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ORIGINAL PAPER

The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities

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Abstract The effect of tetracycline (Tc) and cow manure on soil bacterial community composition and antibiotic resistance gene (ARG) abundance in soil was investigated in the present microcosm study. Effects of repeated applications of cow manure spiked with Tc in two concentrations or without Tc on the bacterial communities of a clayey and a sandy soil with different history of anthropogenic pollution by sewer flooding were investigated. Soil samples were taken 60 days after each of three amendments. Denaturing gradient gel electrophoresis (DGGE) fingerprints of 16S rRNA gene amplicons from total community DNA revealed soil type-dependent changes in the bacterial community composition in response to manure and to Tc, which became more pronounced with repeated applications. Repeated manure amendments and Tc, in particular at high concentration, triggered the further increase of ARGs *tet*(A), *tet*(O), *tet*(Q), *tet*(W), *sul1*, and mobile genetic elements (MGEs) IncP-1ε plasmids and *intI1*, in a soil type-dependent manner. In the clay soil with no anthropogenic history, the ARG and MGE abundances were low or not detectable, while manure amendments caused pronounced increases in their relative abundance. In the sandy soil with a history of anthropogenic impact, ARGs and MGEs were already present at a higher level and strong increases were mainly observed for the relative abundances of *sul2* and MGEs. Here, we show for the first time that effects of repeated cow manure applications might be dependent on soil type and foregoing anthropogenic soil pollution and that the presence of Tc could further increase the abundance of ARGs and MGEs.

Keywords Tetracycline · Soil · Bacterial fingerprints · Antibiotic resistance genes · Class 1 integron · Co-selection

Introduction

Antibiotics are widely used in livestock to treat and prevent infectious diseases or to promote animal growth (Durso and Cook 2014; Sarmah et al. 2006). In EU countries since January 2006 (EC 1831/2003), and in South Korea since July 2011 (Hassan and Ryu 2012), the use of antibiotics as growth promoters in feed for livestock is banned, but they are still a common practice in many countries including the USA, Canada (Kim et al. 2011), and China (Zhu et al. 2013). About 70 % of total pharmaceutical products used worldwide are antibiotics (Kümmerer 2003; Sarmah et al. 2006; Thiele-Bruhn 2003), and about half of the total consumption is attributable to veterinary practices (Winckler and Grafe 2001; Zhu et al. 2013). Antibiotics are normally used in livestock farming to maintain animal health and productivity, but this practice has several consequences for human and environmental health, such as alteration of microbiota composition in livestock and non-target animals, rise of methane emission

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from dung (Hammer et al. 2016), and contributes to spreading human and animal pathogens resistant to antibiotics, and thus poses a significant health threat (Van Boeckel et al. 2015).

Among all antibiotics used in livestock, tetracyclines (Tc) are the most consumed drug (FDA 2014), due to their low price (Chopra and Roberts 2001) and high efficacy against a wide range of bacteria, both Gram-negative and Gram-positive. Tc is able to exert a bacteriostatic effect by interfering with the 30S and 70S ribosome subunits and stopping the protein synthesis (Chopra and Roberts 2001; Nelson and Levy 2011). At present, 42 antibiotic resistance genes (ARGs) are known coding for proteins belonging to three resistance mechanisms able to confer resistance against Tc in bacteria, including efflux pumps, ribosomal protection proteins, and degradation enzymes (Roberts 2011), as well as five mutations of the 16S rRNA that reduce the binding affinity of the drug to the ribosome (Nguyen et al. 2014). Tc resistance genes (RGs) are typically associated with mobile genetic elements (MGEs) such as plasmids, transposons, and associated integrons, and they were detected in different environments, e.g. in manure and soil (Schmitt et al. 2006). Moreover, as in the case of *tet*(A), *tet*(Q) and *tet*(W), they were detected in both Gram-negative and Gram-positive bacteria and were often found associated with sulfonamide ARG (*sul1*), as in the case of *tet*(A) (Roberts 2011). Tc is highly water-soluble, poorly absorbed in the animal gut and quickly excreted, mostly unchanged and still bioactive (O'Connor and Aga 2007). Up to 75–90 % of one administered dose can be excreted via feces or urine (Sarmah et al. 2006). For this reason, high concentrations of Tc can be found in animal feces (Zhu et al. 2013) and their extractable concentration decreases in aged manure after composting, resulting from the microbial degradation or the irreversible sorption of Tc on the soil particles (Kreuzig and Höltge 2005). When manure containing antibiotics is applied to agricultural soil as fertilizer, antibiotics are transported into the soil as well (Jechalke et al. 2014b), and this can affect the composition and function of the resident microbial communities, as recently shown for sulfadiazine (SDZ) introduced via manure into soil (Ding et al. 2014). Although the detection of antibiotics in soil remains experimentally challenging, Hamscher et al. (2002), using high-molar citric acid buffer at low pH, reported Tc concentrations in liquid manure fertilized topsoil ranging from 0.086 to 0.171 mg kg^{-1}, while Qiao et al. (2012) measured 0.78 mg kg^{-1} using McIlvaine-Na_2EDTA buffer and subsequent sonication. Tc is strongly and rapidly sorbed to soil clays and organic matter (Gu et al. 2007; Pils and Laird 2007) but free Tc concentrations might be still bioavailable and potentially affect the microbial communities (Thiele-Bruhn and Beck 2005), depending on soil properties (Jia et al. 2008). In addition to antibiotic residues, manure typically contains high numbers of bacteria carrying ARGs on MGEs, which can further increase the abundance of antibiotic-resistant bacteria in soil when manure is applied (Binh et al. 2008; Heuer et al. 2009; Jechalke et al. 2014b; Smalla et al. 2000). Furthermore, antibiotics such as Tc introduced via manure into soil can select the proliferation of resistant soil bacteria and the spread of ARGs via MGEs from manure bacteria to soil bacteria (Hammesfahr et al. 2008; Heuer et al. 2008). The application of manure several times a year, which is a typical agricultural practice (Montforts et al. 1999), might lead to an accumulation of ARGs and antibiotic compounds, as well as to an accumulation of effects on the soil bacterial community composition, as recently shown for the sulfonamide antibiotic SDZ in soil microcosm experiments (Ding et al. 2014; Heuer et al. 2011b). Moreover, manure application to soil can be important not only as nutrient supplement to increase crop yield but also for biocontrol of fungal pathogens by the use of manure enriched with microbial antagonists (Zhao et al. 2014), in order to stabilize, by integrated agricultural management, soil microbial communities important for soil health and sustainability (Wu et al. 2014). Manure can also have negative effects such as the stimulation of methane production by resident methanogenic soil bacteria, and mitigation strategies to reduce methane emission should be considered (Ho et al. 2015).

Most studies on the effects of organic fertilizers on soil microbial community composition were based on piggery manures. However, in several regions of the world fertilization with cow manure is more relevant. Recently, Udikovic-Kolic et al. (2014) showed an unexpected bloom of ARGs in the response to soil fertilization with cow manure free of antibiotics. Little is known so far on how the presence of antibiotics might change the effects that cow manure applied to soil has on the bacterial community composition and on the abundance of ARGs and MGEs (Kyselková et al. 2015b).

