

## RESEARCH ARTICLE

# Soil texture-dependent effects of doxycycline and streptomycin applied with manure on the bacterial community composition and resistome

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**One sentence summary:** Effects of doxycycline- or streptomycin-spiked manure on soil bacteria are dependent on the antibiotic concentration and the soil type.

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## ABSTRACT

Veterinary antibiotics, bacteria carrying antibiotic resistance determinants located on mobile genetic elements and nutrients are spread on agricultural soil using manure as fertilizer. However, systematic quantitative studies linking antibiotic concentrations and antimicrobial resistance genes (ARGs) in manure and the environment are scarce but needed to assess environmental risks. In this microcosm study, a sandy and a loamy soil were mixed with manure spiked with streptomycin or doxycycline at five concentrations. Total-community DNA was extracted on days 28 and 92, and the abundances of ARGs (*aadA*, *strA*, *tet(A)*, *tet(M)*, *tet(W)*, *tet(Q)*, *sul1*, *qacE/qacEΔ1*) and class 1 and 2 integron integrase genes (*intI1* and *intI2*) were determined by qPCR relative to 16S rRNA genes. Effects on the bacterial community composition were evaluated by denaturing gradient gel electrophoresis of 16S rRNA gene amplicons. Manure application to the soils strongly increased the relative abundance of most tested genes. Antibiotics caused further enrichments which decreased over time and were mostly seen at high concentrations. Strikingly, the effects on relative gene abundances and soil bacterial community composition were more pronounced in sandy soil. The concept of defining antibiotic threshold concentrations for environmental risk assessments remains challenging due to the various influencing factors.

**Keywords:** manure; soil; antibiotic resistance; resistome; mobilome; risk assessment

## INTRODUCTION

By manure fertilization, veterinary antibiotics, antibiotic-resistant bacteria and antibiotic resistance determinants located on mobile genetic elements (MGEs) reach agricultural soils, where they can have significant effects on the soil bacterial community composition and resistome (Jechalke et al. 2014a). Ultimately, the pollution of soil by veterinary antibiotics and resistance determinants could contribute to the increasing threat of multiresistant pathogens. However, for an environmental risk assessment of these components systematic quantitative studies, linking concentrations of antimicrobials and antimicrobial resistance genes (ARGs)/MGEs in manure and in the environment are urgently needed.

In a modeling approach, predicted no-effect concentrations (PNECs) for resistance selection were estimated based on minimal inhibitory concentrations from the EUCAST database resulting in a range from 8 ng/L to 64  $\mu\text{g/L}$  (Bengtsson-Palme and Larsson 2016). First attempts have been made in determining minimal selective concentrations (MSCs) for complex environments such as artificial streams (Quinlan et al. 2011) or aquatic bacterial biofilms (Lundström et al. 2016). However, the concept of MSCs is likely to be limited when applied to complex microbial communities and heterogeneous matrices such as manure-amended agricultural soils, in which the estimation of bioavailable concentrations of antibiotics is challenging and depending on the extraction method and the soil type (Jechalke et al. 2014a; Bengtsson-Palme and Larsson 2016).

