) three times per week for 1 hour. After each aeration step the NaOH solution was exchanged to quantify the trapped ^{14}C-CO$_2$ by taking an aliquot of 2 ml, mixing it with 3 ml Ultima Flo and measuring the radioactivity in the liquid scintillation counter. From the detected ^{14}C-CO$_2$, the amount of SMZ mineralised by the bacteria in the liquid cultures, compared to the total applied SMZ was calculated.

2.7.4   Liquid culture preparation for soil inoculation experiments

Before the start of the soil incubation experiment, in which four soil inoculation variants were compared (2.6), all variants were simultaneously cultivated in liquid medium. The different inocula were cultivated in liquid cultures in the same way liquid cultures for the community enrichment were prepared (20 ml mineral salt medium without vitamins, 10 mg L^{-1} SMZ, shaking at 75 rpm in the dark at 20 °C). Three replicates were generated for each variant.

The four variants consisted of the

Microbial **C**ommunity on **C**arrier **P**articles (MCCP),

the **M**icrobial **C**ommunity without carrier particles in **L**iquid **C**ulture (MCLC),

the **I**solated **S**train on **C**arrier **P**articles (ISCP) and

the **I**solated **S**train without carrier particles in **L**iquid **C**ultures (ISLC).

The MCCP originated from the enrichment (2.7.2); the single strain *Microbacterium* sp. C448 for the ISCP variant originated from the agar plates. The variants containing bacteria without carrier particles were established, by taking aliquots (1 ml) of the MCCP and the ISCP liquid culture, transferring them into fresh liquid medium (19 ml) and not adding carrier particles to these fresh liquid cultures, incubating them exactly like the other liquid cultures before transferring them to soil. In the control soil, no bacteria were added.

For the soil inoculation experiment where only the MCCP was applied, the liquid cultures containing the microbial community were divided into two groups with different initial mineralisation activities, three replicates each. The concentration of SMZ in the liquid cultures was 10 mg L^{-1}. One group received a second dose of SMZ (5 µg ml^{-1}) after 7 days of incubation. Another 8 days later the MCCP were inoculated to the loamy soil for testing the enhanced SMZ mineralisation. The other group did not receive a second SMZ dose on day 7 and was transferred to the soil after 15 days of inoculation. In this way, the two groups showed different mineralisation activities when being transferred to soil samples.

2.8   Four soil inoculation alternatives in sandy loam soil

To identify the most effective method for soil inoculation by transferring SMZ degraders to soil, four different variants were compared;

transfer of degrading bacteria on carrier particles (CP) or

application of liquid culture containing these bacteria (LC) and

application of isolated strain (IS) to soil samples or

application of the microbial community (MC).

Per replicate, 1 µg g^{-1} SMZ was applied to 50 g sandy loam soil (dry weight equivalent). The experimental design is shown in table 8. The soil was equilibrated as described in 2.3, and

before start SMZ was applied to a soil aliquot as reported in 2.4.1. The ^{14}C-CO$_2$ measurement was conducted as described in 2.5.1. Microbes have been added to the equilibrated soils either in liquid or on carrier particles; the water content was set to pF 2.18 and adjusted weekly, gravimetrically, using distilled water. Three replicates for each inoculation variant received a higher amount of microbial inoculum (either in liquid or on clay particles); three replicates received a lower amount of inoculum. To apply the liquid cultures to soil, 1.5 ml and 3 ml of MCCP were applied to soil incubators, 10 ml and 5 ml of ISLC were centrifuged and the pellet was dissolved in 3 ml of water and homogeneously mixed into the soil incubators. These different amounts of inoculum were used to provide comparable experimental setups with similar mineralisation capacities at the start of the experiment. In this way the mineralisation rates were similar at start and the different developments of microbial SMZ mineralisation were compared to identify the most effective soil inoculation method.

Table 8. Experimental design of four soil inoculation variants.

Samples	Inoculated bacteria	Inoculation method	Amount of inoculum replicates in brackets
MCCP	Microbial community	Carrier particles	5 CP (3)
	Microbial community	Carrier particles	10 CP (3)
MCLC	Microbial community	Liquid culture	1.5 ml LC (3)
	Microbial community	Liquid culture	3 ml LC (3)
ISCP	Isolated strain	Carrier particles	10 CP (3)
	Isolated strain	Carrier particles	20 CP (3)
ISLC	Isolated strain	Liquid culture	5 ml LC (3)
	Isolated strain	Liquid culture	10 ml LC (3)
Control (no microbes added)	-	-	- (4)

2.8.1   First phase of incubation with four application variants

The SMZ mineralisation in the four soil inoculation variants (MCCP, MCLC, ISCP, ISLC) in the sandy loam soil samples was monitored continuously by quantifying the evolved ^{14}C-CO$_2$ three times per week during 99 days of incubation in the amber bottles, at 20 °C in the dark (soil incubator shown in fig. 9). In this way, mineralisation rates were calculated. At the end of the experiment, the incubators that received the higher amount of inoculum underwent a drying stress, whereupon a second incubation phase started. The incubators that received the lower amount of inoculum were frozen after the first phase until further usage (second phase).

2.8.2   Second incubation phase with four application variants after drying

The incubators that received the higher amount of microbial inoculum in the first phase (10 carrier particles of MCCP, 3 ml of MCLC, 20 carrier particles of ISCP and 10 ml of ISLC) were opened and the soil was dried to pF 2.5-3. Afterwards, ^{14}C-SMZ was reapplied to these incubators to investigate the reaction of the degrading bacteria to the drying stress and the soil was moistened to pF 2.18 again. No soil aliquot has been taken out for SMZ application this time; after the application the soil was mixed carefully with a spatula, soil moisture was adjusted and mineralisation was quantified via ^{14}C-CO_2 measurement three times per week for 50 additional days. After this second incubation phase, the soil samples have been analysed via extraction and combustion (2.8.4) and the ^{14}C mass balance was computed.

2.8.3   Second phase of incubation with four application variants after freezing

The incubators that received the lower amount of microbial inoculum in the first phase (5 carrier particles of MCCP, 1.5 ml of MCLC, 10 carrier particles of ISCP and 5 ml of ISLC) were frozen after the first incubation phase for 35 days (-20 °C). After thawing for one week in the fridge and one week at room temperature, ^{14}C-SMZ was reapplied to the soil samples to investigate the reaction of the degrading bacteria to the freezing stress. After application the soil was mixed carefully with a spatula, soil water tension was adjusted to pF 2.18 and SMZ mineralisation was measured three times per week for 46 days. After this second incubation phase the soil incubation was finished and the ^{14}C mass balance was calculated.

2.8.4   Soil sample analysis

After the experiment testing the four application variants was finished, extractable ^{14}C-residues in soil samples were determined by mixing 35 g (dry weight) soil with 0.5 g of diatomaceous earth prior to accelerated solvent extraction (ASE 200, Dionex, Dreieich, Germany) with a mixture of methanol and water (80:20) at 100 °C and 14 MPa. To ensure exhaustive extraction, in total nine sequential extractions per soil sample were conducted. Three extracts were collected in one glass vial, so every soil sample resulted in three glass vials containing three extracts each. After extraction, the exact volume of the extracts was measured and two aliquots of 1 ml of each glass vial were mixed with 4 ml Ultima Gold XR and measured by liquid scintillation counting.

To quantify the non-extractable ^{14}C-residues (NER), soil material was homogenized after ASE by grinding it in a mortar and three aliquots (250 mg – 300 mg) were mixed with a

saturated sugar solution for better combustion efficiency and combusted in a Sample Oxidizer (Packard, Dreieich, Germany). During combustion, the generated ^{14}C-CO_2 was trapped in Carbo-Sorb®E, mixed with Permafluor®E+ and quantified in the liquid scintillation counter.

At the end of each experiment, the ^{14}C mass balance was calculated based on the initially applied ^{14}C-SMZ, including the mineralised ^{14}C amount, the extractable and the non-extractable ^{14}C amount.

2.9 Additional soil inoculation with the microbial community on carrier particles

Testing the four different soil inoculation methods (MCCP, MCLC, ISCP, ISLC) led to the conclusion, that inoculation of the microbial community on carrier particles was the most effective variant for SMZ mineralisation in the tested sandy loam soil. Therefore, this soil inoculation was repeated to test the sustainability of the inoculation method. Two additional incubation phases were conducted. After 112 days of incubation, the second phase started, SMZ was reapplied, the microbes were reactivated and the mineralisation was restarted. Thus, the sustainability of the inoculation approach was verified. In the third phase, the mineralisation site was investigated. The experimental setup is shown in fig. 12.

Samples	Phase I 1st SMZ application 112 days	Phase II 2nd SMZ application 49 days	Phase III 3rd SMZ application 49 days	
Control 3 replicates	Soil & SMZ	Soil & SMZ	Soil & SMZ	
MCCP-LA 3 replicates	Soil & SMZ & LA-community on CP	Soil & SMZ & LA-community on CP	Used soil without CP Used CP in fresh soil	Sample analysis
MCCP-HA 3 replicates	Soil & SMZ & HA-community on CP			
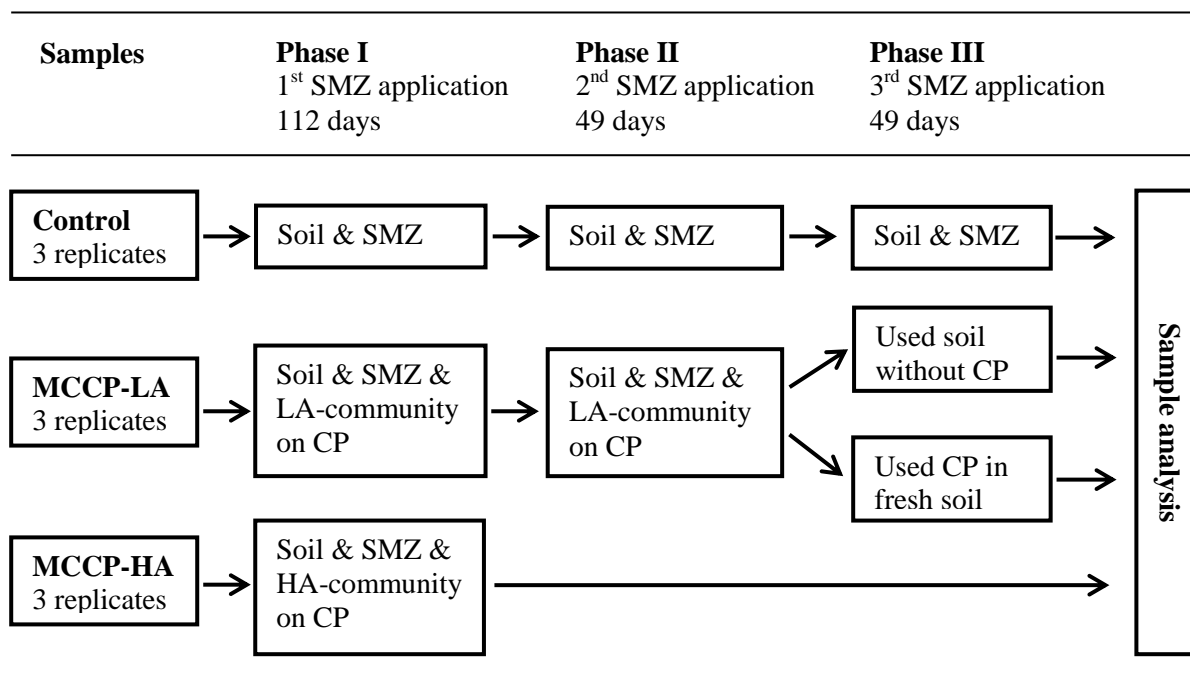
Fig. 12. Experimental setup of the three consecutive soil inoculation phases. The microbial community (two activity levels LA and HA) was applied to soil on carrier particles and ^{14}C-SMZ was applied (phase 1). The SMZ degradation capability of the inoculated microbial community was investigated after 112 days (phase 2) and the degradation site was localised (phase 3).

For this experiment, where only the MCCP was introduced to soil, 30 g sandy loam soil (dry weight equivalent), 12 CP overgrown by the microbial community and 1 µg g^{-1} SMZ per replicate were used. Soil equilibration and SMZ application were conducted as described above (2.3 and 2.4.1). Soil incubators were kept at 20 °C in the dark and the water content was adjusted weekly. Three replicates received carrier particles with a higher initial SMZ mineralisation activity (MCCP-HA) and three replicates received CP with a lower initial SMZ mineralisation activity (MCCP-LA). These differences in activities were obtained by the treatment of the liquid cultures, when preparing the CP for soil inoculation (as described in 2.7.4).

2.9.1 First incubation phase of MCCP

The first phase of soil incubation with MCCP in the sandy loam soil samples lasted for 112 days; SMZ mineralisation was measured three times per week by exchanging the sodium hydroxide and quantifying the ^{14}C-CO$_2$ in the liquid scintillation counter as described in 2.4.2.

2.9.2   Second incubation phase of MCCP

After the first soil incubation phase (112 days), ^{14}C-SMZ was applied a second time to the soil incubators (1 µg g^{-1}) and the incubation restarted. ^{14}C-CO$_2$ evolution was measured three times per week and the samples were incubated for 49 days.

2.9.3   Third incubation phase of MCCP

After the second phase a third incubation phase was started with the variants that showed a lower initial mineralisation rate in the first phase (MCCP-LA). At the end of the second phase, all CP have been taken out of these soil samples to detect whether the microbes, responsible for the SMZ mineralisation, were still located on these CP or not. The CP were taken out of the old soil incubators using a tweezer and were cautiously washed in a petri dish with distilled water for a few seconds to remove any remaining soil fragments attached to the carrier particles. The 12 CP of each soil incubator were transferred together to a new incubator with freshly equilibrated aliquots of the same soil (sandy loam soil). The CP were mixed in the fresh soil very carefully, SMZ was applied (1 µg g^{-1}) to the soil samples and the soil moisture was adjusted.

The soil from the second phase, where the CP have been taken out, remained in the old incubators and received a new SMZ application (1 µg g^{-1}). In this way, it was examined, whether the soil microbes have been proliferating into the surrounding soil habitat or remained on the carrier particles in the previous incubation phases one and two. The new incubators containing the already used CP mixed in fresh soil samples and the old incubators with the used soil where the CP have been taken out, were incubated for 62 additional days at 20 °C in the dark. The soil moisture was adjusted weekly and the SMZ mineralisation was quantified via ^{14}C-CO$_2$ measurement three times per week.

2.10   Data visualisation and statistical analysis

Data calculation, analysis and creation of graphs and tables was realised using Microsoft PowerPoint 2010, Word 2010 and Excel 2010 (Microsoft, Redmond, Washington, USA).

For statistical analysis the t-test was performed to compare mean values of two data sets, utilising IBM SPSS Statistics 20 (IBM, Armonk, New York, USA). Significance was verified at a p value of $p < 0.05$.

3.   Results

3.1   Sorption and desorption behaviour of three antibiotics in soil

Three different antibiotics, frequently used in livestock husbandry, were investigated to select a suitable one for soil experiments concerning microbial degradation of contaminants in soil samples. The antibiotic selected for such experiments should amongst others, be available for biodegradation in the soil pore water. Thus, Tetracycline (TC), Tetracycline*HCl (TC*HCl) and Sulfamethazine (SMZ) have been tested for their sorption and desorption behaviour in the loamy sand soil (soil description in 2.3).

3.1.1   *In situ* bioavailability of three antibiotics in soil

Permanent sorption and desorption regulate the bioavailability for microbial degradation of a substance in soil. The decrease of the *in situ* bioavailable fractions of the three tested antibiotics in the soil pore water is shown in figure 13. The SMZ spiked samples were incubated longer and centrifuged two times more than the others. The results reveal a significantly higher bioavailability of SMZ in soil compared to the other antibiotics (t-test, $p < 0.05$). On the first day, 26.4% of the applied SMZ remained in the soil pore water and was available for biodegradation. The amounts were then constantly decreasing until day 21, when the last centrifugation step took place and 2% of the applied SMZ were still dissolved in the soil pore water. For TC, the bioavailable amount in soil decreased from 3.5% of applied TC to 0.6%. For TC*HCl, only 2.8% of the applied amount were available in the beginning, decreasing to 0.8% at the last centrifugation step.
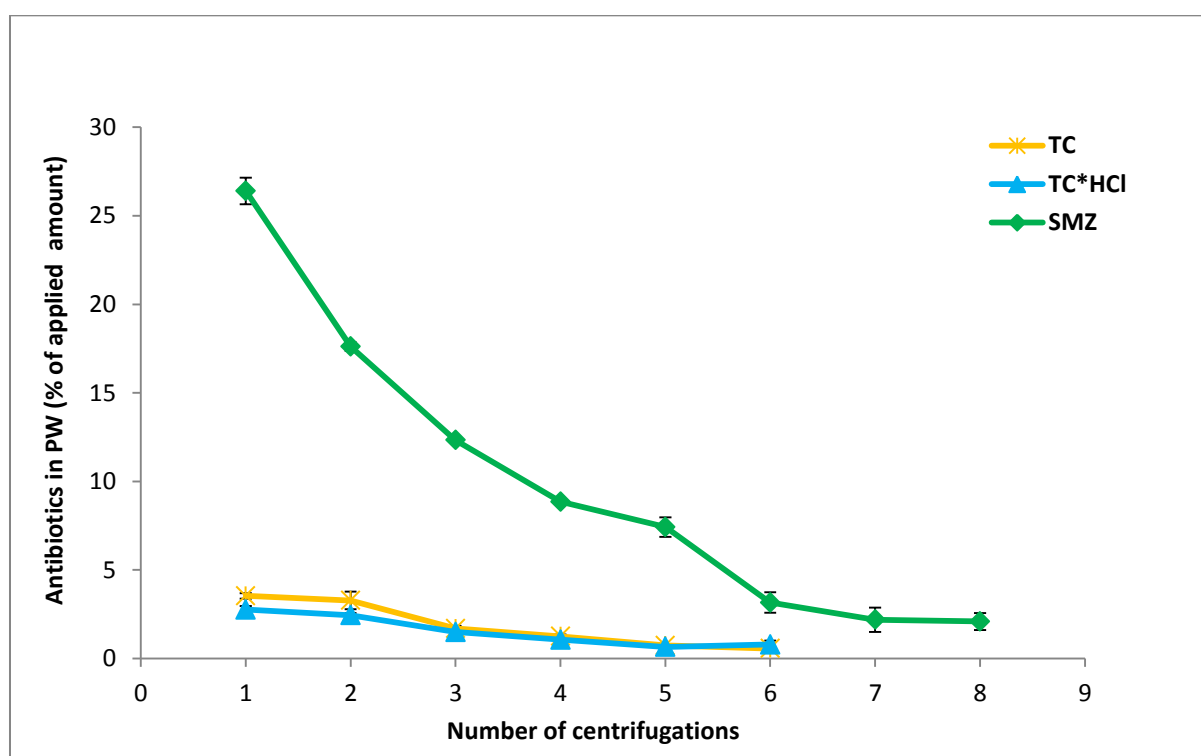
Fig. 13. Decrease of the three antibiotics (Tetracycline (TC), Tetracycline*HCl (TC*HCl) Sulfamethazine (SMZ)), dissolved in soil pore water (PW – refilled after each extraction), available for degradation in the loamy sand soil samples. n=3, bars indicate standard deviation. 1st centrifugation: SMZ in porewater significantly higher than TC and TC*HCl (t-test, $p < 0.05$).

3.1.2   Desorption of three antibiotics from soil

From the *in situ* bioavailable amount of antibiotics, quantified in 3.1.1, the desorbed fraction was calculated for each centrifugation step. For this calculation, the amount of antibiotics in the remaining soil pore water, which could not be extracted by this method, was considered. This amount of non-extracted antibiotics from the former sampling time, which remained in soil, was subtracted from the concentration quantified in the soil pore water, for every sampling day. In this way, only the desorbed SMZ fraction was taken into account (fig. 14).

After day one, where 26.4% of the applied SMZ was available, this calculated desorbed fraction makes up about 2% each day, before it gets close to (and even below) zero. For the other two substances, after the first sampling day, where 2.8-3.5% of the applied antibiotic were available, the desorbed amount stayed below 1% for the other five sampling days.
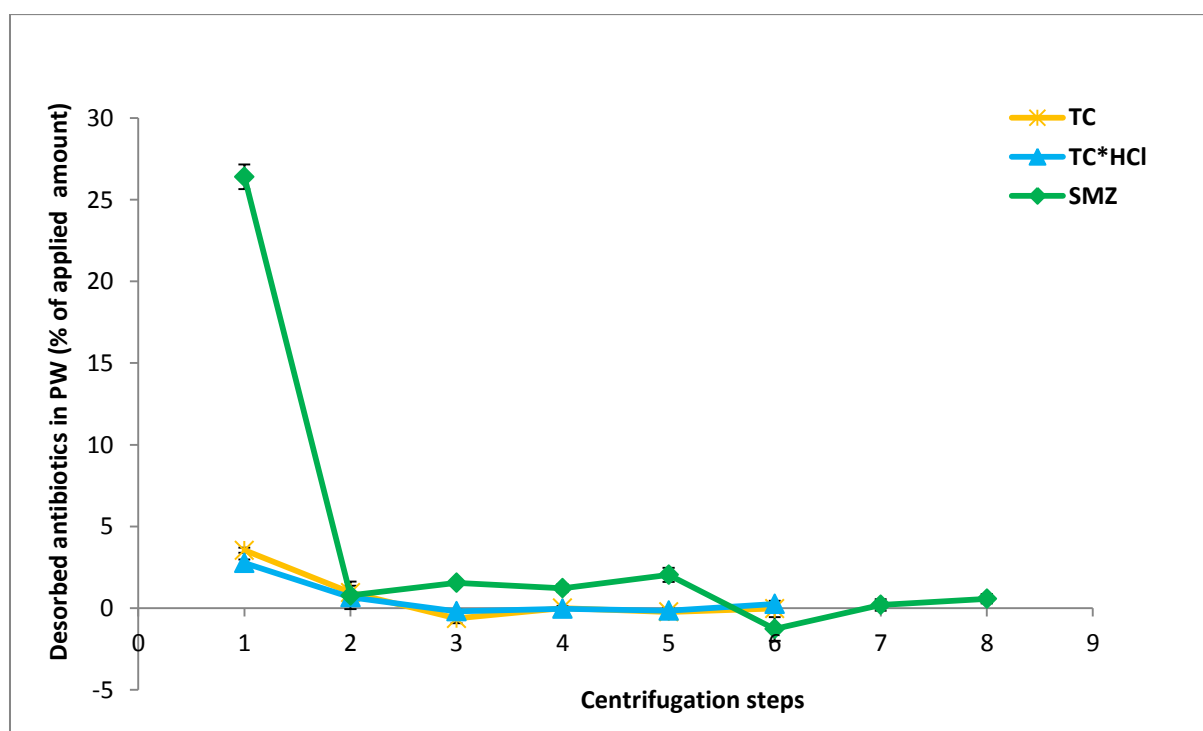
Fig. 14. Calculated desorbed amount of three antibiotics (Tetracycline (TC), Tetracycline*HCl (TC*HCl) and Sulfamethazine (SMZ)) in the soil pore water (PW) of the loamy sand soil at each centrifugation step, n=3, bars indicate standard deviation.

3.2   *In situ* bioavailability of SMZ in three soils

After selection of the antibiotic for all further experiments, SMZ, the soil suitable for the inoculation experiments had to be defined. Therefore, the *in situ* bioavailability of SMZ in three different soils was examined over a period of 21 days, which also lead to more information about the instant and later sorption performance of SMZ in different soils. The three soils (30 replicates each) were spiked with ^{14}C-SMZ and centrifuged 10 times in two weeks to quantify the *in situ* bioavailable ^{14}C-SMZ in the soil pore water. Three replicates were centrifuged each time. Besides their texture (sandy loam, silty loam and sandy soil), these soils also varied in their pH and water regime (table 5). They were incubated at a soil water tension of pF 2.18, which led to complete different soil water contents in the three tested soils, but similar water availabilities. Figure 15 shows the decrease of *in situ* bioavailable ^{14}C-SMZ in the soil pore water over time in the different soils. After an instant severe decrease of ^{14}C-SMZ in the soil pore water for about seven days, the amount of ^{14}C-SMZ in all three soils decreased at a lower rate down to 1-2% of the applied ^{14}C-SMZ. On day one, the three different soil samples vary significantly from each other (t-test, $p < 0.05$). Sandy loam soil samples provided the highest amount of SMZ in the soil pore water.
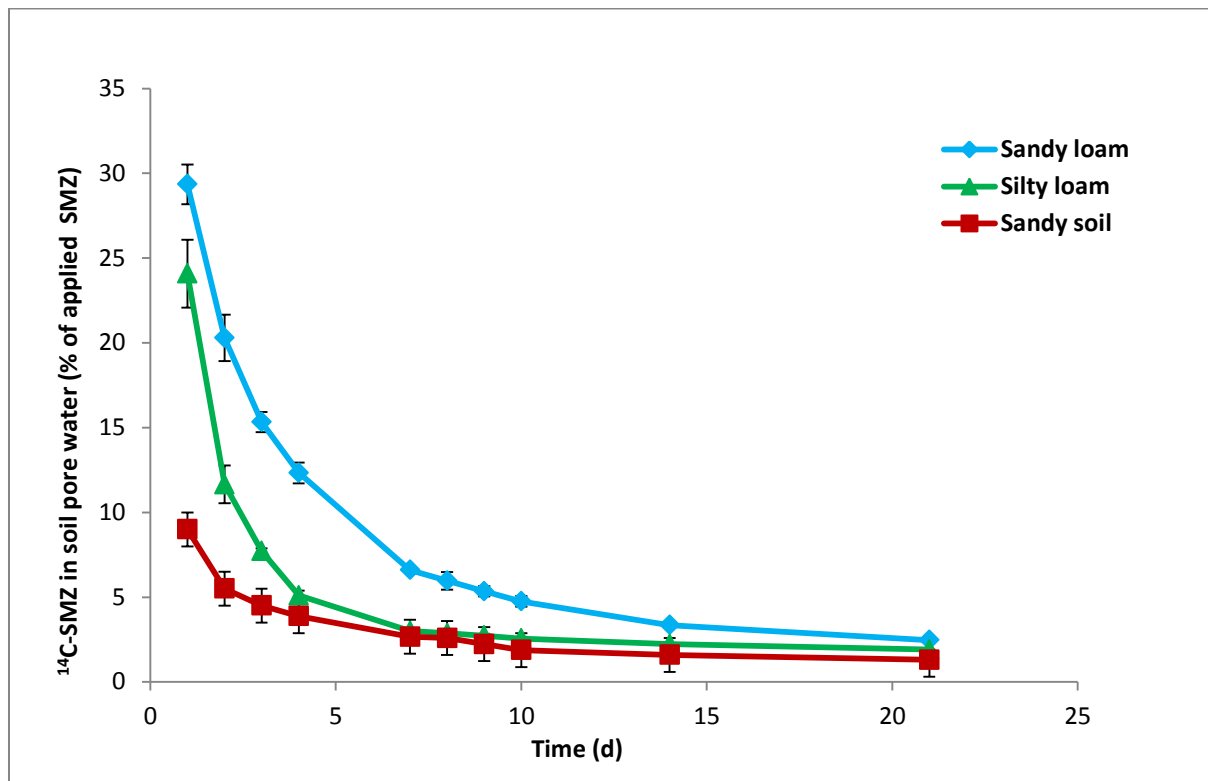
Fig. 15. Decrease of *in situ* bioavailable ^{14}C-SMZ, dissolved in extracted soil pore water over time, in soils differing in texture. 30 replicates were set up at start. Standard deviation was calculated from three replicates per centrifugation step. The three soil samples differ significantly on day one (t-test, p<0.05).

