Structural Diversity of Soil Microorganisms as a sensitive Indicator of adverse Effects from Pharmaceutical Antibiotics

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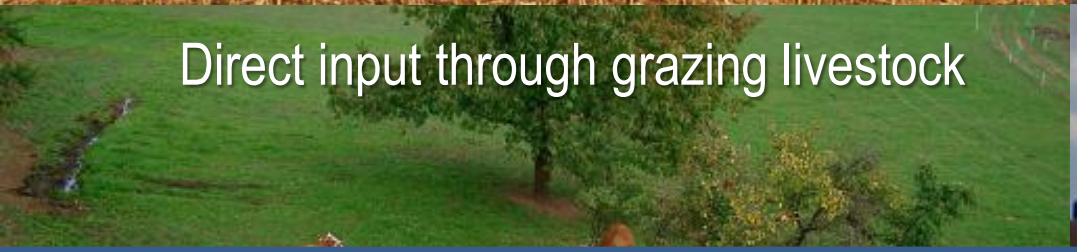
Introduction

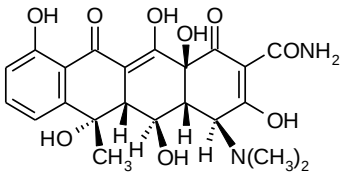


Consumption of antibiotics for livestock, t yr^{-1}

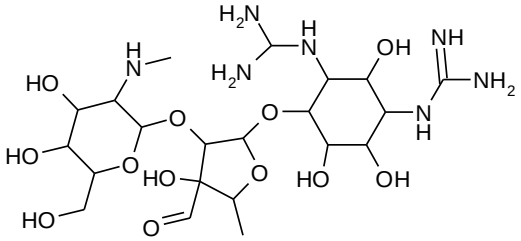
(Kim et al. 2011, Schneidereit 2012, Sarmah et al. 2006)

UK	France	Netherlands	Denmark	Germany	USA	NZ	Korea	Kenya	China	Russia
VMD, 2004	ANMV, 2003	FIDIN, 2004	DANMAP, 2005	BfT, 2012	Benbrook, 2002	MAF 1999	KFDA 2006	1995-99		
476	1,261	453	114	1,734	11,148	19	1,278	13	non-medical drugs	

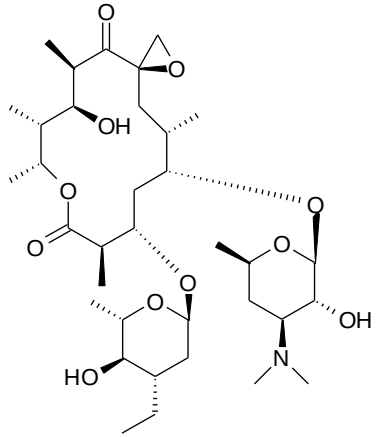
Molecular structures of selected antibiotics from often used structural classes



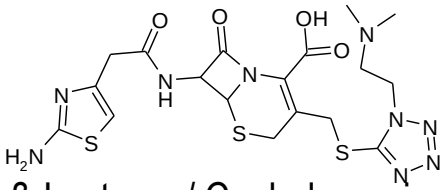
Tetracyclines: Oxytetracycline



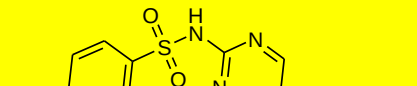
Aminoglycosides: Streptomycine



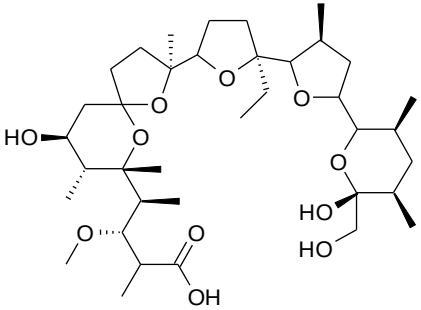
Macrolides: Oleandomycine



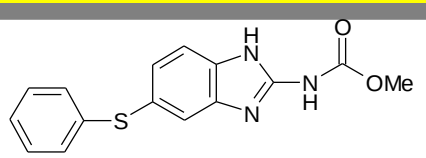
ß-Lactams / Cephalosporines: Cefotiam



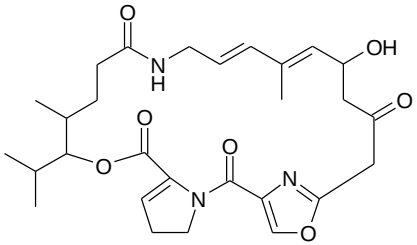
Sulfonamides: Sulfadiazine



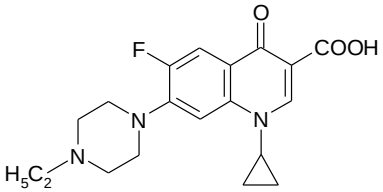
Polyethers: Monensin



Benzimidazoles: Fenbendazole



Polypeptides: Virginiamycine



Fluoroquinolones: Enrofloxacine

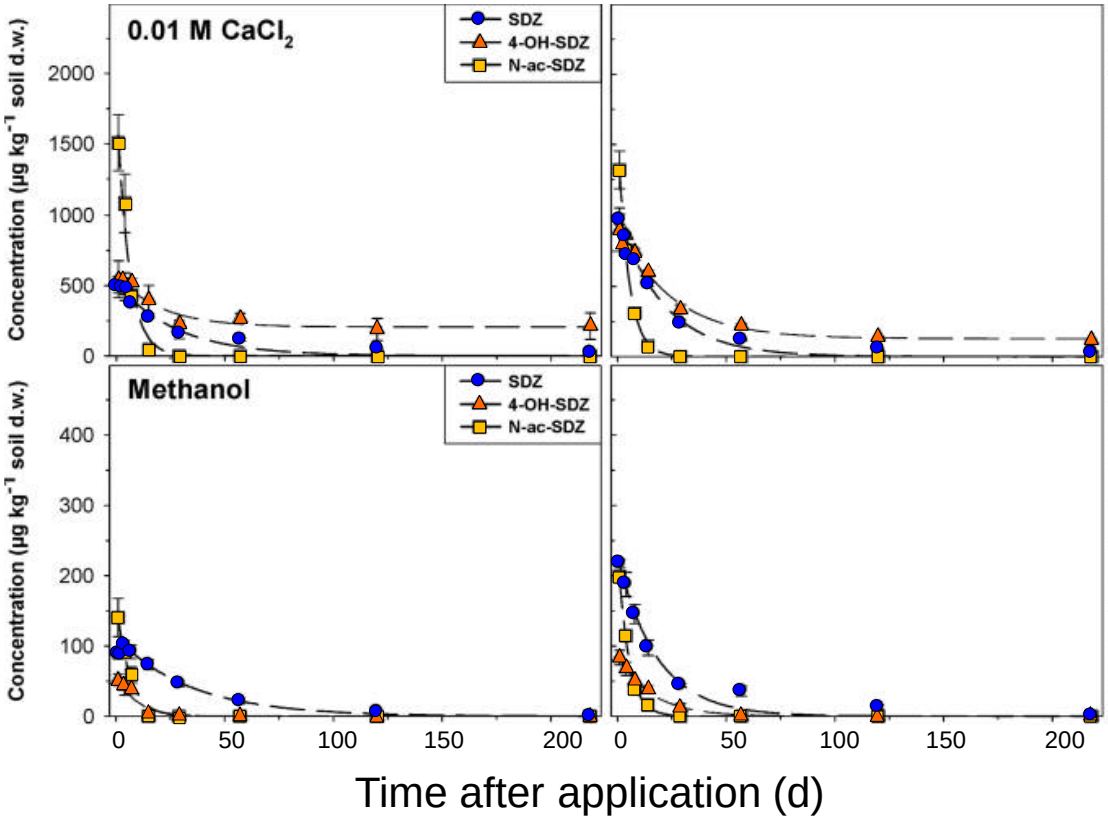
Thiele-Bruhn S. (2003) Antibiotika. In: Blume et al. (eds.) *Handbuch der Bodenkunde*. Chap. 6.5.5, 1-19.

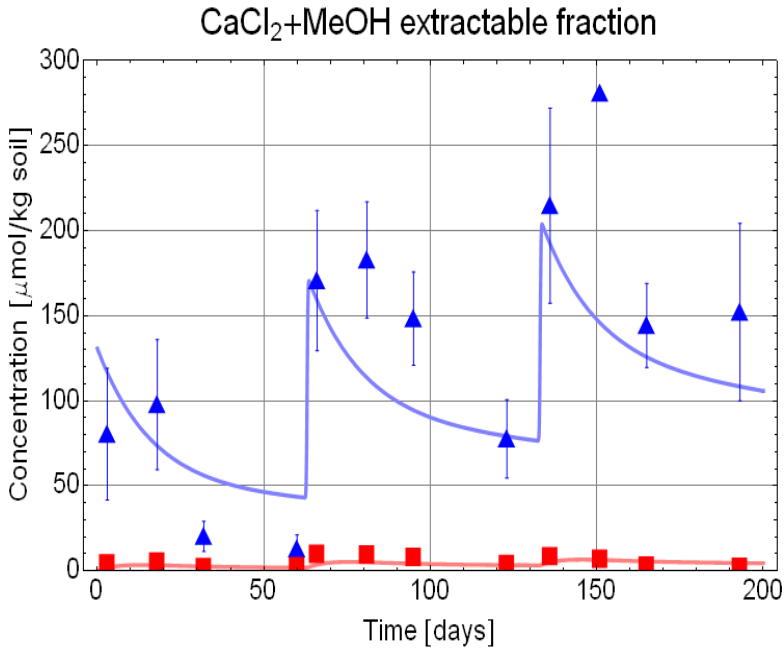
Concentrations of SDZ and major transformation products in $CaCl_2$ and methanol extracts from soil

(model fits = dashed lines).