Doxycycline is a second-generation tetracycline derivate used as veterinary antibiotic in animal husbandry (Szatmari et al. 2012). It is active against a range of Gram-negative and Gram-positive bacteria by inhibiting protein synthesis (Chopra and Roberts 2001; Nguyen et al. 2014). Residues of doxycycline were reported in considerable concentrations in liquid pig manures and digestates of biogas plants with concentrations of up to 381 mg kg<sup>-1</sup> dry weight and 10.5 mg kg<sup>-1</sup> dry weight, respectively (Widyasari-Mehta, Hartung and Kreuzig 2016; Wolters et al. 2016b). Concentrations of up to 22.76 mg kg<sup>-1</sup> were detected in swine manure samples collected from manure pits and farms in West Flanders, Belgium (Van den Meersche et al. 2016). The half-life of doxycycline in manure was calculated to be 25.7 days, while in soil it was from 59.4 to 76.3 days, depending on soil depth (Szatmari, Laczay and Borbely 2011; Szatmari et al. 2012). Among more than 50 known tetracycline resistance determinants, *tet(A)* coding for the Tet(A) efflux pump is the gene most frequently occurring in Gram-negative bacteria (Nguyen et al. 2014) and was frequently detected on IncP-1 $\epsilon$  plasmids associated with class 1 integrons and transposons (Popowska and Krawczyk-Balska 2013; Wolters et al. 2015). Resistance genes *tet(Q)*, *tet(M)* and *tet(W)* were frequently detected in manure and manure-treated soils (Zhu et al. 2013; Wolters et al. 2016a). It was observed that the application of pig manure elevated the occurrence of tetracycline-resistant bacterial isolates in soil but declined over a period of eight months to a level corresponding to the control soil (Sengeløv et al. 2003). However, not much is known about the effects of doxycycline on soil bacterial populations and on the abundance of resistance genes when spread with manure, and about the influence of the soil texture.

Streptomycin belongs to the broad-spectrum aminoglycoside antibiotics used in human and veterinary medicine to treat infections caused by Gram-positive and Gram-negative bacteria. In the year 2015, the amount of aminoglycoside antibiotics delivered to veterinarians in Germany was 25 tons

([www.bvl.bund.de](http://www.bvl.bund.de)). Aminoglycosides are excreted unchanged with the urine (Gehring et al. 2005) and might reach agricultural fields by manure application. Streptomycin and spectinomycin resistance-mediating *aadA* gene cassettes are among the most prevalent gene cassettes in class 1 and 2 integrons (Sunde and Norström 2005) and were, for example, frequently detected on class 1 integrons from field-scale manures (Binh et al. 2009). The *strA-strB* gene pair was reported to be widely disseminated and was frequently detected on small broad-host-range IncQ plasmids or on large conjugative plasmids associated with the transposon Tn5393 (Smalla et al. 2000; Sundin 2000; Sunde and Norström 2005). For example, the *strA-strB* gene pair was also found on LowGC-type plasmids that were observed to be highly prevalent in manure and manured soil (Heuer et al. 2009; Jechalke et al. 2013b). In a study by Sengeløv et al. (2003), only minor variations in abundance of streptomycin-resistant bacteria were observed in soil after application of pig manure slurry, although high numbers of streptomycin-resistant bacteria were found in the manure. However, streptomycin applied with manure to soil is likely to have an effect on the bacterial community composition and might select for antibiotic resistance.

In this microcosm study, five concentrations of streptomycin or doxycycline were applied with manure to a sandy or a loamy soil. Besides a strong manure effect, we hypothesized that correlations could be observed between amounts of antibiotics and effects on the abundance of ARGs and MGEs, but would differ over time and between the soil types and antibiotic compounds. These correlations then could be used to derive threshold concentrations for environmental risk assessments with no effects on the soil ARGs and MGEs. As a second indicator for effects of antibiotics applied with manure on structure and function of soil bacterial communities, we evaluated correlations between amounts of antibiotics and changes in soil bacterial community composition, which were assessed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from total community (TC)-DNA.

## MATERIAL AND METHODS

### Origin and characteristics of manure and soil

In order to investigate the influence of the soil texture and properties on the effects of doxycycline and streptomycin applied with manure, two standard soils were selected (a silty sand and a clayey loam), which were obtained from the Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer (LUFASpeyer, Speyer, Germany). The soils are natural soils of commonly occurring soil textures from selected areas of Germany which were under agricultural use without the application of pesticides, biocidal fertilizers or organic manure for at least 5 years, sampled from 0–20 cm depth and sieved <2 mm ([www.lufaspeyer.de](http://www.lufaspeyer.de)). The characteristics of the standard soils were as follows: pH value (0.01 M CaCl<sub>2</sub>) of 5.1 and 7.2, 0.65 and 2.26% organic carbon, 0.05 and 0.20% N and 4.3 and 31.4 (meq/100 g) cation-exchange capacity for the sandy and the loamy soil, respectively.