In the present study, we investigated how three soil amendments with cow manure, spiked with Tc or not, affected the bacterial community composition of two soils from Sardinia which differed not only in soil mineral composition but also in their history of exposure to anthropogenic inputs. The addition of manure and/or Tc to soil was performed in two-month intervals, a period which can be considered a long-term evaluation of the effects on the microbial populations in soil (Heuer and Smalla 2007). Recently, Chessa et al. (2016) investigated the effects of Tc and cow manure on the soil microbial community composition after a single application in the same Sardinian soils analyzed here. They reported high Tc sorption in both soils and antibiotic bioavailable concentrations ranged between 0.155 and 1.092 mg kg^{-1} soil, and between 0.767 and 4.468 mg kg^{-1} soil in the cow manure-amended clayey (CL) and sandy (SA) soils, respectively. The Tc bioavailability found in the two soils investigated was not proportional to Tc spiked, and these differences could be attributable to soil properties. In fact, Tc preferably interacts with soil clay minerals, and the extent of adsorption decreases

with increasing pH (Li et al. 2010). Therefore, lower bioavailable Tc concentrations found in CL soil could be due to higher clay content and lower soil pH. Indeed, the concentration of Tc in soil aqueous solution measured after the first soil amendment in the study of Chessa et al. (2016) was about five times higher in the SA than in the CL soil. Tc had a short-term detrimental effect and after two days reduced the microbial activity (fluorescein diacetate hydrolysis) and shifted the microbial composition from bacteria to fungi, as revealed by phospholipids fatty acids (PLFA) analysis, respectively. Moreover, the Tc effects were transient, decreased on the seventh day, and had disappeared after 60 days in the SA soil, while in the CL soil, with no history of antibiotic pollution, the utilization of substrates in BIOLOG plates still remained different among the patterns of the different treatments. Given the recent findings of Chessa et al. (2016) on Tc and cow manure effects on soil microbial communities after single amendment, we aimed to test the hypothesis that repeated applications of manure to soil affect the soil bacterial community composition and increase the abundance of ARGs and MGEs in a soil type-dependent manner and that these effects are more pronounced in the presence of Tc.

Materials and methods

Experimental design

Cow liquid manure (M), free of antibiotics, was collected from a beef cattle farm in Sardinian (Italy), dried and stored at room temperature in the dark for one year in order to reduce its water content and to allow the investigation of Tc sorption. Microcosm experiments were performed using two different soils: a CL and a SA soil, sampled in Sardinia, previously characterized by Chessa et al. (2016), as well as the Tc sorption and the resulting potentially bioavailable Tc concentrations in these soils. These bioavailable Tc concentrations, i.e., available for bacterial communities, are not proportional to Tc applied to soil with M but are dependent on soil characteristics and Tc speciation (see "Discussion"). CL is a forest soil that was never used for agriculture or intensive farming. SA soil was collected from a dry river bed in the center of Sassari. Since several decades it was used for orchard cultivation (lemon and orange) and no organic fertilizers or antibiotics were applied. The SA soil was previously exposed to anthropogenic inputs during one sewer flooding period of a few days, one year before the sampling, caused by the split of an underground conduit carrying off drainage water and waste matter, next to the site of study.

Four soil treatments were performed with four independent replicates for each treatment. For each replicate, 200 g of soil sieved at <2 mm were placed in a glass pot (18 × 10 × 6 cm) with the following treatments: (i) Only water was added to the soil (CL/SA); (ii) 8 g of uncontaminated aged M were mixed with soil (CL/SA + M); (iii) and (iv) 8 g of aged M spiked with Tc solution were added to the soil (freshly prepared by dissolving Tc in sterile deionized water and mixed by agitation for 1 h in the dark before addition to soil) to reach final theoretical concentrations of 100 or 500 mg Tc kg^{-1} soil (CL/SA + M + Tc100 and CL/SA + M + Tc500, respectively). Soil and M were carefully mixed in pots and incubated in the dark at a constant temperature of 20 °C and at 50 % of maximum water-holding capacity. Every two days, water was sprayed on the soil surface to compensate weight loss by evaporation. Amendments were performed three times at 60-day intervals, and the soil was mixed after each amendment in order to observe, at microcosm scale, the effect of repeated M and Tc addition to soils. After 60 days, a quadruplicate set of soil samples was collected from each pot. Soil in the pot was mixed and 10 g of soil was transferred to a sterile polypropylene jar for microbiology (Becton Dickinson International, Erembodegem, Belgium). The soil in the jar was mixed again and 1 g of soil was transferred into a sterile Eppendorf tube (1.5 mL volume) and stored at −20 °C until total community DNA extraction (see below). The remaining soil in the jar was put back into the pot and the next amendment was performed.

Total community DNA extraction

Total community DNA was extracted from 0.5 g of soil using the FastDNA®SPIN Kit for Soil (MP-Biomedicals, Solon, OH, USA) with some modifications: Cell lysis in soil samples by the FastPrep® Instrument (MP Biomedicals) was performed twice for 30 s at setting 5.5. Also, two washing steps were performed, by addition of salt/ethanol washing solution (SEWS-M). Finally, DNA was eluted in 100 μL of DNA elution solution (DES). Then, 50 μL of DNA solution from each sample was purified by the Geneclean® Spin Kit (Q-Biogene/MP-Biomedicals) and eluted in 50-μL final volume observing the manufacturers' protocol.

Denaturing gradient gel electrophoresis

Total bacterial 16S rRNA gene fragments were amplified by PCR using primers F984GC and R1378 (Heuer et al. 1997). Denaturing gradient gel electrophoresis (DGGE) analyses were performed, with few modifications, according to Heuer et al. (1997) and Gomes et al. (2001). In the reaction mixture, 0.2 μM of each primer and 0.6 U AmpliTaq DNA Polymerase (Stoffel Fragment, Applied Biosystems, Weiterstadt, Germany) were used. The protocol was previously described by Gomes et al. (2001) except that 35 cycles were performed instead of 30. The amplified 16S rRNA gene fragments were separated for bacterial fingerprinting by DGGE using an Ingeny PhorU system (Ingeny, Goes, The Netherlands) according to Weinert et al. (2009). Polyacrylamide gels were stained by the silver method

described by Heuer et al. (2001), then digitalized, and pairwise analysis was performed by the software GelCompar II® (version 6.5, Applied Maths, Austin, TX, USA) (Smalla et al. 2001) to calculate Pearson correlation indices through the unweighted pair group method using arithmetic averages (UPGMA). Differences between treatments were analyzed by the permutation test described by Kropf et al. (2004) using the Pearson correlation indices for significance ($P < 0.05$) calculation. The permutation tests (10^4 random permutations) for the comparison of groups of lanes based on pairwise similarity measures were applied to calculate the effect of treatments in one soil (CL or SA) at a specific time point and also in one soil 60 days after the first and the third amendments. Differences between community compositions, expressed as *d*-values, were calculated by the average correlation of coefficients within treatments minus correlation of coefficients between treatments.