(Förster et al. 2009. Env. Sci. Technol. 43, 1824-1830)

Luvisol + manure | Cambisol + manure



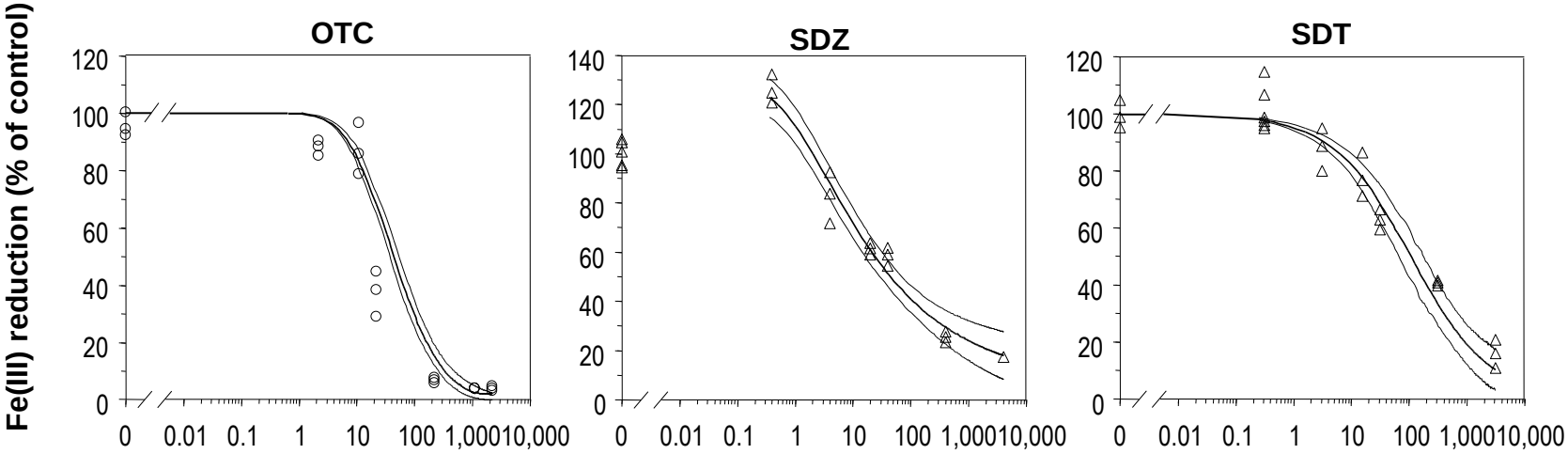


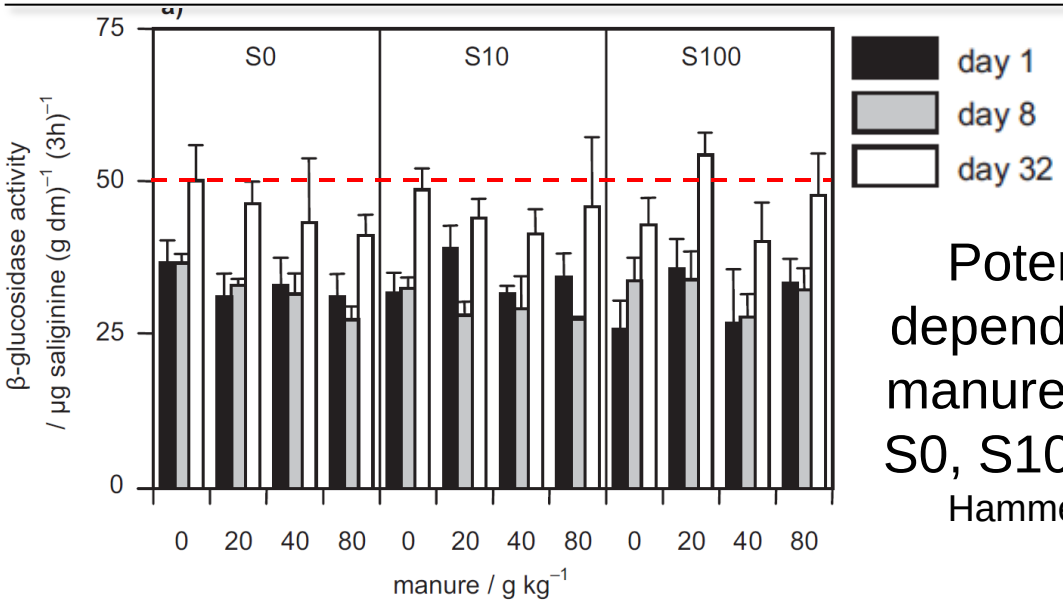
SDZ concentration in soil that was repeatedly treated with contaminated manure.

(Data: DFG FOR566, A. Focks)

Dose-related effects of antibiotics on microbial Fe(III)-reduction in Luvisol-Ah.

Thiele-Bruhn (2005) Environ. Toxicol. Chem. 24:869–876

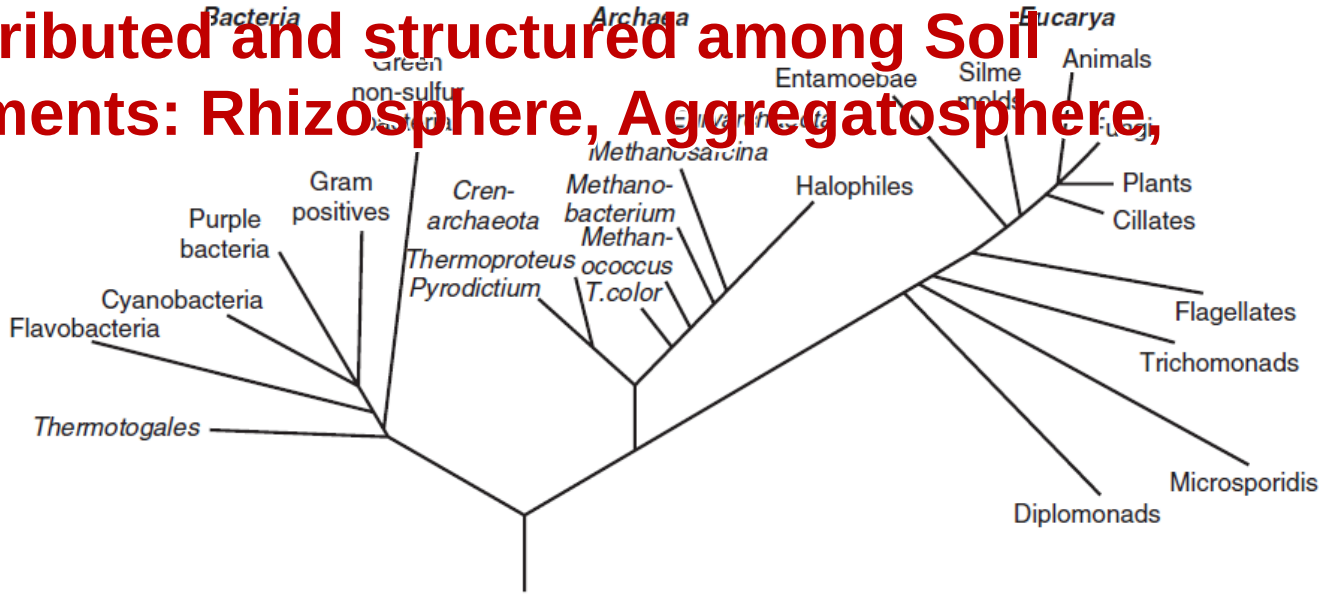




Potential activity of ß-glucosidase depending on sample treatment (liquid manure and SDZ) and incubation time. S0, S10, S100 = SDZ spiking C mg/kg.

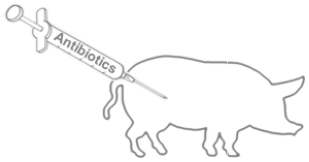
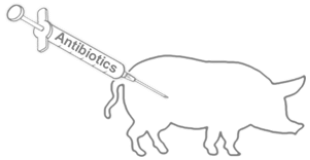
Hammesfahr et al. (2011) JPNSS 174, 614-623.

- Mixed Communities with high Abundance of Microorganisms.
- ➢ Structural Diversity of the Soil Microbial Community.
- Differently distributed and structured among Soil Microcompartments: Rhizosphere, Aggregatosphere, Drillosphere.



lter.kbs.msu.edu/.../ SEM_microbes_in_soil.jpg

Experimental Design

Lab. exp.	Microcosm/Pot exp.	Mesocosm	Field Experiment
---------- Sandy Cambisol and Topsoil of a Luvisol from Loess ----------			
No plants	*Zea mays* L. (Cultivar RR39K13, Pioneer Hi-Bred)		
	Soil + pig slurry (control) ↓		
	Soil + pig slurry with SDZ /DIF ↓		
varied doses	1 / 10 / 100 mg SDZ	0.3 mg SDZ	1 mg SDZ (kg^{-1} soil)
flasks	pots à 0.004 m^3	containers à 0.5 m^3	field plots à 3 m^2
Application↓:			
1 x	1 x 	1x 	3x
Soil sampling (d):			
1, 4, 32, 61, 125	↓, 1, 4, 30, 60	-1, ↓0, 6, 13, 27, 41, 60	↓, 14, 48, ↓ 56, 132, ↓140, 252

Experimental Design - Soil microbial community analysis

DGGE of 16S rRNA genes
(Heuer et al. 2002)
↓
universal bacterial primers
↓
group specific bacterial primers:
pseudomonas, α-proteobacteria, ß-proteobacteria, actinomycetes, streptomycetes
↓
Sequencing
selected bands

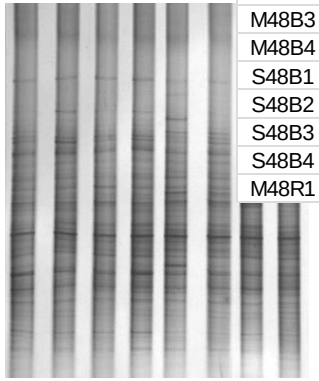
Phospholipid fatty acids (PLFA)
(Zelles & Bai 1993)
→ **GC-FID, GC-MS**
→ **various markers for bacteria & fungi**

Enzyme activities
N-cycle
C-cycle

Microbial biomass

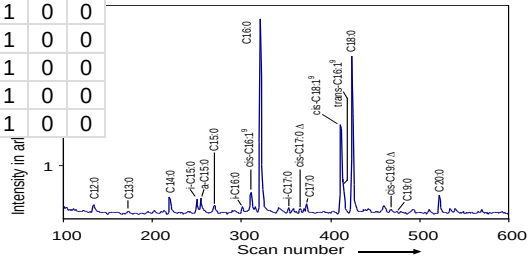
Fluorescence in-situ hybridization (FISH) and **staining methods**
Markers:
EUB338-I: active bacteria
DAPI: DNA

Statistics
ANOVA + HSD post-hoc ($p < 0.05$)
Principal Response Curves (PRC)
DA, CA and PCA



DGGE of 16S rRNA genes

M48B1	1	0	0	0	1	0
M48B2	1	0	0	0	1	0
M48B3	1	0	0	1	0	0
M48B4	1	0	0	1	0	0
S48B1	0	1	0	1	0	0
S48B2	0	1	0	1	0	0
S48B3	0	1	0	1	0	0
S48B4	0	1	0	1	0	0
M48R1	0	1	0	1	0	0