To determine the effect of SMZ degradation by soil microorganisms, the same experiment was conducted with sandy loam soil samples sterilized by gamma radiation. The *in situ* bioavailability time courses of the sterile and the non-sterile soil show analogous curve progressions (fig. 16). On day one, the non-sterile soil showed a slightly higher bioavailability of SMZ (29% compared to 26%). After one week, the SMZ bioavailability in the two soils was equal (6%), whereupon the sterile soil remained at this level (4-5%) whereas the SMZ bioavailability in the non-sterile soil decreased further (down to 2%) until day 21.
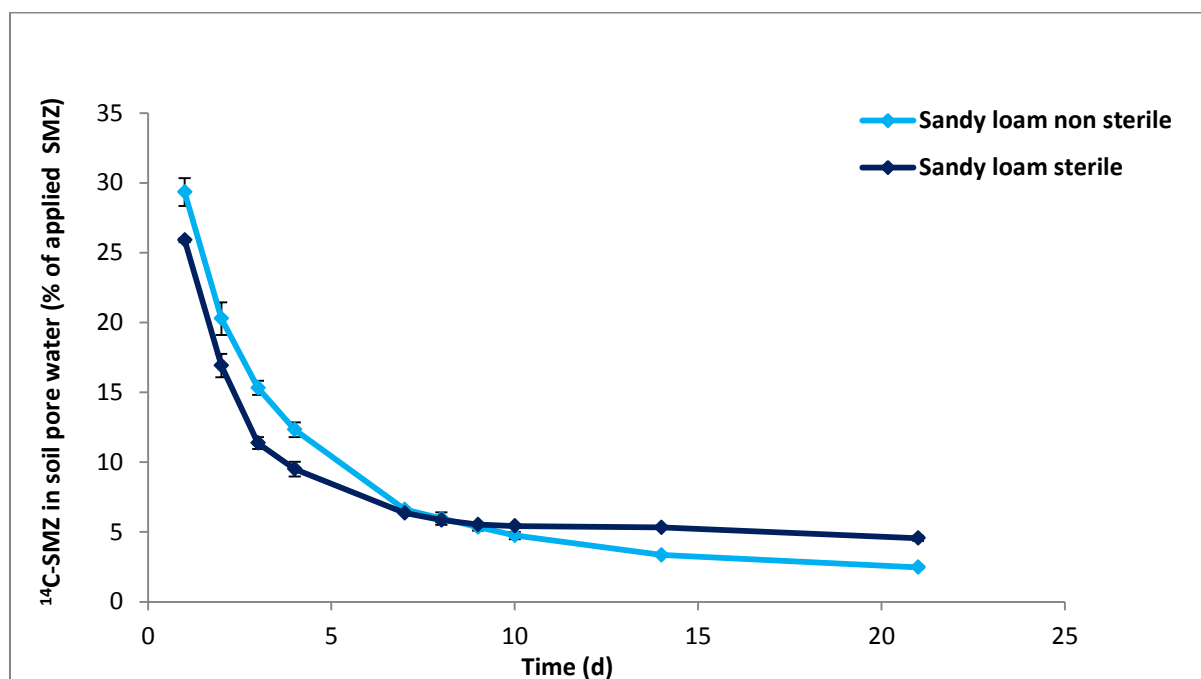
Fig. 16. Decrease of *in situ* bioavailable ^{14}C-SMZ (% of applied ^{14}C-SMZ) in extracted soil pore water over time in sandy loam soil samples; sterilised and non-sterilised. Out of 30 replicates, three were extracted each time. Bars indicate standard deviation, n=3.

3.3   Effect of SMZ application on microbial biomass in soil

After the appropriate antibiotic (SMZ) and soil (sandy loam) were selected, the effect of SMZ on the soil microorganisms in general, was identified. Therefore, the microbial carbon has been quantified via chloroform fumigation and extraction in SMZ spiked and non-spiked soil samples before and after the SMZ application (1 µg g^{-1}). Samples were incubated for 20 days. The microbial carbon in the samples without SMZ was set to 100% and microbial carbon in the SMZ treated soil was calculated according to this. No effect was apparent from the application of SMZ to soil microbial carbon in this concentration (table 9).

Table 9. Microbial carbon in soil samples in µg g^{-1} (dry weight equivalent) and relative in %, whereby each untreated variant (-SMZ) was set to 100%.

Day	- SMZ		+ SMZ	
	C_{mic} (µg g^{-1})	(%)	C_{mic} (µg g^{-1})	(%)
1	934.58	100	969.26	103.7
6	1086.58	100	1039.16	95.6
20	893.51	100	876.34	98.1

3.4 Stimulation of soil microorganisms to degrade SMZ

Stimulating indigenous soil microorganisms to the degradation of a chemical was achieved by increasing its bioavailable amounts in soils. For the herbicide isoproturon, highly increased mineralisation by soil microorganisms occurred after increasing its bioavailability by a triple isoproturon application to soil (Kiesel 2014). For SMZ, multiple applications of high SMZ concentrations over several years led to the desired adaptation effect (Topp et al. 2013). In the present work, the bioavailability of SMZ had to be increased by applying different strategies. SMZ application rate, SMZ concentration as well as soil moisture and soil temperature were varied. All adaptation variants were conducted with 35 g aliquots of the loamy sand soil at a soil water tension of pF 2.18 for constant soil moisture and at pF 2.18 – pF 4.18 for experiments with fluctuating soil moisture. A detailed description of the experimental set up was given in the materials and methods section.

3.4.1   Effect of soil moisture on SMZ degradation at different concentrations

A higher (100 µg g^{-1}) and a lower (10 µg g^{-1}) SMZ concentration were applied to 35 g of the loamy sand soil samples. Both SMZ concentrations were applied to soil incubators with constant soil moisture (fig. 9) and fluctuating soil moisture (fig. 10).

Mineralisation rates in the variants with a lower SMZ concentration of 10 µg g^{-1}, incubated at constant soil moisture (fig. 17) reached 0.1 µg d^{-1} or 0.03% d^{-1} of the applied SMZ. The mineralisation did not lead to a clear peak, but showed a plateau at the beginning and was afterwards constantly decreasing. In the variants with low SMZ concentration incubated at fluctuating soil moisture, SMZ mineralisation rates were also at the level of 0.1 µg d^{-1} or 0.03% d^{-1} (fig. 17).
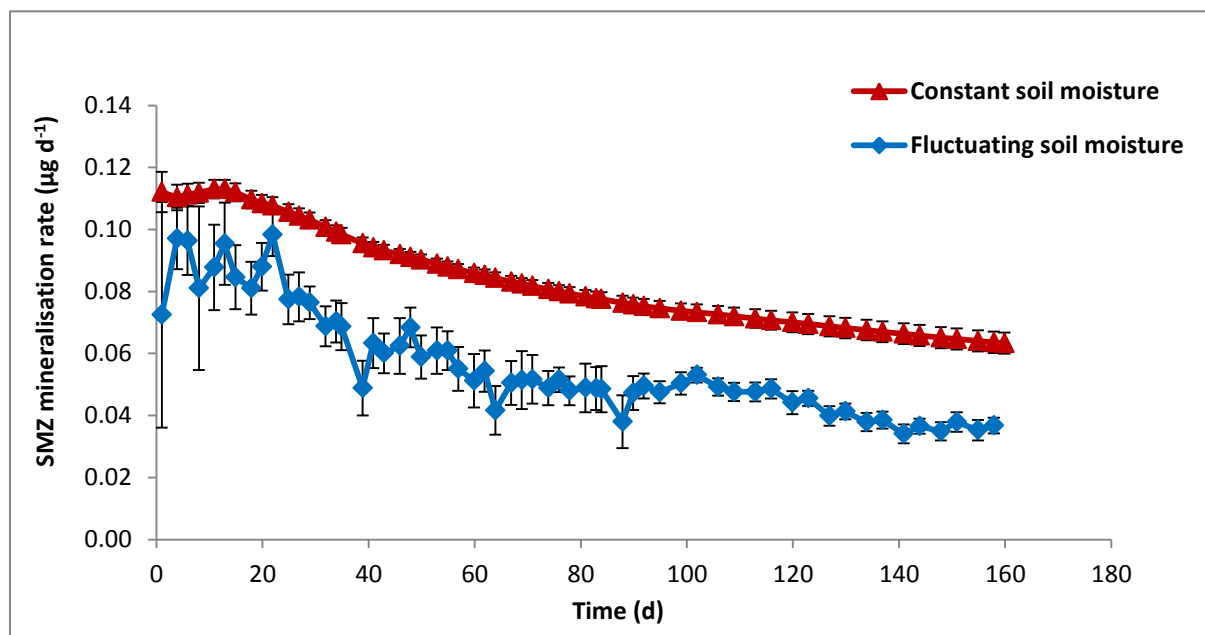
Fig. 17. SMZ mineralisation rates in soil samples, two moisture regimes after single SMZ-application of   10 µg g^{-1}, incubated at 20 °C, no microbes were added.
Soil moisture was varied (blue line) to enhance adaptation to SMZ mineralisation. Fluctuating soil moisture was compared to constant soil moisture at pF 2.18 (red line). Bars indicate standard deviation, n=4.

Mineralisation rates in the variants with higher SMZ concentration (100 µg g^{-1}) were increased only up to 2.5 µg d^{-1} or 0.05% d^{-1} for both, the fluctuating and the constant soil moisture. The curve progression shows a mineralisation peak on day 22 for both variants, but the overall mineralisation was negligible (first 41 days are presented in fig. 18). After day 41 the samples with higher SMZ concentration and fluctuating soil moisture received another treatment with multiple applications, which was explained in 3.4.3.
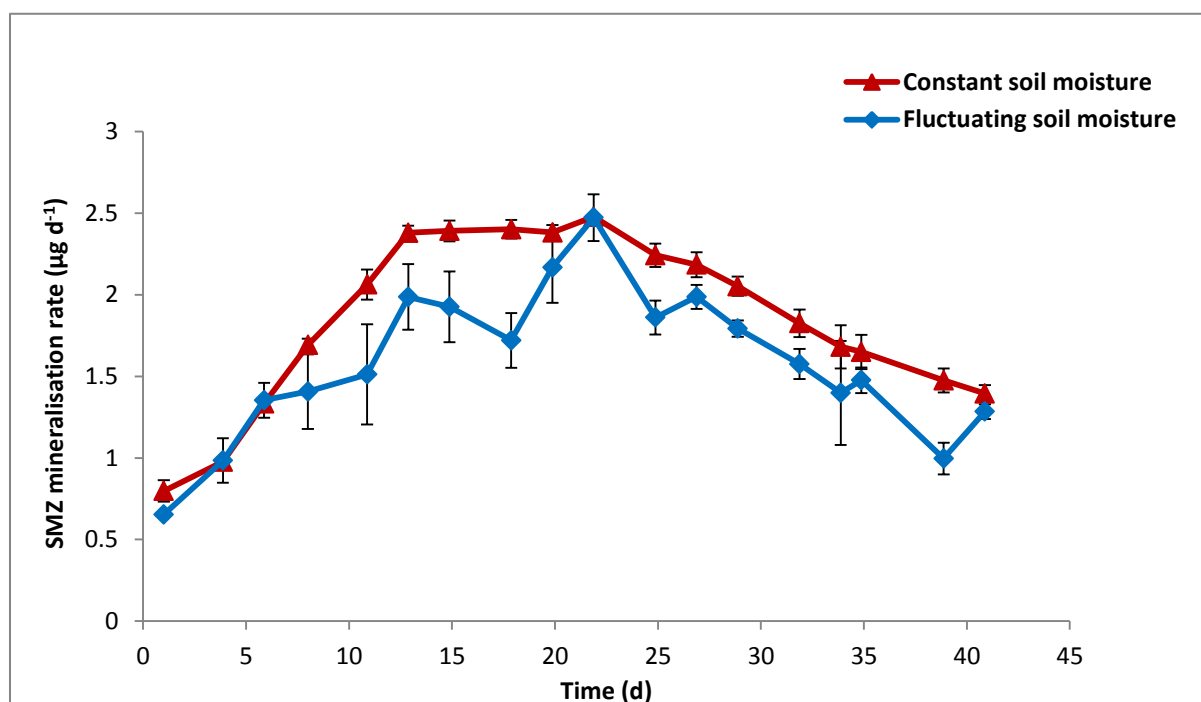
Fig. 18. SMZ mineralisation rates in soil samples, two moisture regimes, after single SMZ-application of a higher SMZ dose of 100 µg g^{-1} incubated at 20 °C, no microbes were added. Graph is cut off at day 41 for better resolution. After day 41 the treatment for the samples with fluctuating soil moisture changed. The whole 160 days are shown in fig. 22 and explained in 3.4.3. Soil moisture was varied (blue line) and SMZ concentration was increased to enhance adaptation to SMZ mineralisation. Fluctuating soil moisture was compared to constant soil moisture and at pF 2.18 (red line). Bars indicate standard deviation, n=4.

3.4.2   Effect of soil temperature on SMZ degradation at different concentrations

The loamy sand soil samples were incubated at two different temperatures (20 °C and 30 °C) and were spiked with two different SMZ concentrations (10 µg g^{-1}, fig. 19 and 100 µg g^{-1}, fig. 20) at a constant soil water tension (pF 2.18). Mineralisation rates in the variants with lower SMZ concentration (10 µg g^{-1}) and 30 °C were not up to 0.3 µg d^{-1} (0.06% of the applied SMZ, fig. 19)

Mineralisation rates in the variants with higher SMZ concentration (100 μg g^{-1}) and 30 °C only reached 3.7 μg d^{-1} which is equivalent to 0.1% of the applied SMZ (fig. 20).
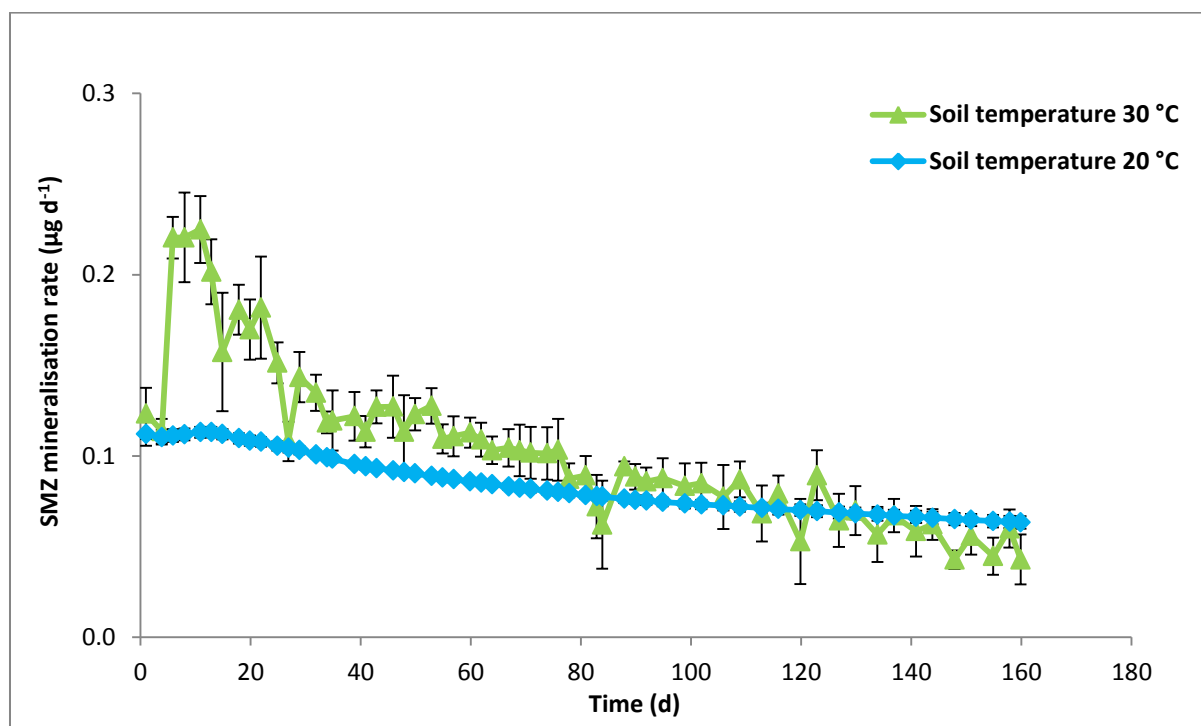
Fig. 19. SMZ mineralisation rates in soil samples after one single SMZ-application of 10 μg g^{-1}, incubated at constant soil moisture (pF 2.18) at two different temperatures, no microbes added.

Soil samples were incubated 30 °C (green line) to enhance adaptation to SMZ mineralisation. Incubation at 30 °C was compared to incubation at 20 °C (blue line). Bars indicate standard deviation, n=4.
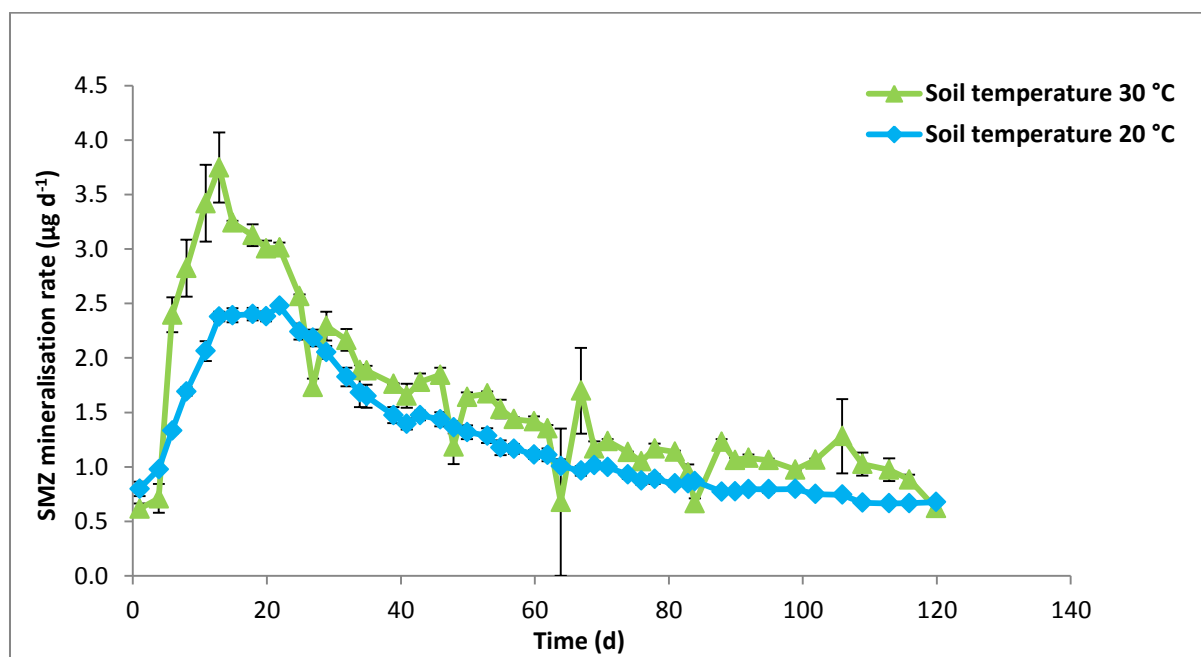
Fig. 20. SMZ mineralisation rates in soil samples after one single SMZ-application of 100 μg g^{-1} at constant soil moisture (pF 2.18), no microbes added.

Soil samples were incubated with a high dose of SMZ and at 30 °C (green line) to enhance adaptation to SMZ mineralisation. Incubation at 30 °C was compared to incubation at 20 °C (blue line). Standard deviation was calculated from four replicates at 20 °C and three replicates at 30 °C.

3.4.3   Sulfamethazine mineralisation after multiple applications

Another attempt to stimulate the soil bacteria to degrade SMZ by increasing the SMZ bioavailability was to conduct multiple SMZ applications (5 µg g^{-1}) to the same soil incubators. The soil samples were incubated at constant and at fluctuating soil water tension, with two different initial SMZ concentrations.

In the incubators with constant soil moisture, the initial SMZ concentration was 5 µg g^{-1} followed by 8 additional SMZ applications (5 µg g^{-1}), within 209 days (fig. 21). Arrows mark the times of the initial and the following SMZ applications. This method did also not lead to the expected higher SMZ degradation, as mineralisation rates accounted only for 0.4 µg d^{-1} (0.03% of the total applied SMZ).
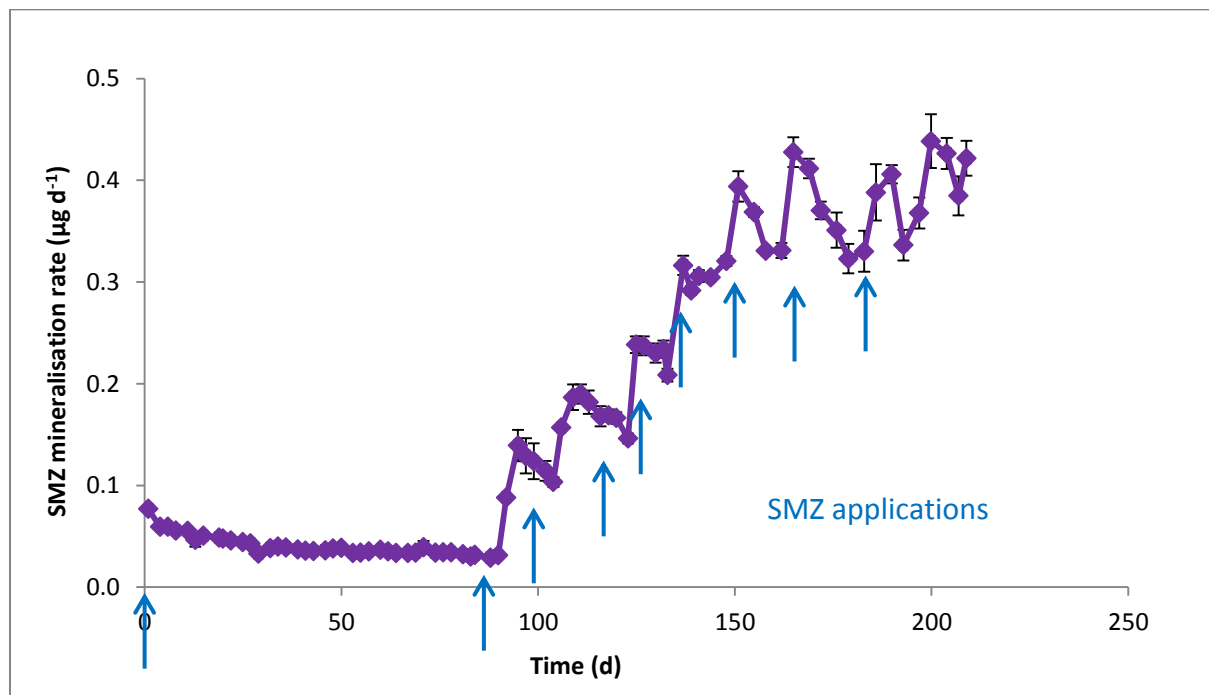
Fig. 21. SMZ mineralisation rates in soil samples incubated at constant moisture (pF 2.18) at 20 °C, with multiple SMZ applications, no microbes added. SMZ applications were repeated to enhance adaptation to SMZ mineralisation. All SMZ applications: 5 µg g^{-1}. Arrows mark times of SMZ-applications. Bars indicate standard deviation, n=3.

The incubators with fluctuating soil moisture received a higher initial SMZ dose of 100 µg g^{-1} followed by 6 reapplications (5 µg g^{-1}) (fig. 22). The highest mineralisation rate (2.5% d^{-1}) was recorded after the first SMZ application of 100 µg g^{-1} but accounted for less than 0.1% of the applied SMZ amount. Hence, this approach did also not lead to an enhanced SMZ degradation.
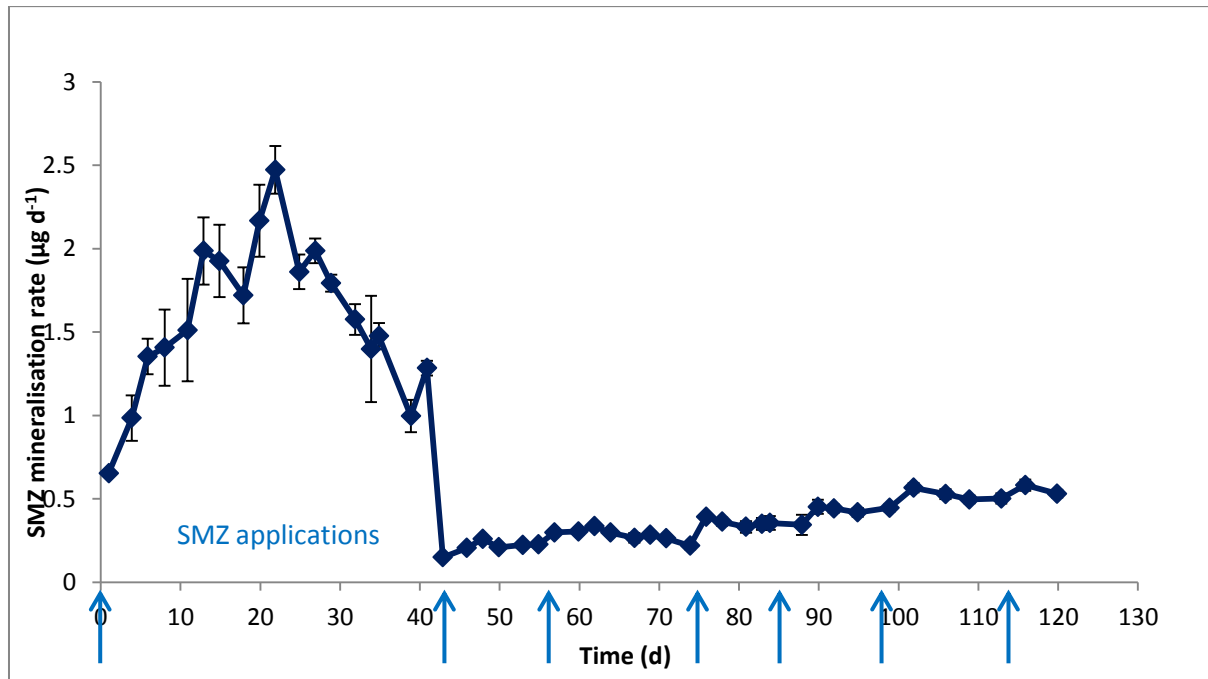
Fig. 22. SMZ mineralisation rates in soil samples with multiple SMZ-applications, fluctuating moisture (pF 2.18-4.18) at 20 °C, no microbes added. Initial supply of SMZ: 100 µg g^{-1}, 6 following SMZ applications with 5 µg g^{-1}, respectively (day 42, 56, 74, 84, 98 and 112). Arrows mark times of SMZ-application. SMZ was applied seven times to enhance adaptation to SMZ mineralisation. Bars indicate standard deviation, n=4.

3.5   Characteristics of the degrading strain *Microbacterium* sp. C448 and its corresponding microbial community

The SMZ degrading strain *Microbacterium* sp. C448 as well as the associated microbial community was further investigated to get important information about the shape, behaviour and growth pattern of the strain. The strain was tested for its morphology, its growth time and additional antibiotic resistances while the soil microbial community was analysed for its metagenome (DNA of community grown on carrier particles in liquid culture).

3.5.1   Morphology of *Microbacterium* sp. C448

The morphology of the SMZ-degrading strain *Microbacterium* sp. C448 was investigated by scanning electron microscopy (fig 23a and b, fig. 24). The length of *Microbacterium* sp. C448 cells was about 2 μm and the width was about 0.5 μm, which is in the expected range for bacteria and the short rod morphology is visible in all pictures. Besides the function to mineralise SMZ, the shape and size of the bacteria *Microbacterium* sp. C448 are in accordance with the description of this strain in the work, which describes its isolation (Topp et al. 2013). This identical morphology can be seen as a proof that after all this experiments we were still working with the desired microorganism.
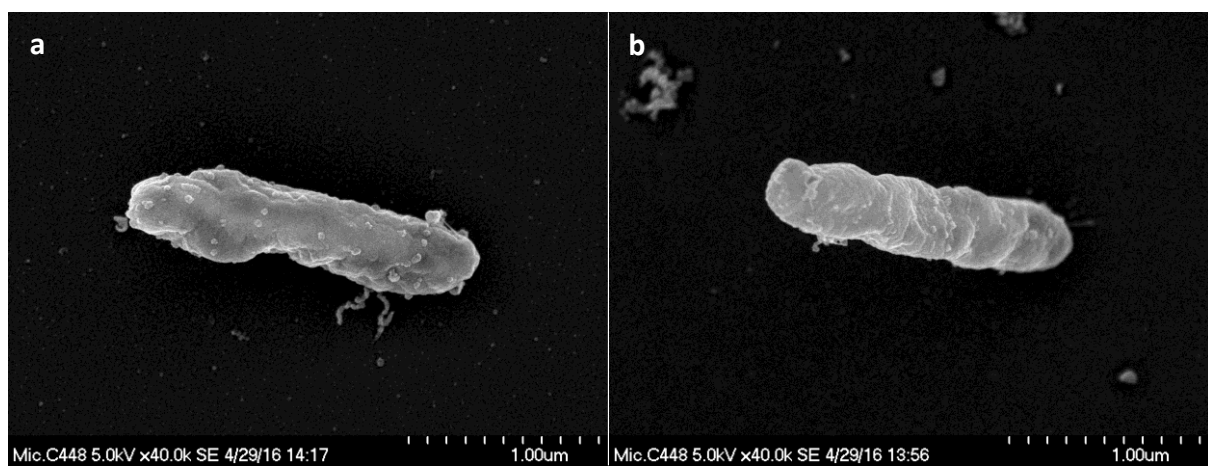
Fig. 23 a+b. Scanning electron microscope pictures of *Microbacterium* sp. C448, showing its rod shape. Magnification: 40 000 times.
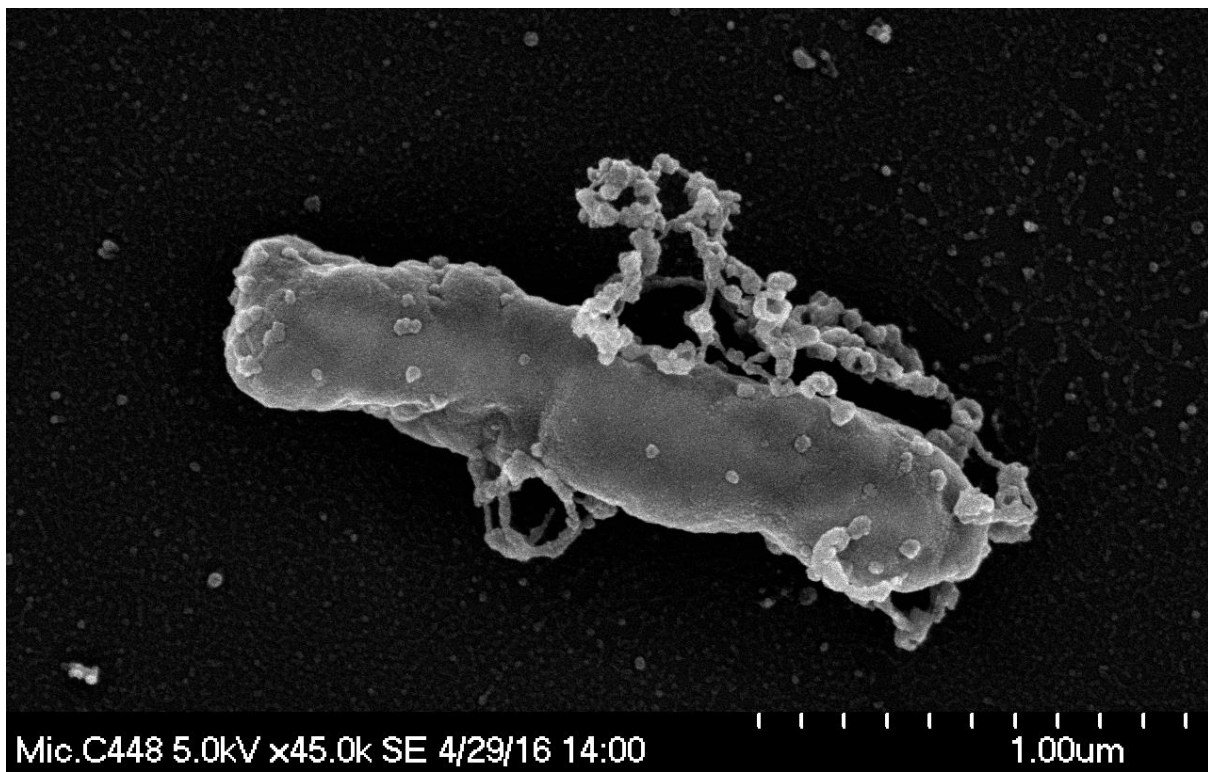
Fig. 24. Scanning electron microscope picture of *Microbacterium* sp. C448, showing its size of about 2 μm in this top view. Magnification: 45 000 times.