Pig manure used in this study was obtained from a pig husbandry farm in Lower Saxony, Germany, keeping 2000 fattening pigs and 220 sows. The manure was stored at 4°C until the beginning of the experiment. The matrix characterization of this manure was performed by LUFASpeyer (Hameln, Germany). Matrix parameters were as follows: 3% dry weight, 3.4 kg N<sub>total</sub> m<sup>-3</sup>, 1.9 kg NH<sub>4</sub>-N m<sup>-3</sup>, 1.3 kg P<sub>2</sub>O<sub>5</sub> m<sup>-3</sup>, 2.2 kg K<sub>2</sub>O m<sup>-3</sup>, 0.5 kg MgO m<sup>-3</sup>, 1.1 kg CaO m<sup>-3</sup>, 0.2 kg S m<sup>-3</sup>, 18.7 g Cu m<sup>-3</sup>,

41 g Zn m<sup>-3</sup>. The concentration of doxycycline in manure was determined by ultrahigh-performance liquid chromatography-tandem mass spectrometry as described previously by Van den Meersche et al. (2016).

### Experimental design

Soil microcosm experiments were established with the following treatments: (i) non-treated soil, (ii) soil treated with manure, soil treated with manure supplemented (iii) with streptomycin and (iv) with doxycycline at different concentrations.

Two kinds of soil microcosms and treatments were performed in four replicates consisting of (i) soil treated with manure spiked with streptomycin (0.1, 1, 10, 100 and 1000 mg kg<sup>-1</sup> manure wet weight) (streptomycin sulfate salt, Sigma-Aldrich, München, Germany); (ii) soil samples amended with manure spiked with doxycycline (1, 10, 100, 1000 and 5000 mg kg<sup>-1</sup> manure wet weight) (doxycycline hyclate, purity >98%, Sigma-Aldrich). This resulted in theoretical concentrations of 0.04, 0.4, 4, 40, 200 mg doxycycline kg<sup>-1</sup> soil (dry weight) and 0.004, 0.04, 0.4, 4 and 40 mg streptomycin kg<sup>-1</sup> soil (dry weight), respectively.

All soil microcosms contained 300 g of soil (dry weight) per replicate. Doxycycline and streptomycin-treated soil samples were prepared by dissolving streptomycin and doxycycline in water separately and then adding them to the amount of manure approximately corresponding to a general application for arable soil (40 g kg<sup>-1</sup> soil). The manure was added to the soils and mixed, and the water content of the soil was adjusted to a maximum water holding capacity of 50%. Unamended and manure-amended soils served as controls to assess the effect of manure and of doxycycline and streptomycin, respectively. The pots were incubated in the dark at 20°C in a greenhouse, for 28 and 92 days. Every 2 days, the amount of evaporated water was measured by weighing and compensated by adding deionized water to maintain constant moisture. On day zero after setup of the microcosms, as well as on days 28 and 92, soil samples (5 g) were taken and stored at -20°C for later extraction of TC-DNA.

### DNA extraction and purification

TC-DNA was extracted from 0.5 g of soil or 0.1 g manure using the FastDNA® SPIN Kit for soil (MP Biomedicals, Heidelberg, Germany). The extracted DNA was purified by the GeneClean® Spin Kit (MP Biomedicals), following the manufacturer's instructions. The quality of extracted TC-DNA was determined by agarose gel electrophoresis.