Real-time quantitative PCR

Several real-time quantitative PCR (qPCR) assays were performed for the detection and quantification of the relative abundance (target gene per 16S rRNA gene (*rrn*) copies) of sequences specific for ARGs and MGEs. Gene abundances in untreated and treated soils were compared at each time point by pairwise comparisons (Tukey-Kramer statistical test; $P < 0.05$). Quantification of *rrn* copies was performed in accordance with Suzuki et al. (2000). All primers and TaqMan probes used are listed in Table 1. Standard dilutions of PCR fragments cloned into pGEM-T vector systems (Promega Corporation, Madison, WI, USA) were used for quantification. The PCR mastermix contained 1.2 μM forward primer Bact1369F, 1 μM reverse primer Prok1492R, 0.5 μM TaqMan-Probe TM1389F, 0.1 mg mL^{-1} of bovine serum albumin (BSA), and 1.25 U TrueStart Taq (Fermentas, St. Leon-Rot, Germany). The PCR protocol was 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 56 °C, and 1 min at 60 °C. A CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used. The qPCR for determination of the abundance of class 1 integron integrase genes *intI1* was performed using the forward primer int1-LC1, the reverse primer int1-LC5, and TaqMan-Probe int1-probe previously described (Barraud et al. 2010). Total reaction volume was 50 μl. Five-microliter DNA template of 1:5 diluted purified DNA solution was used and 1× TrueStart Buffer (Fermentas), 2.5 mM $MgCl_2$ (Fermentas), 0.2 mM dNTPs, 0.08 mg μL^{-1} BSA, 0.3 mM of primers and probe, and 1.25 U TrueStart Taq (Fermentas); 40 cycles were performed according to the protocol described by Barraud et al. (2010). To normalize for different extraction and amplification efficiencies, the relative abundance of target genes was calculated by dividing the copy number of each gene by the *rrn* copy number.

For *tet*(Q) and *tet*(W), the same reaction mixture composition was used. The protocol was as follows: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C. Primers used to quantify *tet*(Q) and *tet*(W) were described by Smith et al. (2004). The qPCRs for *sul1* and *sul2* genes were performed as described by Heuer and Smalla (2007) and Heuer et al. (2008), respectively. The qPCR to measure the abundance of *trfA* genes specific for the ε subgroup of IncP-1 plasmids was performed according to Heuer et al. (2012). Concentrations used in the reaction mixture were the same as described for *intI1* qPCR, and amplification and detection were performed by a 10 min step at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

PCR and Southern blot hybridization of *tet*(A) and *tet*(M) genes

Primers used for the amplification of *tet*(A) and *tet*(M) were described by Lanz et al. (2003) and Ng et al. (2001), respectively (Table 1). Digoxigenin-labeled probes were generated from PCR products as described by Jechalke et al. (2014a) using plasmids RP4 and pAT101 as templates for *tet*(A) and *tet*(M), respectively. For both genes, 25-μL reaction mixture composed of 1× TrueStart buffer (Fermentas), 0.2 mM of deoxynucleoside triphosphates, 2.5 mM $MgCl_2$ (Fermentas), 0.1 mg mL^{-1} bovine serum albumin, 0.5 μM of primers, respectively, and 0.6 U TrueStart Taq (Fermentas) were used. Amplification and detection were performed according to the following protocol: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 64 °C and 1 min at 72 °C, followed by a final 5-min step at 72 °C. PCR products were loaded on an agarose gel with size markers (DIG-labeled DNA Molecular Weight Marker VI, Roche Diagnostics Deutschland GmbH, Mannheim, Germany) in the borders. Southern blotting to a Hybond-N membrane (GE Healthcare Limited, Amersham, UK) and hybridization of PCR products were done as described by Sambrook et al. (1989) and following the standard procedure of Roche Diagnostics for filter hybridization under conditions of middle stringency (Fulthorpe et al. 1995). The exposure times were 20 min for *tet*(A) and 1 h for *tet*(M).

Results

Effects of cow manure and tetracycline effects on soil bacterial communities

The effects of M with or without Tc on the bacterial communities in CL and SA soils were analyzed by DGGE fingerprinting of 16S rRNA gene fragments amplified by PCR from total community DNA. Soil type-dependent treatment effects on the bacterial community composition, which increased with repeated M applications, were revealed. The effects of

Table 1 Sequences of primers and TaqMan probes used for the detection of ARGs and MGEs

Gene target	Forward primer (5' - 3')	Reverse primer (5' - 3')	Probe	Annealing temp. (°C)	Reference
16S rRNA	CGGTGAATACGTTCYCGG	GGWTACCTTGTTACGACTT	5′CTTGTACACACCGCCCGTC	56	Suzuki et al. (2000)
tet(Q)	AGGTGCTGAACCTTGTTTGATTC	GGCCGGACGGAGGATTT	5′FAM-TCGCATCAGCATCCCGCTC-TAMRA	60	Smith et al. (2004)
tet(W)	GCAGAGCGTGGTTCAGTCT	GACACCGTCTGCTTGATGATAAT	5′FAM-TTCGGGATAAGCTCTCCGCCGA-TAMRA	60	Smith et al. (2004)
tet(A)	GGCGGTCTTCTTCATCATGC	CGGCAGGCAGAGCAAGTAGA		64	Lanz et al. (2003)
tet(M)	GTGGACAAAGGTACAACGAG	CGGTAAAGTTCGTCACACAC		64	Ng et al. (2001)
sul1	CCGTTGGCCTTCCTGTAAAG	TTGCCGATCGCGTGAAGT	5′FAM-CAGCGAGCCTTGCGGCGG-TAMRA	60	Heuer and Smalla (2007)
sul2	CGGCTGCGCTTCGATT	CGCGCGCAGAAAGGATT	5′FAM-CGGTGCTTCTGTCTGTTTCGCGC-TAMRA	53	Heuer et al. (2008)
intI1	GCCTTGATGTTACCCGAGAG	GATCGGTCGAATGCGTGT	5′FAM-ATTCCTGGCCGTGGTTCTGGGTTTT-TAMRA	60	Barraud et al. (2010)
incP-1ε/*trfA*	ACGAAGAAATGGTTGTCCTGTTC	CGTCAGCTTGCGGTACTTCTC	5′FAM-CCGGCGACCATTACAGCAAGTTCATTT-TAMRA	60	Heuer et al. (2012)

treatments analyzed 60 days after each of three amendments (Fig. S1, S2, and S3) showed that the bacterial community fingerprints of the control soils (CL or SA) always formed a cluster separate from those of soils treated with M unspiked and Tc-spiked, CL/SA + M and CL/SA + M + Tc, respectively (Fig. 1), except for SA soil 60 days after the first amendment. The amendment of soils with cow M, with or without Tc spiking (CL/SA + M + Tc and CL/SA + M), caused a high variability of the fingerprints among replicates of each treatment in particular after the second amendment, and a clear effect of Tc became only evident 60 days after the third M amendment. Especially in CL soil, the fingerprints of CL + M + Tc100 and CL + M + Tc500 treated soil samples, 60 days after the third amendment, clustered and were clearly separated from the M-treated soil (CL + M); in SA soil only SA + M + Tc500 treatment clustered separately, indicating a minor effect of SA + M + Tc100 on the soil bacterial communities. In addition, 60 days after the second and third amendments, the fingerprint of the bacterial community in the M was rather similar to the bacterial fingerprints of SA + M + Tc100 and SA + M + Tc500. This similarity was not observed between M and CL soils, as here the bacterial community fingerprint of M was, with repeated M applications, increasingly distinct from those of the M-treated soils.