GC-FID/GC-MS of PLFA

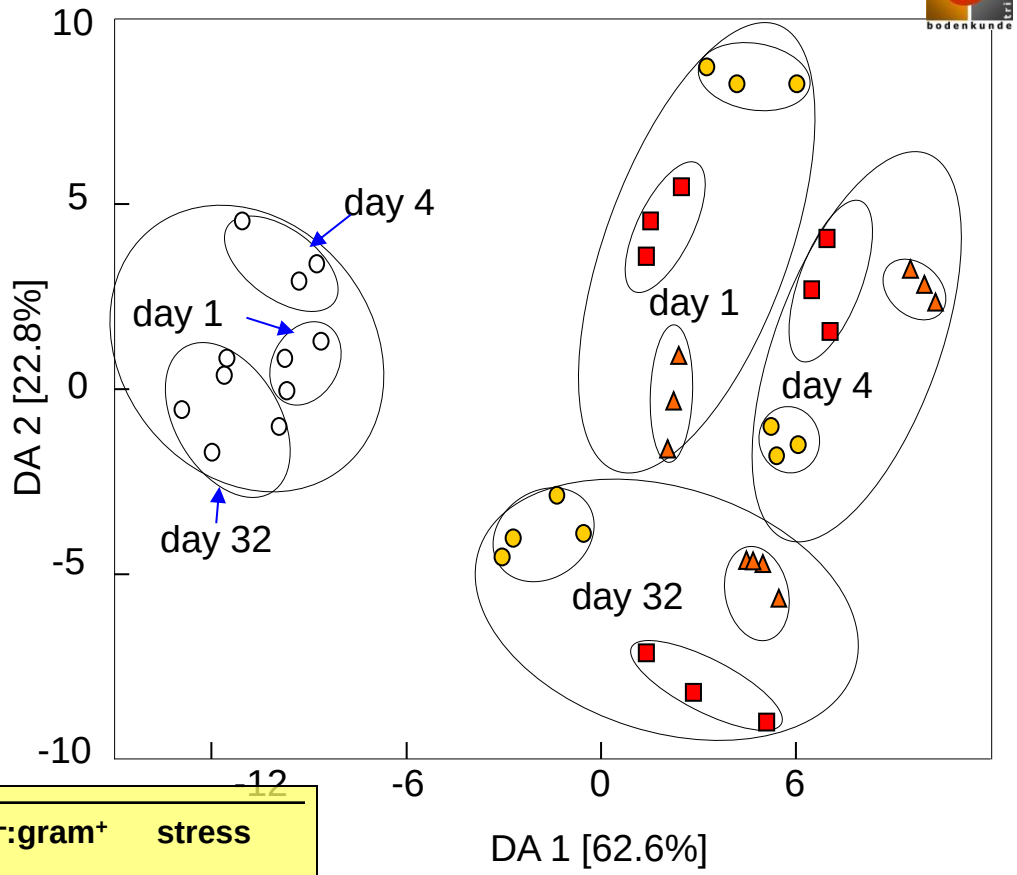
FISH and DAPI staining

DNA sequencing

Discriminant analysis of PLFA from soil treated with manure and SDZ

- unfertilized soil
- + manure
- +manure + 10 mg SDZ kg^{-1}
- +manure + 100 mg SDZ kg^{-1}

(Hammesfahr et al. (2008) Soil Biol. Biochem. 40, 1583-1591)

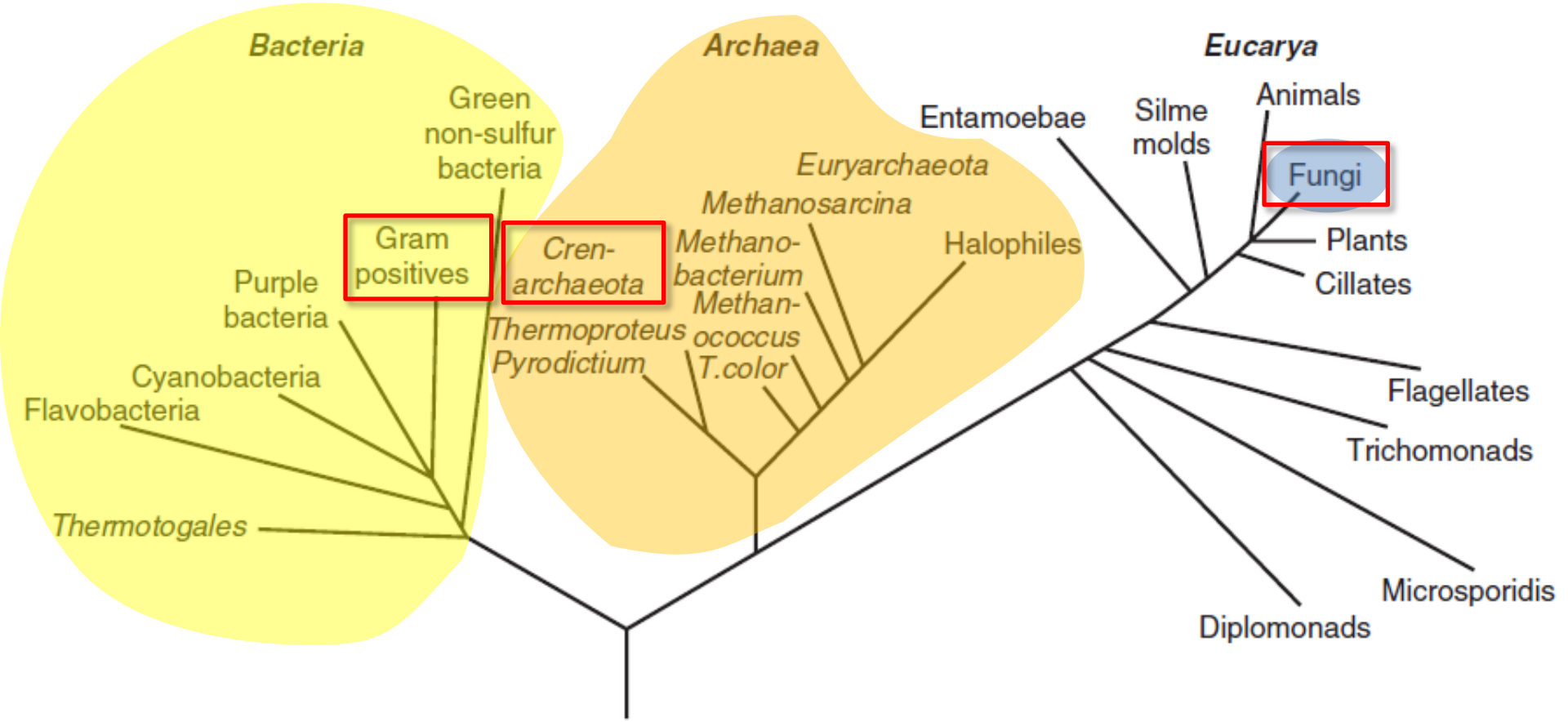


Cambisol KS

3-factorial ANOVA

Factor	$PLFA_{tot}$	bac:fungi	gram⁻:gram⁺	stress
Soil	92.2 ***	59.3 ***	31.1 ***	8.1 **
Treatment	30.0 ***	56.6 ***	3.4 *	13.5 ***
Time	20.3 ***	6.2 **	20.9 ***	2.6
Soil x Treatment	7.1 ***	5.4 **	3.1 *	6.3 ***
Soil x Time	10.9 ***	11.2 ***	1.4	1.0
Treatment x Time	4.2 **	3.6 **	1.0	0.4
Soil x Treatment x Time	2.2	1.3	1.3	0.8

Antibiotic effects on soil microbial community structure

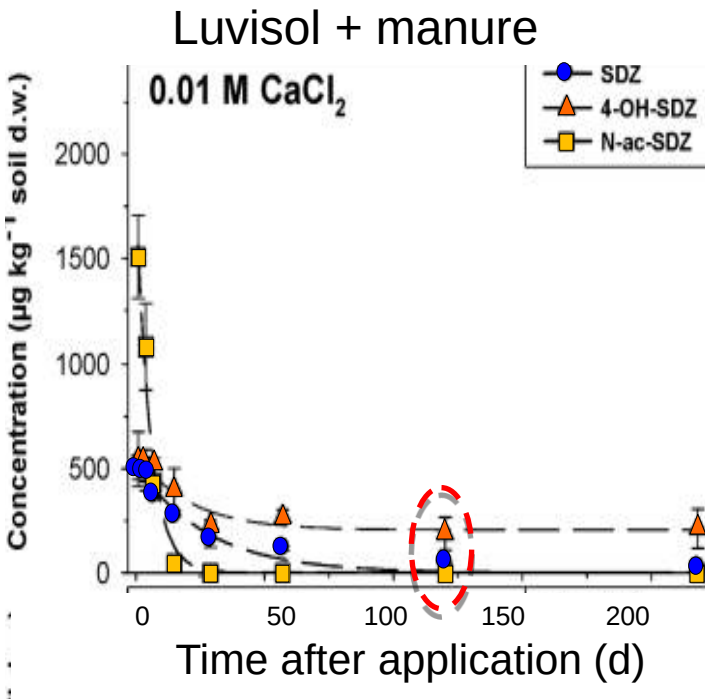


The universal tree of life

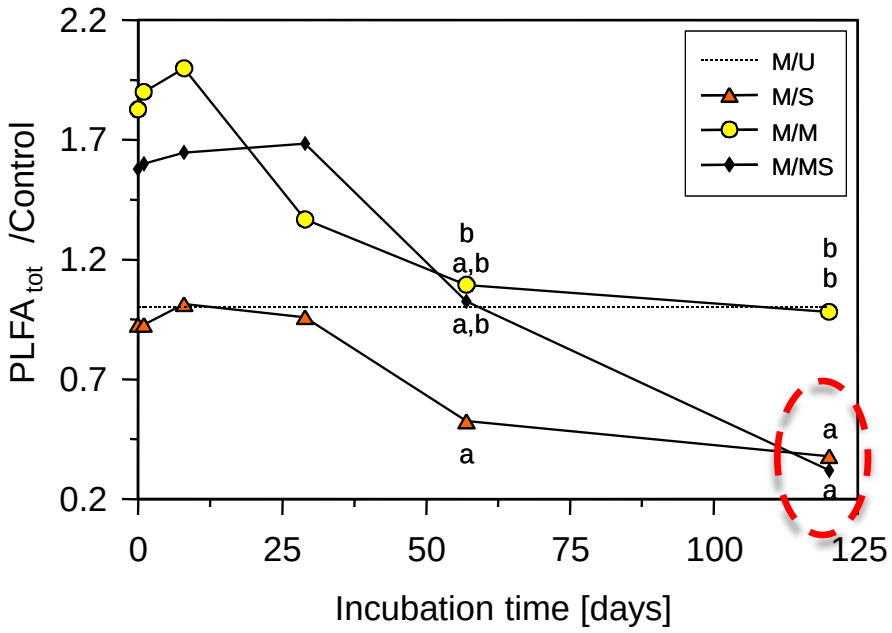
Paul E.A. (2007) Soil Microbiology, Ecology, and Biochemistry. 3rd ed.

- **Shift from bacteria to fungi**
- **Shift from bacteria to archaea**
- **Effects on pseudomonads and ß-proteobacteria**
- **Shifts from Gram- to Gram+ bacteria**