3.5.2   Growth curve of *Microbacterium* sp. C448

The growth curve of *Microbacterium* sp. C448 was observed for seven days, the OD and the pH was measured regularly. The result is presented in figure 25. While the microbes were growing in the test tube, the pH was constantly increasing, turning the liquid medium more and more basic.
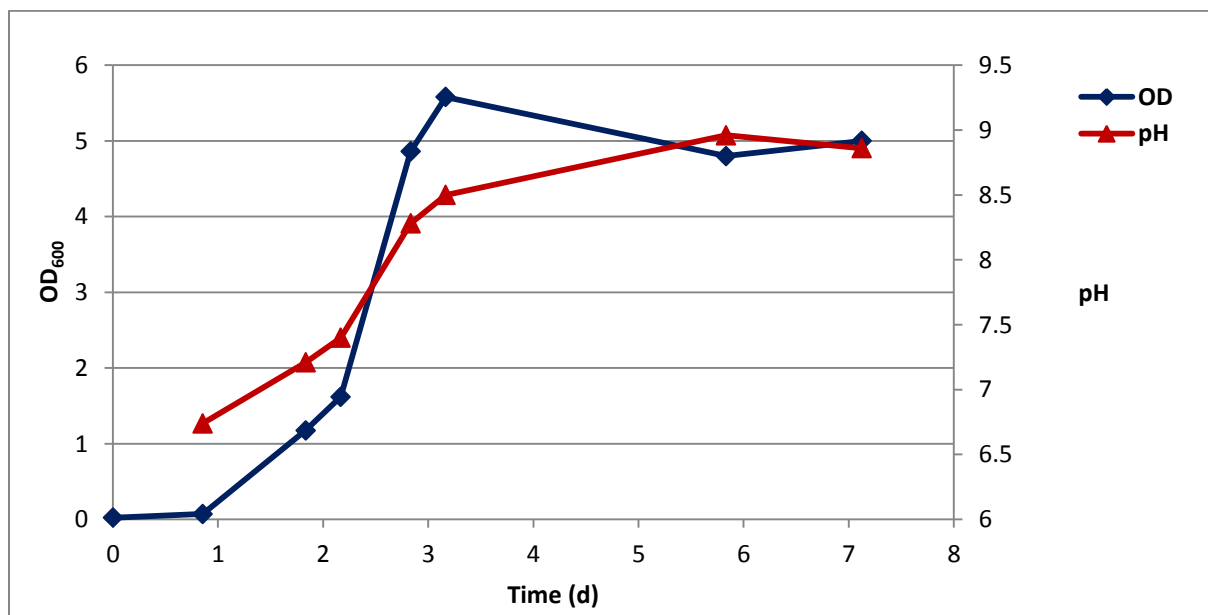
Fig. 25. Growth curve of *Microbacterium* sp. C448 and pH measurement in one liquid culture, incubated for one week at 37 °C on the orbital shaker without replicates.

3.5.3   Antibiotic resistances of *Microbacterium* sp. C448

Antibiotic resistances of the SMZ degrading strain, additional to SMZ resistance, were tested using antibiotic test rings on agar plates.

In addition to the SMZ resistance, the strain *Microbacterium* sp. C448 showed multiple antibiotic resistances, including other groups of antibiotics. Fig. 26 shows two antibiotic test rings. Some bacteria-free areolas are visible, where bacterial growth was inhibited, while other antibiotic spiked areas are overgrown by the strain.
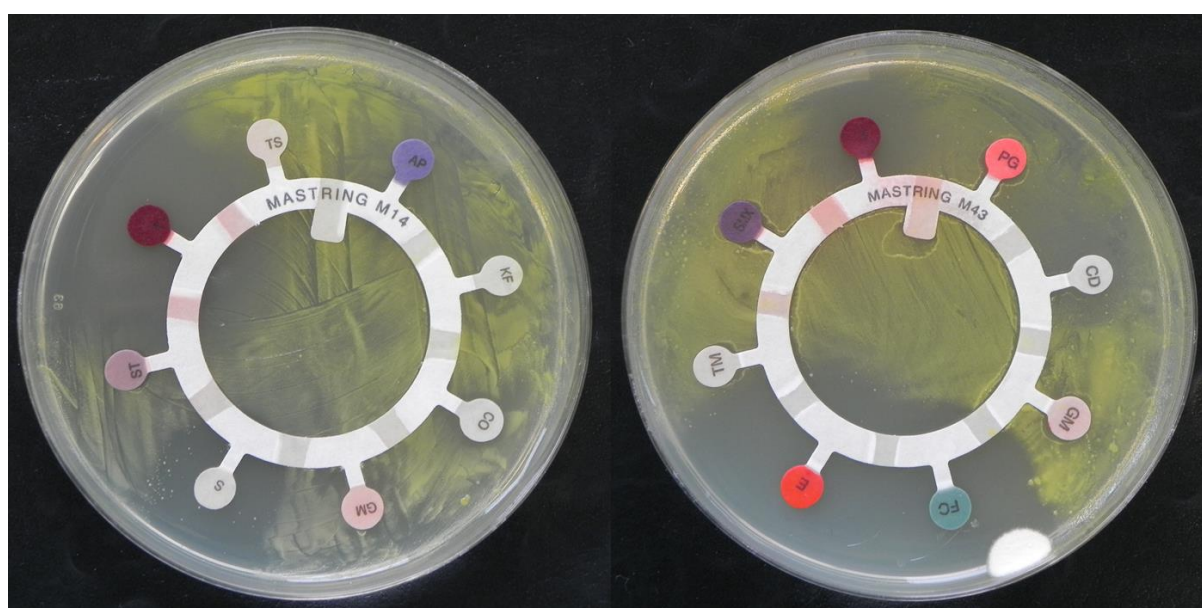
Fig. 26. The yellowish *Microbacterium* sp. C448 growing on LB medium with antibiotic test-rings. The strain was sensitive to five antibiotics and not to the other 10 antibiotics tested.

Sensitive: right hand side:	tetracycline (dark red T), Clindamycin (grey CD),
	fusidic acid (green FC), erythromycin (red E),
left hand side:	streptomycin (grey S), tetracycline (dark red T)
Not sensitive: right hand side:	penicillin G (pink PG), gentamicin (light pink GM),
	trimethoprim (grey TM), sulfamethoxazole (purple SMX),
left hand side:	co-trimoxazole (mixture of trimethoprim and sulfamethoxazole,
	grey TS), ampicillin (purple AP), cephalothin (grey KF),
	colistin sulphate cephalothin (grey CO),
	gentamicin (light pink GM), sulphatriad (light purple ST)

3.5.4   DNA analysis of the microbial community

The microbial community including the degrading strain *Microbacterium* sp. C448 was enriched from the Canadian soil, cultivated on carrier particles. From these carrier particles which were overgrown by the microbial community, their DNA was extracted.

After DNA extraction, sequencing and alignment, most sequences were analysed to genus level, the different levels of phylogenetic taxonomy are marked in the diagram. The Microbacteriaceae, where *Microbacterium* sp. C 448 is included, was present to only 0.6%; therefore it is listed under "others" in this circle diagram (fig.27).
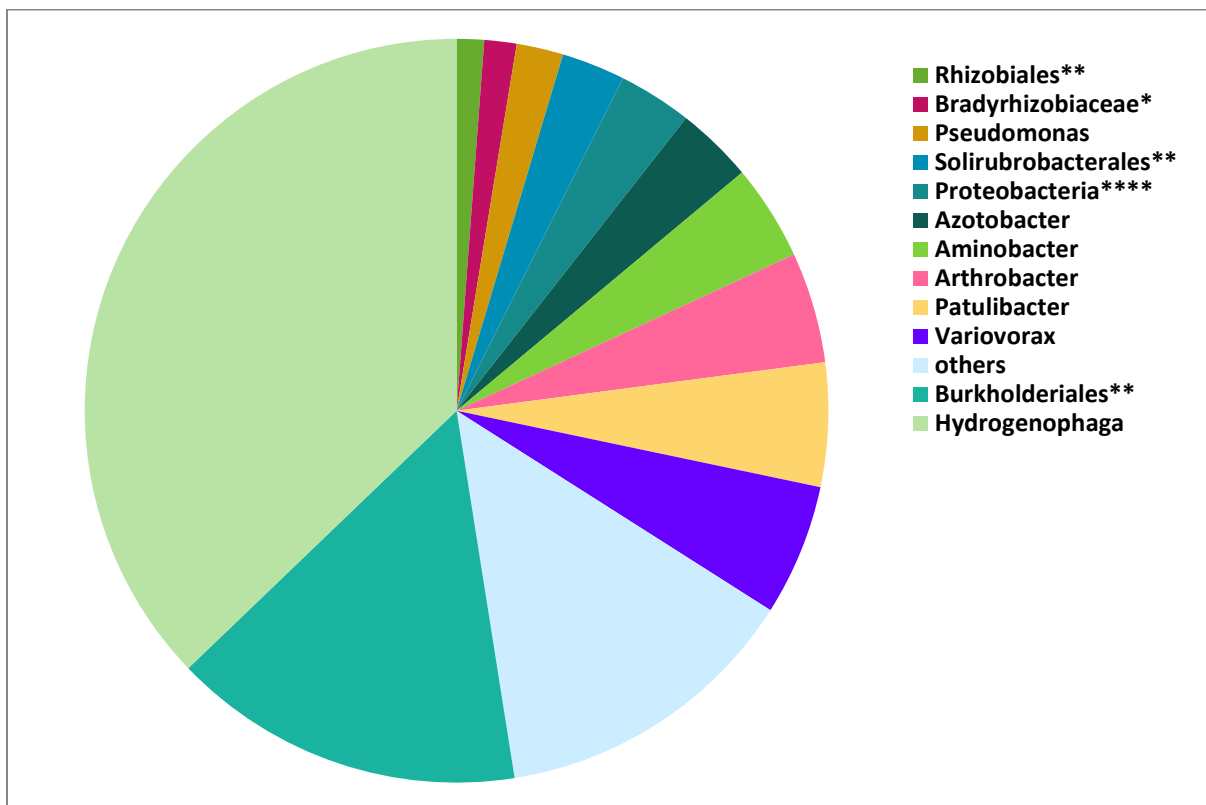
Fig. 27. The composition of the microbial community analysed by determination of the 16S rRNA-gene sequence using illumina sequencing. All findings smaller than 1% of the total amount were summarised under "others".
*alignment ends at family level
** alignment ends at order level
**** alignment ends at phylum level

3.6   SMZ mineralisation capacity in liquid culture

Prior to soil inoculation, the microbes were always cultivated in liquid media (mineral salt medium) with or without carrier particles. The mineralisation rates and the cumulative mineralisation of the bacteria in liquid culture, prepared for comparison of four soil inoculation variants, are shown in fig. 28 and 29. The four variants in liquid cultures were **M**icrobial **C**ommunity, established on **C**arrier **P**articles (MCCP), the **M**icrobial **C**ommunity without carrier particles in **L**iquid **C**ulture (MCLC), the **I**solated **S**train on **C**arrier **P**articles (ISCP) or without carrier particles in **L**iquid **C**ultures (ISLC). After the mineralisation rates in all liquid cultures were below or close to 1% d^{-1} of the applied SMZ, the microbes were transferred to the soil samples to have similar starting conditions at the beginning of the soil inoculation experiments.
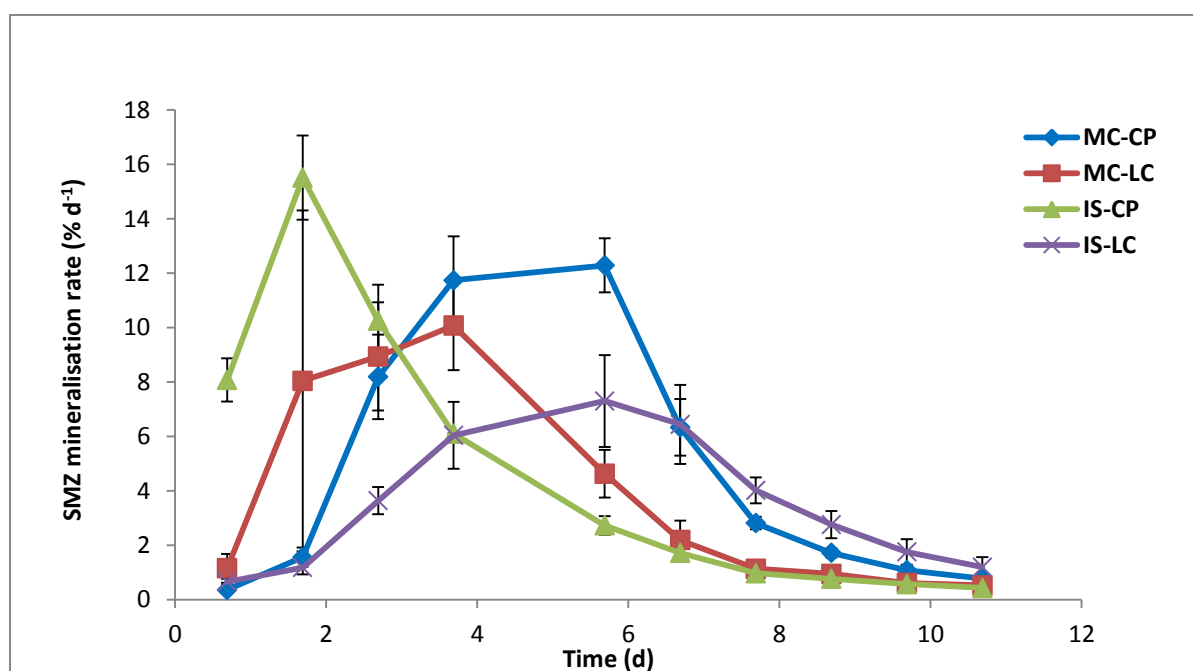
Fig. 28. SMZ mineralisation rates (% of applied amount per day) of the four different variants in liquid cultures before soil inoculation. Soils were inoculated after the mineralisation peak, when mineralisation rates were low again. Bars indicate standard deviation, n=3.
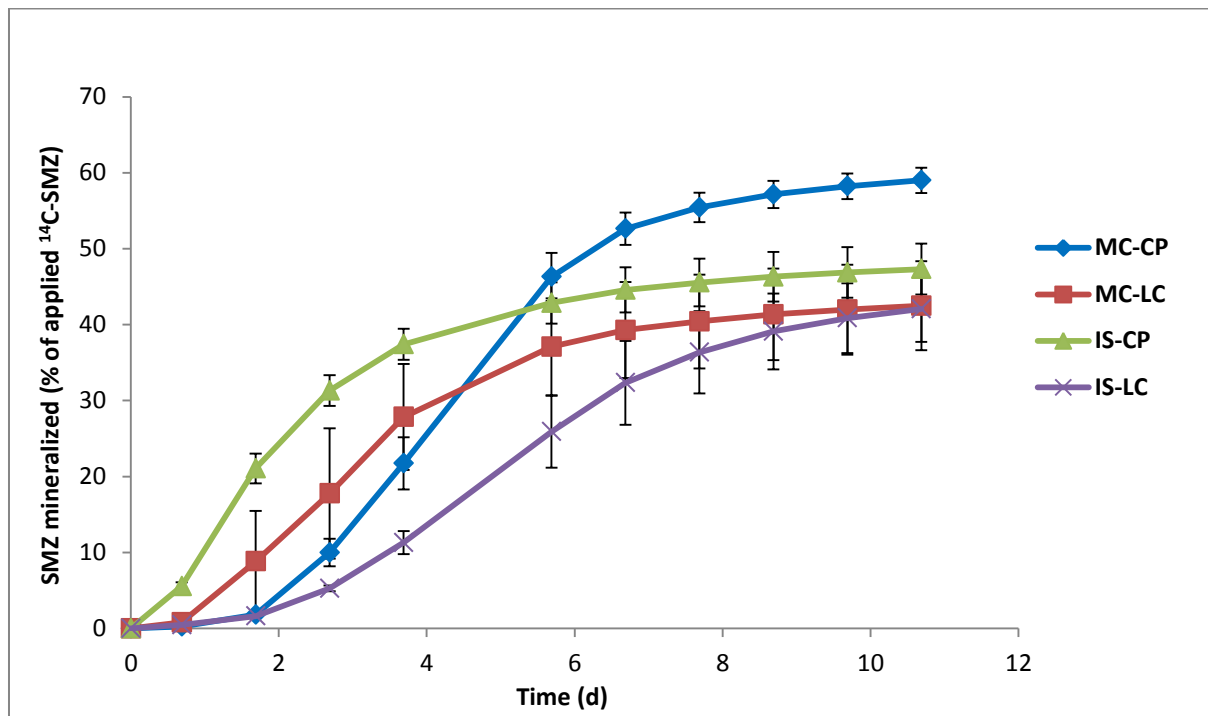
Fig. 29. Cumulative SMZ mineralisation of the four variants in liquid cultures before being transferred to soil samples. Bars indicate standard deviation, n=3.

For the soil inoculation experiment where the MCCP was applied (3.8), the liquid cultures containing the microbial community were treated differently with SMZ before soil inoculation. The two groups of MCCP showed different mineralization activities in the liquid cultures: one group, which received a second SMZ application in the liquid culture, showed a higher (HA, $2.3 \pm 0.5\%$ d^{-1}) and the other one with only one SMZ application showed a lower SMZ mineralization rate (LA, $1.1 \pm 0.1\%$ d^{-1}, fig. 30). On day 16, both variants were transferred to soil incubators. In the control, no bacteria were introduced. Thereby, the effect of different initial mineralization activities at the beginning of the soil inoculation experiment was tested.
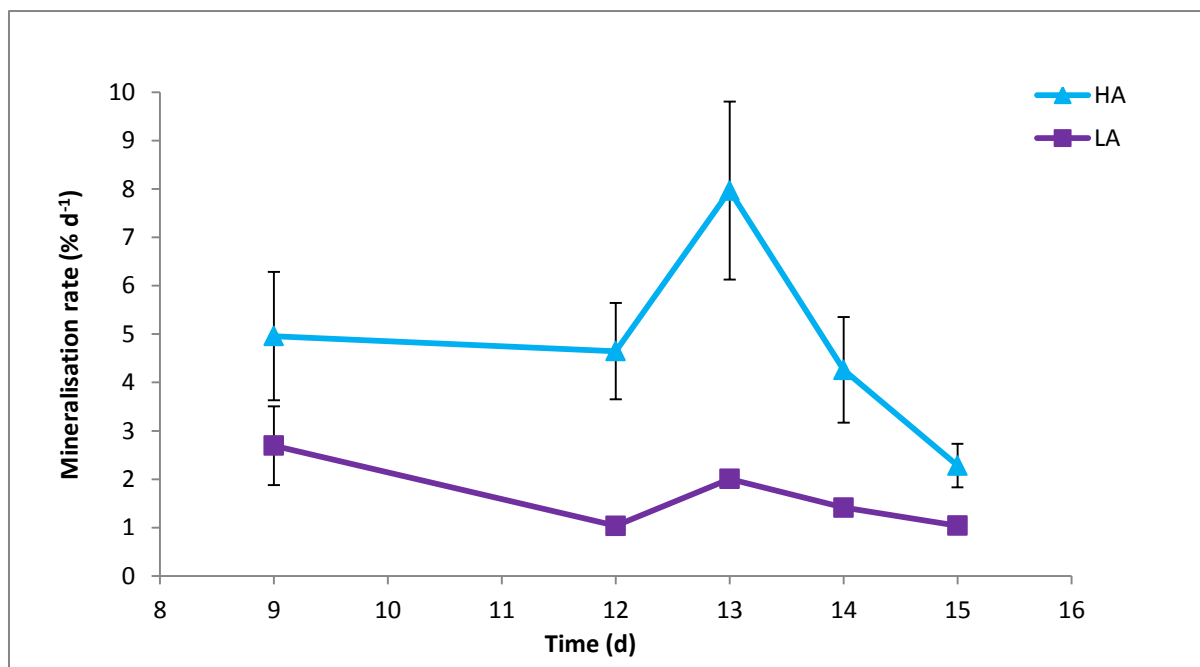
Fig. 30. SMZ mineralisation rates (% of applied amount per day) in liquid cultures, n=3, bars indicate standard deviation. Two treatments:

HA (higher mineralisation activity): liquid culture was spiked with a second 5 µg g^{-1} of SMZ on day 8.

LA (lower mineralisation activity): liquid culture received only the first SMZ application in the liquid culture on the first day.

Transfer of bacteria (on CP) to soil on day 16 to test influence of different initial mineralisation activities.

In the graph showing the cumulative mineralisation (fig. 31), the effect of the two different treatments is clearly visible as the HA treatment mineralised 43% of the applied SMZ on day 15 and the LA treatment, which was not spiked for a second time, only mineralised 13% of the applied SMZ in total.
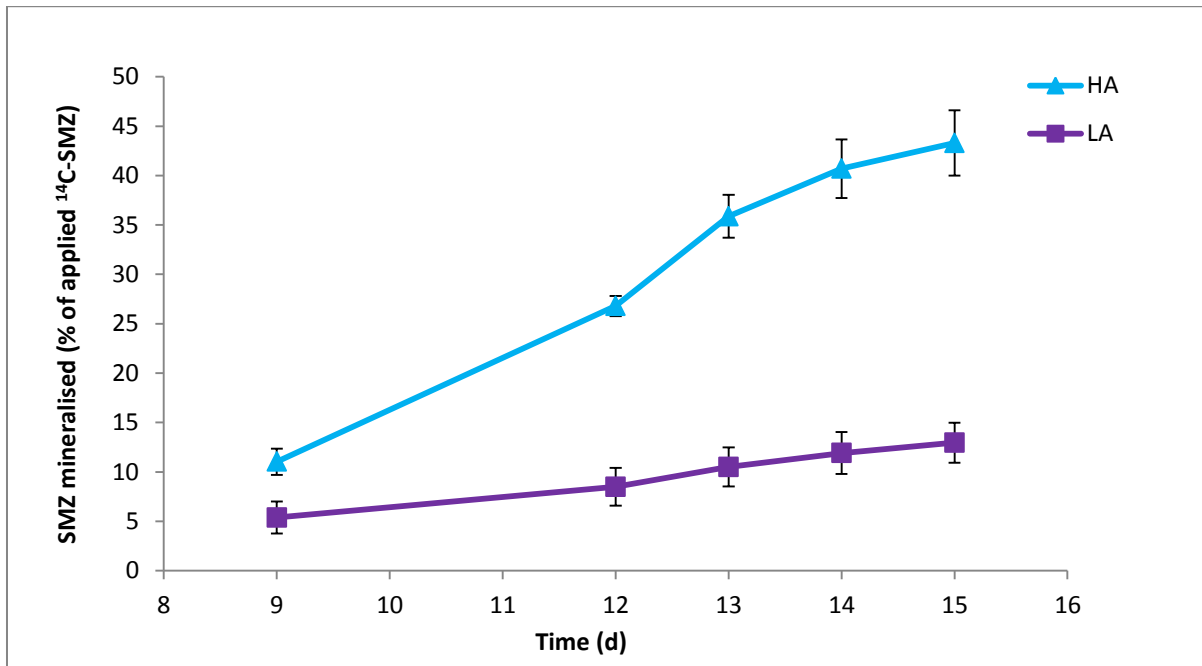
Fig. 31. Cumulative SMZ mineralisaion in liquid cultures, bacteria transfer (on CP) to soil on day 16, n=3, bars indicate standard deviation. Two treatments in liquid cultures to test the influence of different initial mineralisation activities in soil samples:

HA (higher mineralisation activity): liquid culture was spiked with a second 5 µg g^{-1} of SMZ on day 8.

LA (lower mineralisation activity): liquid culture received only the first SMZ application in the liquid culture on the first day.

3.7   Most effective soil inoculation variant for SMZ biodegradation

Four soil inoculation methods were applied to identify the most effective method to support SMZ mineralisation in sandy loam soil. Soil was inoculated either with

the **M**icrobial **C**ommunity, established on **C**arrier **P**articles (MCCP),

the **M**icrobial **C**ommunity without carrier particles in **L**iquid **C**ulture (MCLC),

the **I**solated **S**train on **C**arrier **P**articles (ISCP) or with

the **I**solated **S**train without carrier particles in **L**iquid **C**ultures (ISLC).

The soil samples were incubated with bacteria applied in these four variants for 99 days in a first phase. After this time, the best inoculation method regarding the highest SMZ mineralisation was evaluated. To verify the inoculation method, two more incubation phases were conducted, whereupon two different extreme scenarios were represented. After the first phase was finished, for half of the samples the soil was dried; the other half of the soil samples was frozen. Afterwards, SMZ was applied to both (rewetted and thawed) variants and a second incubation phase was started.

3.7.1   Mineralisation capacities of the four application variants

In the first step, the sandy loam soil samples were incubated with the four different soil inoculation variants (MCCP, MCLC, ISCP, ISLC) to identify the most effective one. Every bacterial inoculum was added to soil in two different sizes, respectively (two different amounts of CP and two different volumes of LC) to provide the same starting conditions for the different soil inoculation methods. In this way, it was possible to obtain matching initial mineralisation rates on day one of the different treatments to ensure, that the incubation of the different treatments started with the same capability to degrade SMZ.

Fig. 32 and 33 show the mineralisation rates of the soil inoculated with microbes via CP (5 vs 10 and 10 vs 20 CP) and via LC (1.3 vs 3 and 5 vs 10 ml cell suspension). For better visibility of the differences in the mineralisation rates during the early phase of the experiment, the two graphs were cut at day 30 even though incubation lasted for 99 days. When comparing the different inoculation methods, it can be seen that 10 CP overgrown by the MC (MCCP 10, red line, fig 32), 10 CP overgrown by the IS (ISCP 10, dark purple line, fig. 33) and 5 ml of the IS in LC (ISLC 5, dark blue line, fig. 33) showed initial mineralisation rates of about 2% d^{-1} on day one, indicating similar starting conditions. Both MCLC variants started with lower

mineralisation rates (0.1% d^{-1}) but were increasing from day one to day seven, performing a SMZ mineralisation peak, before decreasing again.

Comparing the soil inoculated with bacteria in liquid cultures on day one, the mineralisation rates of the ISLC (light purple and dark blue, fig. 33) started with higher mineralisation rates than the ones of the MCLC (light and dark green, fig. 32). From there on, the soil inoculated with the MC showed increasing mineralisation rates, while the rates in the soil inoculated with the IS decreased. For the carrier particles, this curve progression is also clearly visible. The MCCP showed increasing mineralisation rates, while the ISCP showed decreasing mineralisation rates from the start.