The permutation test revealed significant differences ($P < 0.05$) between the DGGE fingerprints of CL control soil and the M-treated CL soil at all sampling times (Table 2). After the first M amendment, the bacterial community composition of CL soil was clearly more affected than the community of SA soil where the differences (*d*-values) between control SA soil and M-treated SA soils were small and not significant. However, after the second M amendment, the differences between control soil and the M-treated soil became also significant for SA soil. For both soils, the effects of M on the bacterial community composition increased with repeated M amendment and the differences observed 60 days after the third M amendment were remarkably high. Unexpectedly, for both soils, the fingerprints of S + M + Tc100 treatments were not significantly different from those of the M treatments (S + M), even after repeated application of M + Tc100. In contrast, the fingerprints of the M + Tc500 treated soil were significantly different from those of the M-treated soil at all time points except for the M + Tc500-treated CL soil 60 days after the second treatment.

Treatment-dependent changes in the relative abundance of tetracycline and sulfonamide resistance genes

tet(Q)

The relative abundance of *tet*(Q) genes, 60 days after the first amendment, was below detection limit in the control CL. The addition of M, which contained approximately −5.7 Log *tet*(Q) per *rrn* copy numbers, significantly increased the abundance of *tet*(Q) in this soil (Fig. 2a). In contrast, 60 days after the first amendment, *tet*(Q) was detected in DNA extracted

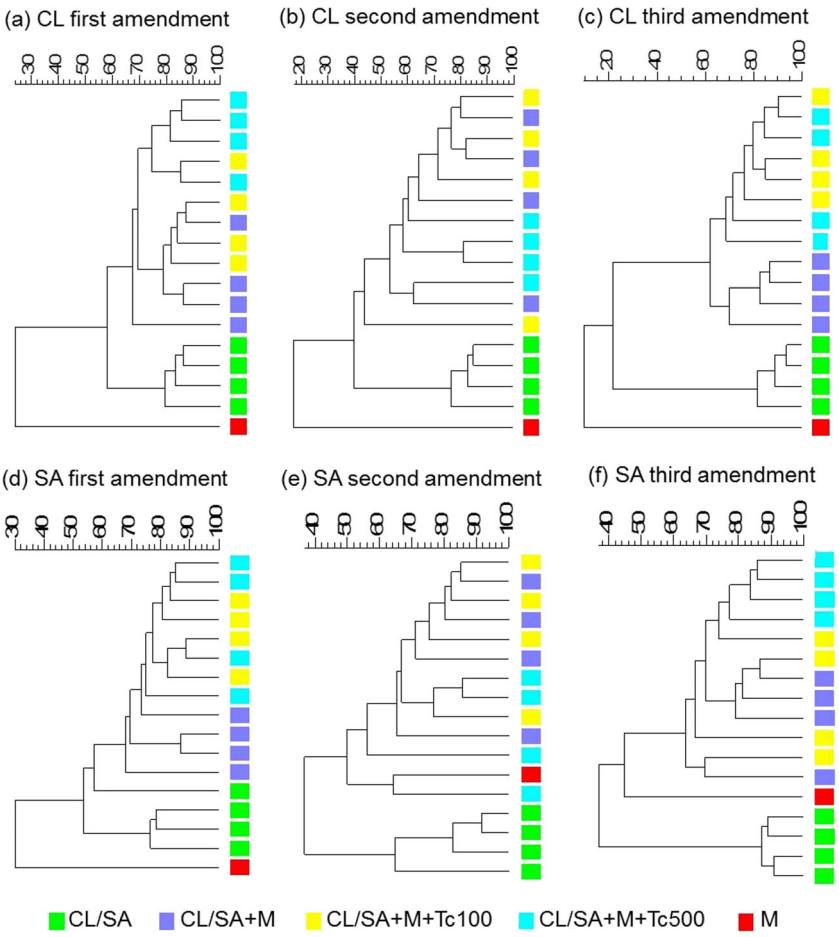

Fig. 1 UPGMA cluster analysis of DGGE fingerprints for 16S rRNA gene amplicons from CL and SA soils 60 days after the first amendment (**a** and **d** respectively); 60 days after the second amendment (**b** and **e** respectively); 60 days after the third amendment (**c** and **f** respectively). *Horizontal axis* indicates the % of similarity. UPGMA cluster analysis was based on Pearson correlation indices to compare the effect of treatments in each soil at a specific time point. *S* soil CL or SA, *M* manure, *Tc100* 100 mg Tc kg^{-1} soil dry weight, *Tc500* 500 mg Tc kg^{-1} soil dry weight

from the SA and in SA + M soil treatments and its relative abundance was not significantly ($P < 0.05$) different between these treatments (Fig. 2b). Also the addition of M + Tc100 or M + Tc500 did not alter the abundance of *tet*(Q) in both soils, compared to the soil amended with M alone. As the relative abundance of *tet*(Q) in the control SA soil significantly ($P < 0.05$) decreased over time, 60 days after the third amendment, the abundance of *tet*(Q) was higher in all M treatments, both unspiked and Tc spiked. Repeated amendments of M and M + Tc did not further increase the relative abundance of *tet*(Q) in both soils, with the only exception of CL + M + Tc100 after the third amendment.

tet(W)

Similar to *tet*(Q) genes, also *tet*(W) genes were not detected in CL soil. The addition of M containing −3.3 Log *tet*(W) rrn^{-1} copy number increased the relative abundance of this gene in CL + M, 60 days after the first amendment (Fig. 2c). In SA soil, 60 days after the first amendment, *tet*(W) genes were detected also in the control SA and their relative abundance was not significantly ($P < 0.05$) different to SA + M (Fig. 2d). Moreover, in both soils, the first addition of M + Tc did not increase the relative abundance of *tet*(W) genes, compared to M treatments. As observed for *tet*(Q) gene in SA soil, also the relative abundance of *tet*(W) progressively decreased over time in SA soil. However, the continuous addition of M with or without Tc spike significantly increased the relative abundance of *tet*(W) in both soils.

tet(A) and *tet*(M)

Due to the low abundance of *tet*(A) and *tet*(M) genes, it was not possible to measure their relative abundance by qPCR. Therefore, these genes were amplified by PCR and detected through Southern blot hybridization 60 days after the first and after the third amendments (Fig. 3). Southern blot hybridizations revealed that cow M contained *tet*(A) carrying bacteria and, 60 days after the first amendment, *tet*(A) was also detected in CL + M, CL + M + Tc100 and CL + M + Tc500, while it was not detected in total community DNA from control CL soil (Fig. 3). At this time point, Southern blot hybridization indicated that *tet*(A) abundance was low in all treatments of SA soil and was only detected in some of the replicates. After the