Long-term effects on microorganisms



apparent concentration independence

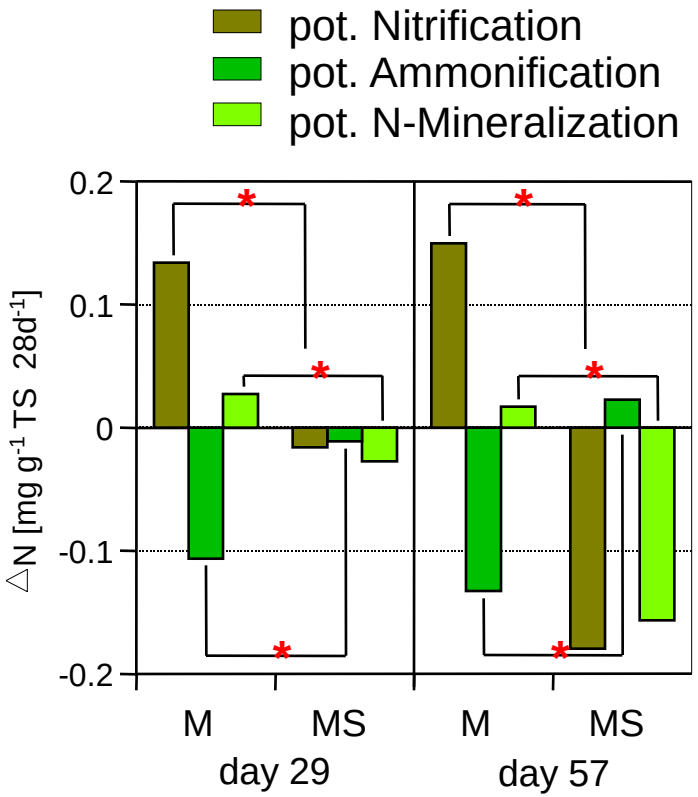


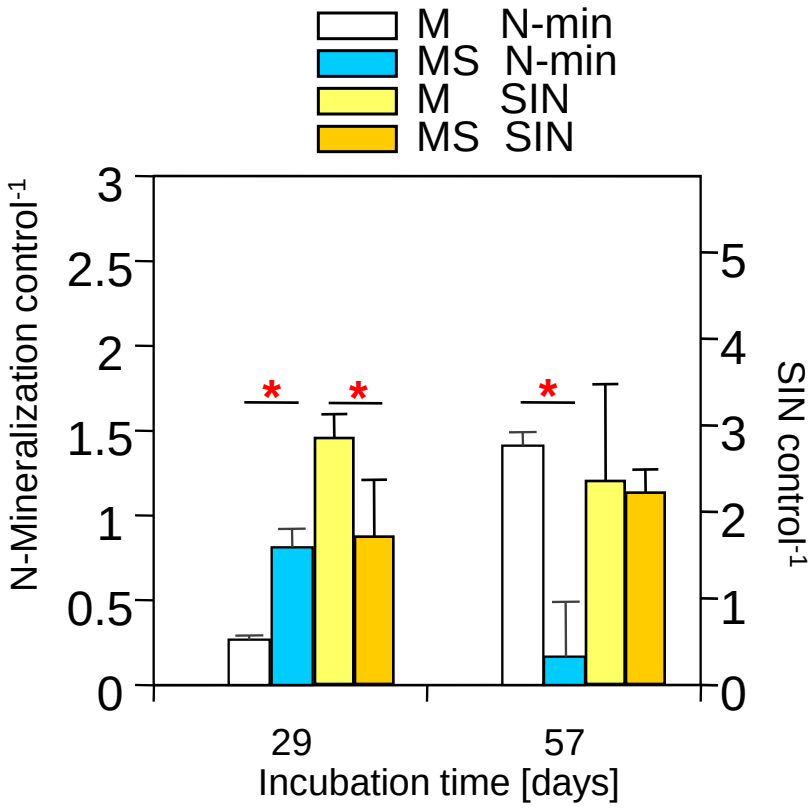
Total-PLFA from Luvisol-Ah Merzenhausen treated with manure and SDZ

- Control
- + 8.6 mg SDZ
- +manure
- +manure + 8.6 mg SDZ

(Hammesfahr 2011, PhD thesis)

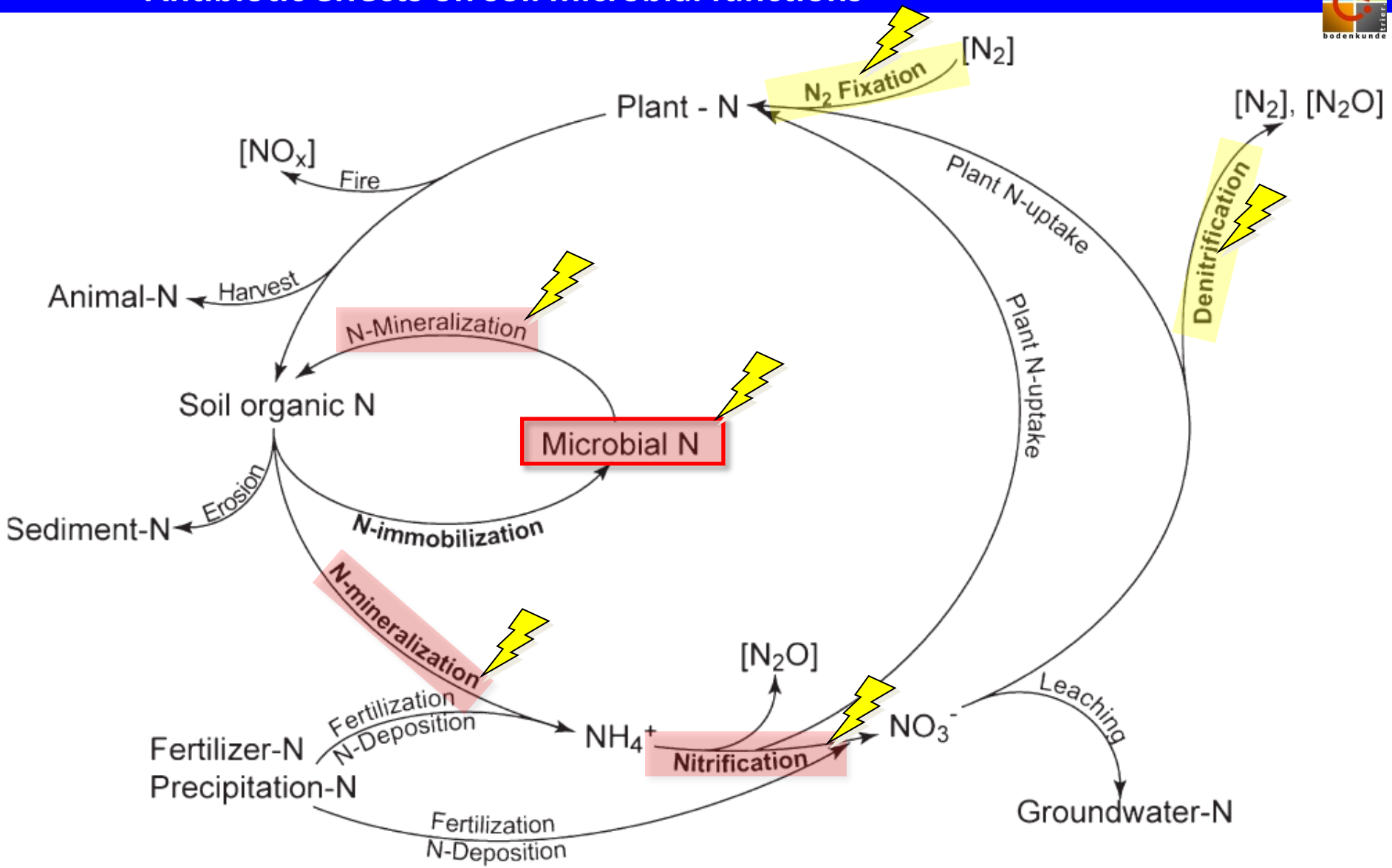
Effect of SDZ in manured soil – lab. experiments – functions





(Hammesfahr et al. (2011) Europ. J. Soil Biol. 47, 61-68)

Factor	pot. Nitrification	pot. Ammonification	pot. N-Mineralization	N-Mineral. control^{-1}	SIN control^{-1}
+/- SDZ	**120*** **	**2162*** **	**9.4** **	**111*** **	**15.6*** **
Storage	0.3	9.9**	0.0	98.6***	9.5**
Time	3.0	1.4	3.6	127**	1.4
Storage x +/-SDZ	0.8	104***	6.6*	39.4***	1.4
+/- SDZ x Time	3.0	27.3***	0.7	4.9*	1.4
Storage x Time	0.0	16.5***	0.4	16.9***	4.0
Storage x +/-SDZ x Time	7.3*	12.1**	4.3*	8.2**	14.7***



Major elements of the terrestrial nitrogen cycle

(from Paul, E.A. (2007) Soil Microbiology, Ecology, and Biochemistry, Elsevier – AP, 3rd ed.)

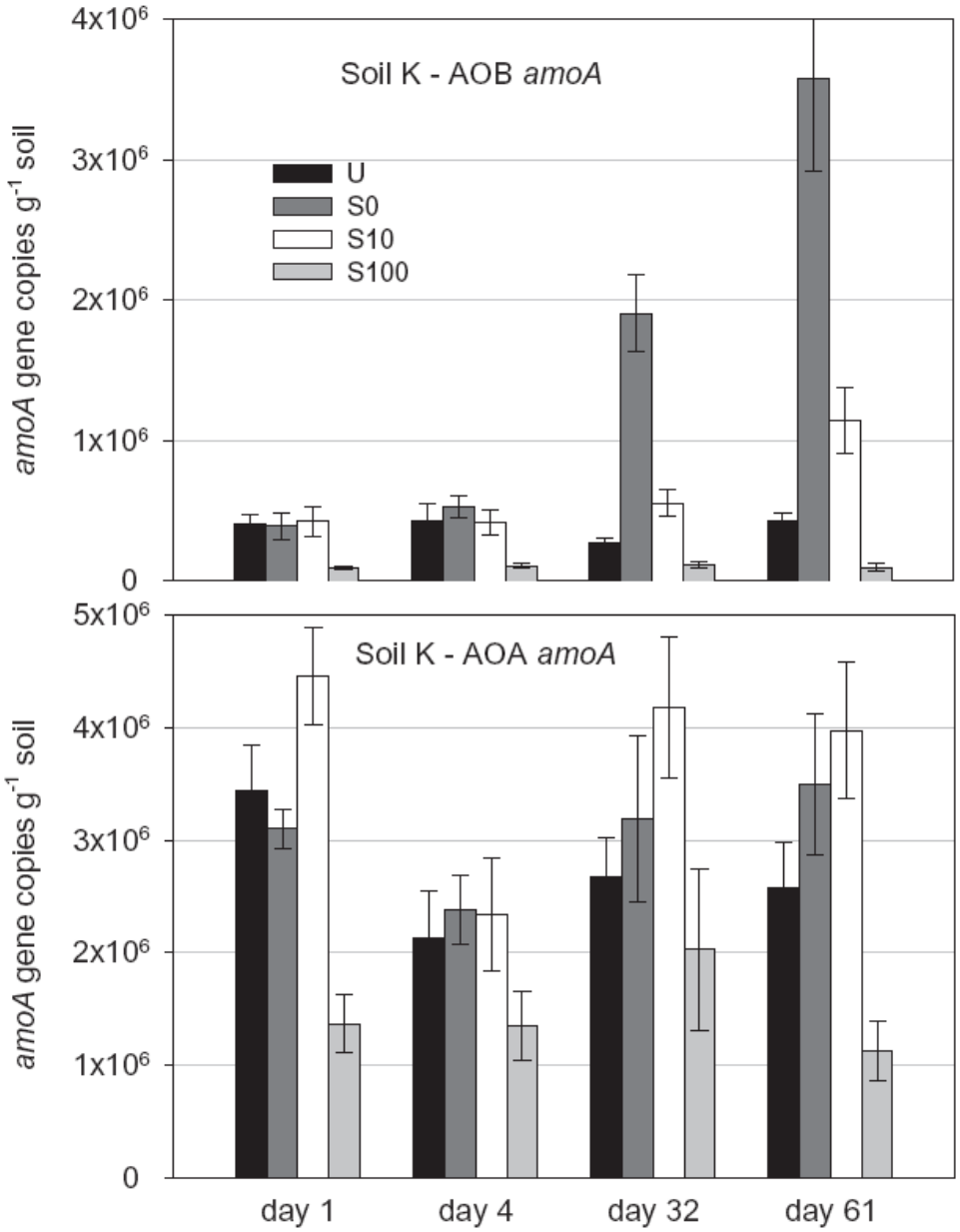
Effects of SDZ on different microbial endpoints in a sandy Cambisol