Generally, a big difference is visible, comparing the effect of the soil inoculated with the microbial community to the soil containing the isolated strain. The rates of all variants supplied with the microbial community came to a mineralisation peak in the first week before decreasing (fig. 32), whereas the mineralisation rates of all variants containing the isolated strain decreased immediately after the start, clearly shown in fig. 33. The mineralisation rates of the control samples stayed below 0.05% d^{-1} at all times.
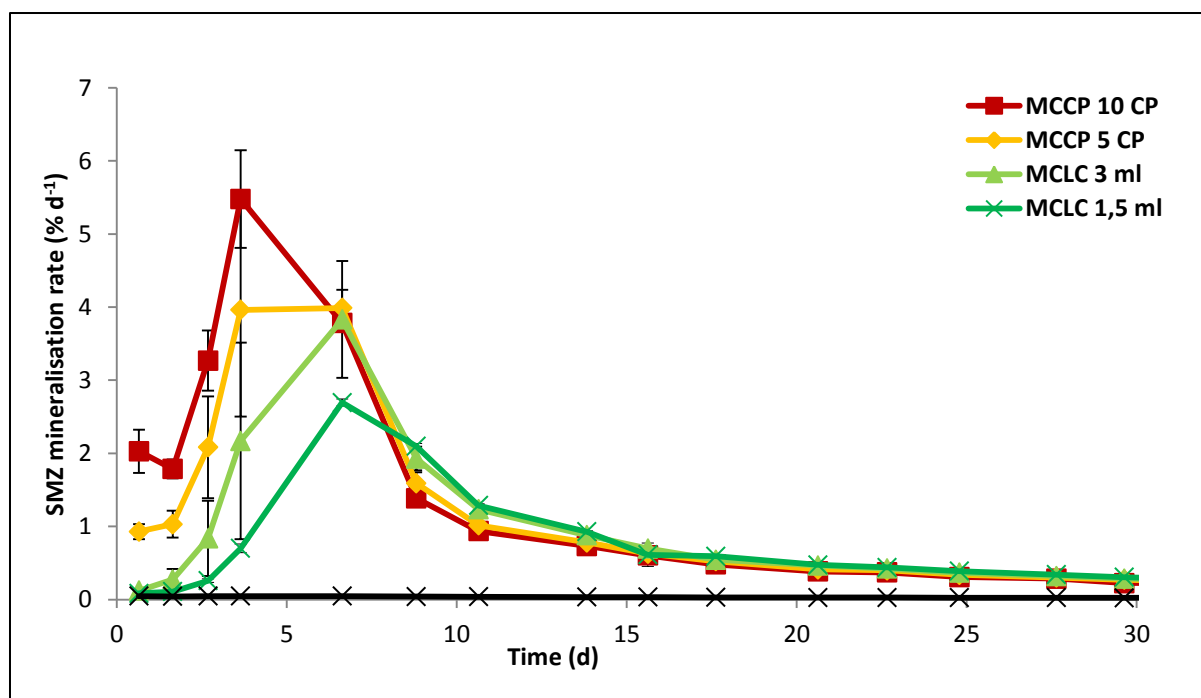
Fig. 32. SMZ mineralisation rates (% of applied amount per day) in soil samples after ^{14}C-SMZ application. Soil inoculation variants: microbial community on carrier particles (MCCP) and microbial community in liquid culture (MCLC). For better visibility of the mineralisation rates within the first days, the graph was cut at day 30. Bars indicate standard deviation, n=3.
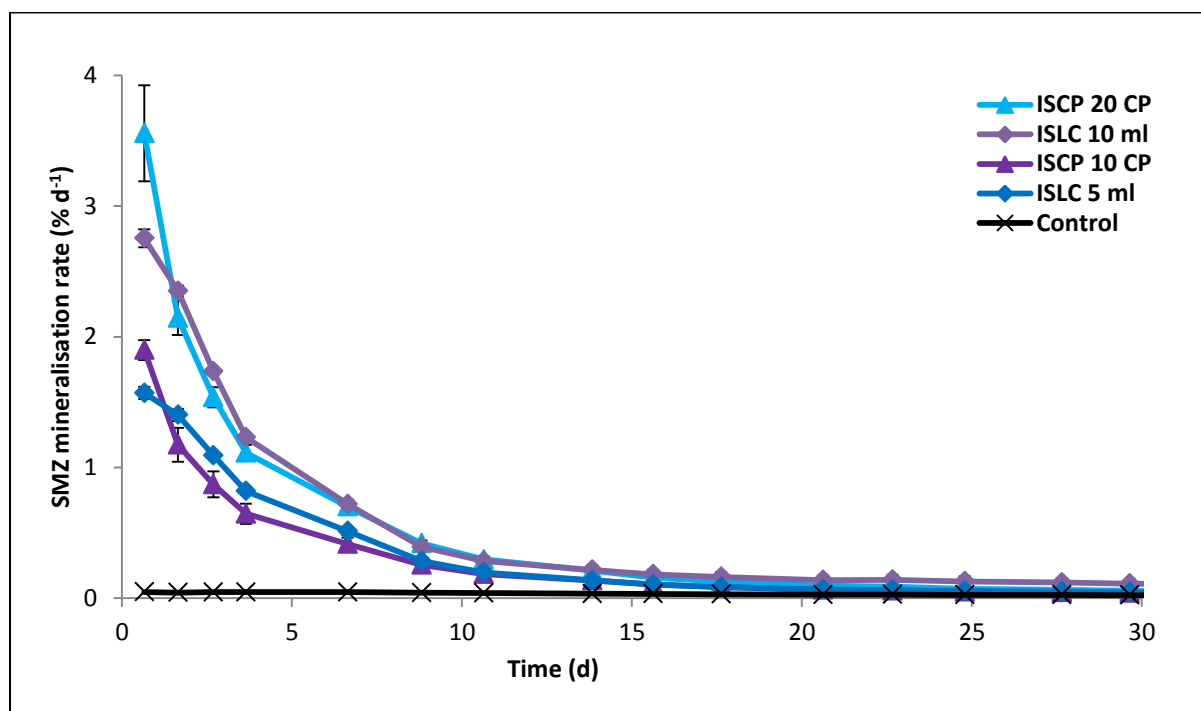
Fig. 33. SMZ mineralisation rates (% of applied amount per day) in soil samples after ^{14}C-SMZ application. Soil inoculation variants: isolated strain on carrier Particles (ISCP) or isolated strain in liquid cultures (ISLC) after ^{14}C-SMZ application. For better visibility of the mineralisation rates within the first days, the graph was cut at day 30. Bars indicate standard deviation, n=3.

After 99 days of incubation the cumulative SMZ mineralisation of the four soil inoculation variants showed clear differences in the soil inoculated with MC compared to IS (fig. 34). While the IS mineralised 9% to 18% after 99 days, the MC mineralised 34% to 43% in the same time, depending on the soil inoculation method (with or without CP). All treatments significantly increased the SMZ mineralisation in the soil samples (t-test, $p < 0.05$). The MC variants mineralised significantly more SMZ than the IS variants.
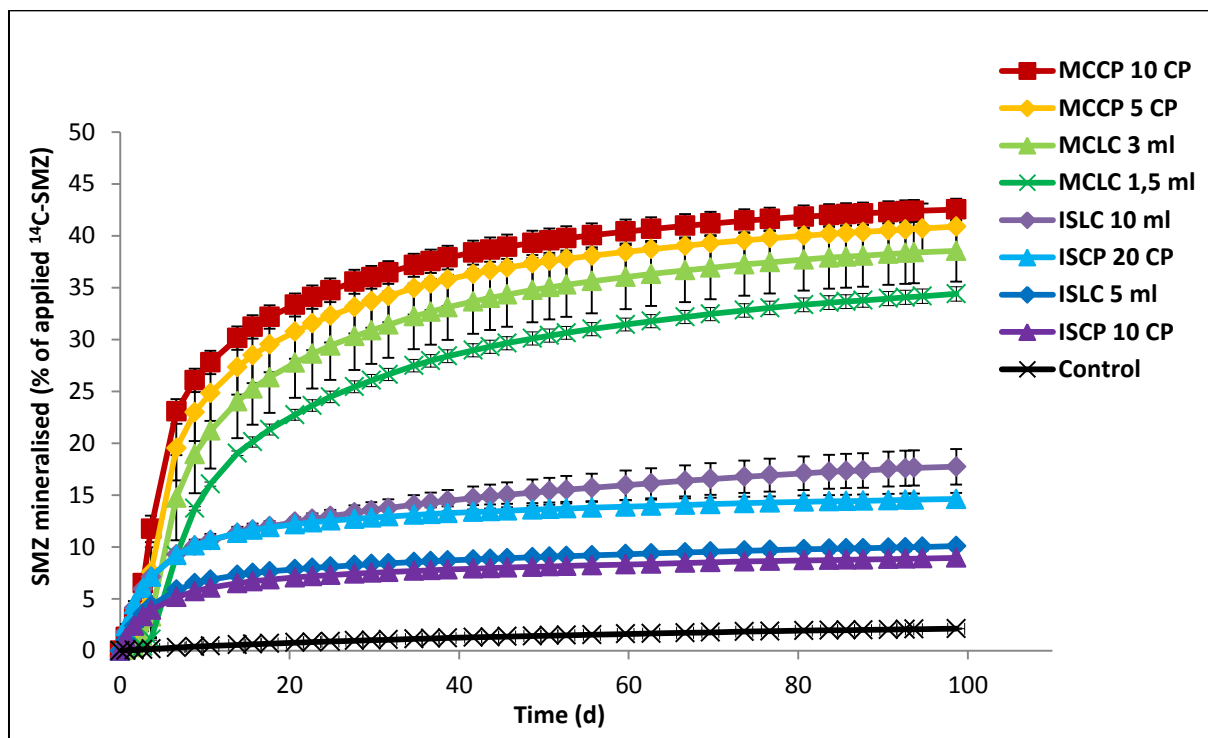
Fig. 34. Cumulative SMZ mineralisation of the four soil inoculation variants (MCCP, MCLC, ISCP, ISLC) with two different amounts of inoculum, added to SMZ-spiked soil samples. Bars indicate standard deviation, n=3. At day 99 all samples differ significantly from the control and MC variants differ significantly from IS (t-test, $p < 0.05$).

3.7.2   Effect of drying the soil samples on microbial SMZ mineralisation

After the first incubation time of 99 days had passed, the effect of a drying period on the mineralisation capacity of the soil microorganisms was tested. Half of the soil samples were air dried to pF 3.0-3.5 by opening the soil incubators; afterwards they were wetted and supplied with a second dose of ^{14}C-SMZ. The bacteria were not added again in the second phase, only SMZ was applied for a second time. The samples with the higher amount of inoculated CP (MCCP 10, ISCP 20) and LC (MCLC 3, ISLC 10) were selected for this second incubation phase, which lasted for 50 days. After the drying period, none of the four variants achieved the SMZ mineralisation rates they showed in the first phase. None of the rates exceeded 1% mineralisation per day (fig. 35).

The second half of the soil samples underwent a freezing event instead of drying; results are shown in 3.7.3.
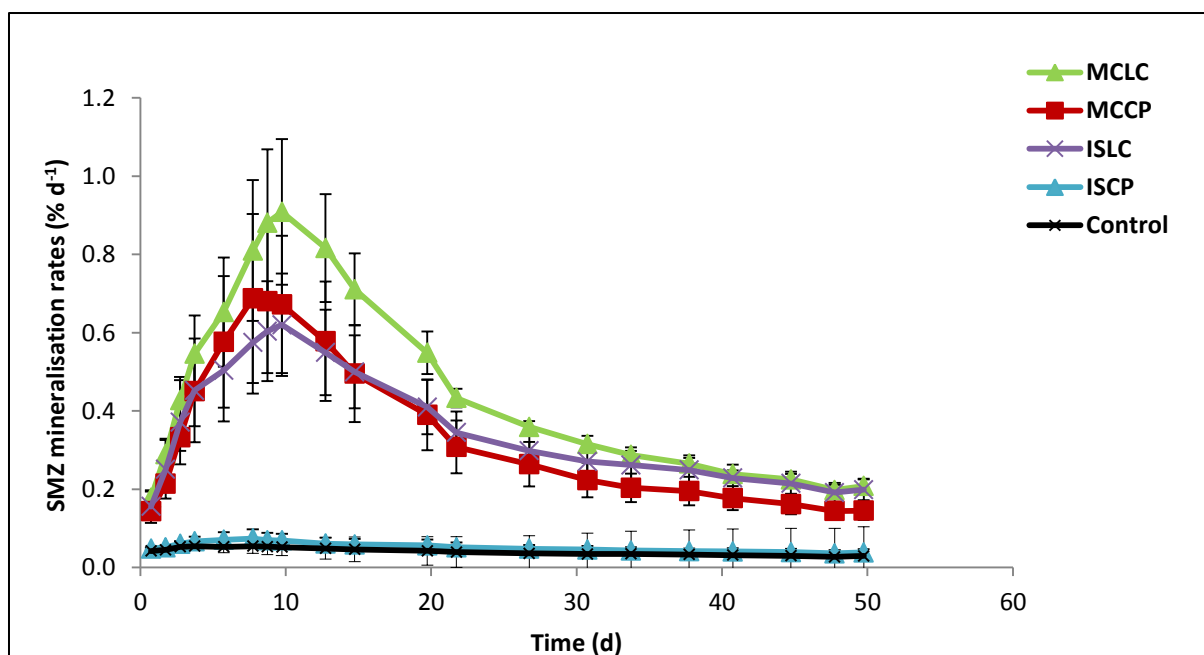
**Fig. 35.** SMZ mineralisation rates (% of applied amount per day) in soil samples after ^{14}C-SMZ application. Soil inoculation variants (MCCP, MCLC, ISCP, ISLC) incubated after drying and re-wetting of the soil samples and after a second ^{14}C-SMZ application. Bars indicate standard deviation, n=3.

The cumulative mineralisation of SMZ in the second phase was lower than in the first incubation phase in all samples except one: The variant ISLC which mineralised 15% of the applied SMZ at day 49 in the first phase mineralised 16% at day 48 in the second phase (fig. 36), which is however, a very similar result. The mineralisation capacities of the LC and the CP were inverted. The MCLC variant mineralised 21% SMZ after 50 days in the second phase, whereas the MCCP was only able to mineralise 16%. Same for the IS; ISLC mineralised 17% of the applied SMZ on day 50 whereas ISCP only mineralised 3% which was close to the control samples (1.9%). In summary, the most effective variant (MCLC) was still able to mineralise 21% of the applied SMZ, even after this soil drying process (fig. 36). The bacteria in the two variants ISLC and MCCP survived the drying treatment and successfully enhanced the SMZ mineralisation compared to the control.
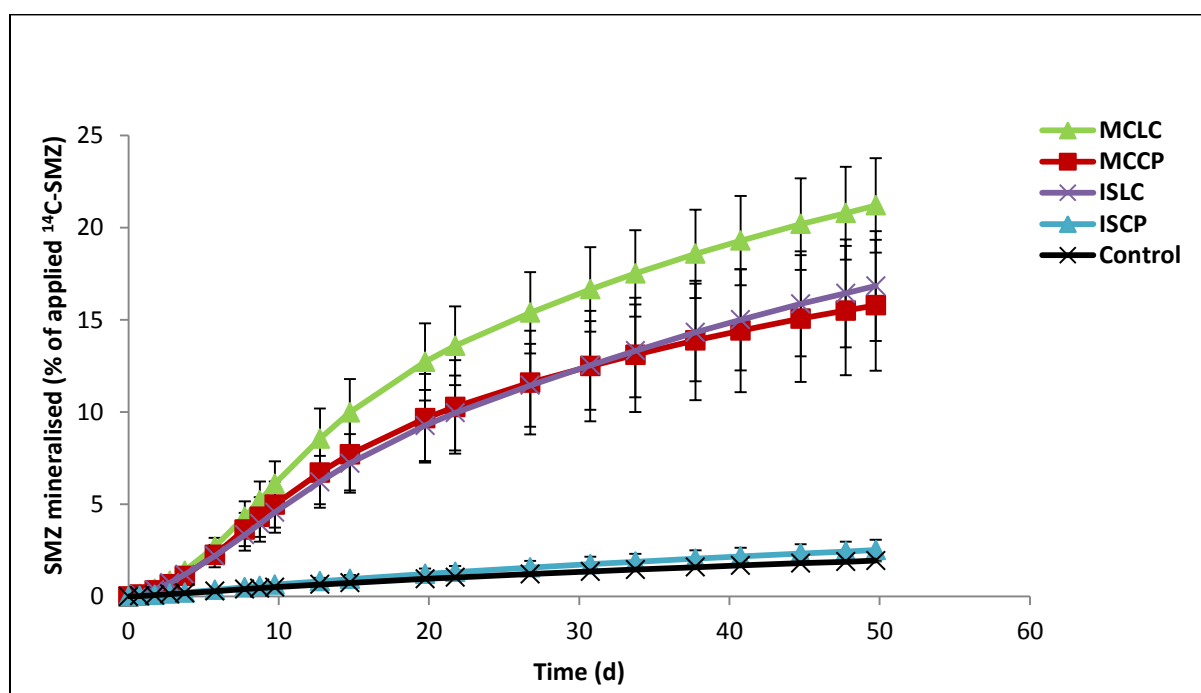
Fig. 36. Cumulative SMZ mineralisation after a second ^{14}C-SMZ application. The four soil inoculation variants (MCCP, MCLC, ISLC, ISCP) were incubated after drying and rewetting of the soil samples. Bars indicate standard deviation, n=3.

3.7.3   Effect of freezing of soil samples on microbial SMZ mineralisation

To test the impact of freezing stress on the bacteria inoculated to soil, the second half of the soil samples, used to identify the most effective soil inoculation variant (3.7.1), was frozen at -20 °C. Samples with the lower inoculant in the first incubation phase (MCCP 5, ISCP 10, MCLC 1.5, ISLC 5) which were not used for the soil drying experiment (3.7.2), were selected. After thawing, the ^{14}C-SMZ was applied for a second time to investigate whether the bacteria kept their ability to degrade SMZ. The microbes have not been added again. The SMZ mineralisation rates of this second phase of the experiment didn't achieve the maximum SMZ mineralisation rates that were achieved in the first phase. An increase in the mineralisation rates was clearly visible for the variants containing the microbial community (MCCP and MCLC) leading to a peak in the mineralisation rates on day 10. The highest rate was 1.1% d^{-1}, in the variant of the MC applied in LC. SMZ mineralisation rates of the other treatments did not exceed 0.5% d^{-1} (fig. 37)
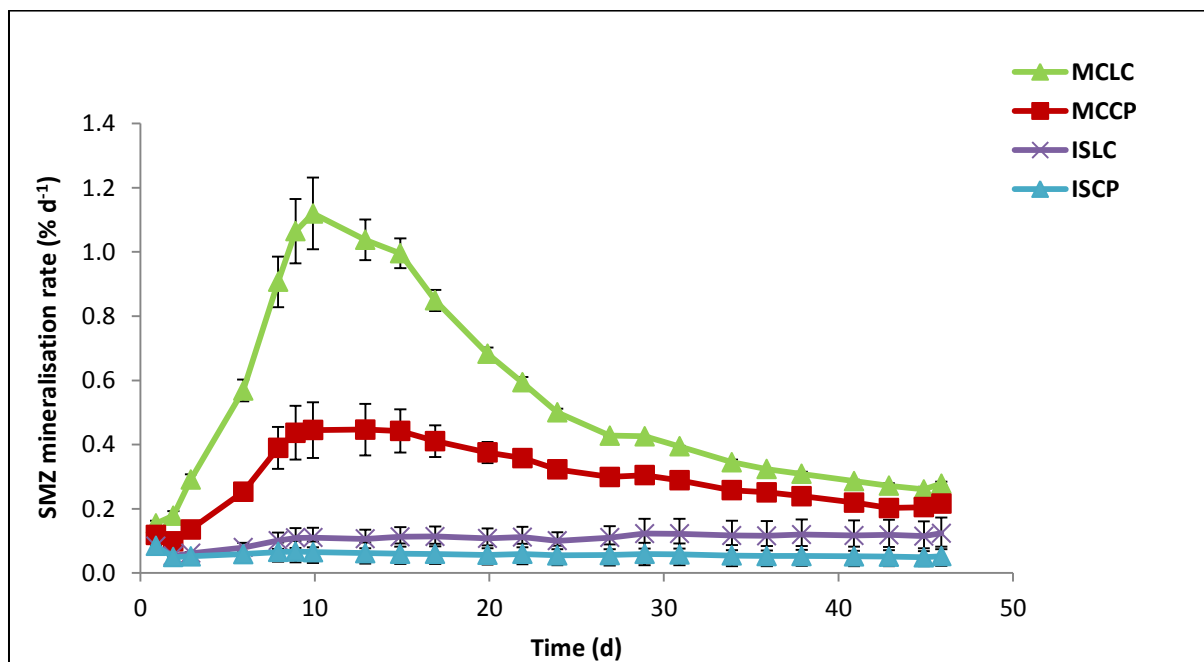
Fig. 37. SMZ mineralisation rates in soil samples (% of applied amount per day) after a second ^{14}C-SMZ application. Four soil inoculation variants (MCCP, MCLC, ISCP, ISLC) were incubated after freezing and thawing of the soil samples. Bars indicate standard deviation, n=3.

After an extreme freezing event, the soil incubators containing the MCLC were still able to mineralise in total 25% of the applied SMZ at the end of the second incubation phase (fig. 38). The soil incubators containing the MCCP mineralised 14% of the applied SMZ after 46 days. The two variants of the IS stayed below 6%. All variants showed a lower cumulative mineralisation in the second phase, compared to the first phase, but still SMZ mineralisation was visibly enhanced in the variants containing the microbial community, compared to the samples containing the isolated strain (fig. 38).
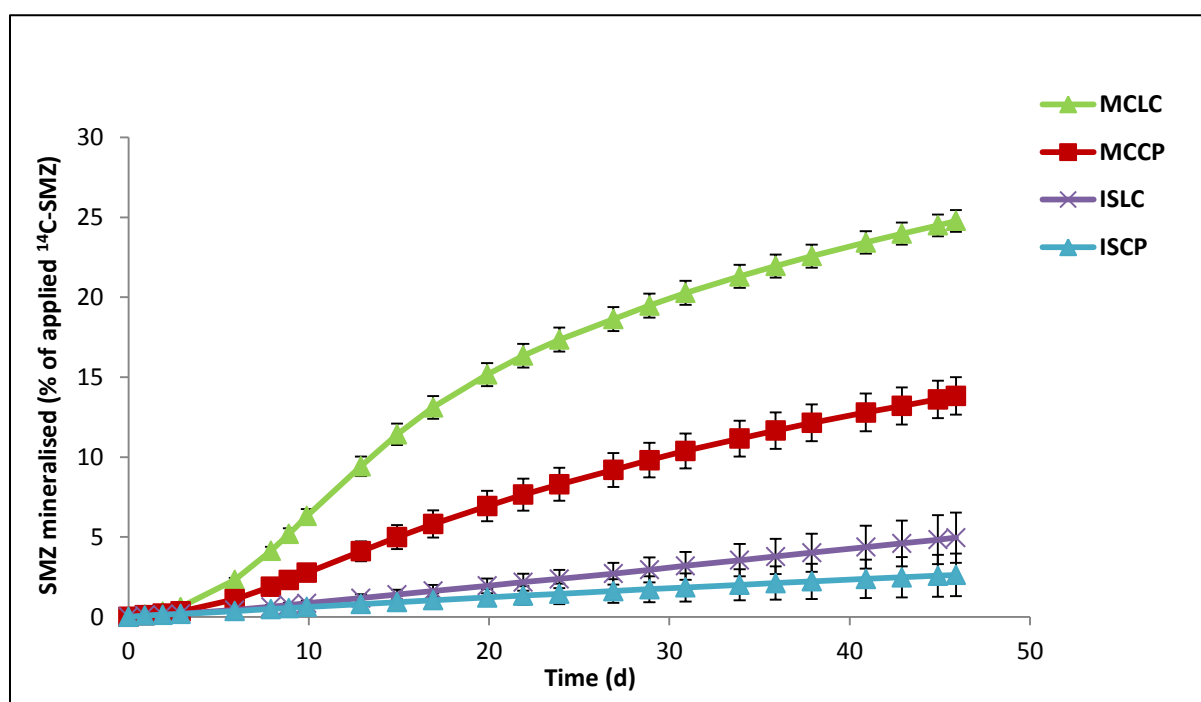
Fig. 38. Cumulative SMZ mineralisation after a second ^{14}C-SMZ application. Four soil inoculation variants (MCCP, MCLC, ISLC, ISCP) were incubated after freezing at -20 °C and thawing. Bars indicate standard deviation, n=3.

3.7.4 ^{14}C recovery in soil inoculated with four different variants

After the second incubation phase, the experiment was finished off. Therefore, the soil from all soil incubators was ASE-extracted separately to determine the extractable amount of ^{14}C. The non-extractable amount of ^{14}C in the extracted soil samples was determined by combustion. Together with the mineralised amount of ^{14}C-SMZ, these masses result in about 100% and are presented in fig. 39 and 40. Fig. 39 shows the ^{14}C mass balance of the soil samples that have been dried in the second phase; fig. 40 shows the ^{14}C mass balance of the ones that were frozen.

Both figures show a higher percentage of mineralised SMZ (measured as ^{14}C-CO$_2$) in the soil samples containing the microbial community, compared to the samples inoculated with the isolated strain, regardless of the treatment in the second phase (drying or freezing). In the samples containing the isolated strain, the percentage of non-extractable residues is higher than in the ones containing the microbial community. The extractable residues made up 10 to 16 percent in all soil samples. The soil samples containing the microbial community showed a significantly lower amount of NER than the samples containing the IS and the control samples (t-test, p<0.05).
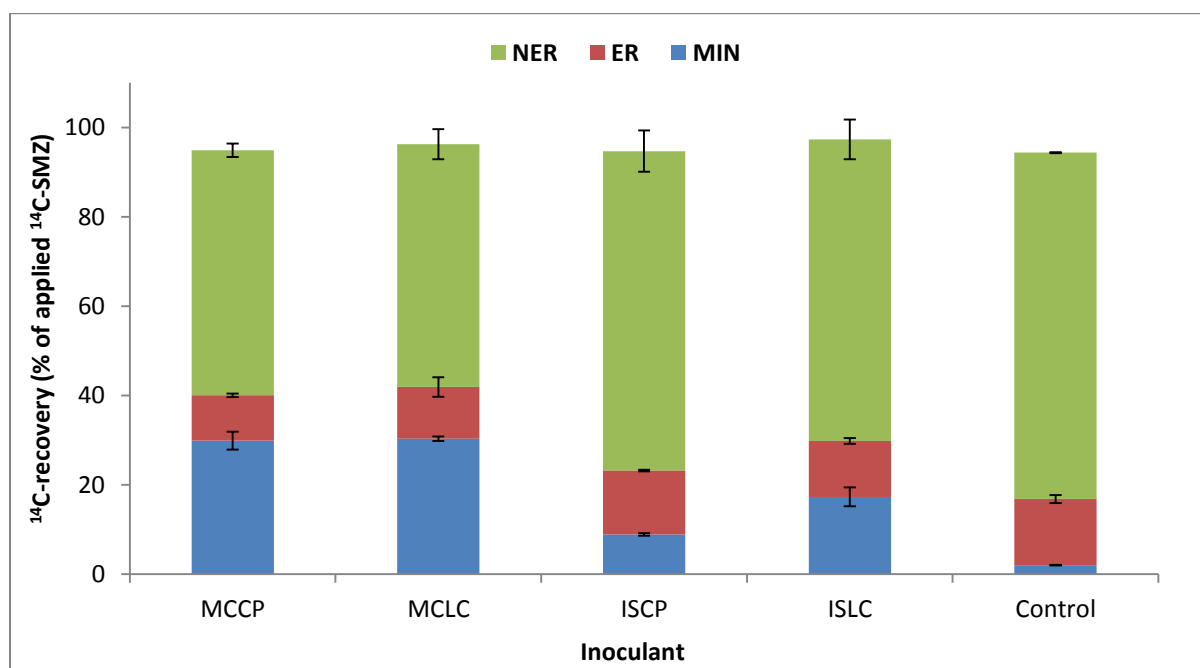
Fig. 39. ^{14}C mass balance of samples with higher amount of inoculated CP (MCCP 10, ISCP 20) and LC (MCLC 3, ISLC 10) after the two incubation phases, including drying of soil samples. Bars indicate standard deviation, n=3. NER fraction was significantly smaller in samples containing the microbial community (MCCP and MCLC) compared to samples containing the isolated strain (ISCP and ISLC), t-test, p<0.05.
NER = non-extractable residues, ER = extractable residues, MIN = mineralised ^{14}C-SMZ
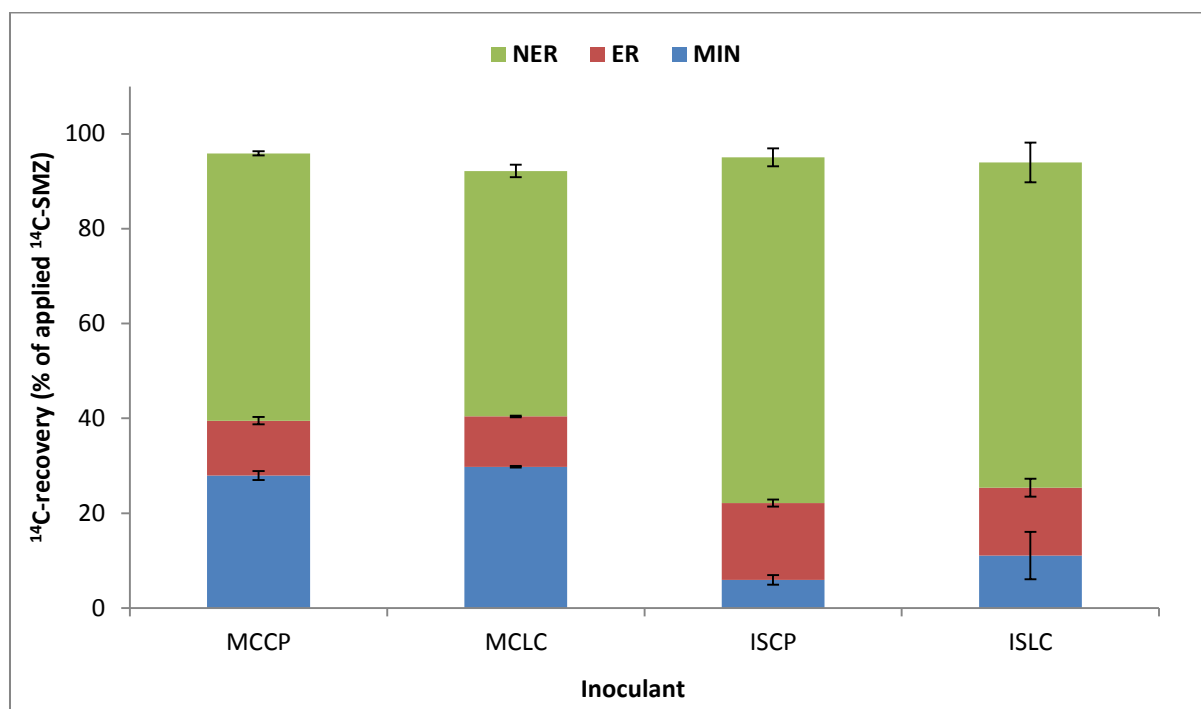
Fig. 40. ^{14}C mass balance of samples with the lower inoculant in the first incubation phase (MCCP 5, ISCP 10, MCLC 1.5, ISLC 5) after the two incubation phases, including freezing of soil samples. Bars indicate standard deviation, n=3. NER fraction was significantly smaller in samples containing the microbial community (MCCP and MCLC) compared to samples containing the isolated strain (ISCP and ISLC), t-test, $p<0.05$.
NER = non-extractable ^{14}C-residues, ER = extractable ^{14}C-residues, MIN = mineralised ^{14}C-SMZ

3.8 Efficiency, sustainability and localisation of the most effective soil inoculation variant: MCCP

According to the former results, where the four inoculation methods have been compared and the most promising one was identified (the **M**icrobial **C**ommunity, containing the key degrader *Microbacterium* sp. C448, established on **C**arrier **P**articles, MCCP), this soil inoculation variant was repeated using the same soil (sandy loam). Three consecutive incubation phases with different treatments were conducted.