### Quantification of target genes

PCR amplifications for the quantification of target genes in TC-DNA from soil and manure samples were performed with quantitative real-time PCR 5'-nuclease TaqMan assays (qPCR) in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) as previously reported for integrase genes *intI1* and *intI2* of class 1 and 2 integrons (Barraud et al. 2010), *korB* specific for IncP-1 plasmids (Jechalke et al. 2013a), quaternary ammonium compound resistance genes *qacE* and the *qacEΔ1* variant (Jechalke et al. 2014c), the streptomycin/spectinomycin resistance genes *aadA* and *strA* (Walsh et al. 2011), tetracycline resistance genes *tet(W)* and *tet(Q)* (Smith et al. 2004), *tet(A)* (Guarddon et al. 2011), *tet(M)* (Peak et al. 2007) and sulfonamide resistance gene *sul1* (Heuer and Smalla 2007). The 16S rRNA gene (*rrn*) copies were quantified using the primers BACT1369F and PROK1492R and the probe TM1389F (Suzuki, Taylor and DeLong 2000). Serial

dilutions of quantification standards were used to calculate copy numbers of genes. The relative abundances of ARGs were calculated by dividing the copy numbers of the respective genes by the *rrn* copy numbers and the results were log transformed. The Pearson correlation coefficient (*r*) and *P*-values between the relative abundance of antibiotic resistance genes and concentrations of antibiotics were tested by means of the CORR procedure of the SAS statistical package (*P* < 0.05; SAS 9.3; SAS Institute Inc., Cary, NC, USA). Pairwise comparisons of mean values were performed using the GLIMMIX procedure (Tukey test, *P* < 0.05; SAS 9.3).

### Denaturing gradient gel electrophoresis

Bacterial 16S rRNA gene fragments were amplified by PCR and separated by DGGE, which was performed as described previously (Weinert et al. 2009). Bacterial primers used (F984-GC, R1378) were described by Heuer et al. (1997). DGGE gels were scanned with high-resolution settings (Epson 1680 Pro, Seiko-Epson Corp. Suwa, Nagano, Japan). After scanning of the DGGE banding patterns and alignment, a Pearson correlation was performed with the program GelCompar II® (version 6.5, Applied Maths, Sint-Martens-Latem, Belgium). The resulting similarity matrix was used to test for significant effects between treatments as described previously (Kropf et al. 2004; Jechalke et al. 2013b).

Non-metric multidimensional scaling analyses were performed with the function *metaMDS* of the package *vegan* implemented in R (R Core Team 2015), which is based on the dissimilarity matrix. Gene relative abundance data were fitted onto the ordinations using the function *envfit* of the R package *vegan*. Significance of the associations was determined by 10 000 random permutations.

## RESULTS

### Concentration of doxycycline in manure

The background concentration of doxycycline in the manure for this experiment was determined in four biological replicates. The mean concentration was 0.6 ± 0.1 mg kg<sup>-1</sup> wet weight. The background concentration of streptomycin was not measured in this study.

### Background relative abundance of resistance genes and MGEs in untreated soils and manure

In untreated soils on days 28 and 92, all of the tested genes were below or close to the detection limit (Figs 1 and 2; Tables S1–S4, Supporting Information). In manure, all tested ARGs as well as the class 1 integron integrase gene *intI1* were detected in high relative abundance of -2.8 until -1.2 (Figs 1 and 2). The class 2 integron integrase gene *intI2* and *korB* genes specific for IncP-1 plasmids were detected in manure on average in relative abundance of -4.1 and -3.7, respectively.

### Manure effects on the relative abundance of resistance genes and MGEs in soil

All tested genes were detected in manure-treated soil on days 28 and 92 except for *korB* in the loamy soil and *intI2*, which were below the limit of quantification (Figs 1 and 2; Tables S1–S4). Because the measured gene abundances in the manure for the two experiments (doxycycline/streptomycin) were slightly different, comparisons between the soil types were made for each experiment separately. In general, the effect of the manure