Table 2 Percent difference (*d*-values) of soil bacterial community structure based on Pearson correlations of background-subtracted densitometric curves from DGGE analysis (60 days after the first, second, and third amendment)

		CL soil		SA soil	
		d-value	*P*	*d*-value	*P*
First amendment	S / S + M	11.8	0.018	7.9	0.099
	S / S + M + Tc100	15.1	0.027	9.5	0.139
	S / S + M + Tc500	25.9	0.022	12.6	0.053
	S + M / S + M + Tc100	−1.4	0.660	1.5	0.262
	S + M / S + M + Tc500	5.7	0.029	10.1	0.030
	S + M + Tc100 / S + M + Tc500	3.9	0.119	1.9	0.156
Second amendment	S / S + M	28.7	0.035	30.9	0.029
	S / S + M + Tc100	29.0	0.037	35.4	0.030
	S / S + M + Tc500	33.2	0.028	37.9	0.048
	S + M / S + M + Tc100	1.1	0.345	2.0	0.098
	S + M / S + M + Tc500	6.0	0.054	13.8	0.029
	S + M + Tc100 / S + M + Tc500	1.1	0.397	9.7	0.021
Third amendment	S / S + M	62.2	0.027	45.5	0.034
	S / S + M + Tc100	61.7	0.042	41.1	0.027
	S / S + M + Tc500	52.7	0.025	44.2	0.025
	S + M / S + M + Tc100	0.1	0.398	2.5	0.138
	S + M / S + M + Tc500	16.2	0.029	12.9	0.030
	S + M + Tc100 / S + M + Tc500	1.8	0.163	4.1	0.029

Significance is indicated by the respective *P* value

S soil CL or SA, *M* manure, *Tc100* 100 mg Tc kg^{-1} soil dry weight, *Tc500* 500 mg Tc kg^{-1} soil dry weight

third amendment, the abundance of *tet*(A) decreased in CL + M soils. Thus, *tet*(A) was detected only in three of four replicates of the CL + M + Tc100 and in all replicates of CL + M + Tc500, whereas it was below the detection limit in CL + M. In SA soil, a remarkably increased abundance of *tet*(A) was detected in the total community DNA from all replicates of SA + M + Tc500, indicating a selective effect of Tc (Fig. 3). The *tet*(M) was neither detected in soil, treated soil, nor in M (data not shown).

sul1 and *sul2*

Cow M contained −2.5 Log per *rrn* copy number of *sul*1 and *sul*2. In CL soil, the relative abundances of *sul1* and *sul2* genes were, respectively, −4.6 and −6.3 Log per *rrn* copy number and 60 days after the first amendment, and the relative abundance of *sul1* and *sul2* genes significantly increased after application of M, compared to control CL (Fig. 4a, c). In contrast, 60 days after M addition to SA soil, an increased relative abundance was only observed for *sul2* but not for *sul1* (Fig. 4b, d). In CL + M + Tc100 and CL + M + Tc500, a significantly increased relative abundance of *sul1* was observed, compared to CL + M, while *sul2* was only increased in the CL + M + Tc500 treatments. The effect of the presence of Tc was even less pronounced in SA soil. Here, only the relative abundance of *sul1* was significantly increased in SA + M + Tc500, compared to SA + M after the third amendment. Repeated addition of M + Tc caused an accumulation of *sul1* and *sul2* for both Tc concentrations, except for the *sul1* in SA + M + Tc100 soils, while repeated additions of M did not.

Changes in the relative abundance of class 1 integrons and IncP-1ε plasmids

intI1

The relative abundance of class 1 integron integrase genes (*intI1*) significantly ($P < 0.05$) increased in CL + M, compared to control CL, 60 days after the first amendment (Fig. 5a), while the relative abundance of *intI1* gene was not significantly increased in SA + M, compared to SA soil (Fig. 5b). The presence of Tc500 caused a significant increase in the relative abundance of *intI1* in CL soil at all sampling times, while in SA soil, an increased abundance was only observed 60 days after the third amendment. In the SA + M + Tc100 treatments, no increase in the relative abundance of *intI1* for SA soils was observed at all sampling times, while an increase was found after the third amendment in the CL + M + Tc100 treatment.

IncP-1ε trfA

Quantification of the *trfA* gene was used to detect and quantify the ε subgroup of IncP-1 plasmids in response to the treatments. The abundance of IncP-1ε plasmids in CL soil was