The microbial community was prepared for soil inoculation in two ways (one vs two SMZ applications in the liquid culture). The ^{14}C-SMZ spiked soil was inoculated for 112 days with these degrading microbial communities exhibiting two different initial mineralisation activities (MCCP-LA, microbial community on carrier particles showing lower initial mineralisation activity and MCCP-HA, microbial community on carrier particles showing higher initial mineralisation activity).

After these 112 days, a second incubation phase was started to verify the long term sustainability of the inoculation approach. In the second phase of the experiment, ^{14}C-SMZ

was reapplied to the same soil incubators to examine, whether the microbes in the soil were still able to degrade SMZ after more than 100 days. The second incubation lasted for 49 days, before a third phase was started. In this third phase, the carrier particles were extracted from the bulk soil and transferred to fresh soil samples. The soil where the CP were extracted, and the separated CP (in fresh soil) received a third SMZ application, separately, to localize the site of mineralisation in the incubators (in the bulk soil or on the CP).

3.8.1 Efficiency of MCCP in two different states of mineralisation activity

The soil was inoculated with the MCCP in two variants differing in the initial mineralization rates at the beginning of the soil inoculation experiment (high and low initial mineralisation capacity, MCCP-HA and MCCP-LA). Each soil replicate received 12 CP. This difference in the mineralisation rates was caused by the different treatments in the liquid cultures, where the MCCP received one (MCCP-LA) or two (MPPC-HA) SMZ applications before they were transferred to soil. Further explanations are given in the materials and methods section. Incubation lasted for 112 days in this first phase. After four days of incubation, both variants performed a SMZ mineralisation peak, showing a highly enhanced SMZ mineralisation, compared to the control samples. Especially the variant with the higher initial mineralisation activity (MCCP-HA) performed a remarkably high peak in the mineralisation rate of 12.3% d^{-1}. The variant with the lower initial mineralisation activity (MCCP-LA) was mineralising 5.5% d^{-1} (fig. 41). Compared to the control samples that stayed below 0.1% SMZ d^{-1}, the mineralisation rates were successfully increased in both treatments.
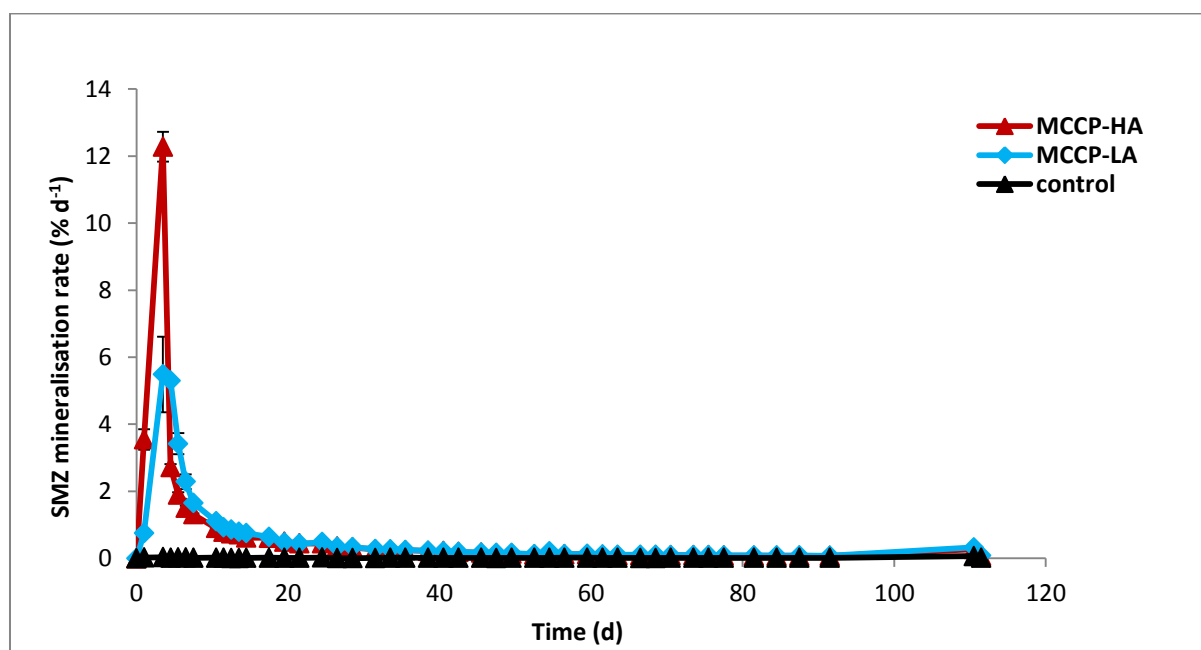
Fig. 41. ^{14}C-SMZ mineralisation rate (% of applied amount per day) of the microbial community on carrier particles (MCCP) in soil samples. The MCCP was applied with two different initial mineralization activities (HA, high initial mineralization activity; LA low initial mineralization activity). Bars indicate standard deviation, n=3.

After 112 days, the first incubation phase was finished. Both inoculations significantly increased the SMZ mineralisation in the treated soil samples (t-test, p<0.05). The soil inoculated with MCCP-HA showed a cumulative SMZ mineralisation of 62.4%, the soil inoculated with MCCP-LA mineralised 49.8% of the SMZ and in the control samples only 1.5% of the applied SMZ was mineralised (fig. 42).
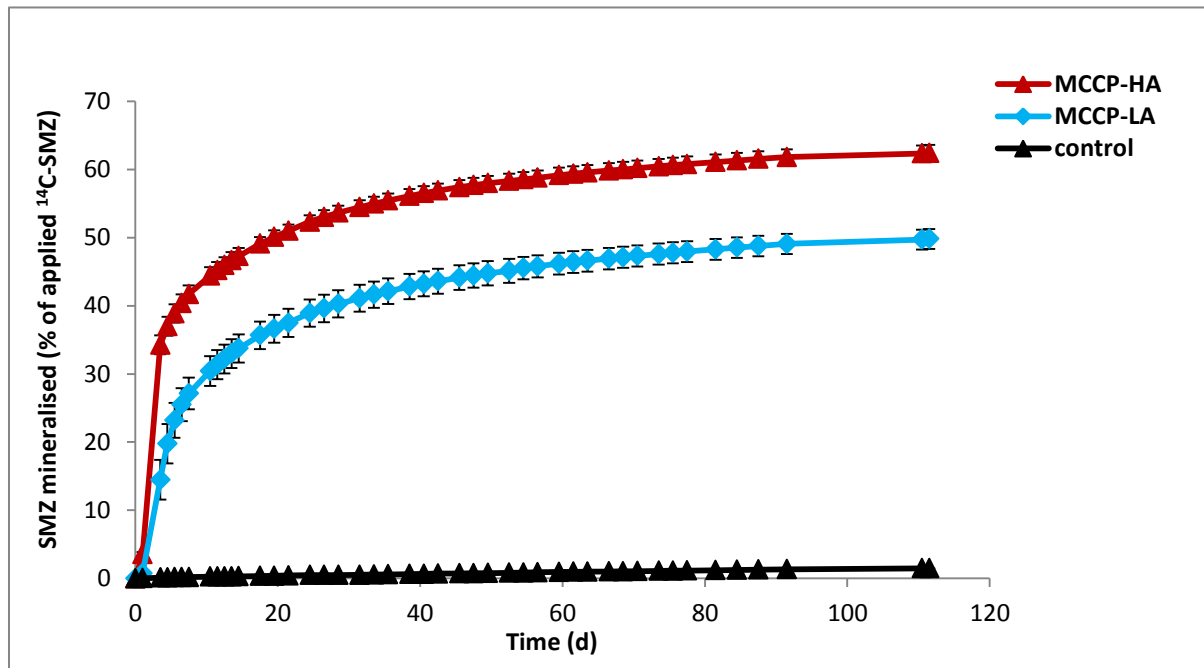
Fig. 42. Cumulative SMZ mineralisation of the MCCP in soil samples, with two different initial mineralization activities (HA, high initial mineralization activity; LA low initial mineralization activity). Bars indicate standard deviation, n=3. SMZ mineralisation was significantly increased in both treatments compared to control samples (t-test, p<0.05).

3.8.2   Sustainability of soil inoculation with MCCP

After the first SMZ application and soil incubation phase (112 days), ^{14}C-SMZ was reapplied
and a second phase was started to verify the sustainability of the soil inoculation approach. It
was tested, whether the microbes can be reactivated and restart the SMZ mineralisation. Both
variants (MCCP-HA and MCCP-LA) were treated similarly in phase two, microbes were not
added again. The soil incubators received a second dose of SMZ in the same concentration as
the first (1 µg g^{-1}) and the mineralisation resumed. The SMZ mineralisation rates came to a
peak after 6-8 days at 3-4% (fig. 43) and showed a clear improvement in the SMZ
mineralisation compared to the control samples (0.1% d^{-1}). The difference in the
mineralisation rates due to the different treatment in the liquid culture preparation was
reduced in this second phase of incubation.
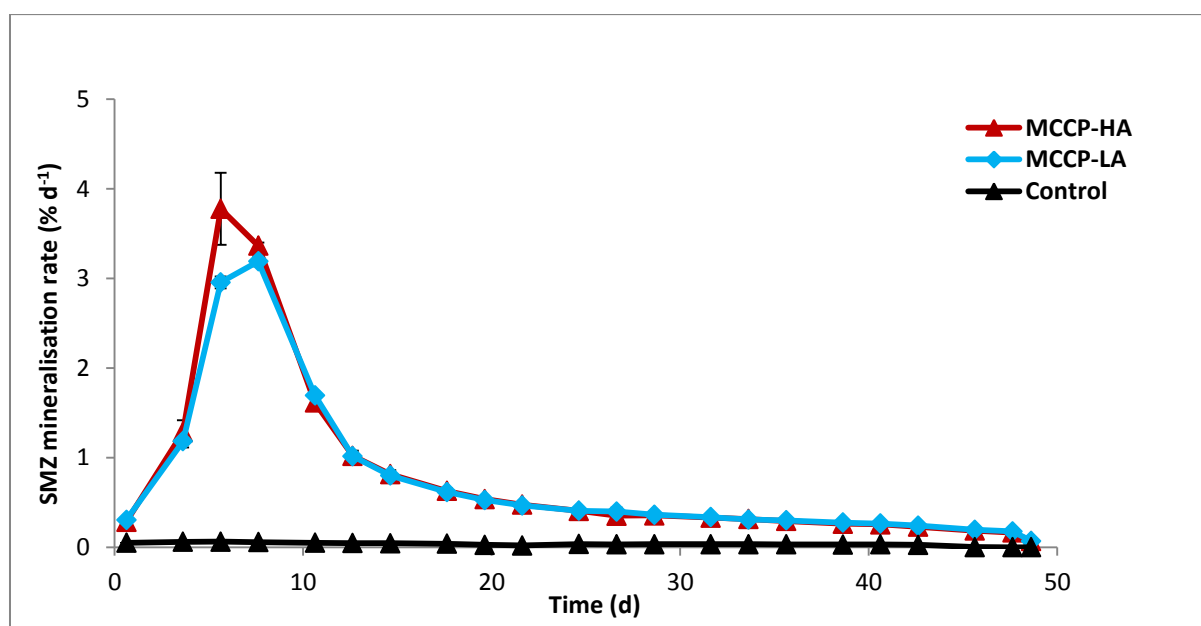
Fig. 43. SMZ mineralisation rates (% of applied amount per day) in soil samples after the second application of
^{14}C-SMZ on both variants, the higher and lower initial mineralisation rates (MCCP-HA and MCCP-LA). Bars
indicate standard deviation, n=3. This second phase with a second SMZ application was started 112 days after
the first SMZ application, to verify the sustainability of the inoculation approach.

The cumulative mineralisation of the second phase revealed that the different mineralisation rates, measured in the first phase, have equalized here. After 49 days the MCCP-HA mineralised 38.2% and the MCCP-LA mineralized 36.3% of the applied SMZ while the control samples stayed below 2% (fig. 44). Even though the mineralisation rates were not as high as in the first incubation phase, the improvement in SMZ mineralisation in the soil inoculated with the MCCP is still very effective. Even after 112 days the mineralisation was restarted and significantly increased (t-test, $p<0.05$).
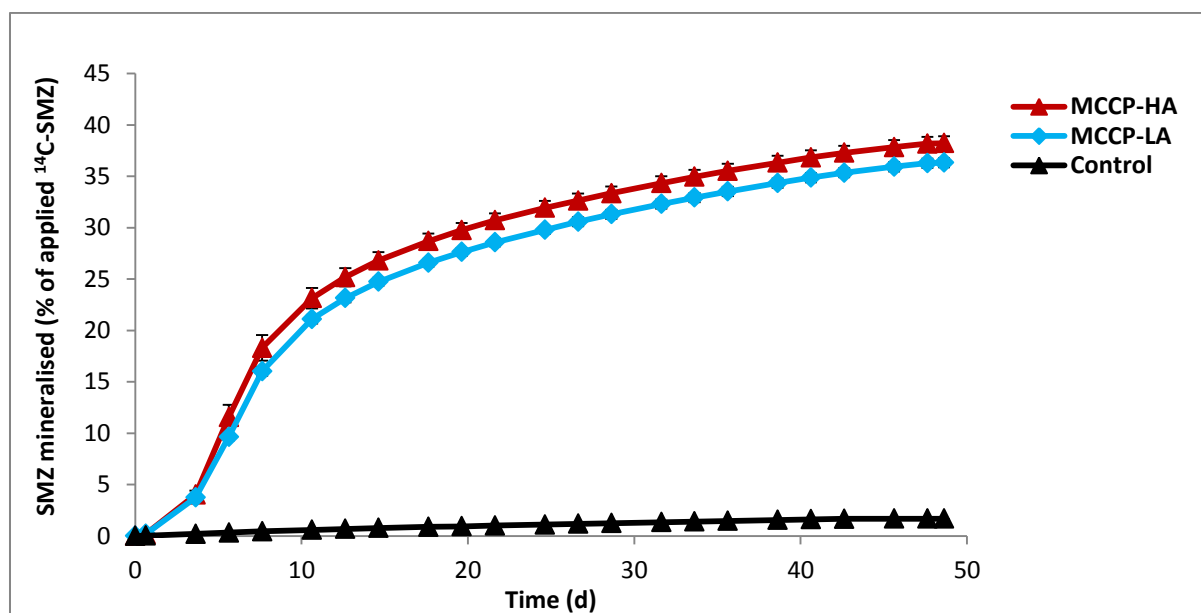
Fig. 44. Cumulative ^{14}C-SMZ mineralisation after the second SMZ application on both soil treatments (MCCP-HA and MCCP-LA). Bars indicate standard deviation, n=3. This second phase with a second SMZ application was started 112 days after the first SMZ application, to verify the sustainability of the inoculation approach. SMZ mineralisation was significantly increased in both treatments compared to control samples (t-test, $p<0.05$).

3.8.3   Localisation of the degradation site by separating CP from soil matrix

In the third incubation phase of this soil inoculation experiment, the carrier particles that had been introduced to transfer the microbial community into the soils, have been extracted from the bulk soil (only the variant of the lower initial mineralisation rate, MCCP-LA was used for this third phase). The separated carrier particles were then transferred into fresh soil, yet, the (old) bulk soil from phase one (and two) was also kept. Both variants (CP in fresh soil and bulk soil without CP) received a third ^{14}C-SMZ application to prove where the SMZ mineralisation is located.

The soil samples without the carrier particles (referred to as "soil only") showed a mineralisation rate of 5.5% d^{-1} on day 6 (fig. 45), which is even higher than mineralisation rates in the second phase (fig. 43 and 44). The freshly prepared soil that received the carrier particles from phase one and two ("CP only") showed a mineralisation rate of 0.6% d^{-1} on day 6 (fig. 45). Only the variant of the lower initial mineralisation rate (MCCP-LA) was selected for this third phase, whereas the MCCP-HA replicates have been finished off by extraction, combustion and calculation of the ^{14}C mass balance.
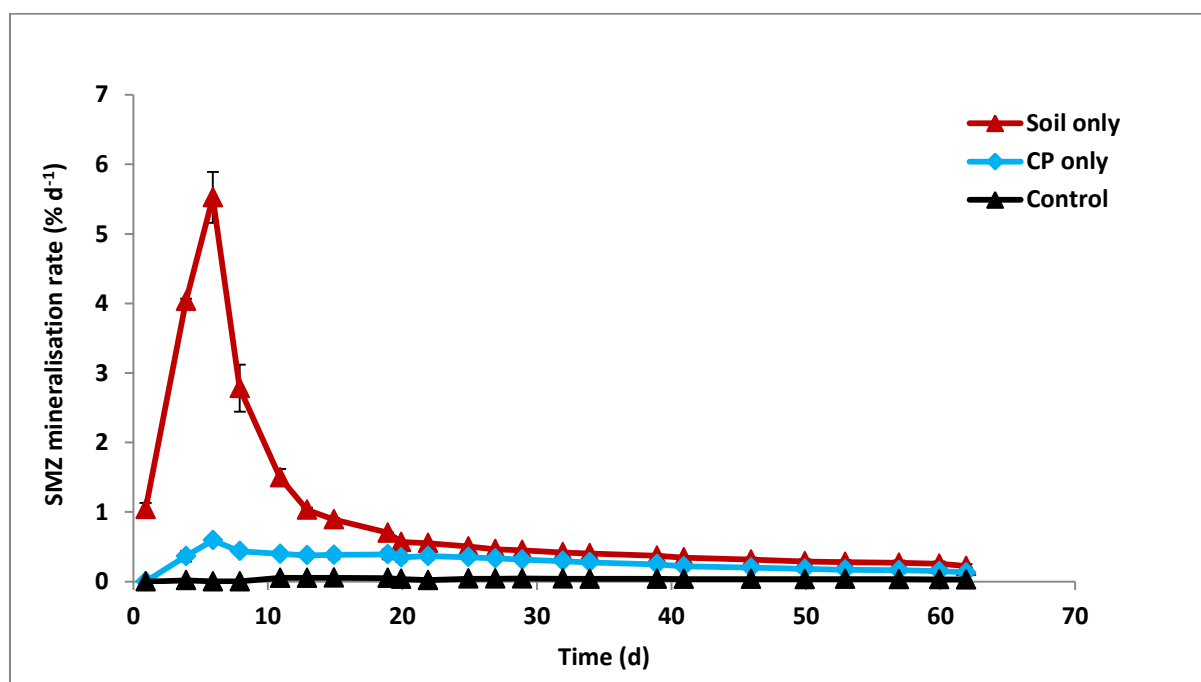
Fig. 45. Mineralisation rates of the separated bulk soil and carrier particles in fresh soil samples during the third phase of the experiment. Bars indicate standard deviation, n=3.
Soil only: soil from phase one and two, where carrier particles had been extracted.
CP only: the carrier particles, used for inoculation in phases one and two, transferred into fresh soil samples.

After 62 days, the soil samples where the carrier particles have been extracted, resulted in a cumulative SMZ mineralisation of 56.3%, whereas the fresh soil that received the old carrier particles mineralised 17.6% ("soil only" and "CP only", fig. 46). The microbial community, still present in the "soil only", was mineralising three times more SMZ than the microbial community located on the carrier particles. The carrier particles still contained the microbial community in an amount, high enough to mineralise about nine times more SMZ than the control samples, which only mineralised 2.2% after 62 days (fig.46). Differences in between treatments and between treatments and control were significant (t-test, $p<0.05$).
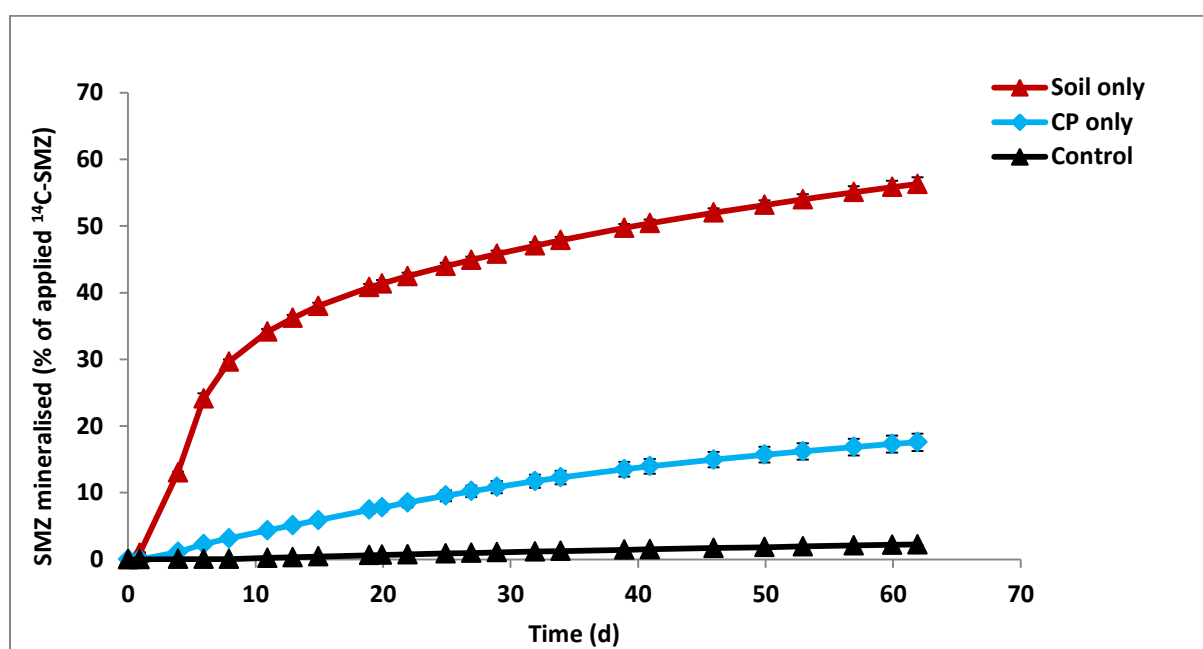
Fig. 46. Cumulative SMZ mineralisation during the third phase of the experiment. Bars indicate standard deviation, n=3.
Soil only: soil from phase one and two, where carrier particles have been extracted.
CP only: the carrier particles, used for inoculation in phases one and two, transferred to fresh soil samples.
SMZ mineralisation was significantly increased in both treatments compared to the control samples (t-test, $p<0.05$).

### 3.8.4 ^{14}C recovery in soil inoculated with MCCP

After the second incubation phase, the higher initial mineralisation rate soil samples (MCCP-HA) have been finished off by extraction, combustion and calculation of the ^{14}C mass balance. The lower initial mineralisation rate replicates (MCCP-LA) passed a third incubation phase, before being processed for the ^{14}C mass balance. The extractable amount of ^{14}C in the soils was quantified via measuring of volume and radioactivity of the ASE extracts, the non-extractable residues were determined by combustion. Together with the mineralised amount of ^{14}C-SMZ, these masses result in about 100% and are presented in fig. 47.
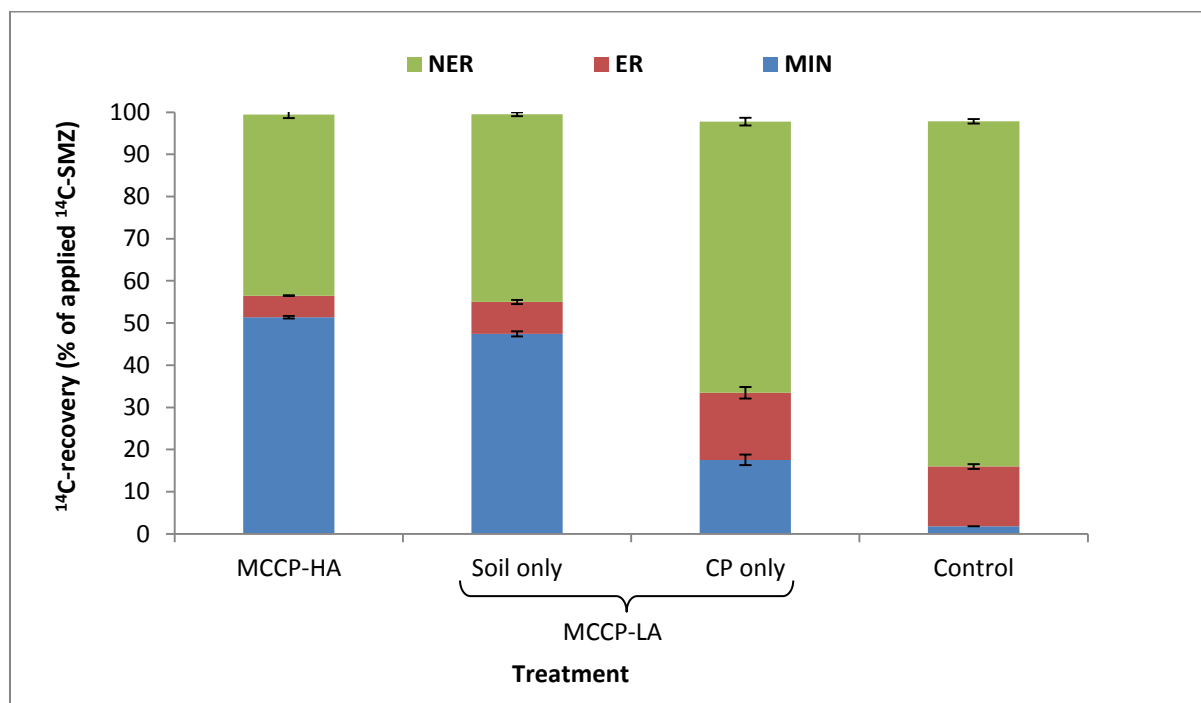
Fig. 47. ^{14}C mass balance of soil samples with higher (MCCP-HA) and lower initial mineralisation activity (MCCP-LA). MCCP-HA samples were ended after the second incubation phase, MCCP-LA were ended after a third incubation phase, where they were separated into "soil only" and "CP only". Bars indicate standard deviation, n=3.
NER = non-extractable ^{14}C-residues, ER = extractable ^{14}C-residues, MIN = mineralised ^{14}C-SMZ

The experiments concerning the bioavailability of SMZ in three soils, the DNA analysis of the microbial community, the first phase and the liquid cultures of the experiment "comparing the four soil inoculation variants" and the three phases of the MCCP in soil, are published in *Chemical and Biological Technologies in Agriculture* (Hirth et al. 2016). This publication is attached at the end of this thesis.

4.   Discussion

The aim of this study was to select an antibiotic as a model compound that is frequently used in veterinary medicine and suitable for biodegradation in soil incubation experiments. Next, a bacterium with the ability to degrade this compound was to be identified. Subsequently, further soil inoculation experiments should show whether this degrading soil-borne microorganism can decontaminate soils from the foreign and contaminating antibiotic with or without its indigenous microbial community. Finally, a suitable method to decontaminate soils from this antibiotic in the long term and in the most effective way was to be determined.

4.1   Bioavailability of sulfamethazine in soil samples

SMZ experiments described in literature, concerning the distribution constant K_d, describing the sorption equilibration behaviour of SMZ in a soil-water-mixture, are usually obtained from batch experiments, conducted at a certain time point. These results are not considering the long-term sorption effects, as well as the effect of manure or soil structure on the sorption behaviour. Therefore, they are not as significant as sorption experiments under natural soil conditions and water content (Thiele-Bruhn and Aust 2004). The applications of these batch experiments are differing in the host material (for example soil-water-slurries with or without manure) and the results are always only valid for this material, which makes it difficult to compare K_d values and use the information for other substrates (Bailey et al. 2016).