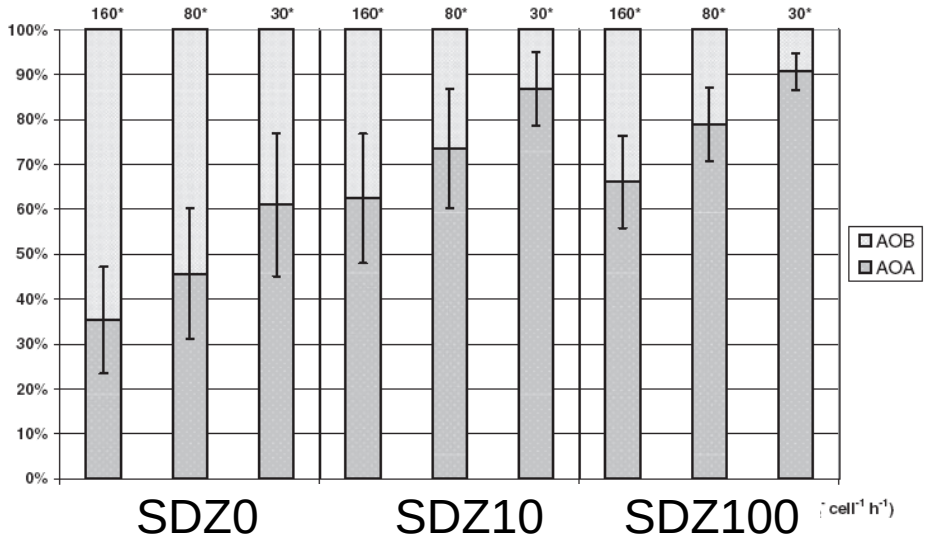
Table 3: Three-way ANOVA of the chemical parameters NH_4-N and NO_3-N, the microbial parameters basal respiration (bas. resp.), microbial biomass C (MBC), the potential activities of the enzymes β-glucosidase (β-gluco.), urease, and protease. Factor 1: liquid-manure concentration (manure) (0, 20, 40, 80 g [kg dm]$^{-1}$), factor 2: SDZ concentration (SDZ) (0, 10, 100 mg [kg dm]$^{-1}$), factor 3: time (1, 8, 32 d). All values are F values with significance level *p* as indicated by asterisks.

Factor	NH_4-N	NO_3-N	bas. resp.	MBC	β-gluco.	urease	protease
Manure	3402***	25.5***	108***	5.3**	6.6***	43.0***	80.2***
SDZ	0.4	384***	0.3	57.6***	0.2	2.6	2.8
Time	816***	117***	36.2***	80.2***	132***	1.9	73.1***
Manure × SDZ	3.1**	97.7***	0.9	17.4***	4.9***	1.2	1.6
Manure × time	134***	12.2***	5.5***	5.8***	1.7	2.6*	13.5***
SDZ × time	5.4***	4.3**	0.3	21.0***	2.2	1.1	5.5***
Manure × SDZ × time	1.7	2.9**	0.6	6.8***	0.9	0.9	1.2

(Hammesfahr et al. (2011) J. Plant Nutr. Soil Sci. 4, 614-623)

Effect of SDZ in manured soil – lab. experiments





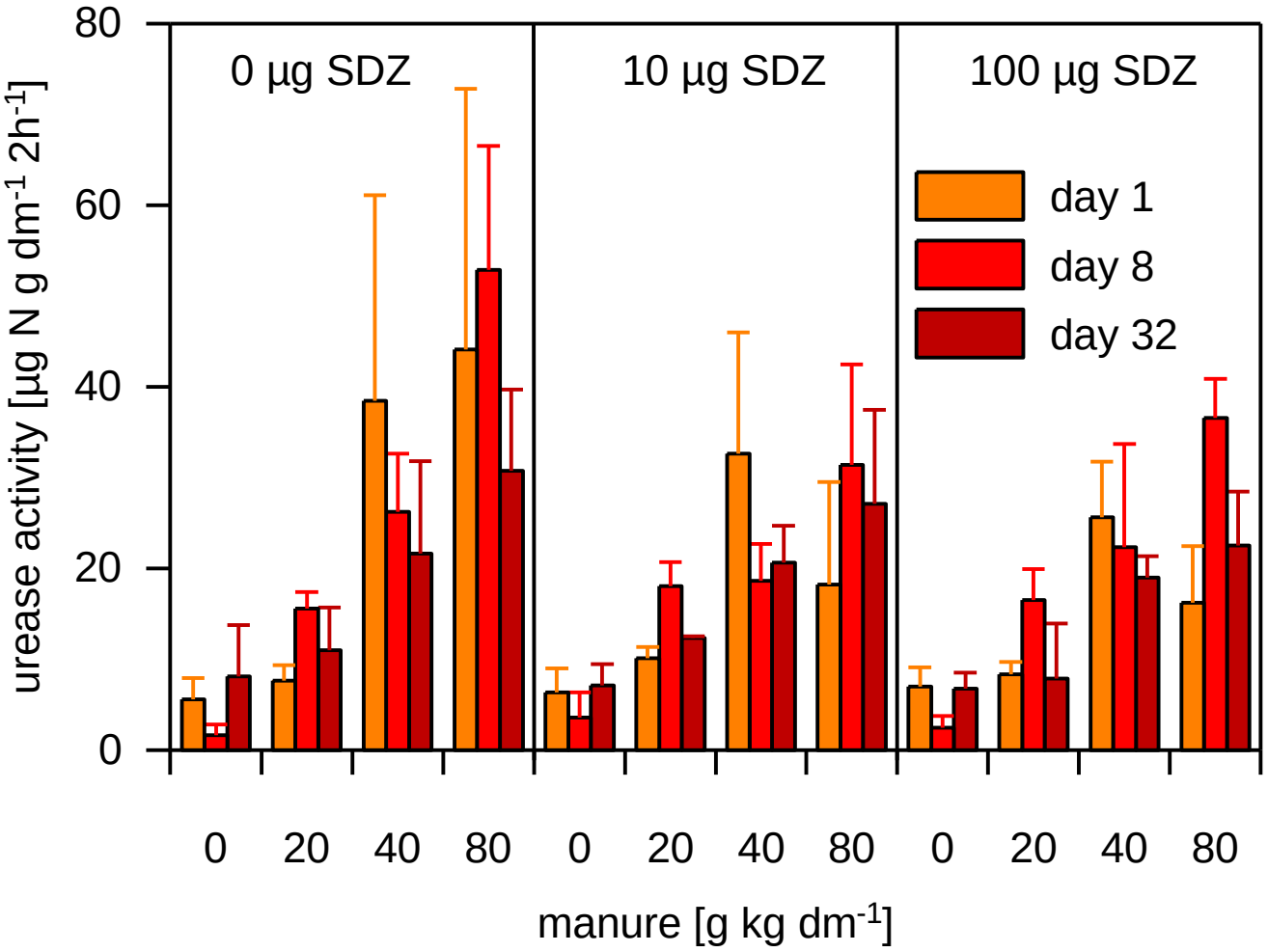
Proportion of AOA and AOB in total simulated nitrate production.

Abundances of AOB and AOA *amoA* genes in soil K in treatments.

Schauss et al. (2009) Environ. Microbiol. 11, 446–456

Functional redundancy

Manure: Influence on SDZ effects in soil – pot experiments – functions

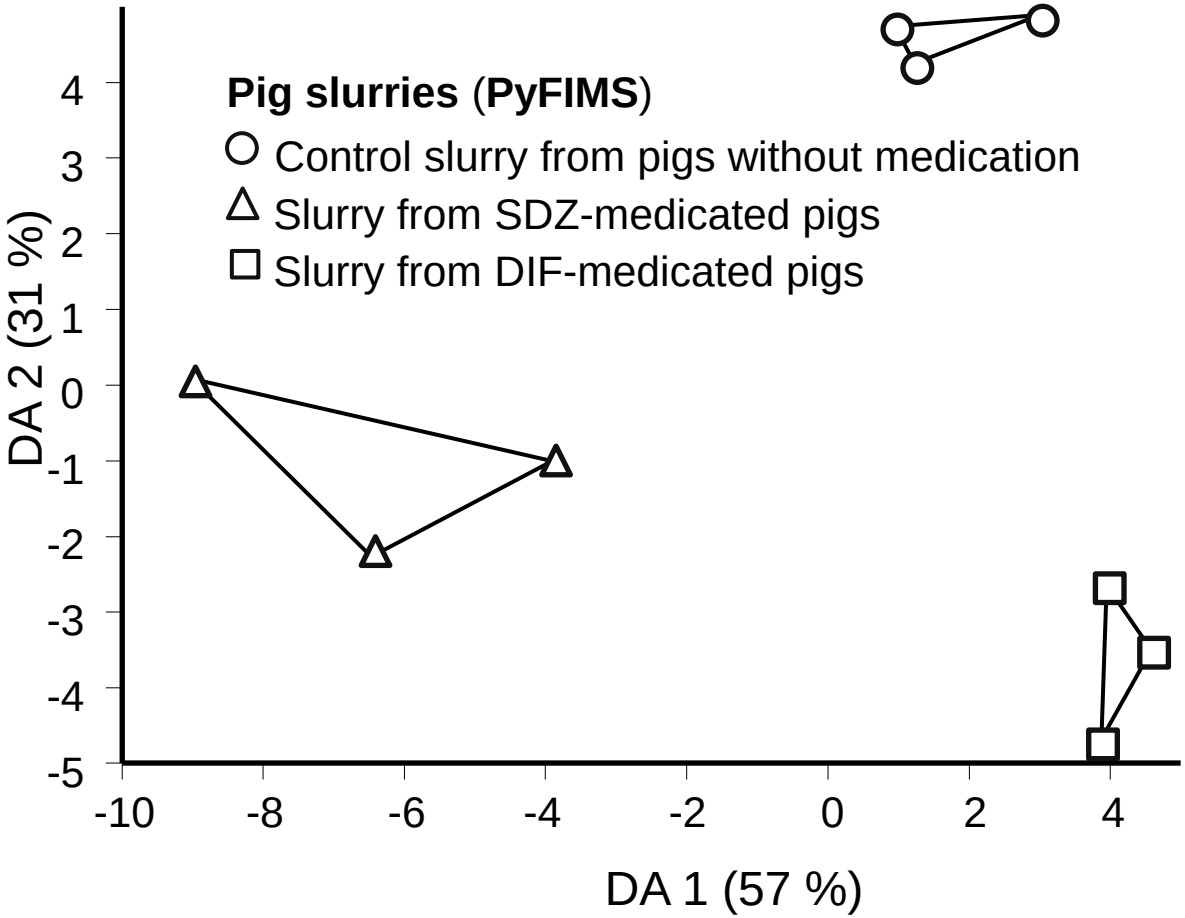


Potential activities of urease depending on the sample treatment (SDZ spiking concentration in mg kg^{-1} soil).

(Hammesfahr et al. (2008) J. Plant Nutr. Soil Sci. 4, 614-623)

→ Manure and SDZ interact in their effects on microorganisms

- **Antibiotic medication affects digestive tract system and molecular composition of excreta/slurry**.



Discriminant analysis of slurry composition determined by pyrolysis-field ionization mass spectrometry (Py-FIMS) obtained from medicated and control pigs.