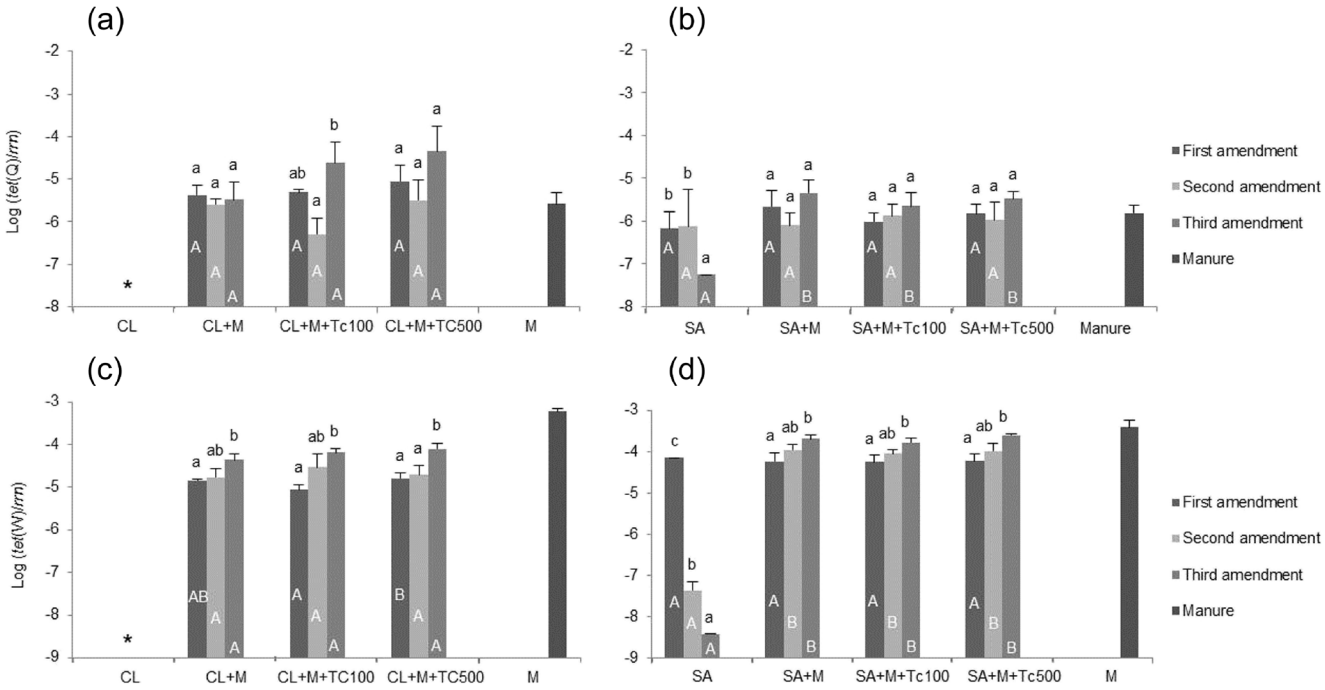

Fig. 2 Relative abundances of *tet*(Q) and *tet*(W) genes in CL (**a**, **c**) and SA (**b**, **d**) soils, respectively, were measured by qPCR 60 days after the first, second, and third amendments. Tukey-Kramer post hoc test ($P < 0.05$) was used to compare the relative abundances of the target genes in total community DNA at the same time point or within the treatment after repeated amendments. *M* manure, *Tc100* 100 mg Tc kg^{-1} soil dry weight, *Tc500* 500 mg Tc kg^{-1} soil dry weight. Below the detection limit (*asterisk*). For each time point, average values which share the same *white capital letter within columns* do not differ significantly at the 5 % level. For each treatment, average values which share the same *letters above columns* do not significantly differ at the 5 % level. *Error bars* indicate the standard deviation of four replicates

low, and although the M added to the soil contained a high abundance of IncP-1ε plasmids, the relative abundance of these plasmids in CL + M remained low, even after repeated M additions (Fig. 5c). Even the presence of Tc did not lead to an increase in the relative abundance of IncP-1ε plasmids. In contrast, in SA soil, M increased the relative abundance of *trfA* in SA + M already 60 days after the first amendment, compared to the SA soil (Fig. 5d). The presence of Tc increased the relative abundance of *trfA*, which was significant for SA + M + Tc500 already after the first amendment while a significantly increased abundance was observed for SA + M + Tc100 treatment, compared to SA + M, only after the second amendment. After the third amendment, the relative abundance of *trfA* in SA + M + Tc100 and SA + M + Tc500 was even higher than in the M. An accumulation of the *trfA* gene with repeated application was only observed in SA + M + Tc500.

Discussion

The fate of antibiotics in soil and likely also their effects are influenced by the physicochemical properties of the antibiotic, by soil properties (Kong et al. 2012) and soil history, intended as foregoing antibiotic soil pollution due to anthropogenic

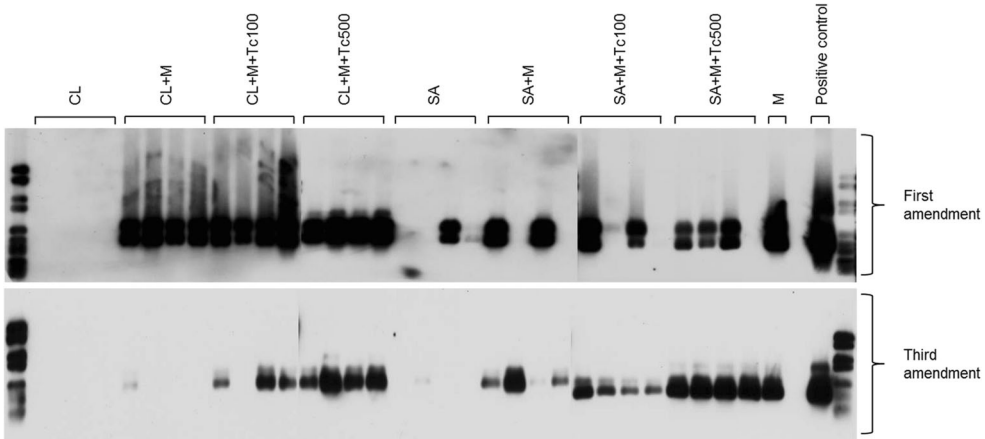

Fig. 3 PCR Southern blot hybridization of the *tet*(A) gene in CL and SA soils 60 days after the first and third amendments. Four replicates for each treatment are shown. The *lanes* at the *utmost right* and *left* are size markers. The figure is composed of six hybridized membranes (three membranes per soil) which were reassembled by Photoshop

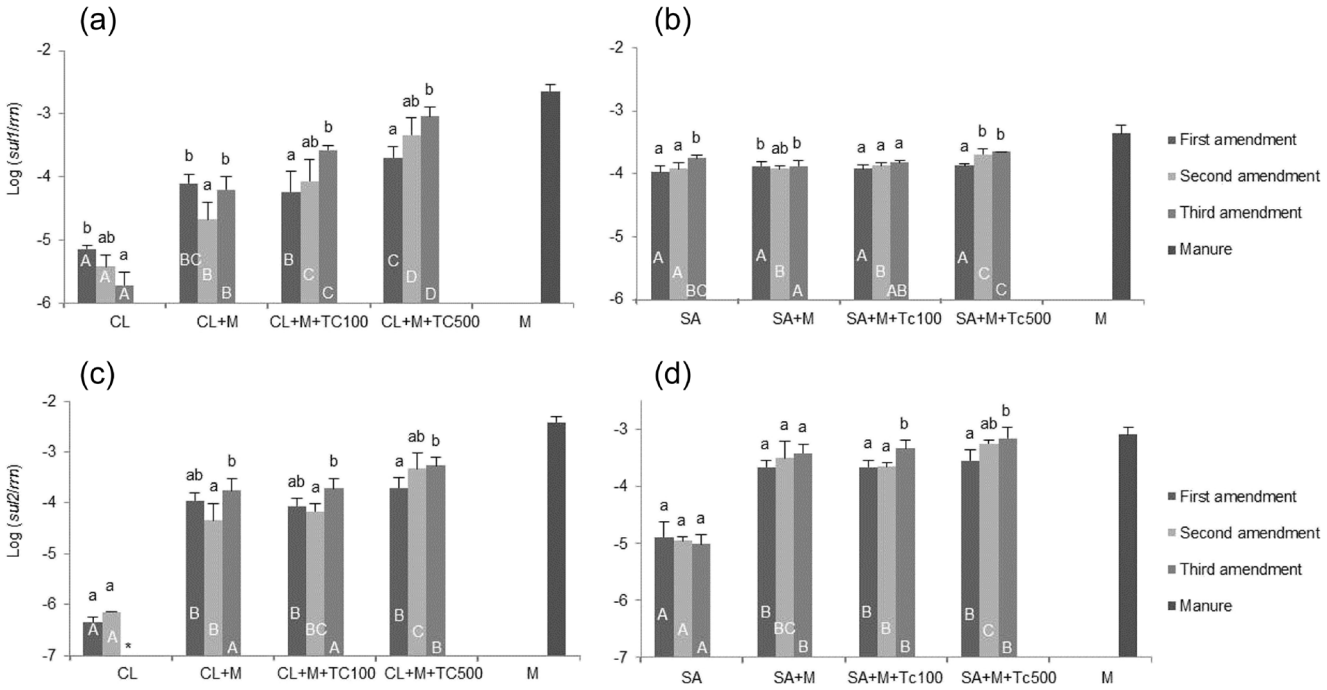

Fig. 4 Relative abundances of *sul1* and *sul2* genes in CL (**a**, **c**) and SA (**b**, **d**) soils, respectively, measured by qPCR 60 days after the first, second, and third amendments. The Tukey-Kramer post hoc test ($P < 0.05$) was used to compare the relative abundances of the target genes in the total community DNA at the same time point or within the treatment after repeated amendments. *M* manure, *Tc100* 100 mg Tc kg^{-1} soil dry weight, *Tc500* 500 mg Tc kg^{-1} soil dry weight. Under the detection limit (*asterisk*). For each time point, average values which share the same *white capital letter within columns* do not differ significantly at the 5 % level. For each treatment, average values which share the *same letters above columns* do not differ significantly at the 5 % level. *Error bars* indicate the standard deviation of four replicates