The soil pore water extraction, conducted in the present work is similarly only valid for the investigated soils. However, the conditions of the extracted soils regarding the water content and the water, air and soil particle distribution in the incubators was set up as close as possible to the natural soil conditions. Therefore, the results presented here are better comparable to the native actual behaviour of SMZ in the field than any batch experiment.
The fast decrease of dissolved SMZ in the soil pore water of the sandy loam soil (sterilised and non-sterilised) indicates that most of the SMZ is adsorbed to the soil matrix within the first seven days. To exclude degradation no microbes were added.
The high stability of SMZ in the sandy loam soil samples and the strong sorption after this first incubation period is in agreement with Stoob et al. (2007), who also investigated the behaviour of sulphonamides by analysing soil pore water. Stoob et al. (2007) also came to

high sorption results, which indicated stronger sorption than the K_d values, formerly reported in the literature, obtained from batch experiments (Tolls 2001; Pavlović et al. 2014).

The high stability of SMZ in monitored soil experiments is important when considering the characteristic of an antibiotic, to stimulate resistances in soil microorganisms. Even non-lethal antibiotic concentrations enrich resistant microorganisms in the environment and in lab experiments (Gullberg et al. 2011; Andersson and Hughes 2012). Via gene transfer, these resistances can subsequently be transferred to other microorganisms in soils, including human pathogens (Kemper 2008; Baran et al. 2011; Finley et al. 2013; Gaze et al. 2013; Wellington et al. 2013).

A total of three soil samples from agricultural fields differing in their properties as described in table 5, were tested for their SMZ fraction dissolved in the soil pore water, which was defined as *in situ* bioavailable for mineralising microorganisms. The amount of dissolved SMZ in the soil pore water fraction was decreasing in this order:

Sandy loam > silty loam > sandy soil

The sandy loam showed the highest amount of SMZ in the soil pore water and was therefore chosen for the degradation experiments. Since several studies detected cross-coupling between SMZ and the humid acid fraction in soils (Bialk et al. 2005; Bialk and Pedersen 2008; Gulkowska et al. 2013), the result of the lowest SMZ availability in the sandy soil samples was unexpected. This finding, however, might be explained by the lowest water content in the sandy soil material. The incubation of all soil samples at the same water potential of -0.015 MPa, (equals pF 2.18, pF = potential of the soil matrix to hold water in the soil against gravity) resulted in completely different soil water contents. This water potential represents the optimal moisture for organic xenobiotic degradation (Schroll et al. 2006). The sandy soil was the one with the lowest water content, as described in the materials and methods section. Hence, we compared SMZ in the soil pore water of the sandy soil (water content: 0.1 g g^{-1} soil) with the silty loam (water content: 0.2 g g^{-1} soil) and the sandy loam (water content: 0.2 g g^{-1} soil). Probably due to the lower water content, the sandy soil samples were not able to dissolve and desorb as much SMZ from the soil particles into the pore water as the other two soil samples with higher water content.

Different SMZ sorption behaviour in natural soils can also be steered by different soil acidities, expressed as pH values, which are known to play an important role in the SMZ adsorption to soil (Boxall et al. 2002; Thiele-Bruhn et al. 2004; Kurwadkar et al. 2007). In

former studies it was shown, that sorption of sulphonamides in the soil matrix is increasing with lower soil pH. These results fit well to the results obtained here, as the soil samples with the highest pH value (sandy loam, pH 7.8) conserved the highest amount of SMZ in the soil pore water and therefore exhibit the lowest SMZ adsorption to soil particles. The other two investigated soil samples with lower pH values (5.4 and 5.8, respectively) showed higher SMZ adsorption and lower SMZ solubility in the soil pore water, representing a lower availability of the antibiotic.

To exclude any SMZ degradation by soil microorganisms during the soil extraction experiments, sterile sandy loam samples were investigated for their sorption behaviour and compared to the non-sterile extraction results. Gamma radiation was chosen for sterilising soil samples (9 Gy/min for 72 h), because it has been formerly evaluated as a practical method, which is efficient and implicates only minimum alteration of soil properties (McNamara et al. 2003). The differences between SMZ in the soil pore water of the sterile and non-sterile soil samples are negligible. The SMZ fraction in the soil pore water was slightly lower in the sterile soil within the first eight days in comparison to the non-sterile soil samples. This finding proves that no degradation effect occurred while conducting this solubility/bioavailability experiment in this sandy loam soil.

Even though SMZ sorption to the sandy loam soil samples was strong in the first seven to ten days, low desorption processes were also detected, due to SMZ equilibrium between the solid phase and the water phase of the soil samples. After the inoculated microorganisms mineralised a considerable quantity of the SMZ in the sandy loam soil within the first days, and the *in situ* bioavailability in the soil pore water was supposed to be low, SMZ was mineralised at a rate of 0.5% d^{-1} of applied SMZ (equals 0.005 µg SMZ d^{-1} g^{-1} soil) on day 20 (see results fig. 43). As sorption and mineralisation by microbes both happen intensely right after SMZ application, it can be concluded that they are competing processes when removing SMZ from soil pore water.

4.2   Effect of SMZ application on soil microbial biomass and activity

To improve the understanding of the SMZ impact on the soil microorganisms in general, the microbial carbon was quantified as indicator for microbial biomass amounts before and after the SMZ-application in the concentration as used for soil incubation experiments (1 µg g^{-1}). In my experiments, the applied SMZ concentration was not affecting the natural soil microbes in respect of soil microbial biomass within the error tolerance. It is still possible, that the composition of the soil microorganism community had changed, but the amount of soil microbiological biomass stayed constant during these tested 20 days of incubation.

In the literature, other studies with higher SMZ concentrations revealed a toxic effect on the bacterial soil community. After SMZ application of 53.6 µg g^{-1} to soil samples, Pinna et al. (2012) revealed a negative short-term effect on the number of cultivable bacteria, on enzyme activities and even on the bacteria/fungi ratio. Liu et al. (2009) exhibited an effective concentration of SMZ to influence soil respiration to be 13 µg g^{-1}.

Instead of soil respiration, Gutiérrez et al. (2010) measured bacterial growth in soil samples taken at a dairy farm. On basis of phospholipid fatty acids (PLFA) profiles and 16S rDNA analysis a negative SMZ impact on enzyme activities were found at 0.9 µg g^{-1} soil.

4.3   Stimulation of indigenous soil microorganisms to SMZ degradation

According to Topp et al. (2013) the adaptation process of the soil microbes to SMZ took 10 years of periodic SMZ application (once per year). The SMZ degrading strain *Microbacterium* sp. C448 could be isolated from the treated soil only after this long exposition time. This strain not only had the ability to degrade SMZ, but to use it as the sole carbon source in liquid cultures. Mineralisation of SMZ by *Microbacterium* sp. C448 in pure cultures, as conducted in chapter 3.6 proves that the strain is able to perform all the degradation steps and no syntrophic degradation is given.

In this study, a similar SMZ adaptation process was supposed to be accelerated under laboratory conditions (chapter 2.5). Many different enrichment efforts failed (different SMZ concentrations (10 and 100 µg g^{-1} soil), incubation at elevated temperature (30 °C) and multiple SMZ applications (nine consecutive SMZ applications of 5 µg g^{-1} soil within 209 days)) to induce the indigenous soil microbes in the soil incubators to the degradation of SMZ. Therefore, the many different treatments conducted here, lead to the assumption that developing the adaptation to degrade SMZ in soil samples is not as easy and fast as for other

organic compounds. This assumption is supported by Mohring et al. (2009), who conducted a fermentation experiment of swine manure and detected degradation of five other sulphonamides, but no SMZ degradation at all after 34 days. Other studies also show difficulties in genetic adaptation of soil microorganisms to the decontamination of soils by degradation of synthetic compounds in several degradation steps (van der Meer 1994; Janssen et al. 1995).

While SMZ degradation was reported recently (Ingerslev and Halling-Sørensen 2000; García-Galán et al. 2011; Oliveira et al. 2016) only Topp et al. (2013) isolated a degrading microorganism (*Microbacterium* sp. C448) from soil.

Microbacterium sp. C448 as well as a soil aliquot, from which the degrader was isolated, were kindly provided by E. Topp (Topp et al. 2013), to continue this work and to conduct the intended soil inoculation experiments.

4.4   Analysis of *Microbacterium* sp. C448 and its microbial community

To get adequate knowledge about the bacteria used in this study, different analysis of the SMZ degrading strain *Microbacterium* sp. C448 and its soil inherent community were conducted.

4.4.1   Electron microscopy of *Microbacterium* sp. C448

The electron microscopical analysis of strain *Microbacterium* sp. C448 exhibited its rod shape morphology as described likewise in Topp et al. (2013). In this way, it was confirmed, that we were working with the same organism, described in Topp et al. (2013), also verified by its SMZ mineralisation capacity. The length of the bacterium turned out to be about 2 µm, which is on the average for common bacteria being 0.3- 10 µm (Munk 2000).

4.4.2   Growth curve of *Microbacterium* sp. C448

The growth curve revealed a fast growth of *Microbacterium* sp. C448 in liquid full medium. As the pH in the liquid medium was rising to almost 9 on day 6, it was supposedly too alkaline for *Microbacterium* sp. C448 to grow any further. Probably the microbes built NH_3, when metabolising the SMZ and also the peptides of the growth medium. From the growth curve a doubling time of about 10-12 h can be deduced, however, this conclusion is only valid

for the full medium. Certainly in the mineral salt medium, which I used for the liquid culture experiments, the strain was growing slower, as the mineral salt medium is lacking any C and N-sources except from SMZ.

4.4.3   DNA parameters of the microbial community

The analysis of the microbial community enriched from the Canadian soil aliquot, revealed members of many different families of the phylum proteobacteria. The most abundant genus was the *Hydrogenophaga* (37.2%). From this genus, some strains were able to degrade organic pollutants, for example the *Hydrogenophaga palleronii*, which was able to grow on several toxic compounds (Reddy et al. 2015).

The order of *Burkholderiales*, which belongs to the betaproteobacteria, was the most abundant order in this analysis. The genera Variovorax, Patulibacter, Arthrobacter and Aminobacter showed abundance between about six to four percent each. The SMZ degrading strain *Microbacterium* sp. C448 belongs to the family of *Microbacteriaceae*, which showed abundance below 1% here, and is therefore included in the group of "Others" in the results chapter. The DNA of *Microbacterium* sp. Strain C448 was already investigated in a draft genome sequence, provided by Martin-Laurent et al. (2014). In this investigation, the SMZ resistance gene *sulI* was detected among several other resistance genes.

4.4.4   Antibiotic resistances

Many different resistance mechanisms to different antibiotics are known. Resistant microbes can for example provide special transport mechanisms to export the antibiotic out of the cell, antibiotic substances can be inactivated by the organism, or the affected proteins can be transformed, to avoid any impact of the antibiotic; also the affected biosynthesis pathway can be altered to avoid any influence of the antibiotic (Munk 2000).

In general, resistances to sulphonamides were discovered in the 1950s. They are located on plasmids, which are small DNA fragments that usually appear as rings in many prokaryotic cells (Madigan 2013). These DNA fragments carry information that is not essential for cell survival, but can have other helpful functions for the cells. Generally, thousands of different plasmids are known but the ones that are investigated the best are the resistance plasmids (R plasmids), which enclose DNA that encodes resistances against different antibiotics and other substances (Madigan 2013). For example the plasmid R100, which can be easily transferred to other bacteria, contains genes for resistances proteins against different antibiotics:

sulphonamides, streptomycin, spectinomycin, fusidic acid, chloramphenicol and tetracycline (Madigan 2013).

To degrade an antibiotic, the degrading bacterial strain has to be resistant to this drug at first. When cultivated on LB agar plates with different antibiotic test substances, *Microbacterium* sp. C448 revealed resistances against SMZ and many other antibiotics from different groups, presented in the results (3.5.2).

Four genes are known to encode a slightly altered dihydropteroate synthase, *sul1*, *sul2*, *sul3* and *sulA* (Zhang et al. 2009) and are therefore sulphonamide resistance genes. This alteration leads to an enzyme, which is still binding to para-aminobenzoic acid (PABA) but unaffected by sulphonamides (meaning not binding to them). So the dihydropteroic acid can be formed and folic acid can be produced (Sköld 2000). *Sul1*, which is encoding dihydropteroate synthase type 1 was detected in the sulfamethazine resistant strain *Microbacterium* sp. C448 by Martin-Laurent et al. (2014). These resistance genes *sul1* and *sul2* were found in 190 clinical isolates from all over the world, probably developed in a time, when sulphonamides were used in human medicine more frequently (Rådström et al. 1991).

4.5   Soil inoculation leading to enhanced SMZ mineralisation

In the first step, the most effective soil inoculation method was identified. For this purpose, four different inoculants were compared (microbial community with and without carrier particles and isolated strain *Microbacterium* sp. C448, with and without carrier particles). Clear differences in the soil inoculation variants were visible right after the start of the experiment (3.7).

The mineralisation rates of the treatments containing the isolated strain were decreasing constantly from the beginning. This leads to the assumption, that the isolated strain *Microbacterium* sp. C448 was not able to survive in the new soil habitat for longer periods. Whether the single strain was transferred to the soil incubators in liquid cultures or attached on the protective carrier particles made no difference in this regard. The first mineralisation measuring point was higher in the variant where carrier particles were used, but still all variants were constantly decreasing from the start. In principle, bacterial strains newly introduced into an already established biodiverse soil habitat may have severe disadvantages. They are competing for nutrients and space with the indigenous soil microorganisms, which

are perfectly adapted to the existing chemical and physical soil conditions and may even live in communities in biofilms (Burns and Stach 2002; van Elsas et al. 2012). Additionally, as biosystem foreign organisms, they fall prey to microfaunal predators.

In contrast, in the soil inoculated with the microbial community, the mineralisation rates were increasing in the first days, leading to the assumption that the microbes were surviving, establishing and proliferating in the new soil habitat. After the mineralisation rates came to a maximum within the first week, they were decreasing; mineralisation was continued on a very low level, probably due to a decrease in cell number. Usually in a bacterial growth curve, after the stationary phase comes a death phase. The cells are dying because the nutrients are used up; also some cells built toxins which are increasing in concentration as the cell number is increasing. Such growth curves are developed in the lab under optimised conditions with single strains in liquid cultures, though. It is not possible to measure the growth curve of a whole microbial community. Nevertheless we can compare the bioavailability of SMZ in the same soil with the SMZ mineralisation rates. The SMZ bioavailability in soil in the first seven to ten days is rapidly decreasing and the consequence seem to be decreasing mineralisation rates of the microbial community after this period since most of the SMZ is adsorbed to the soil matrix until then, and cannot be mineralised anymore. Therefore, low SMZ availability is presumably also a reason for decreasing mineralisation rates. Due to SMZ desorption processes however, the mineralisation in soil can continue on a lower level.

These results indicate a clear advantage in using a natural soil borne microbial community for SMZ degradation in soil, over the use of the degrading isolated single strain.

4.6   Long-lasting effect of the inoculation approach

After the inoculated soil samples were incubated for 112 days in the lab, ^{14}C-SMZ was applied for a second time. It was mineralised fast and efficient by the introduced microbial community, demonstrating the long-term sustainability of this inoculation approach. SMZ was not available in large amounts after these 3.5 months due to rapid mineralisation and due to sorption processes, investigated in the bioavailability experiment (3.1.1). Hence, the SMZ degrading bacteria have not been living on SMZ as a carbon source in this period. They survived in soil in dormancy or by living on other carbon sources than SMZ, provided by the soil environment. Yet, the bacterial community sustained its SMZ-degrading capability for at least 112 days to restart the degradation process after this second SMZ application.

In field conditions, where manure is typically applied twice per year, the veterinary residues are reaching the grounds irregularly. The situation between the manure applications is similar to the one we created in the lab, with low available amounts of SMZ. After some months, the available amount of SMZ is presumably not sufficient to provide the necessary energy for the SMZ-degrading microbes or in concentrations too low to initiate their specific activity. For the laboratory experiments, we confirmed the sustainability of this soil inoculation approach and therefore recommend it for sustainable, long-term soil remediation.

There are some hints that with this approach not only SMZ but also other sulphonamides might be degraded. In an activated sludge study, Ingerslev and Halling-Sørensen (2000) showed that after degradation of one sulphonamide, the same microbes were also able to degrade several other sulphonamides. The authors therefore conclude that for environmental risk assessment tests, not every single substance has to be tested.

4.7 ^{14}C-residues in incubated soil samples

The different ^{14}C-residues were analysed via measuring of ^{14}C-CO_2, extraction of soil samples with methanol and combustion of extracted soil aliquots at the end of the experiments.

The fraction of ^{14}C-CO_2 originated from the mineralisation of ^{14}C-SMZ by the degrading microbes. The extractable fraction can be composed of ^{14}C residues that are dissolved in the soil pore water as well as soil adsorbed residues, which are extractable. The ^{14}C-fraction detected in the combusted soil aliquots after extraction is the fraction, not extractable via the accelerated solvent extraction.

The aromatic ring of the SMZ molecule was radioactive labelled, therefore, when ^{14}C-CO_2 was detected, ring cleavage had taken place and compounds of the molecule were used as substrate by the bacteria and oxidized by energy gaining metabolism to ^{14}C-CO_2. The ASE extracted radioactivity from soil samples can consist of the SMZ parent compound, and/or its metabolites, resulting from incomplete degradation by biological, chemical or physical processes or it can be ^{14}C in newly synthetized biomass. Some metabolites of SMZ have already been identified, with N4-acetyl-SMZ being the most common one in the excreted manure of pigs (Haller et al. 2002; García-Galán et al. 2008).

The non-extractable ^{14}C residue (NER) fraction in soil samples can represent the parent compound, the metabolites and ^{14}C in synthesised biomass. In the control samples, the

indigenous soil microorganisms did not degrade SMZ and therefore, the non-extractable ^{14}C-residues (more than 80% of the applied ^{14}C-SMZ), are presumably consisting of SMZ and some other SMZ degradation products, generated by non-biological degradation, physico-chemically bound to the soil matrix. These results are in accordance with degradation results by Lertpaitoonpan et al. (2015) showing a very high amount of NER (70 to 91% of the applied ^{14}C) in soil samples with very low SMZ mineralisation (0.1-1.5%, also measured via ^{14}C-CO_2 production).

For the formation of non-extractable ^{14}C residues, different binding mechanisms can occur. Bialk et al. (2005) verified cross coupling of SMZ to model humic substances. Other studies with different sulphonamides also showed the fast formation of NER in high quantities (Kreuzig and Holtge 2005; Heise et al. 2006; Lertpaitoonpan et al. 2015). Although the SMZ-NER uptake by earthworms and by plants was shown to be negligible, and the affinity of NER to soil matrix seemed to be high (Heise et al. 2006), a certain risk for human health cannot be excluded. Compounds of the NER fraction can be remobilized during natural turnover processes of soil organic matter, whereby they can become bioavailable and consequently contaminate other environmental compartments.

The soil samples with the highest SMZ mineralisation (MCCP-HA, microbial community on carrier particles showing higher initial mineralisation activity, results in section 3.8) removed most of the SMZ from soil by microbial mineralisation (62.4% of applied SMZ in phase one). In this case, it can be assumed that the high NER fraction mainly consisted of biogenic residues.

In general, when microorganisms mineralise xenobiotics in aerobic systems and use them as carbon source, the assimilation of the compound derived carbon not only leads to CO_2 production, which was released in high amounts in this work, but can also be used for the cell metabolism and for synthesis of cell components like fatty acids, amino acids, sugars and others. After cell death, these components can be transferred to soil organic matter and become NER (Nowak et al. 2011).

It can be concluded, that the inoculation approach used here, not only improves the soil remediation from SMZ by biodegradation, but also lowers the risks caused by NER in soil. The NER produced in the inoculated soil samples with high mineralisation, consist of synthesized biomolecules rather than of the parent compound, unlike the NER in the control

samples. Therefore these NER don't harm the environment, if ever remobilized by natural soil turnover processes.

5.   Conclusion

The conducted laboratory soil experiments revealed the most successful inoculation variant for SMZ degradation being the transfer of an adapted soil-borne microbial community located on carrier particles to SMZ contaminated soil samples. This approach was very efficient as SMZ mineralisation was significantly improved in the inoculated soil samples despite the considerably low bioavailability, in comparison to the un-inoculated control samples, where the mineralisation was negligible.

The successfully restarted significantly enhanced SMZ mineralisation after 112 days of incubation and a second SMZ application, verified the sustainability of this approach. In this way, common field situations were simulated, when manure is applied to soil twice per year, and degrading bacteria have to survive for several months without this substance, but should still be able to degrade it, when it is reapplied.

The separated mineralisation in soil matrix and carrier particles after incubation showed that the degrading bacteria was not only established on the carrier material, but also proliferated into the new habitat in the surrounding soil matrix. Under field conditions this means, the carrier particles would have to be applied to the field only once, and the microbes would be able to proliferate in the soil and continue the degradation from the soil compartment.

For this three positive effects of the inoculation approach (high efficiency, sustainability, proliferation to soil matrix), and also the soil preserving mode of action, we conclude that this is a very promising approach for soil SMZ decontamination and should definitely be verified with other contaminants and in outdoor conditions. In this case however, one has to be careful when applying the bacteria to the fields, because the applied strain *Microbacterium* sp. C448 showed additional resistances to other antibiotics. More knowledge about the abundance of natural antibiotic resistances in soils would be necessary to estimate the risk of distributing such kind of organism in the fields. Apart from that, we strongly recommend this method for soil bioremediation.

As this approach has been verified for other organic compounds before (herbicide isoproturon and environmental chemical 1,2,4-TCB), and was proven to be very efficient for SMZ soil remediation, we conclude that this method is probably also effective for many other organic soil contaminations. The usage of microbial communities is definitively recommended over isolated strains, due to the higher efficiency and sustainability, as shown in this work and the

former studies. It has to be pointed out, that the decontaminated soil is not destroyed or mis-functioning by this approach, and is immediately utilisable for food production.

List of abbreviations

DW   Dry weight

ISCP   Isolated Strain on Carrier Particles

ISLC   Isolated Strain without carrier particles in Liquid Cultures

MCCP   Microbial Community, established on Carrier Particles

MCLC   Microbial Community without carrier particles in Liquid Culture

MCCP-HA   Microbial Community on carrier particles with a higher initial SMZ mineralisation activity

MCCP-LA   Microbial Community on carrier particles with a lower initial SMZ mineralisation activity

NER   non-extractable ^{14}C-residues

PABA   para-aminobenzoic acid

SMZ   Sulfamethazine

TC*HCl   Tetracycline Hydrochloride

TC   Tetracycline

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Publication

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RESEARCH

An effective bioremediation approach for enhanced microbial degradation of the veterinary antibiotic sulfamethazine in an agricultural soil

Natalie Hirth[1] , Edward Topp[2], Ulrike Dörfler[1], Erhard Stupperich[3], Jean Charles Munch[4] and Reiner Schroll[1*]

Abstract

Background: The veterinary antibiotic Sulfamethazine (SMZ) contaminates soils via manure applications. Like other soil contaminants (herbicides, fungicides, and nematicides), it has to be degraded. The main challenge is that SMZ biodegradation with bacteria is impeded, since SMZ is a bacteriostatic antibiotic, designed to block microbes in their growth.

Results: In this study, we enriched the indigenous soil microbial community (including the single strain *Microbacterium* sp. C448, adapted to SMZ degradation) from a Canadian soil and we present a suitable approach, for soil remediation by inoculating a German soil with this microbial community established on carrier particles, at environmentally relevant concentrations of 1 mg kg^{-1}. When compared with the isolated SMZ-degrading strain (also obtained from Canada), the microbial community outperformed the mineralization rates of the isolated strain in soil. The negligible soil native SMZ mineralization was successfully increased to 44 and 57 % within 46 days, by the microbial community. The sustainability of this increased SMZ mineralization capacity was proven by the rapid mineralization of a second application of ^{14}C-SMZ 112 days after the first.

Conclusions: The pronounced SMZ mineralization and the high amount of non-extractable ^{14}C-residues (NER) in the inoculated soil indicate that the NER are mainly of biogenic origin (metabolically fixed ^{14}C). Therefore, the applied inoculation approach decreased the risk of persistent non-extractable SMZ residues. Together with our former studies, this specific soil inoculation approach was tested for three substances with different physico-chemical properties, indicating that this soil bioremediation technique might also be used for other substances.

Keywords: Sulfamethazine, Enhanced biodegradation, Microbial community, Carrier particles, Soil, Remediation

Background

Sulfamethazine (4-Amino-*N*-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide; SMZ) is a veterinary sulfonamide antibiotic used against infections of the respiratory tract, mainly in swine farming [1, 2]. It hampers the production of folic acid in target microbes. In the USA, no monitoring of the antibiotic consumption is in place, so reliable data are scarce, and in most cases, they are estimated [3, 4]. In Europe, a cumulative usage of 2855.2 tons of antibiotics considering 7 European countries in 2004 is reported [5]. In Germany, 162 tons of sulfonamides have been sold to veterinarians in the year 2012, which makes them the third most sold group of antibiotics after tetracyclines and penicillin [6].

After administration to farm animals, parent compounds as well as metabolites of the veterinary antibiotics are excreted and reach agricultural soils either directly by grazing animals or via the application of manure to land after a storage period. Haller et al. [2] quantified SMZ in

*Correspondence: schroll@helmholtz-muenchen.de
[1] Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany
Full list of author information is available at the end of the article

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the manure of treated pigs and calves of six farms mainly in the range of mg kg^{-1}. By application of liquid manure to fields, the risk of contaminating other environmental compartments is thus heightened [7, 8]. Christian et al. [9] found SMZ residual concentration both in surface water (7 ng L^{-1}) and soil samples (15 µg kg^{-1}, dry weight) 7 months after a liquid manure application, indicating a high stability of SMZ in soil. These non-lethal concentrations can select resistant microorganisms [10, 11] which can then potentially transfer resistance to other soil bacteria, including human pathogens, via gene transfer [12–16].

To reduce these risks for the environment and human health, an effective long-term approach is strived, to decontaminate soils from SMZ, without destroying soils, instead preserving them for further agricultural usage. To decontaminate soils from antibiotics, which are applied to the fields regularly, the bioremediation approach should not only be efficient, but also sustainable. In this case, "sustainability" means that the microbes should be applied to the soil only once and they should sustain their degradation ability over time and degrade the contaminant again, when next it is applied.

Until now, studies on SMZ removal have been focusing on the decontamination of waste water reactors using activated sludge [17–20] electrochemical SMZ removal from aqueous solutions [21–23], SMZ removal from water and soil using biochar [24, 25], gamma irradiation in sewage and aqueous solution [26–28], and other adsorption removal techniques [29]. All of these techniques are not developed for large-scale soil remediation of whole agricultural areas.

Oliveira et al. [20] reported that SMZ degradation was mostly studied in activated sludge systems and anaerobic waste water treatment; nevertheless, information about successful SMZ degradation is limited and where a high success was reported, the study was conducted with disproportional high concentrations of SMZ (90 mg L^{-1}). In their study, SMZ was biodegraded at the environmental concentration of 100 µg L^{-1} in anaerobic conditions. Since it was dependent on the availability of easily degradable organic matter, a cometabolic degradation of SMZ was suggested.

All of these studied techniques focused on waste water or sludge and did not consider decontamination of polluted agricultural soils.

The only study showing a metabolic SMZ degradation conducted by indigenous soil microbes was done by Topp et al. [30]. High SMZ degradation by indigenous soil microbes was observed in laboratory experiments after long-term application of SMZ to the field (one time per year for 10 years). Topp et al. found out that for the success of this study, it was necessary to use higher SMZ concentrations than the ones reported in soil (10 mg kg^{-1}).

As the decontamination studies, mentioned before, focused on SMZ degradation in aqueous and sometimes anaerobic systems, we saw the need to provide an approach that effectively accelerates the SMZ mineralization in soil. This approach should be successful at a concentration of 1 mg kg^{-1} which is close to environmental conditions [2], and should be directly applied to soil and avoid destruction of soil structure and relocation as performed by chemical soil extraction, soil combustion, or other harsh ex situ soil remediation techniques. In this soil inoculation approach, not only a single strain capable to degrade a soil contaminant is applied, but a microbial community. The microbial community was enriched by us from an aliquot of this Canadian soil, from which Topp et al. [30] isolated the single SMZ-degrading strain earlier. Furthermore, this microbial community was attached to a protective material (defined clay particles) to improve the survival of the microbes in the new and foreign soil environment. We already presented this very efficient approach, for enhanced biodegradation of other organic chemicals in soils, which ensures that the introduced function of accelerated mineralization survives and establishes in the new soil environment [31–33]. This time, we successfully applied the approach to even decontaminate soil from an antibiotic, which makes it more difficult for the microbial community to survive and support the degrading strain, because all microbes that are sensitive are affected by the SMZ application.

The aim of this study was to apply this soil inoculation approach, for the successful mineralization of an antibiotic, SMZ, in soils. Our objective was, therefore, to enrich the microbial community, which also includes the degrading strain, from SMZ contaminated soil, establish it on carrier particles, introduce it to another ^{14}C-SMZ contaminated soil, and quantify the SMZ mineralization via trapping of $^{14}CO_2$.