Reichel et al. (2013) Soil Biology Biochemistry 62, 82-91

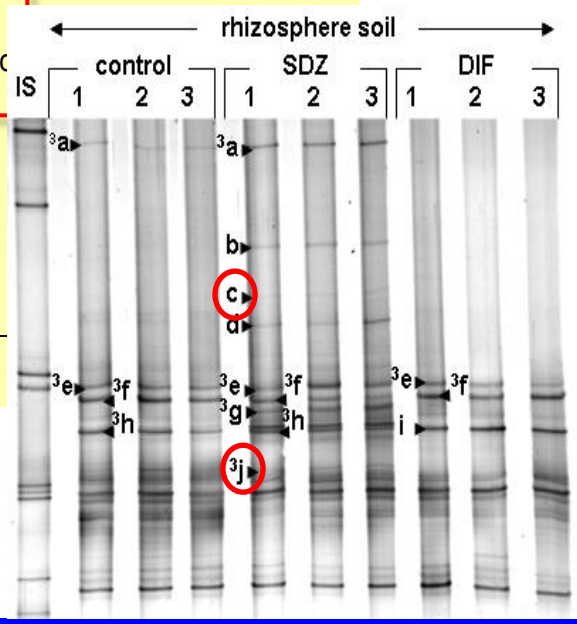
Manure: Effects on community structure – 16S rRNA gene sequencing

- **Change of excreted and survival of manure-borne**, possibly antibiotic **resistant microorganisms in soil**.

Tab. Sequencing results of excised DGGE bands and most closely related bacterial sequences

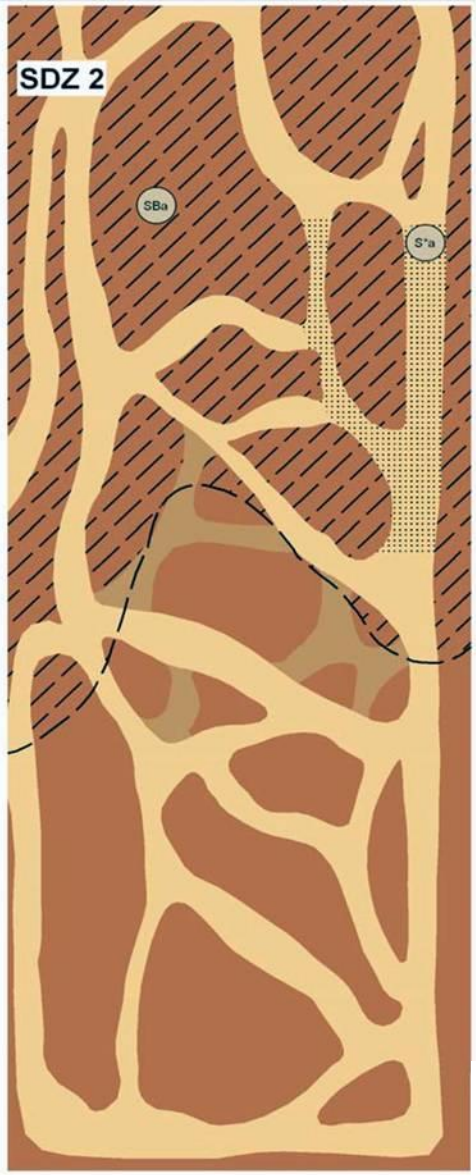
DGGE band	Genus (otu) and most related bacterial sequence(s)#	% Identity (bases of match)#	Accession no.#	Source and notes
3f	**Pseudomonas (otu_3227)**			
	Pseudomonas pseudoalcaligenes str. W-20	100.00 (435) 100.00 (435)	EU187489.1 EU395787.1	Pseudomonas strains with special degradation potentials
	…	…	…	
3g	**Pseudomonas (otu_3227)**			
	Pseudomonas sp. BBTR25	97.70 (435)	DQ337603.1	Swine effluent amended soil
	Pseudomonas sp. str. 91S1	97.70 (435)	EU370417.1	Pig manure
	Pseudomonas sp. str. HY-14	97.70 (435)	EU620679.2	-
	Pseudomonas sp. str. d130 (unclassified)	97.25 (406)	FJ950669.1	Treated oxytetracycline productic wastewater
3h	**Pseudomonas (otu_3227)**			
	Pseudomonas sp. str. SKU	97.47 (413)	AY954288.1	-
	Pseudomonas sp. BBTR25	97.47 (413)	DQ337603.1	Swine effluent amended soil
	Pseudomonas sp. str. 91S1	97.47 (413)	EU370417.1	Pig manure
	Pseudomonas sp. str. 98S1	97.47 (413)	EU370416.1	Pig manure
	Pseudomonas sp. str. HY-14	97.47 (413)	EU620679.2	-

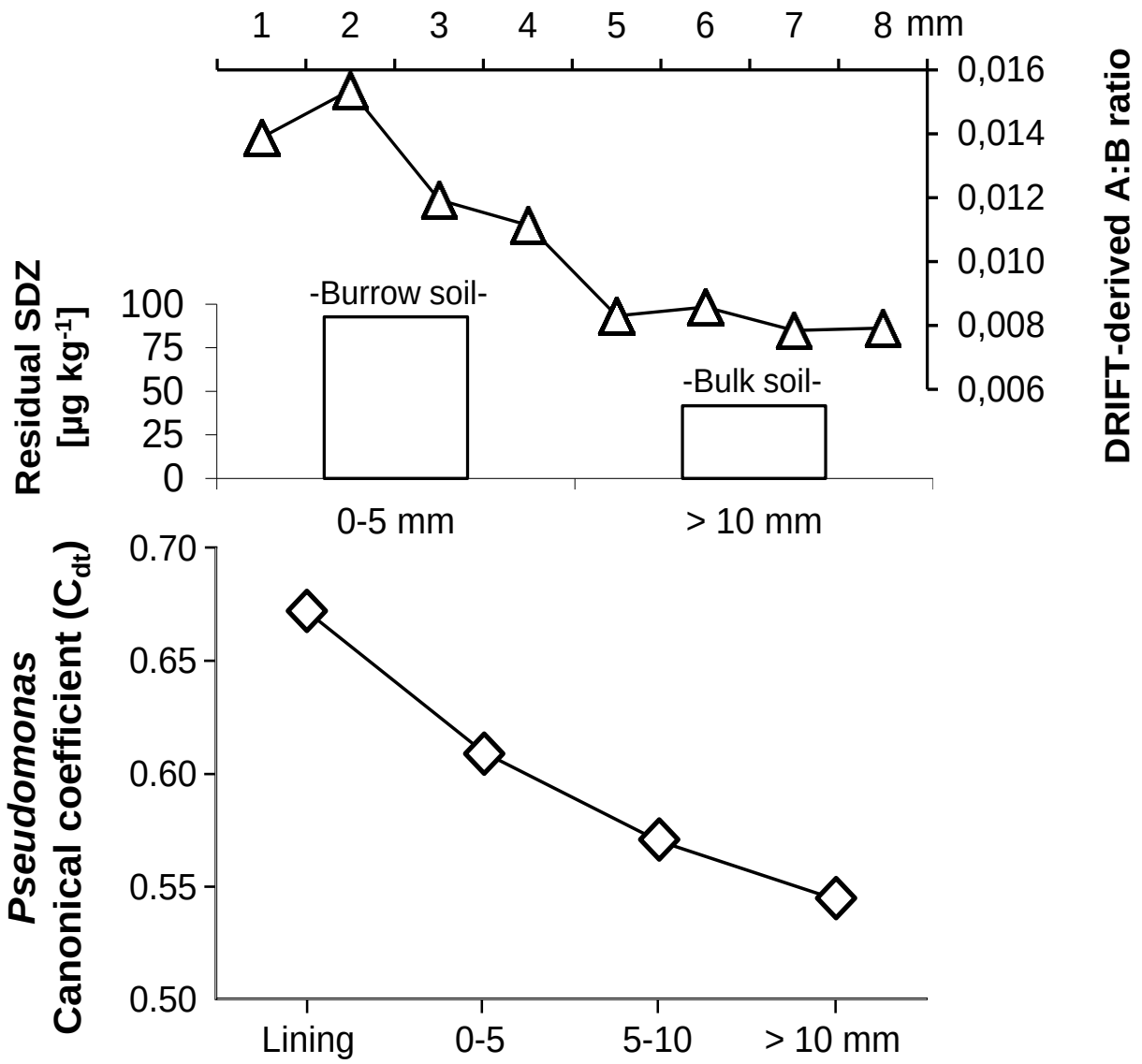
a Classification and identification according to the BlastN analysis of the Greengenes database



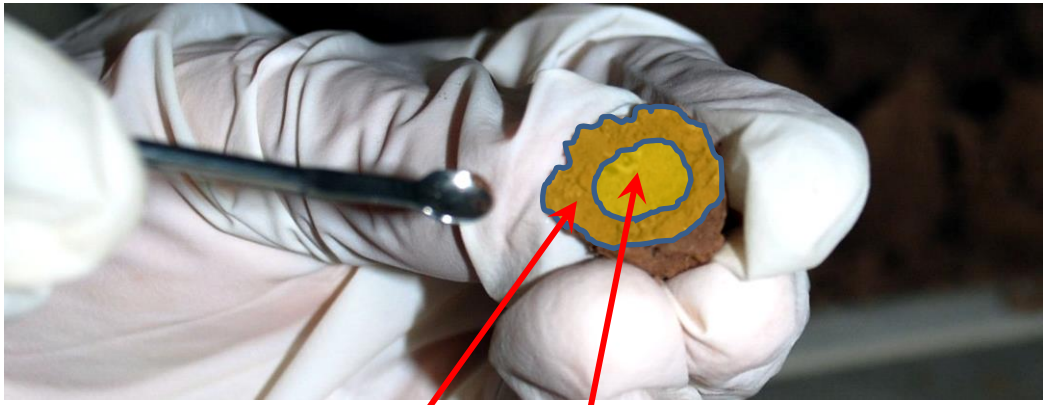
Pseudomonas 16S rRNA gene DGGE from mesocosm experiments; 13 d