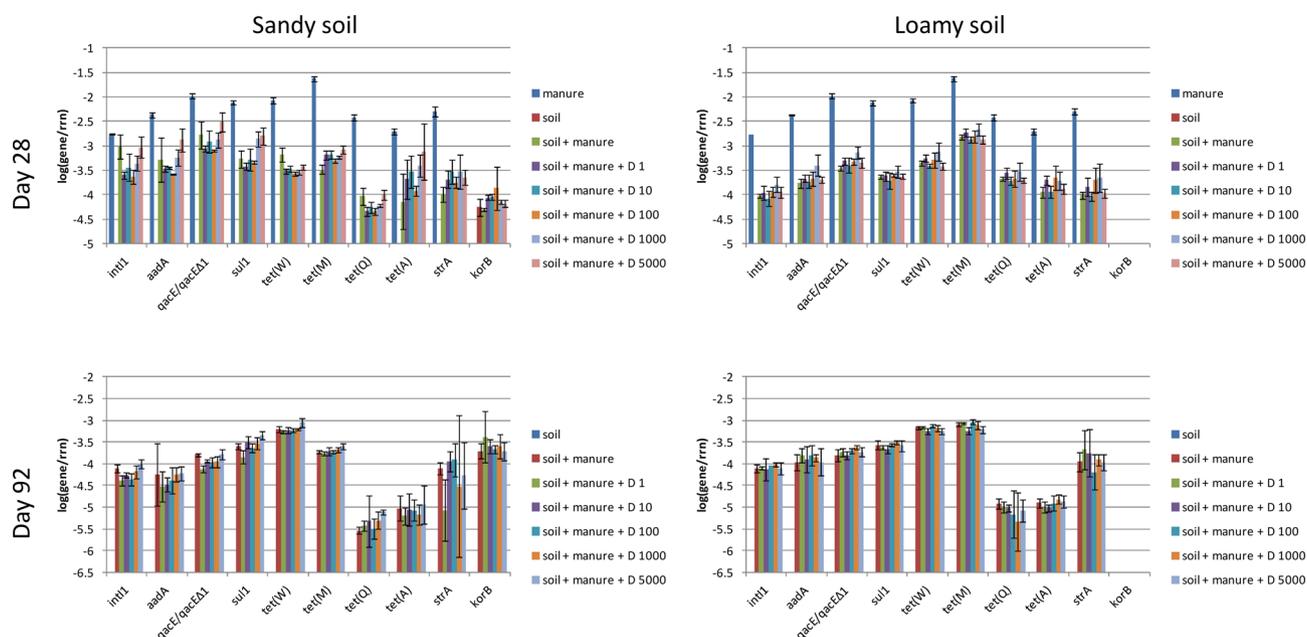


Figure 1. Abundance of genes relative to 16S rRNA gene abundance on days 28 and 92. Results for manure, soil and soil treated with manure spiked with different concentrations of doxycycline (D, mg kg<sup>-1</sup>) and respective standard deviations are shown.