Methods

Chemicals

Uniformly, ^{14}C-ring-labeled sulfamethazine (4-Amino-*N*-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide, SMZ, CAS Number: 57-68-1) was purchased from Campro Scientific GmbH (Veenendaal, The Netherlands) with a specific radioactivity of 673.4 MBq mmol^{-1}, and a radiochemical purity of >98 %. Non-labeled SMZ was obtained from Sigma Aldrich (Taufkirchen, Germany). The ^{14}C-SMZ was blended with non-labeled SMZ to produce the ^{14}C-SMZ application standards (spec. radioactivities ranging from 80 to 500 MBq mmol^{-1}). Carbo-Sorb®E and the scintillation cocktails (Permafluor®E+, Ultima Gold™ XR and Ultima Flo™) were purchased from PerkinElmer (Waltham, USA). All

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other chemicals and solvents were purchased in analytical grade from Merck (Darmstadt, Germany).

Soils

Two soils (Hohenwart and Scheyern1) were sampled from agricultural fields in Germany (0–10 cm depth), air dried, sieved (<2 mm), and stored at −20 °C. Prior to each experiment, the soils were thawed at 4 °C for 1 week, then moistened to a water tension of −15 kPa [34] and equilibrated at room temperature (20 ± 1 °C) for another week to gently activate the native soil microorganisms from the frozen state. None of the soils had the ability to degrade SMZ naturally.

Soil Hohenwart (13 % clay, 19 % silt, 68 % sand, 1.0 % organic carbon, 0.1 % total N, pH 6.7, 48.600° latitude, 11.383° longitude, 392 m altitude) was used for the experiments stimulating the indigenous soil bacteria to degrade SMZ by applying it in different concentrations and frequencies ("adaptation experiments"). In the last 10 years before sampling, this soil was not treated with pesticides or antibiotics and received a barley, maize, and wheat crop rotation, and finally, the field was let to rest for three years.

Soil Scheyern1 (sandy loam: 20 % clay, 40 % silt, 40 % sand, 1.5 % organic carbon, 0.2 % total N, pH 7.8, 48.493° latitude, 11.432° longitude, 497 m altitude) was inoculated with the microbial community for the enhanced SMZ mineralization. This soil was sampled on the agricultural research farm Scheyern in Germany. It has an organic farming crop rotation containing potatoes, winter wheat, sunflower, winter rye, and grass-clover-alfalfa as intertillage and cover crop.

The Canadian soil aliquot, where we enriched the microbial community (including the SMZ-degrading strain) from, was sent to us from Canada and is described precisely in the paper of Topp et al. [30]. The two soils only used for the in situ bioavailability experiment are described in the Additional file 1: Table S1.

In situ bioavailability of SMZ

To investigate the in situ bioavailable ^{14}C-SMZ over time in soil, the ^{14}C-radioactivity in the soil pore water of three different soils (Scheyern1, Scheyern2, and Neumarkt) was determined according to Folberth et al. [35] over a period of 21 days. The characteristics of all 4 soils used in this work are described in the Additional file 1: Table S1. The three soils (35 g dry weight equivalent) were spiked with aqueous ^{14}C-SMZ application solution (1 μg g^{-1}) and incubated in 250 ml amber bottles (30 replicates). For spiking the soil, 200 μl of ^{14}C-SMZ application standard was applied dropwise to a 5 g oven dried (105 °C, 24 h) soil aliquot and carefully mixed. The spiked aliquot was transferred to fresh, equilibrated soil (30 g,

dry weight) in the amber bottle, and thoroughly stirred with a spatula. The soil was then adjusted to a density of 1.3 g cm^{-3} and a soil water tension of −15 kPa [34]. The incubators were closed with parafilm and stored in a desiccator with water at the bottom to prevent drying of the samples. After different incubation times (1, 2, 3, 4, 7, 8, 9, 10, 14, and 21 days), three replicates of each soil were subjected to centrifugation to determine the current in situ bioavailability of SMZ. For this purpose, 30 g (dry weight) of the incubated soil were centrifuged for 90 min at 9000 rpm and 20 °C. After centrifugation, two aliquots of 1 ml of the soil pore water were each mixed with 4 ml Ultima Gold XR and the ^{14}C-radioactivity was measured in a liquid scintillation counter (Tricarb 2800TR, PerkinElmer, Waltham, USA). The amount of ^{14}C extracted from the soil pore water was calculated as percentage of the applied ^{14}C-SMZ and was considered to be the amount of SMZ, available for in situ biodegradation by soil microbes.

Adaptation of soil microorganisms to SMZ degradation

To stimulate the adaptation process of native soil microbes under laboratory conditions, different experimental soil environment scenarios were applied to soil Hohenwart. No microbes were added to soil, only SMZ was applied at the start of the scenarios, to stimulate the indigenous soil microorganisms to degrade SMZ. The soil was incubated at constant soil water tension (−15 kPa), at fluctuating soil water tension (drying and rewetting cycles with a range from −15 to −700 kPa) and at different soil temperatures, achieved by storing soil incubators at room temperature (20 ± 1 °C) and in a water bath (30 ± 1 °C). These different scenarios were conducted in two different SMZ concentrations (10 and 100 μg g^{-1}) and some variants received eight further SMZ applications. The incubation time lasted 160 days. Two samples with 100 μg g^{-1} SMZ, two samples with 10 μg g^{-1} SMZ, and two samples where SMZ was applied several times at 5 μg g^{-1} were incubated longer (430 days) to enhance the chance for adaptation.

Each of the scenarios was conducted with 35 g (dry weight) of soil Hohenwart. SMZ was applied to the soil according to the procedure described in "In situ bioavailability of SMZ" section and the soil samples were incubated in the dark at a soil density of 1.3 g cm^{-3}. Soil samples for the approaches with fluctuating soil water tension were placed in 100 ml flasks, moistened to a water tension of −15 kPa, connected to a laboratory CO_2-trapping system by Lehr, Scheunert [36] (modified), and dried for 7 days to a water tension of −700 kPa by continuously passing dried air through the system (1.3 l h^{-1}). After the drying cycle, the soil samples were remoistened to −15 kPa and the next drying cycle was

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started. Soil samples with constant soil water tension were incubated in 250 ml amber glass bottles at a water tension of -15 kPa in the dark. To survey the effect of the exposition scenarios, the mineralization of ^{14}C-SMZ was quantified in the different treatments, by determining the evolved $^{14}CO_2$.

Enrichment and cultivation of SMZ-degrading bacteria

The SMZ-degrading *Microbacterium* sp. strain C448 was isolated by Topp et al. [30] from a Canadian soil, that was exposed to an annual dose of 1 and 10 mg kg^{-1} SMZ for 10 years (1 mg kg^{-1} for 5 years, 10 mg kg^{-1} for 5 years). This degrading strain was sent to us on agar plates together with an aliquot of this Canadian soil. Hence, in this study, we enriched the microbial community (including the SMZ-degrading *Microbacterium* sp. strain C448) from this soil.

For enrichment, 2 g soil material (<2 mm, wet weight) was added to 19 ml culture medium plus 1 ml of aqueous ^{14}C-SMZ in 100 ml Erlenmeyer flasks (10 mg L^{-1}). The mineral salt medium was prepared according to Topp et al. [30] with the exception that vitamins (biotin and thiamin) and trisodium citrate were excluded. To establish the microbial community on the carrier material, 40 sterilized carrier particles (2–4 mm sized clay particles, total N: 3–8 mg L^{-1}, P$_2$O$_5$: 5–10 mg L^{-1}; K$_2$O: 100–120 mg L^{-1}, expanded for high pore volume (>80 %), fired, Seramis GmbH, Mogendorf, Germany) were added to the liquid culture. To avoid microbial contamination of these liquid cultures, filters (0.20 μm, Sartorius, Göttingen, Germany) were installed at the air inlet and outlet of the Erlenmeyer flasks and the liquid cultures were incubated at 75 rpm in the dark at 20 °C. Three times per week, the $^{14}CO_2$ evolved from the mineralization of ^{14}C-SMZ was quantified. After 6–9 days of incubation the microbial community, established on carrier particles (MCCP) was transferred to fresh ^{14}C-SMZ-spiked liquid medium.

The enriched microbial community from the Canadian soil has been analyzed by isolating the DNA from the carrier particles using the FastDNA™ SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA, USA). The DNA has been prepared for Illumina sequencing according to the Illumina standard protocol. The results were identified mostly to genus level and are attached in the Additional file 2: Fig S1.

The pure *Microbacterium* sp. strain C448 from Canada was transferred from the agar plates to the same mineral salt medium containing the sterile carrier particles and the ^{14}C-SMZ in the same concentrations, volumes, and filters on both sides of the Erlenmeyer flasks. In total, ten subsequent transfers were carried out for the single strain and the community in liquid cultures.

Soil inoculation with the microbial community compared with the single strain

Two methods of soil inoculation were compared to identify the more effective in mineralizing SMZ (50 g soil dry weight equivalent, 1 mg kg^{-1} SMZ). The soil Scheyern1 which did not have the native capacity to degrade SMZ was inoculated with the isolated strain established on carrier particles (ISCP), as well as the MCCP, and the mineralization was measured by trapping the $^{14}CO_2$ in the soil incubators (250 ml amber bottles) for 49 days.

Two different amounts of inoculum were tested for each variant, to overcome possible differences in the amount of applied degrading bacteria on the CP and have comparable conditions in the ISCP and MCCP application. Therefore, 5 and 10 carrier particles of MCCP as well as 10 and 20 carrier particles for ISCP were added to the ^{14}C-SMZ-spiked soil in triplicates.

Soil samples were incubated in the dark at 20 ± 1 °C, at a soil density of 1.3 g cm^{-3}, and a water tension of -15 kPa for 49 days. Three times per week, $^{14}CO_2$ was collected and quantified. The control samples did not receive a microbial inoculum.

SMZ mineralization in soil inoculated with the microbial community

First phase of SMZ mineralization

For testing the sustainability of this soil inoculation approach, soil Scheyern1 with the SMZ-degrading MCCP was incubated for 112 days, before SMZ was reapplied to the soil.

Prior to soil inoculation, the liquid cultures containing the microbial community were divided into two groups: one group (three replicates) received a second dose of SMZ (5 μg ml^{-1}) after 7 days of incubation (higher initial mineralization activity, HA). Another 8 days later, the soil Scheyern1 was inoculated with the MCCP for testing the enhanced SMZ mineralization. The other group (three replicates, lower initial mineralization activity, LA) did not receive a second SMZ dose and was transferred to the soil after 15 days of inoculation. Before being transferred to the soil, the two groups of MCCP showed different mineralization activities in the liquid cultures: the first group showed a higher (2.3 ± 0.5 % days^{-1}) and the latter one a lower SMZ mineralization rate (1.1 ± 0.1 % days^{-1}) (see Additional file 3: Fig S2 for mineralization rates and Additional file 4: Fig S3 for cumulative mineralization of both groups in the 8 days before soil inoculation). Thereby, we tested two MCCP variants with different initial mineralization activities at the beginning of the soil inoculation experiment.

Six replicates of soil Scheyern1 (35 g, dry weight) were spiked with ^{14}C-SMZ (1 μg g^{-1}) and mixed with 12 particles of MCCP. Three replicates were inoculated

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with MCCPs of the HA treatment and three replicates received MCCPs of the LA treatment to test the effect of different initial activity states of the microbial community on its SMZ mineralization capacity. Soil samples were incubated in the dark at 20 ± 1 °C, at a soil density of 1.3 g cm^{-3}, and a water tension of -15 kPa for 112 days. Three times per week $^{14}CO_2$ was collected and quantified. The control samples did not receive a microbial inoculum.

Second phase of SMZ mineralization

At the end of the first phase (after 112 days), a second application of ^{14}C-SMZ was performed to ensure the stability of the approach and to test the sustainability of the enhanced SMZ mineralization capability of the soil over time. The aqueous ^{14}C-SMZ solution (210 µl, 1 µg g^{-1} soil) was applied to the soil in the incubators and mixed carefully. The soil was re-adjusted to a density of 1.3 g cm^{-3}, moistened to a soil water tension of -15 kPa, and incubated in the dark at 20 ± 1 °C for another 49 days. SMZ mineralization was quantified three times per week according to 2.8.2. The controls also received a second ^{14}C-SMZ application.

Third phase of SMZ mineralization

After the sustainability of the inoculation approach was tested, a third phase was conducted, where the carrier particles were separated from the bulk soil, before SMZ was applied for a third time. The 12 carrier particles, transferred to soil in the first phase of the experiment, were taken out of each incubator and transferred to new soil incubators containing freshly equilibrated aliquots of the same soil (Scheyern1). SMZ was applied (1 mg kg^{-1}), and the soil was incubated as in the other two phases. The soil, where the carrier particles were taken out, also received a third SMZ application (1 mg g^{-1}) and was incubated without carrier particles in the same way. All incubators were stored in the dark at 20 °C and mineralization and water content were measured as in the other two phases.

Measurement of $^{14}CO_2$ in the different incubation systems
Soil samples with fluctuating water content

The following aeration system was used to measure the $^{14}CO_2$ generated in the soil samples of the adaptation experiment with fluctuating soil water tension and in the liquid cultures. The soil samples, incubated in 100 ml glass flasks, were connected to a CO_2-trapping system consisting of two wash bottles, each filled with 10 ml of a 0.1 N NaOH solution for collecting $^{14}CO_2$ [36] (modified). The soil was aerated continuously (1.3 l h^{-1}) with dried air to enable drying of the soils for the fluctuating soil water tension experiment. The NaOH solution was

exchanged three times per week to quantify the trapped $^{14}CO_2$ by taking an aliquot of 2 ml, mixing it with 3 ml Ultima Flo and measuring it in a liquid scintillation counter. The liquid cultures, enriched in Erlenmeyer flasks and incubated on a shaker (75 rpm), were connected to this trapping system and aerated (1.3 l h^{-1}) three times per week for 1 h. After each aeration step, the NaOH was exchanged and a 2 ml aliquot was measured for radioactivity as described above.

Soil samples with constant water content

This system was used for soil incubation experiments with a constant soil water tension of -15 kPa, namely the treatments with constant soil water tension of the adaptation experiment ("Adaptation of soil microorganisms to SMZ degradation" section), the IS and CP comparison experiment ("Soil inoculation with the microbial community compared with the single strain" section), and the experiment, where SMZ mineralization was enhanced by inoculating the microbial community ("SMZ mineralization in soil inoculated with the microbial community" section). The soil samples were incubated in 250 ml amber bottles. These bottles were closed with a rubber stopper through which a hollow needle was fed to guarantee a constant oxygen supply. At the atmospheric side, the needle was connected to a soda lime filled syringe for adsorbing atmospheric CO_2. At the bottom of the rubber cap, a 25 ml plastic beaker was installed and filled with 9 ml of 0.1 N NaOH to trap the $^{14}CO_2$ evolved from ^{14}C-SMZ mineralization in the soil. Three times per week, the NaOH solution was exchanged and an aliquot of 2 ml was taken and mixed with 3 ml Ultima Flo, and the $^{14}CO_2$ was measured in a liquid scintillation counter.

^{14}C-mass balances

At the end of the inoculation experiments, ^{14}C-mass balances were calculated. For the experiment where the sustainability of the inoculation approach was tested, soil pore water was extracted to determine the in situ bioavailability of SMZ as described in "In situ bioavailability of SMZ" section. For the inoculation of the community compared with the single strain, pore water was not extracted. Extractable ^{14}C-residues in soil were determined by mixing 35 g (dry weight) soil with 0.5 g of diatomaceous earth prior to accelerate solvent extraction (ASE 200, Dionex, Dreieich, Germany) with a mixture of methanol and water (80:20) at 100 °C and 14 MPa. To ensure exhaustive extraction, three sequential extraction steps for three times were conducted. Two aliquots of 1 ml of each extract were mixed with 4 ml Ultima Gold XR and measured by liquid scintillation counting. To quantify the non-extractable ^{14}C-residues (NER), soil material after ASE was homogenized and three aliquots (250–300 mg)

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were combusted in a Sample Oxidizer (Packard, Dreieich, Germany). The generated $^{14}CO_2$ was trapped in Carbo-Sorb®E, mixed with Permafluor®E + and quantified in a liquid scintillation counter.

^{14}C mass balance was calculated based on the initially applied ^{14}C-SMZ, including the mineralized ^{14}C amount, the extractable, and the non-extractable ^{14}C amount.

Results

In situ bioavailability of SMZ in soil Scheyern1
In the comparison of three soils, differing in their texture, soil Scheyern1 revealed the highest in situ bioavailability of SMZ, and was, therefore, chosen for soil inoculation experiments. The day after ^{14}C-SMZ application, 29.4 % of the applied radioactivity was found in the soil pore water of soil Scheyern1 (Additional file 5: Fig S4). Thereafter, the ^{14}C content in the pore water decreased continuously with a rapid reduction until day seven (6.6 %) and a slighter decrease until day 21 (2.5 %). This indicates that more than 90 % of the in situ available SMZ are adsorbed to the soil matrix during the first week, and after that, less than 10 % of the applied SMZ is present in soil pore water and thus in situ available for biodegradation.

Attempts to adapt indigenous soil microorganisms to SMZ degradation
It was not possible to accelerate the adaptation process of soil microorganisms and to increase the SMZ mineralization in soil Hohenwart, by any of our tested approaches. It can be summarized that even after an "adaptation" period of 430 days, the cumulative mineralization of SMZ did not exceed 6.9 % of the applied SMZ. The SMZ mineralization rates remained below 0.2 % days^{-1} for more than 400 days of incubation. The longsome natural adaptation process could not be enhanced under the selected laboratory conditions.

Enhanced SMZ mineralization in soil Scheyern1 by inoculation
Soil inoculation with microbial community compared with isolated strain
We used an aliquot of the Canadian soil that had been adapted to SMZ biodegradation under field conditions [30], to enrich the microbial community, able to degrade SMZ in our lab. To identify the more efficient variant for SMZ mineralization in soil Scheyern1, soil inoculation with the MCCP was compared with inoculation with the ISCP. In both variants, two different amounts of carrier particles were introduced to soil (ISCP: 10 and 20 CP, MCCP: 5 and 10 CP). Figure 1 shows the different mineralization rates of MCCP and ISCP.

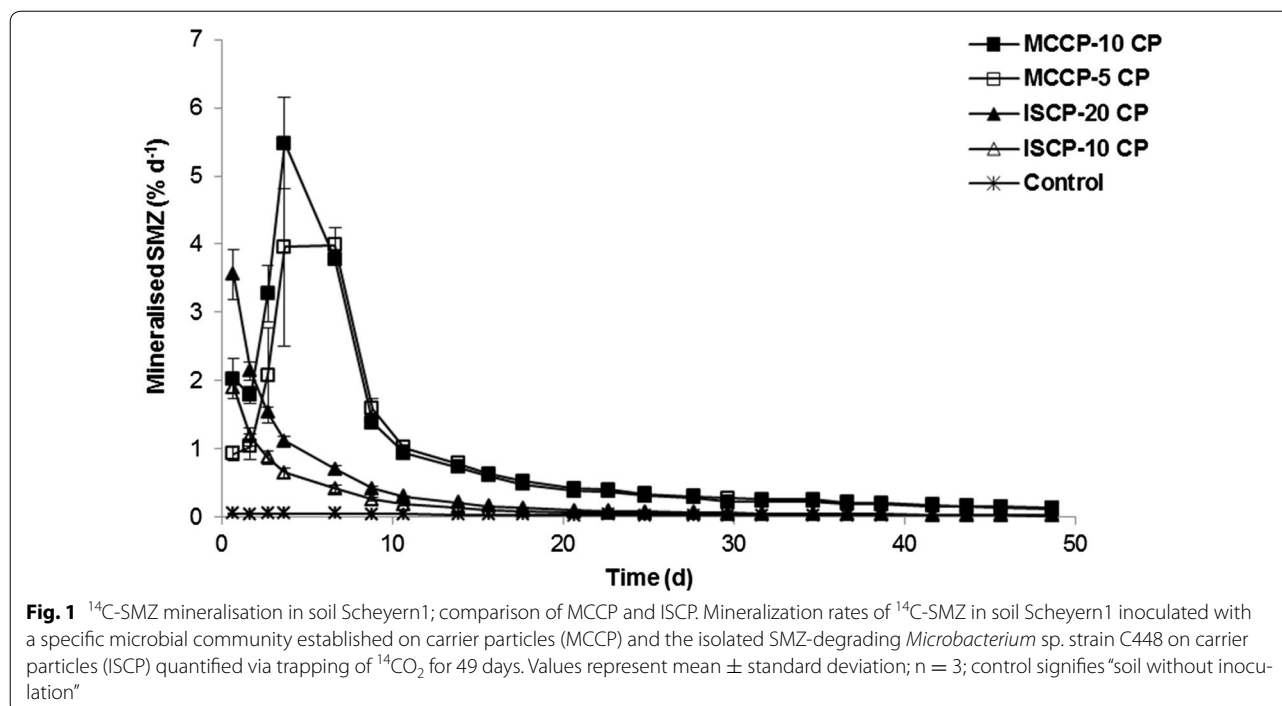The microbial community was able to mineralize SMZ efficiently and showed higher mineralization rates

compared with the isolated strain and a higher cumulative mineralization after 49 days [MCCP, 37 and 39 % of applied SMZ depending on the amount of introduced carrier particles (5 and 10 CP) compared with ISCP, 8 and 14 % of applied SMZ (10 and 20 CP)]. The measurement on the first day showed similar mineralization rates for the soil inoculated with ISCP 10 CP and MCCP 10 CP. Afterwards, the mineralization rates of both variants of the IS were decreasing rapidly, while the mineralization rates of the MC were increasing to come to a peak between day 4 and 7.

Soil inoculation with MCCP to test the sustainability of enhanced SMZ mineralization
After the inoculation of the MCCP turned out to be the more effective bioremediation technique, when compared with the isolated single strain, we repeated the soil inoculation with this community. A second phase was conducted, to find out if this approach is sustainable and the mineralization capacity is stable in soil over time. Finally, in a third phase, the mineralization site was investigated.

The soil Scheyern1, used in our study for soil inoculation experiments, revealed a native SMZ mineralization rate of 0.03 % days^{-1} (Fig. 2). This low natural SMZ mineralization capacity in soil Scheyern1 was increased considerably by inoculation with the SMZ-degrading MCCP in two different states of activity (MCCP-LA and MCCP-HA). Within 46 days, 44 % of the applied ^{14}C-SMZ was mineralized in total in the soil inoculated with the MCCP that showed a lower SMZ mineralization activity (MCCP-LA). The SMZ mineralization rate was at its maximum of 5.5 % days^{-1} of the applied SMZ, after 4 days of inoculation (Fig. 2a). When soil Scheyern1 was inoculated with the microbial community of higher SMZ mineralization activity (MCCP-HA), the inoculation effect was even more pronounced, resulting in a cumulative SMZ mineralization of 57 % after 46 days and a maximum SMZ mineralization rate of 12.3 % days^{-1} at day 4 (Fig. 2a).

To test the sustainability of this enhanced SMZ mineralization, 112 days after the first SMZ application, a second SMZ application was conducted. In this second phase, the two groups of inoculants (MCCP-HA and MCCP-LA) showed no substantial differences in their SMZ mineralization activities (Fig. 2b). SMZ mineralization was considerably enhanced compared with the control, although not as high as in the first phase when the MCCP-HA and MCCP-LA were freshly inoculated into the soil. After 46 days, 36 % (MCCP-LA) to 38 % (MCCP-HA) of the applied ^{14}C-SMZ were mineralized and the mineralization rates peaked on day 6 in 3 % days^{-1} (MCCP-LA) and on day 8 in 4 % days^{-1} (MCCP-HA)

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Fig. 1 ^{14}C-SMZ mineralisation in soil Scheyern1; comparison of MCCP and ISCP. Mineralization rates of ^{14}C-SMZ in soil Scheyern1 inoculated with a specific microbial community established on carrier particles (MCCP) and the isolated SMZ-degrading *Microbacterium* sp. strain C448 on carrier particles (ISCP) quantified via trapping of $^{14}CO_2$ for 49 days. Values represent mean ± standard deviation; n = 3; control signifies "soil without inoculation"

(Fig. 2b). In contrast, the SMZ mineralization rates in the control stayed below 0.1 % days^{-1} and after 46 days, only 1.7 % of the applied ^{14}C-SMZ was obtained as $^{14}CO_2$.

^{14}C-residues in soil

After the second phase, the soil samples MCCP-HA were analyzed for ^{14}C-residues in soil pore water, extractable ^{14}C-residues, and non-extractable ^{14}C-residues. The ^{14}C-residues found in the soil pore water of the inoculated soil samples amounted to 0.5 % (Table 1). In the control, 14.2 % of the initially applied radioactivity was found to be methanol-extractable ^{14}C residues, whereas in the inoculated soil samples, the methanol-extractable ^{14}C-residues were almost three times lower (5.1 %, Table 1). The inoculated samples also showed lower non-extractable residues (42.9 %, Table 1) compared with the control samples (81.9 %, Table 1). The total recovery of ^{14}C-radioactivity was 100.0 ± 0.6 % and 97.8 ± 1.1 % in the inoculated and control samples, respectively.
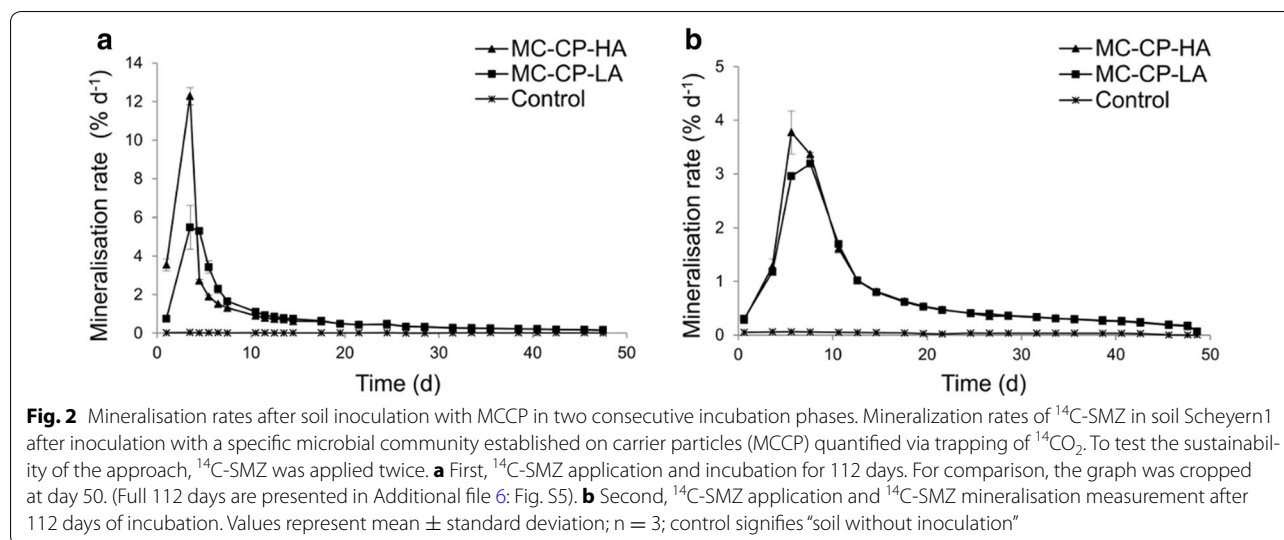
Discussion

Enhanced mineralization of SMZ in soil by inoculation

The SMZ-degrading microbial community was enriched on carrier particles, transferred to soil, and was able to mineralize SMZ efficiently. Hence, the microbial degraders survived and established in the new soil environment. Soil Scheyern1 was inoculated with the SMZ-degrading microbial community as well as the isolated SMZ-degrading *Microbacterium* sp. strain C448, both

established on carrier particles. The MCCP was able to mineralize SMZ efficiently and showed higher mineralization rates compared with the ISCP and a higher cumulative mineralization. These results lead to the assumption that the microbial community supports the degrading strain. When inoculated as isolated strain, mineralization rates were decreasing from the beginning. This indicates a lower cell activity and maybe also a reduction of cell material of the isolated strain. When inoculated as the whole soil born microbial community, the mineralization rates were increasing which indicates an increasing cell activity and probably also a growth of cells. The MCCP was, therefore, selected for the following experiment where the sustainability of the inoculation approach was tested.