- **Specific properties of earthworm burrows and sampling.**

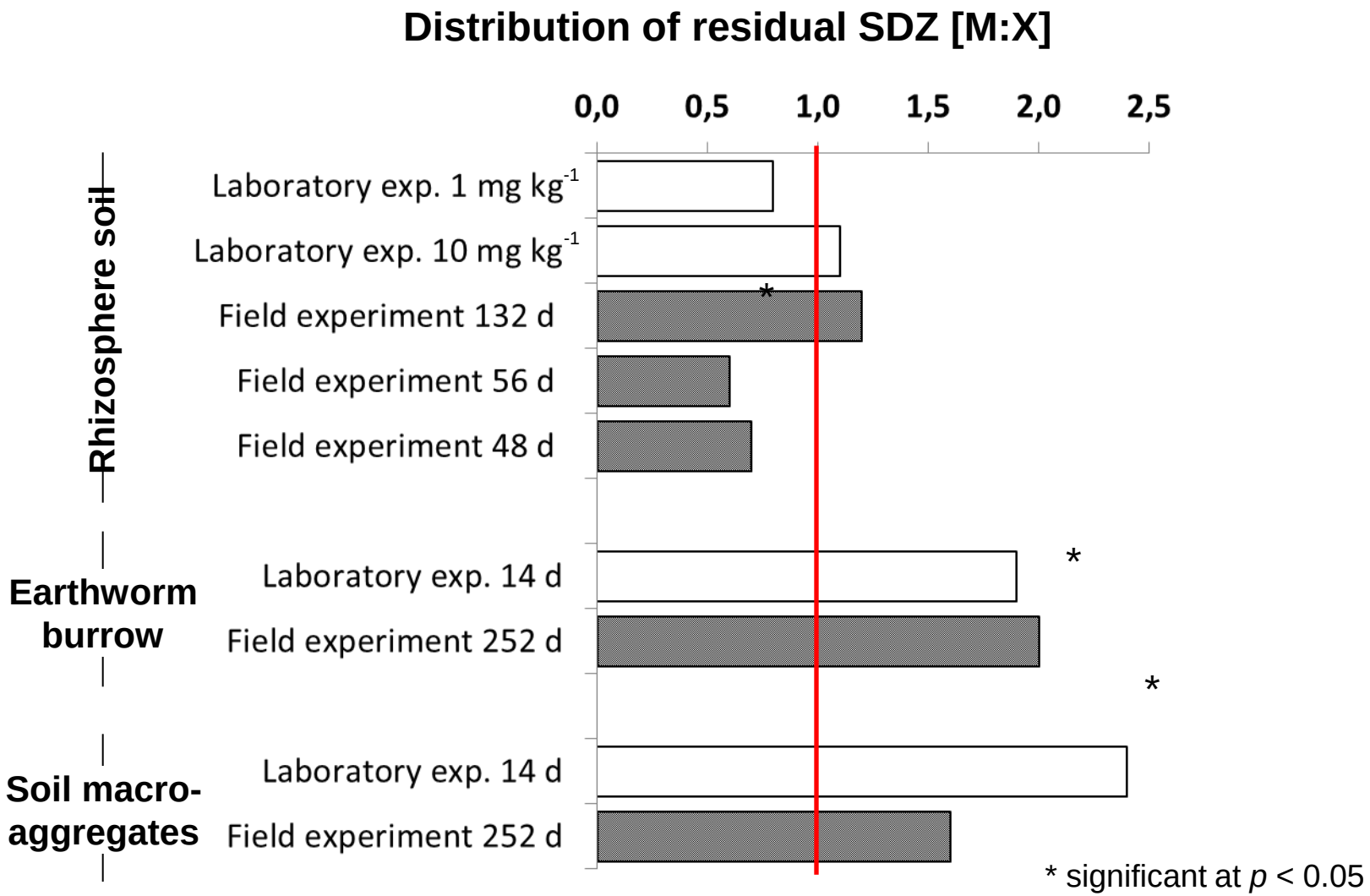




- **Sampling of aggregates and rhizosphere soil.**



- **Ratio of extractable SDZ in rhizosphere and earthworm burrows vs. bulk soil and macroaggregate shell vs. core.** Reichel et al. (submitted)

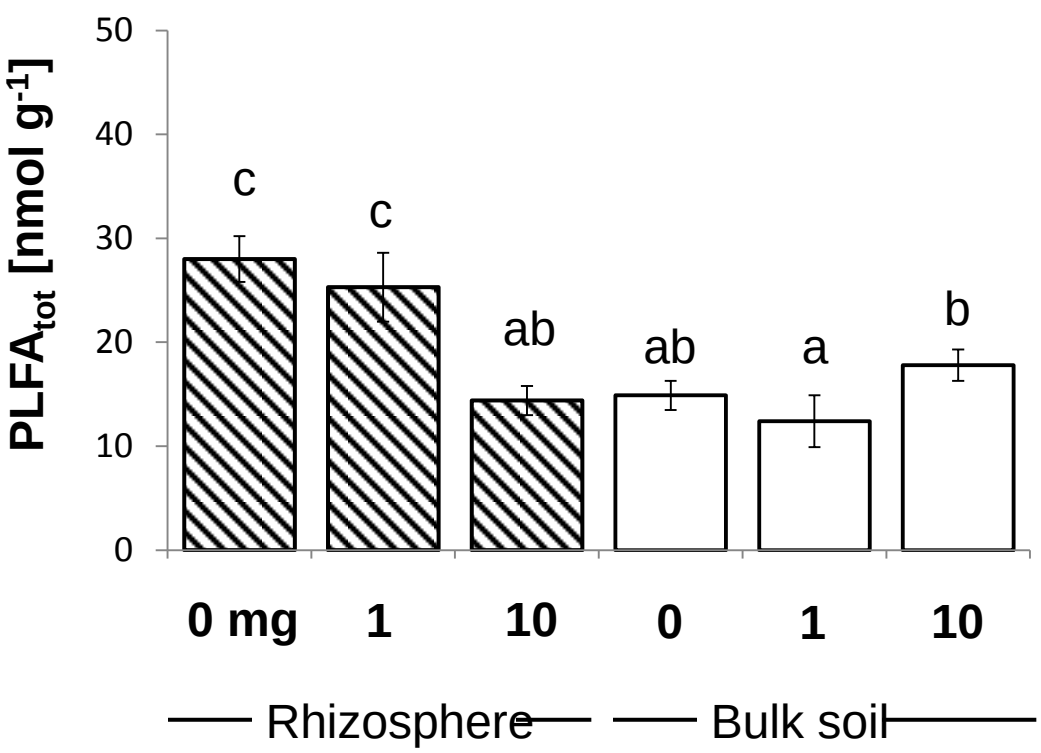


- **Total PLFA in rhizosphere and bulk soil.**

  Microbial biomass (C_{mic}): decreasing with SDZ.

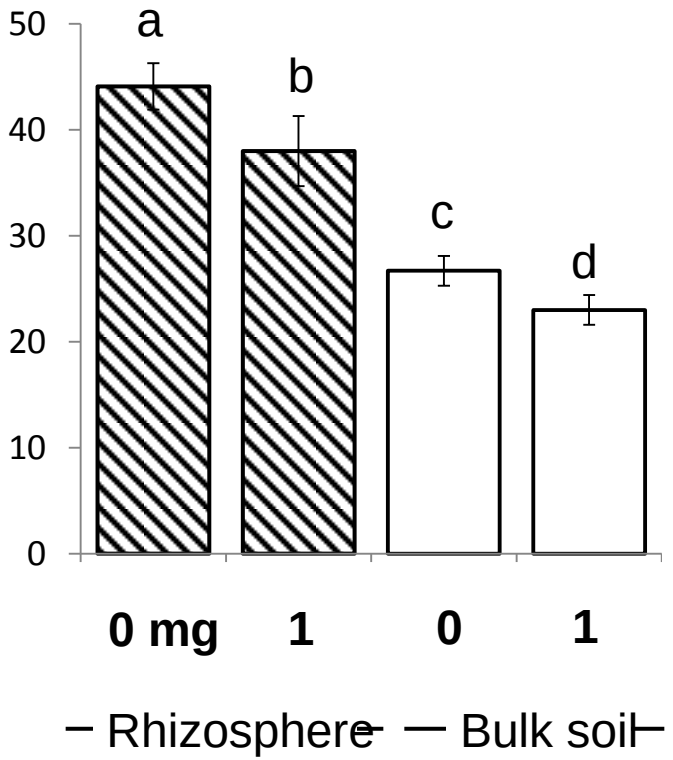
Laboratory experiment

63 d / SDZ / spiked manure



Field experiment

132 d / SDZ / medication



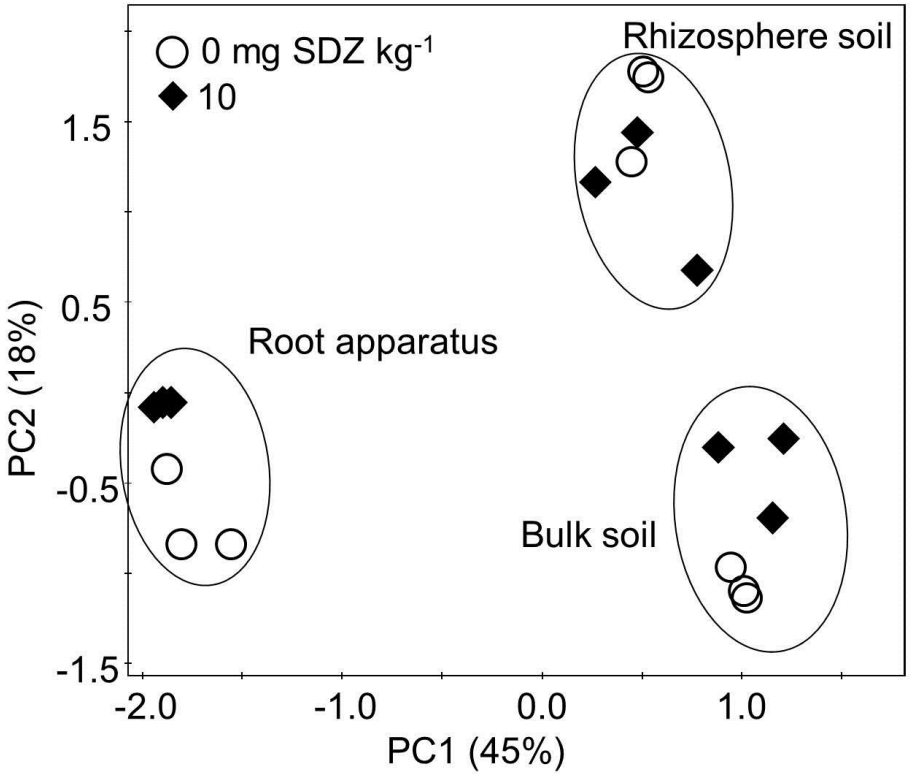
Microbial biomass (C_{mic}): decreasing with SDZ

Reichel et al. submitted.

- **Principal component analysis of Pseudomonas 16S rRNA gene DGGE fingerprints from rhizosphere and bulk soil.**