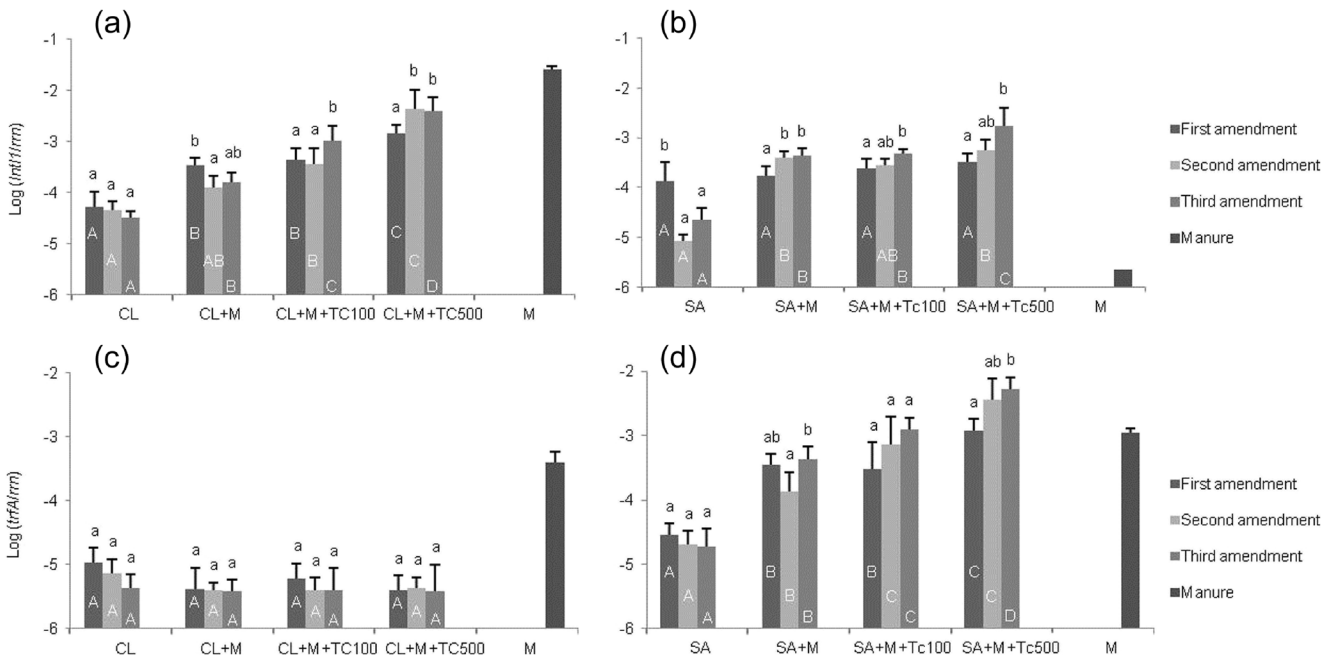

Fig. 5 Relative abundances of *intI1* and *trfA* genes in CL (**a**, **c**) and SA (**b**, **d**) soils, respectively, measured by qPCR 60 days after the first, second, and third amendments. The Tukey-Kramer post hoc test ($P < 0.05$) was used to compare the relative abundances of the target genes in the total community DNA at the same time point or within the treatment after repeated amendments. *M* manure, *Tc100* 100 mg Tc kg^{-1} soil dry weight, *Tc500* 500 mg Tc kg^{-1} soil dry weight. For each time point, average values which share the same *white capital letter within columns* do not differ significantly at the 5 % level. For each treatment, average values which share the same *letters above columns* do not differ significantly at the 5 % level. *Error bars* indicate the standard deviation of four replicates

activities. The present study is part of a project aiming to better understand the effects of Tc and cow M on the microbial community composition and on the antibiotic resistance in soil. In this study, two soils with different history of anthropogenic pollution and physicochemical characteristics, described by Chessa et al. (2016) and listed in Table S1, were used. The soils used were primarily selected for their differences in history of anthropogenic pollution and also for differences in physicochemical composition (pH, clay, sand, and organic matter). The bioavailability of Tc, measured only after the first amendment by Chessa et al. (2016), was 0.155 and 1.092 mg kg^{-1} for treatments 100 and 500 mg kg^{-1} in CL soil and 0.767 and 4.468 mg kg^{-1} in SA soil, respectively. The Tc concentrations used for spiking cow M were higher compared to those used in other studies. However, as Tc quickly, and almost completely, adsorbs to soil clay minerals and organic matter, high amounts of Tc were applied to the soil in order to reach soluble and potentially bioavailable concentrations, i.e. Tc available for the bacterial uptake (Zhang et al. 2014) that were previously described for agricultural soils which are continuously polluted (Hamscher et al. 2005; Qiao et al. 2012). In fact, the Tc concentrations not adsorbed and hence potentially bioavailable found in the M + Tc100 soils were 0.155 and 0.767 mg kg^{-1} for CL and SA soil, respectively (Chessa et al. 2016). Comparable concentrations were previously reported for Tc in M-treated soils and ranged between 0.15 and 0.8 mg Tc kg^{-1} soil (Hamscher et al. 2005, 2002; Qiao et al. 2012). Furthermore, we tested Tc spiked at a fivefold higher concentration (M + Tc500) as a possible worst case scenario, since previous studies already indicated that soil microbial populations were not affected by Tc in lower concentrations, as reported by Hund-Rinke et al. (2004) where no significant effect on the bacterial composition and Tc resistance genes was found in soil polluted with 5 and 50 mg Tc kg^{-1}, whereas Tc significantly affected the microbial community composition at the spiked concentration of 500 mg kg^{-1}.