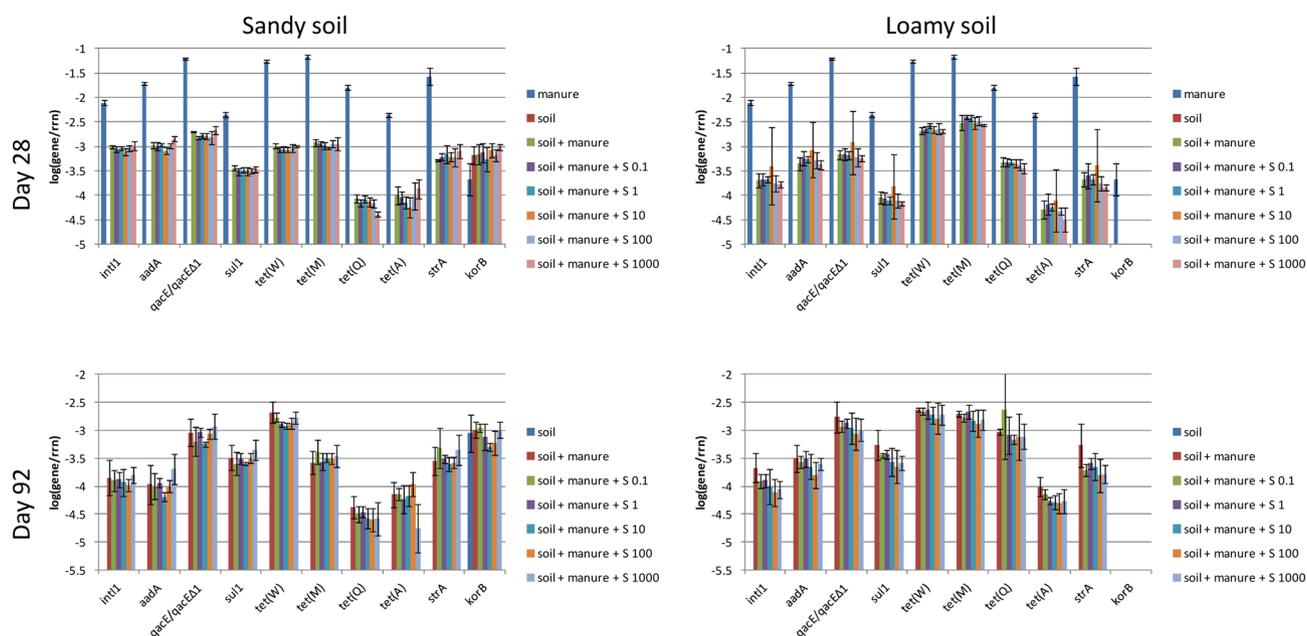


Figure 2. Abundance of genes relative to 16S rRNA gene abundance on days 28 and 92. Results for manure, soil and soil treated with manure spiked with different concentrations of streptomycin (S, mg kg<sup>-1</sup>) and respective standard deviations are shown.

application on the relative abundance of tested genes differed between the two soil types. The relative abundance of tested genes on day 28 was significantly higher (*t*-test,  $P < 0.05$ ) in manure-treated sandy than in loamy soil for the genes *intI1*, *aadA* (only streptomycin experiment), *qacE/qacEΔ1* and *sul1* (Figs 1 and 2). A significantly lower relative abundance in manured sandy compared to loamy soil was observed for *tet(M)* and *tet(Q)*. The relative abundance of *korB* genes could not be compared because it was below the limit of quantification in the loamy soil.

On day 92, most of the gene abundances in the sandy soil were decreased compared to day 28. Furthermore, the relative gene abundances were not significantly higher anymore in the

manured sandy soil compared to the manured loamy soil. Only the genes *tet(M)* and *tet(Q)* were still significantly lower in relative abundance in the sandy soil than in the respective loamy soil (Figs 1 and 2).

### Correlation between antibiotic concentrations and relative abundance of tested genes in sandy and loamy soil

On day 28 in sandy soil, doxycycline concentrations were significantly correlated with class 1 integrons, *aadA*, *qacE/qacEΔ1*, *sul1*, *tet(M)*, *tet(Q)* and *tet(A)* (Table 1). Furthermore,

**Table 1.** Pearson correlation coefficients of tested genes and antibiotic concentrations for soils treated with manure spiked with different concentrations of doxycycline or streptomycin.