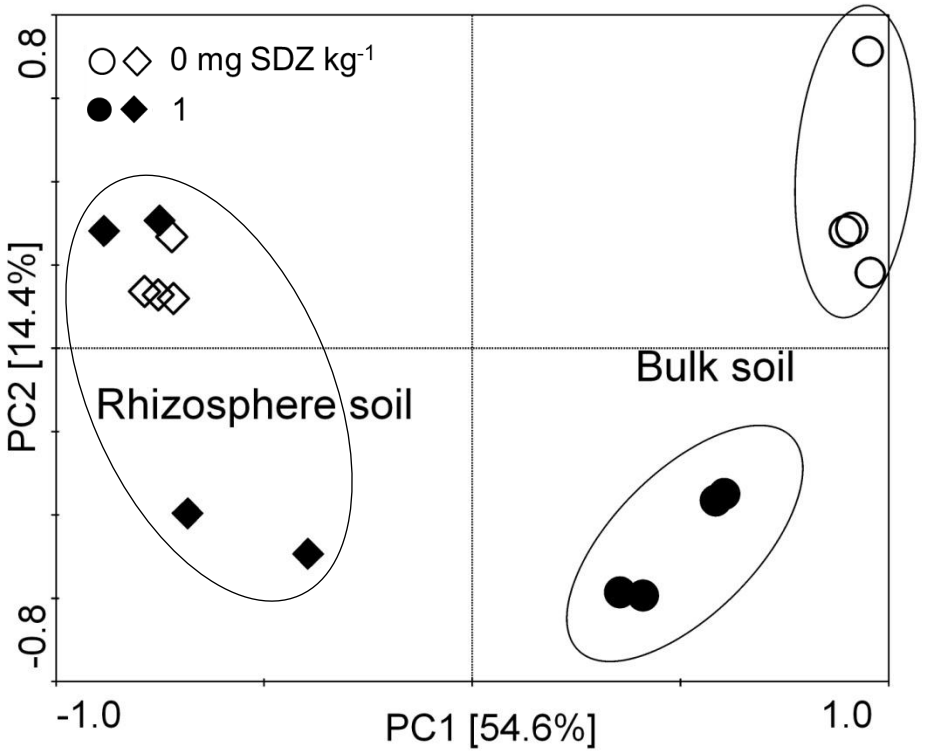
Laboratory experiment

40 d / SDZ / no manure



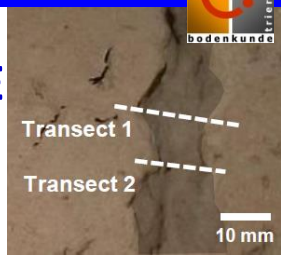
Field experiment

132 d / SDZ / medication



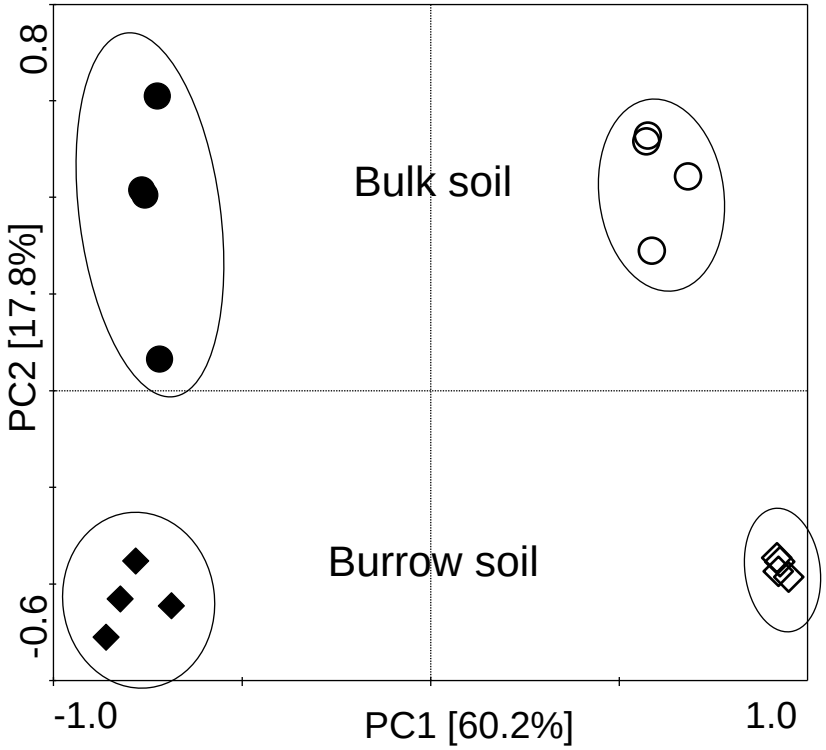
Reichel et al. submitted.

- **Principal component analysis of Pseudomonas 16S rRNA gene DGGE fingerprints from earthworm burrows and bulk soil.**



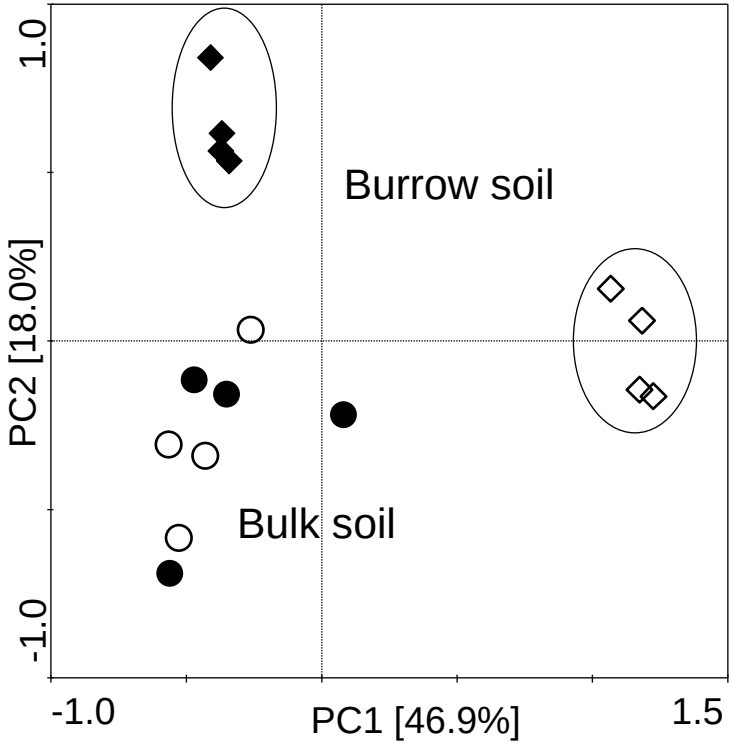
Laboratory experiment

14 d / SDZ / spiked manure



Field experiment

252 d / SDZ / medication

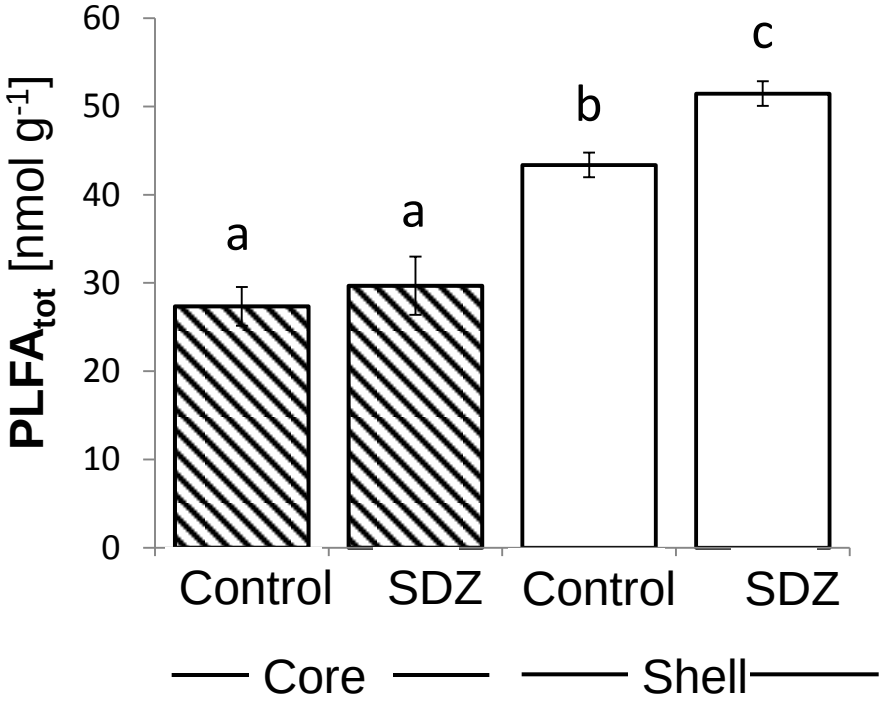


Reichel et al. submitted.

- **Total PLFA and enzyme activity, respectively, in soil macroaggregate shell and core soil.**

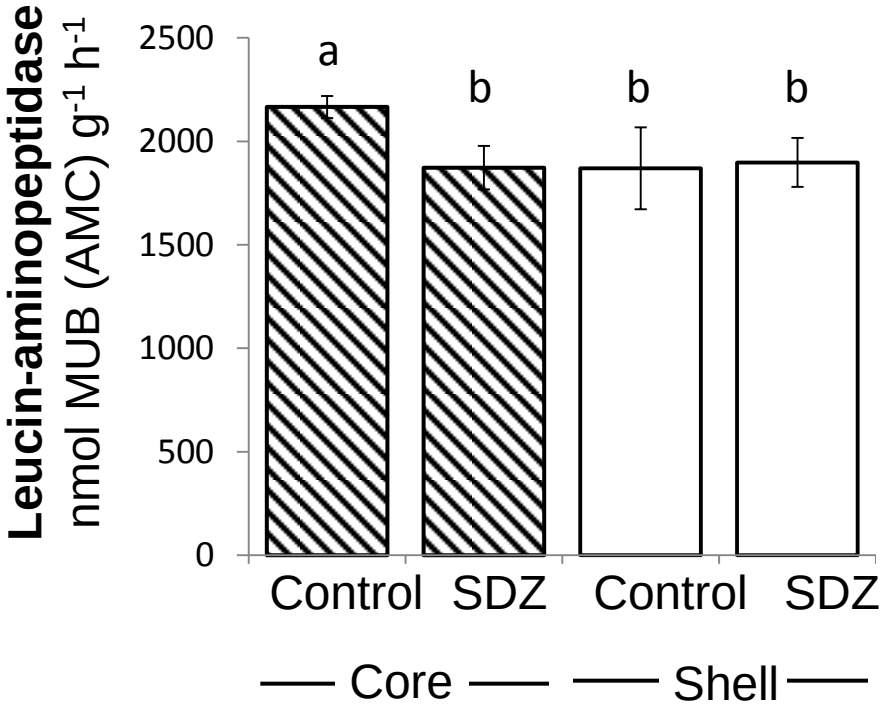
Laboratory experiment - Structure

14 d / SDZ / spiked manure



Field experiment - Function

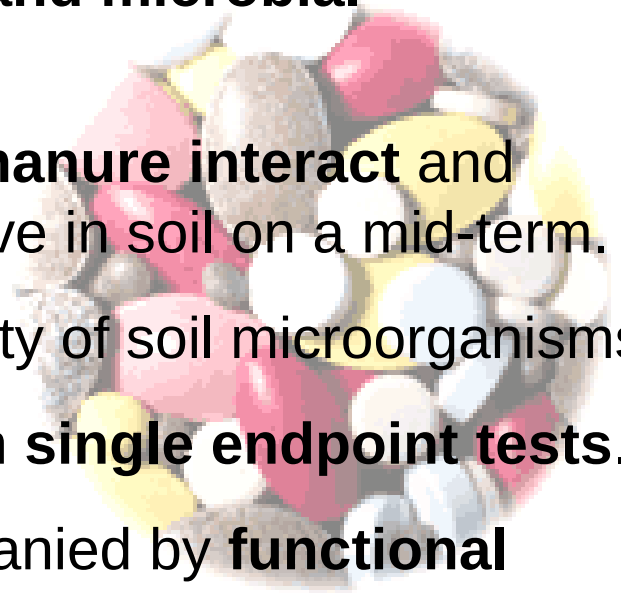
252 d / SDZ / medication



…Shift to **fungi** in shells of SDZ treated aggregates.

Reichel et al. submitted.

- **Antibiotics** reach the soil environment via excrements in **considerable amounts**.
- Antibiotic medication **alters the molecular and microbial composition** of excreta.
- Mid- to long-term effects of antibiotics and **manure interact** and **manure borne microorganisms** may survive in soil on a mid-term.
- Effects on **functional** and **structural** diversity of soil microorganisms.
- Tests on biodiversity are more sensitive than **single endpoint tests**.
- Structural community shifts may be accompanied by **functional redundancy**. **Community structure is a more sensitive parameter.**
- Long-term effects occur → **apparent concentration independence**.
- Accumulation and effects are different in **soil microcompartments**.



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