The higher SMZ mineralization capacity of the inoculant MCCP-HA is most probably due to an increased cell growth in the liquid culture induced by a second SMZ dose before the inoculant was transferred from the liquid culture to the soil. Thus, more active cells were introduced into the soil, resulting in a higher initial SMZ mineralization activity. The inoculant MCCP-LA where the cell growth was not stimulated by a second SMZ dose showed a lower initial SMZ mineralization activity in the soil. The reason why the LA group did not attain the SMZ mineralization capacity of the HA group can be explained by the bioavailability of SMZ. The highest amount of SMZ in the soil pore water was found in the first 7 days after application, thereafter, most of the

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Fig. 2 Mineralisation rates after soil inoculation with MCCP in two consecutive incubation phases. Mineralization rates of ^{14}C-SMZ in soil Scheyern1 after inoculation with a specific microbial community established on carrier particles (MCCP) quantified via trapping of $^{14}CO_2$. To test the sustainability of the approach, ^{14}C-SMZ was applied twice. **a** First, ^{14}C-SMZ application and incubation for 112 days. For comparison, the graph was cropped at day 50. (Full 112 days are presented in Additional file 6: Fig S5). **b** Second, ^{14}C-SMZ application and ^{14}C-SMZ mineralisation measurement after 112 days of incubation. Values represent mean $\pm$ standard deviation; n = 3; control signifies "soil without inoculation"

available SMZ was adsorbed to the soil. For this reason, the MCCP-HA group with its higher initial SMZ mineralization activity had an advantage over the MCCP-LA group: it was able to mineralize more of the available SMZ within the first 7 days—when the SMZ bioavailability was highest—than the MCCP-LA group with its lower initial SMZ mineralization activity. Independent from the differences between the two inoculants, the results present the efficiency of the inoculation approach in enhancing the SMZ mineralization.

Sustainability of the inoculation approach
After 7 days, most of the SMZ applied has adsorbed to the soil matrix as shown in the bioavailability experiment (Additional file 5: Fig S4), only less than 10 % of the initially applied SMZ is available for rapid degradation. Our data show that even after 112 days, when SMZ was applied again, the SMZ-mineralizing function was still present, which emphasizes the long-term sustainability of the inoculation approach. As the manure is typically applied to the fields twice per year, the veterinary residues are reaching the grounds discontinuously. In between, the bioavailability of SMZ is very low due to sorption processes, and the amount of SMZ is probably not sufficient for energy support of the SMZ-degrading microbes. Hence, they have to survive for a long time

under limited SMZ concentrations using other carbon and energy sources, provided by the soil environment. Yet, the bacterial community conserves its SMZ-degrading capability for at least 112 days to restart the degradation process after another SMZ application.

Since there was no difference in the SMZ mineralization capacity of the inoculants MCCP-LA and MCCP-HA after the second SMZ application, it can be concluded that on the long term, the SMZ mineralization capacity of the inoculants mainly depends on the SMZ concentration as compared with its initial SMZ mineralization activity.

The enhanced SMZ mineralization, measured after the first ^{14}C-SMZ application, as well as after the second application is exclusively due to the inoculated microbial community, since the "adaptation" experiments have clearly excluded adaptation processes as a reason for accelerated SMZ degradation. In a third phase, the carrier particles were separated from the bulk soil, before SMZ was applied for a third time (Additional file 7: Fig S6; Additional file 8: Fig S7). In the bulk soil without carrier particles, 56 % of the applied SMZ was degraded after 62 days. These results reveal that microbes were able to proliferate in the soil matrix and continue the SMZ degradation there, which is a very important factor to ensure the success of this application approach. There is no need to apply the carrier particles again, after the microbes

Table 1 ^{14}C mass balance at the end of the incubation experiment

Treatment	$^{14}CO_2$	^{14}C in soil pore water	^{14}C extractable	^{14}C non-extractable	^{14}C recovery
MCCP-HA	51.4 ± 0.3	0.5 ± 0.0	5.1 ± 0.0	42.9 ± 0.8	100.0 ± 0.6
Control	1.8 ± 0.0	not measured	14.2 ± 0.5	81.9 ± 0.5	97.8 ± 1.1

Percent distribution and mass balance of ^{14}C in soil Scheyern1 after 161 days of incubation with MCCP-HA in % of applied ^{14}C-radioactivity. MCCP-HA = Microbial community attached on carrier particles, group of higher initial SMZ mineralization activity; values represent mean $\pm$ standard deviation; n = 3

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have established in the bulk soil. Therefore, we strongly recommend this very effective, sustainable, and stable soil inoculation approach, using a microbial community which supports the degrading strain, and carrier particles for a stable soil inoculation, for remediating soils.

Formation of non-extractable ^{14}C-residues (NER)

In the control samples, the indigenous microbial community was unable to degrade SMZ in a considerable amount and more than 80 % of the applied ^{14}C were found in the NER fraction, which is likely to consist of SMZ and/or SMZ degradation products physico-chemically bound to the soil matrix. The type of binding was not investigated in this study. Several binding mechanisms can play a role in the formation of non-extractable residues, for example, Bialk et al. [37] have proven covalent cross coupling between SMZ and model humic constituents. The rapid formation of high amounts of NER was confirmed by other studies for different sulfonamides [38–40]. Though high affinity to the soil matrix and negligible uptake of sulfonamide-NER by plants and earthworms was shown [39], it cannot be excluded that the NER poses a certain risk for human health, since they can be remobilized during the natural turnover processes of soil organic matter and subsequently contaminate environmental compartments and become bioavailable.

In the inoculated soil samples, the main removal process of SMZ from soil was mineralization, followed by formation of NER. According to the high SMZ mineralization, it can be concluded that the NER in this case are mainly of biogenic origin. Principally, degrading microorganisms can use a xenobiotic as energy and carbon source. When used as carbon source, the degrading bacteria assimilate carbon derived from the xenobiotic to form cellular components, such as sugars, amino acids, etc., and after death of the bacteria, such biomolecules will be fixed in soil organic matter [41]. Thus, the applied inoculation technique enhances the mineralization of SMZ and decreases the risks caused by NER, because they consist to a large amount of biomolecules instead of SMZ or its degradation products.

Résumé of the inoculation approach

The approach for enhanced mineralization of a soil contaminant by inoculating the soil with a degrading microbial community established on carrier material has already been successfully applied in our previous studies for the herbicide isoproturon and the environmental chemical 1,2,4-TCB [31–33]. Accelerated mineralization of the herbicide isoproturon was shown not only in laboratory experiments, but also under field conditions [31].

This study confirms our hypothesis, as this approach could remarkably enhance the mineralization of the veterinary antibiotic SMZ in soil, despite the considerably low bioavailability of the tested substance SMZ (Additional file 5: Fig S4). The microbial community was successfully established on carrier particles and survived in the new soil habitat, enhancing the SMZ mineralization.

The success of this approach can be mainly explained by three reasons:

1. The results of our former studies [32, 33] indicate that the introduced bacteria can establish a protective biofilm on the carrier material. It is known that cells in biofilms have a distinct advantage over submerse bacteria, for example, they have a better chance of survival especially under unfavorable conditions [42–44]. We also found hints for an inter-species communication within the biofilm on the carrier material that promotes the contaminant's mineralization [33].
2. There are strong indications that the contaminant in soils can be transported to the degrading community via diffusion, resulting in mass transfer of the substance to the degradation location on the particles [31].
3. As shown in the present in situ study, this approach is sustainable and the SMZ-degrading function can be established in the new soil environment. Transfer of genetic information from the introduced degraders to the native soil microorganisms is a slow-acting process and happens primarily under high selection pressure, i.e., high concentration of the contaminant, which is normally not the case in agricultural soils. Therefore, it can be assumed that the SMZ-degrading function is performed by the introduced degraders which are able to survive and establish in the new habitat; as has been already verified for the 1,2,4-TCB degrader *Bordetella* sp. F2 for a time span of 30 days [32].

Conclusions

Our specific approach of inoculating microbial communities on carrier material was successful for the antibiotic SMZ, as well as for two other substances with different physico-chemical properties and other microbial communities in various soils. As this inoculation approach is successful and sustainable even for SMZ as an antibiotic used against bacteria which is also showing a relatively low bioavailability, we conclude that it is probably also applicable for many other contaminants in soils. Due to the highly enhanced mineralization conducted by the microbial community containing the degrading strain *Microbacterium* sp. C448, we strongly recommend the utilization of the appropriate microbial community instead of an isolated degrading strain when developing soil remediation methods.

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Additional files

Additional file 1: Table S1. Soil characteristics of all soils used in this work. The main characteristics, the natural site name and the soil classification are provided, as well as the specific soil water content (%) equivalent to the soil water tension of pF 2.18 for every soil.

Additional file 2: Fig S2. Gene sequencing of the soil microbial community used for soil inoculation.The composition of the microbial community enriched from soil was analysed by DNA extraction and determination of the 16S rRNA-gene sequence by illumina sequencing. The degrading strain *Microbacterium* sp. C448 can be found within the group of "others", as the DNA of the *Microbacteriaceae* appeared to less than 1 % of the totally analysed DNA in this sample. Most sequences were analysed to genus level. *Analysis only allows identification on family level. **Analysis only allows identification on order level. ****Analysis only allows identification on phylum level.

Additional file 3: Fig S3. Sulfamethazine mineralization rates in liquid cultures. Mineralization rates were measured via $^{14}CO_2$. Bacteria were transferred from liquid culture to soil (on CP) on day 15. The two variants were: HA: the liquid cultures were incubated with another 5 µg g^{-1} of SMZ on day 8. LA: the liquid cultures did not receive another SMZ application. n = 3, bars indicate standard deviation.

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Additional file 5: Fig S5. In situ bioavailability of SMZ in soils. Decrease of in situ bioavailable ^{14}C-SMZ was measured via soil pore water extraction of three soil types over time.

Additional file 6: Fig S6. Mineralization rates after soil inoculation with MCCP. The ^{14}C-SMZ mineralization results after the first SMZ application and soil inoculation with microbial community on carrier particles (MCCP), which are also presented in Fig. 2a in the manuscript. Here, the whole incubation time of 112 days is shown. Values represent mean ± standard deviation; n = 3; control signifies "soil without inoculation".

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Abbreviations
ISCP: isolated strain established on carrier particles; MCCP: microbial community established on carrier particles; MCCP-HA: microbial community with higher initial mineralization activity in soil; MCCP-LA: microbial community with lower initial mineralization activity in soil; NER: non-extractable ^{14}C-residues; SMZ: sulfamethazine.

Authors' contributions
NH, writing—original draft preparation, conceptualization, project administration and realization. ET, validation, provision of soil samples, and

Microbacterium sp. strain C448. UD, writing—review and editing, validation. ES, supervision, validation. JCM, writing—review and editing, supervision. RS validation, conceptualization, and supervision. All authors read and approved the final manuscript.

Author details
[1] Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany. [2] Agriculture and Agri-Food Canada, London Research and Development Centre, London, ON N5V 4T3, Canada. [3] Institute of Microbiology and Biotechnology, Ulm University, 89069 Ulm, Germany. [4] Lehrstuhl für Grünlandlehre, Technische Universität München, 85354 Freising, Germany.

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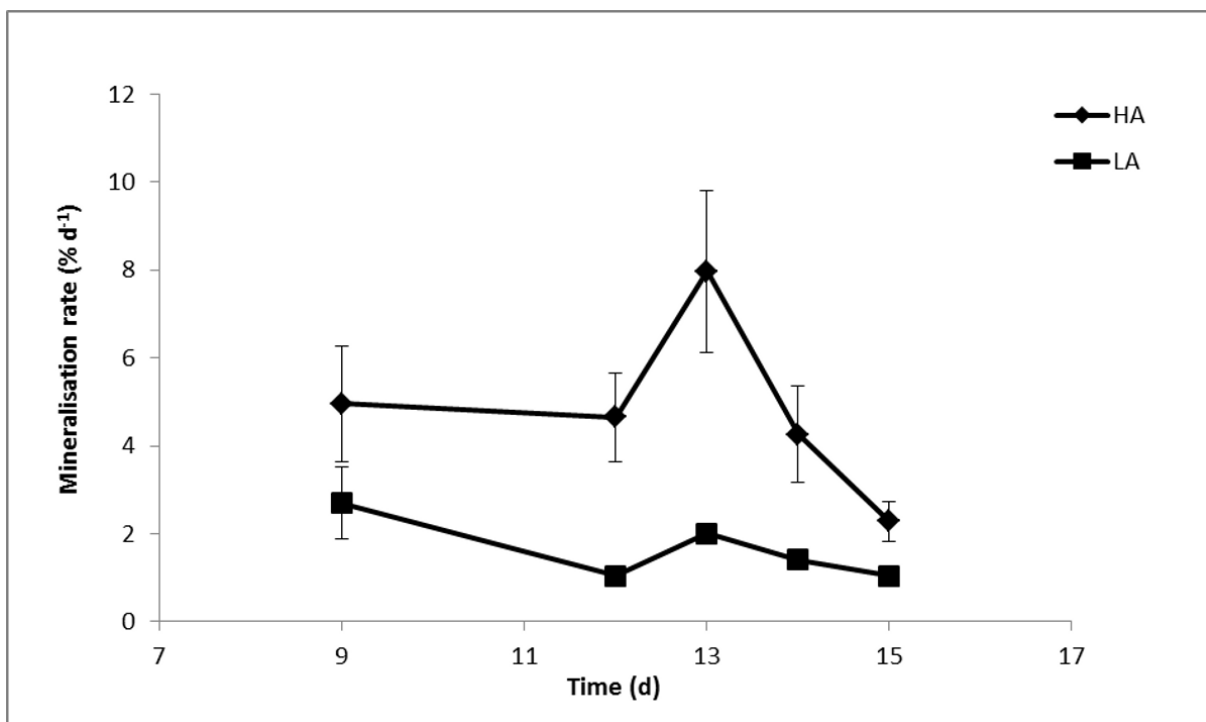
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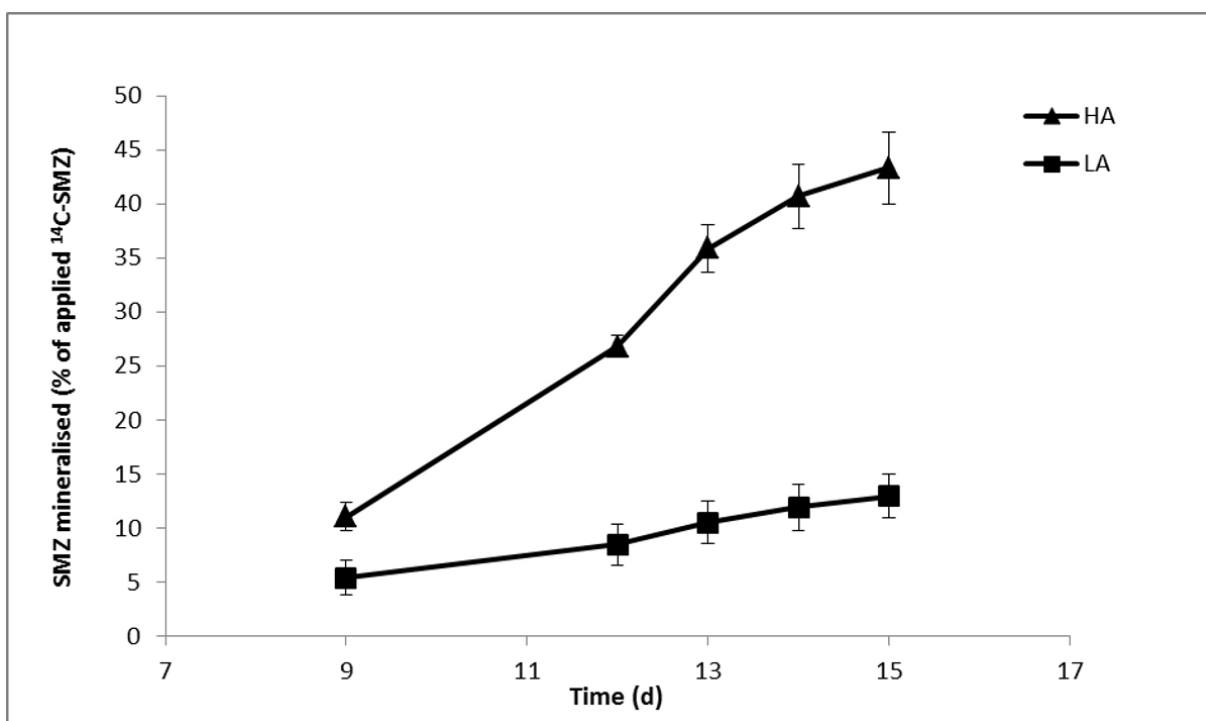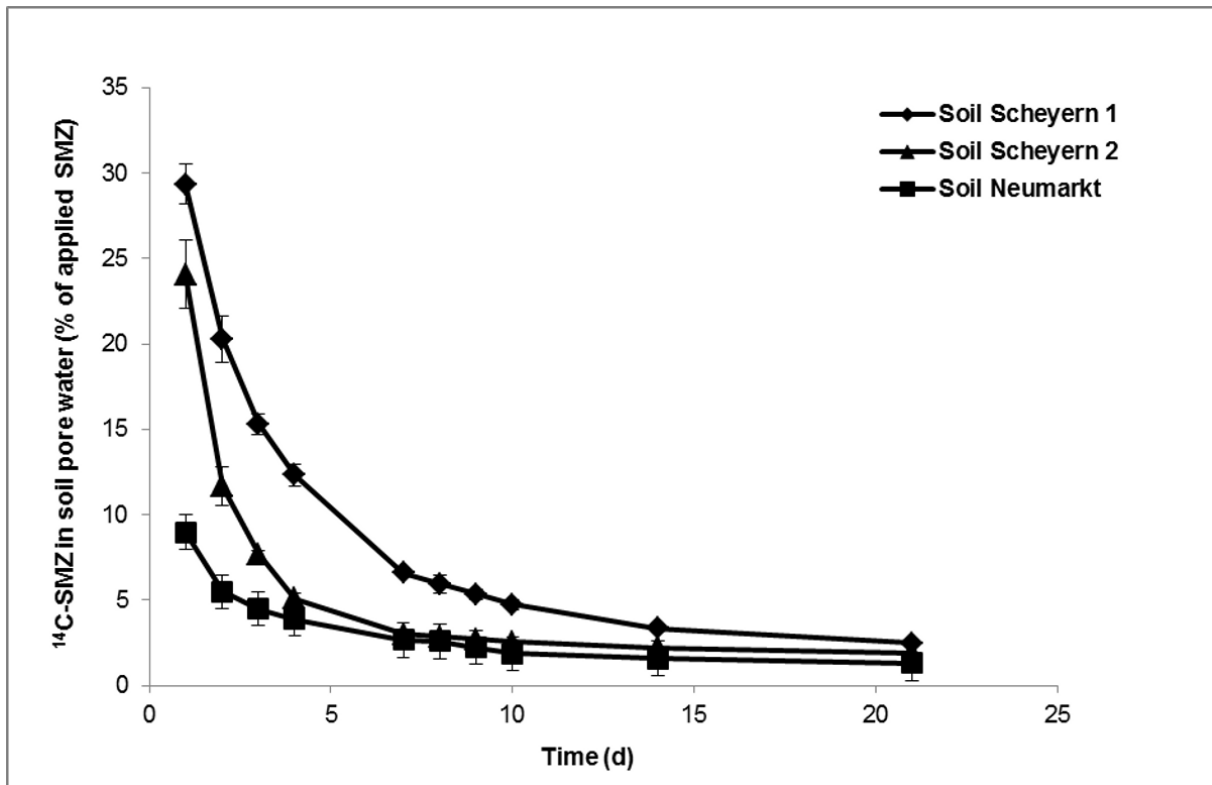| Natural site name classification	Scheyern 1 aric anthrosol sandy loam	Scheyern 2 mollic gleysol silty loam	Neumarkt haplic arenosol sandy soil	Hohenwart aric anthrosol loamy sand
Clay (%)	20	22	4	13
Silt (%)	40	60	8	19
Sand (%)	40	18	88	68
pH CaCl2	7.8	5.4	5.8	6.7
TOC	1.5	1.5	1.0	1.0
Total N	0.2	0.2	0.1	0.1
Water content at pF 2.18 (%)	27.38	29.57	10.79	16.14

Additional file 2: Fig S2. Gene sequencing of the soil microbial community used for soil inoculation. The composition of the microbial community enriched from soil was analysed by DNA extraction and determination of the 16S rRNA-gene sequence by illumina sequencing. The degrading strain *Microbacterium* sp. C448 can be found within the group of "others", as the DNA of the *Microbacteriaceae* appeared to less than 1 % of the totally analysed DNA in this sample. Most sequences were analysed to genus level. *Analysis only allows identification on family level. **Analysis only allows identification on order level. ****Analysis only allows identification on phylum level.
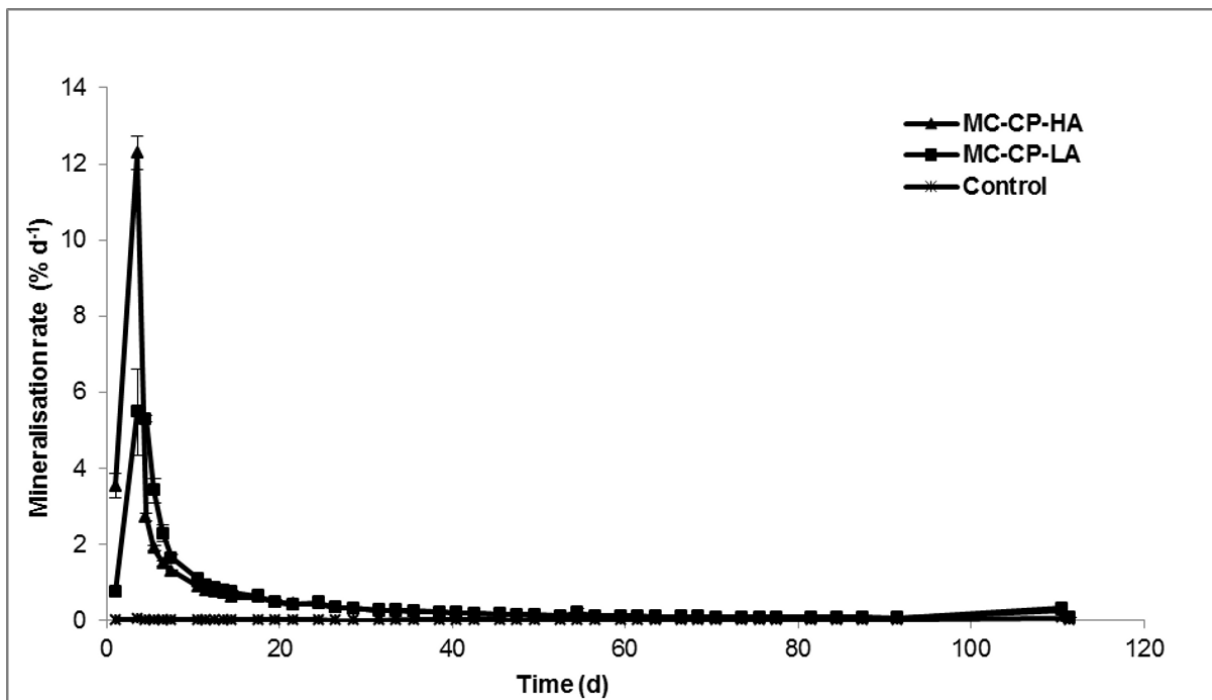
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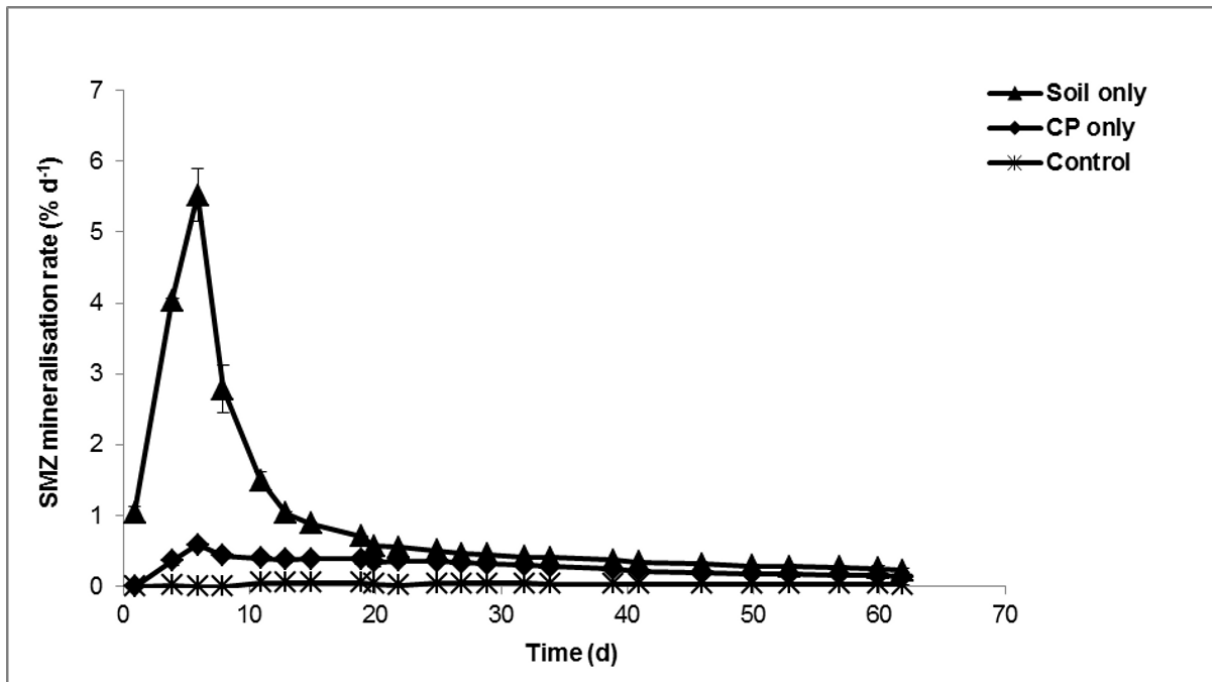

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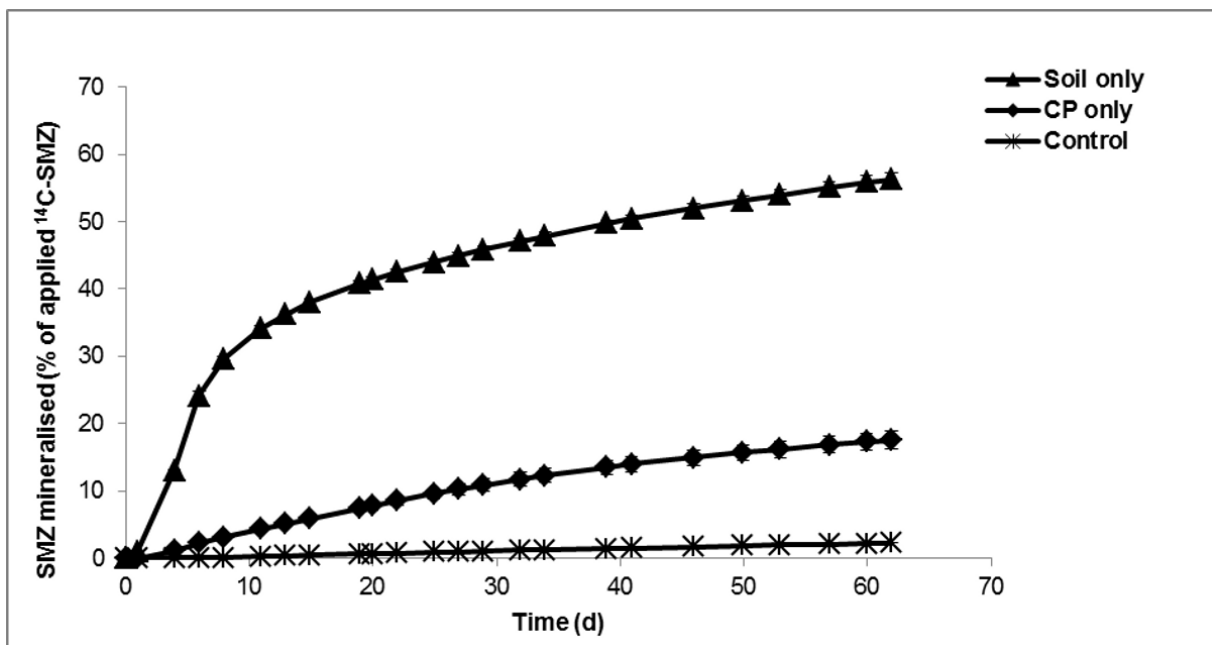
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Curriculum vitae

Personal information

Name:	Natalie Seyfried, née Hirth
Address:	Oberesslingerstraße 15, 73732 Esslingen
Date and place of birth:	29.08.1984, Stuttgart

Tertiary education

12/2012-10/2016	PhD project at the Technische Universität München, Chair of Soil Ecology and the Helmholtz Zentrum München, Institute of Soil Ecology, working group of Dr. Reiner Schroll, with the title "Accelerated microbial degradation of the veterinary antibiotic sulfamethazine in agricultural soil", doctoral advisor: Jean Charles Munch
10/2005-09/2011	Study of Biology at the University of Ulm (degree: Diploma) Main subjects: **Ecology, Microbiology, Pharmacology and Toxicology.** Diploma thesis „Elektronenmikroskopische Untersuchung von Chloroplasten in *Elysia timida* (Opisthobranchia, Sacoglossa) mithilfe von Kryo-Präparationsmethoden" samples taken on Giglio (Italy) at the Institute for Marine Biology (ifmb)
06/2010	Hands-on training in „environmental microbiology", investigating antibiotic resistant bacteria, DNA isolation, determination of germ total
12/2008	Hands-on training in „Watercourse", evaluating water quality classes using saprobic index and characteristic organisms

Stay abroad

08/2009	Marine biological excursion to Denmark
06/2009	Ecological excursion to Croatia
10/2004-02/2005	Stay in Australia with work & travel

Secondary education

09/1995-06/2004	Allgemeine Hochschulreife am Schelztor Gymnasium Esslingen