In the present microcosm study, we showed that the effect of cow M on the bacterial community composition increased with repeated application (Table 2), while the effects of the Tc were far less pronounced. After a single M or M + Tc application, significant differences to DGGE fingerprints of untreated soil were only observed for CL soil. In contrast to CL soil, DGGE fingerprints did not reveal such effects 60 days after the first application of M or of M + Tc to SA soil. We assume that transient effects of M or M + Tc might have occurred also in SA soil but had disappeared already at the time of sampling 60 days after the first amendment, as previously was also observed by Selvam et al. (2012). In accordance with other studies (Ding et al. 2014; Marschner et al. 2003; Sun et al. 2004), we observed that repeated application of M, with or without Tc, enhanced the effects on the bacterial community composition in both soils as indicated by increasing differences (*d*-values) between the DGGE fingerprints of untreated and M-treated soils (Table 2). Chessa et al. (2016) already reported that a single cow M application changed the bacterial community composition to increased PLFA ratio of Gram-positive to Gram-negative bacteria in both soils, and this effect was still observed in CL soil after 60 days but not in SA soil. The cow M used for the present study, the same as already used by Chessa et al. (2016), was long-term stored and air-dried, and this, together with divergent soil properties, might explain differences compared to the findings of other studies performed with piggery M and silt loam soil (Ding et al. 2014; Heuer et al. 2008). In comparison to SDZ used in these studies, the effects of Tc spiked to the cow M were far less pronounced which was likely caused by the stronger sorption of Tc compared to SDZ and due to different properties of soils studied: clayey and sandy soil compared to silt loam. Tc100 spiked to the cow M did not cause significant shifts of the soil bacterial fingerprints, compared to soil treated with unspiked M. After three amendments with M + Tc500, the fingerprints significantly differed from those of the M treatments for both soils. In contrast, the presence of SDZ in piggery M spiked at two concentrations (10 and 100 mg kg^{-1}) caused significant changes of the bacterial community composition in the two soils compared to unspiked M (Ding et al. 2014; Heuer et al. 2011a).

Although the cow M was collected from free ranged cows, which to the best of our knowledge were not treated with antibiotics, all the ARGs and MGEs analyzed in the present study, except *tet*(M), were detected in the M. Thus, with the cow M, not only nutrients and spiked Tc were introduced into the soils but also bacteria containing ARGs and MGEs. The abundances of ARGs and MGEs were below the detection limit or at very low abundance in the CL soil, which had no history of anthropogenic antibiotic pollution by human or animal wastes. Thus, a striking increase in the relative abundance of ARGs and MGEs was observed for CL soil in response to the M amendment. In contrast, all ARGs except *tet*(M) and MGEs analyzed in the present study were detected in the SA soil which was in the past frequently affected by flooding of a river and by orchard farming practice. River water was previously reported as a carrier of ARGs (Amos et al. 2014a, b, 2015). Thus, the differences found between the CL and SA soil likely do not only result from differences in soil properties such as clay content and pH but also result from their previous history of anthropogenic pollutants. This pollution might have resulted in an adaptation of the bacterial community through proliferation of resistant bacteria and horizontal gene transfer (HGT) (Heuer and Smalla 2012).

The ARGs and MGEs analyzed in the present study were previously reported to occur in piggery and cow M (Alexander et al. 2011; Binh et al. 2008). Recently, Kyselková et al. (2015a) proposed that *tet*(Q) and *tet*(W) genes belong to the resistome stably associated with cow M. Here, we also found that cow M from free ranged animals contained bacteria carrying the ARGs *tet*(Q), *tet*(W), *tet*(A), *sul1* and *sul2*. In contrast

to our hypothesis, the M + Tc100- and M + Tc500-treated soils did not show significantly increased abundance of *tet*(Q) and *tet*(W), compared to the M-treatments. However, with repeated M applications, an accumulation of *tet*(Q) and *tet*(W) could be observed irrespective of the presence of Tc. Kyselková et al. (2013) also found that Tc did not show additive effects on the abundance of *tet*(Q) and *tet*(W) genes, compared to unspiked M. Likely these genes were hosted in bacteria that did not proliferate in soil, and thus, neither M nor selective pressure exerted by Tc increased their abundance. In contrast, *tet*(A) likely carried by other bacterial hosts was clearly increased in the M + Tc500 treatments in both soils. In the control soils, the relative abundance of *tet*(Q) and *tet*(W) tended to decrease over the time of our experiment. Probably, the populations carrying these genes decreased in relative abundance due to the lack of nutrient input in the untreated control soils. In cow M and in both soils the abundances of *tet*(Q) genes were, in tendency, lower than those of *tet*(W) (Wolters et al. 2016).

Since *tet* genes were often reported to co-occur with *sul* genes on plasmids (Heuer et al. 2009, 2012; Roberts 2011), the relative abundances of *sul1* and *sul2* genes were also determined in the present study. Our results showed that also the *sul1* and *sul2* genes increased in abundance with repeated M application and that Tc500 clearly co-selected for *sul1* and *sul2* genes in CL soils. Furthermore, our results showed that for the M + Tc500 treatments, a synergistic effect of M and Tc500 occurred, especially in CL soil, and Tc500 spiking caused an accumulation of *sul* genes as well as of *tet*(W) genes. However, M + Tc500 did not cause a pronounced increase in the relative abundance of *sul1* genes compared to the M treatment in SA soil. In contrast, the relative abundance of *sul2* genes, which are typically carried on plasmids reported from piggery M, e.g. on LowG + C (Heuer et al. 2009) or IncQ (Smalla et al. 2000), was increased due to the repeated M amendments in SA soil. An accumulation of *sul1* and *sul2* genes was also reported for repeated soil applications of piggery M spiked with SDZ by Heuer et al. (2011b). In the present study, Tc co-selected for *sul* genes likely due to the co-localization on the same MGE. An example for such a co-localization of *tet*(A) and *sul1* was reported for IncP-1ε plasmids that were recently captured by exogenous isolation from M-treated arable soils, from M or digestates (Bahl et al. 2007; Binh et al. 2008; Heuer et al. 2012; Wolters et al. 2015), and sequencing revealed that these IncP-1ε plasmids often carried *tet*(A) upstream and *sul1* downstream of the class 1 integron.

The presence of Tc in M selected for IncP-1ε plasmids in SA soil, while no such increase was observed in CL soils. Probably bacterial populations carrying IncP-1ε plasmids applied with M were not well adapted to conditions present in the CL soil and thus rapidly decreased in relative abundance. Several previous studies showed that repeated amendments of M enhanced the HGT of MGE within microbial communities (Ghosh and LaPara 2007; Heuer et al. 2011a; Jindal et al. 2006; Popowska et al. 2012; You et al. 2012), whereas the effect of a single M application on tetracycline resistance levels in soil bacterial communities may be only transient (Chessa et al. 2016; Sengeløv et al. 2003).

In the present study we demonstrated a soil type- and history-dependent impact of M amendment on soil bacterial communities. The physicochemical characteristics of the soils likely determined the Tc sorption capacity and antibiotic availability for bacterial uptake, while the different history of pollution of the two soils reflected the different microbiological responses to repeated M and Tc application. Effects of Tc spiked to the M on the bacterial community composition of both soils were surprisingly negligible while more pronounced effects, in particular in response to the application of a high amount of Tc, were found on the resistome and on the mobilome. To allow an appropriate risk assessment of the application of cow M containing antibiotics to agricultural soil, future research should include a broader range of soils to disentangle the influence of different physicochemical soil properties and histories of anthropogenic pollution on the microbial response and use of molecular techniques determining not only dominant but also rare microbial species.

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