Antibiotic	Soil type	Day	<i>intI1</i>	<i>aadA</i>	<i>strA</i>	<i>qacE/qacEΔ1</i>	<i>sul1</i>	<i>tet(W)</i>	<i>tet(M)</i>	<i>tet(Q)</i>	<i>tet(A)</i>	<i>korB</i>
Doxycycline	Sandy	28	0.48*	0.69*	0.13	0.66*	0.78*	0.01	0.53*	0.52*	0.52*	-0.18
		92	0.6*	0.2	-0.14	0.42*	0.61*	0.78*	0.67*	0.43*	0.19	-0.18
	Loamy	28	0.13	0.03	-0.13	0.09	-0.01	-0.27	-0.25	-0.14	-0.14	nd
		92	-0.06	-0.16	-0.09	0.13	0.13	-0.44*	-0.37	-0.12	0.24	nd
Streptomycin	Sandy	28	0.28	0.65*	0.36	0.44*	0.11	0.32	0.09	-0.8*	0.41*	0.33
		92	0.21	0.51*	0.24	0.37	0.42*	0.15	0.11	-0.19	-0.68*	0.2
	Loamy	28	-0.17	-0.2	-0.27	-0.18	-0.2	-0.24	-0.38	-0.5*	-0.36	nd
		92	-0.25	-0.04	-0.28	-0.16	-0.23	-0.11	-0.16	-0.1	-0.18	nd

Significance is indicated by asterisk ( $P < 0.05$ ).  
nd: below limit of quantification.

**Table 2.** Percent difference (d-values) of soil bacterial community composition based on Pearson correlations of background-subtracted den-  
sity curves from DGGE analyses (days 28 and 92).

	Day 28		Day 92	
	Sandy soil	Loamy soil	Sandy soil	Loamy soil
Soil/soil + manure	41.0*	5.7*	32.5*	2.4*
Soil + manure/soil + manure + D 1	9.6*	4.8	1.6*	1.7
Soil + manure/soil + manure + D 10	18.0*	6.1*	1.6*	2.3*
Soil + manure/soil + manure + D 100	8.1*	9.6*	1.7*	1.7*
Soil + manure/soil + manure + D 1000	3.5*	3.3*	4.1*	2.0*
Soil + manure/soil + manure + D 5000	5.4*	2.6*	6.4*	6.7*

Differences are shown between untreated and manure-treated soils as well as between soils treated with unspiked manure and manure spiked with different concentrations of doxycycline (D, mg kg<sup>-1</sup> manure). P-values below 0.05 are indicated by asterisk.

a significant increase in relative abundance of tetracycline resistance genes compared to manured soil was observed for *tet(M)* at all applied concentrations of doxycycline and for *tet(A)* at the highest concentration of doxycycline. In doxycycline-treated loamy soil on day 28, no tested gene was significantly correlated with doxycycline concentration (Table 1).

For streptomycin on day 28, significant positive correlations between antibiotic concentration and the relative abundance of tested genes were only observed in the sandy soil for *aadA*, *qacE/qacEΔ1* and *tet(A)* (Table 1). A significant increase in relative abundance of resistance genes compared to soil treated with unspiked manure was observed for *aadA* at the highest streptomycin concentration.

On day 92, significant positive correlations between doxycycline concentration in the applied manure and relative abundance of tested genes were observed again in the sandy but not in the loamy soil for genes *intI1*, *qacE/qacEΔ1*, *sul1*, *tet(W)*, *tet(M)* and *tet(Q)* (Table 1). A significant increase in relative abundance of genes compared to manured soil was observed for *sul1*, *tet(M)*, *tet(Q)* and *tet(W)* at the highest concentration of doxycycline.

For streptomycin on day 92, significant positive correlations between streptomycin concentration and tested genes were observed again only in the sandy soil for *aadA* and *sul1* (Table 1). A significant increase in relative abundance of genes compared to manured soil was not observed for these genes at any streptomycin concentration. Further correlations between ARGs and class 1 integron integrase gene *intI1* for all soils and treatments are shown in Tables S5–S12 (Supporting Information).

### Effects of manure and antibiotic application on the soil bacterial community composition

Compared to the control, the application of manure had a high impact on the bacterial community composition in the sandy but not in the loamy soil (Tables 2 and 3). The effect of doxycycline-spiked manure compared to the control manure on the bacterial community composition was significant for all concentrations except 1 mg doxycycline/kg manure in the loamy soil (Table 2). On day 92, the effect of doxycycline was less pronounced in both soils at low concentrations, while it was slightly increased at the higher concentrations.

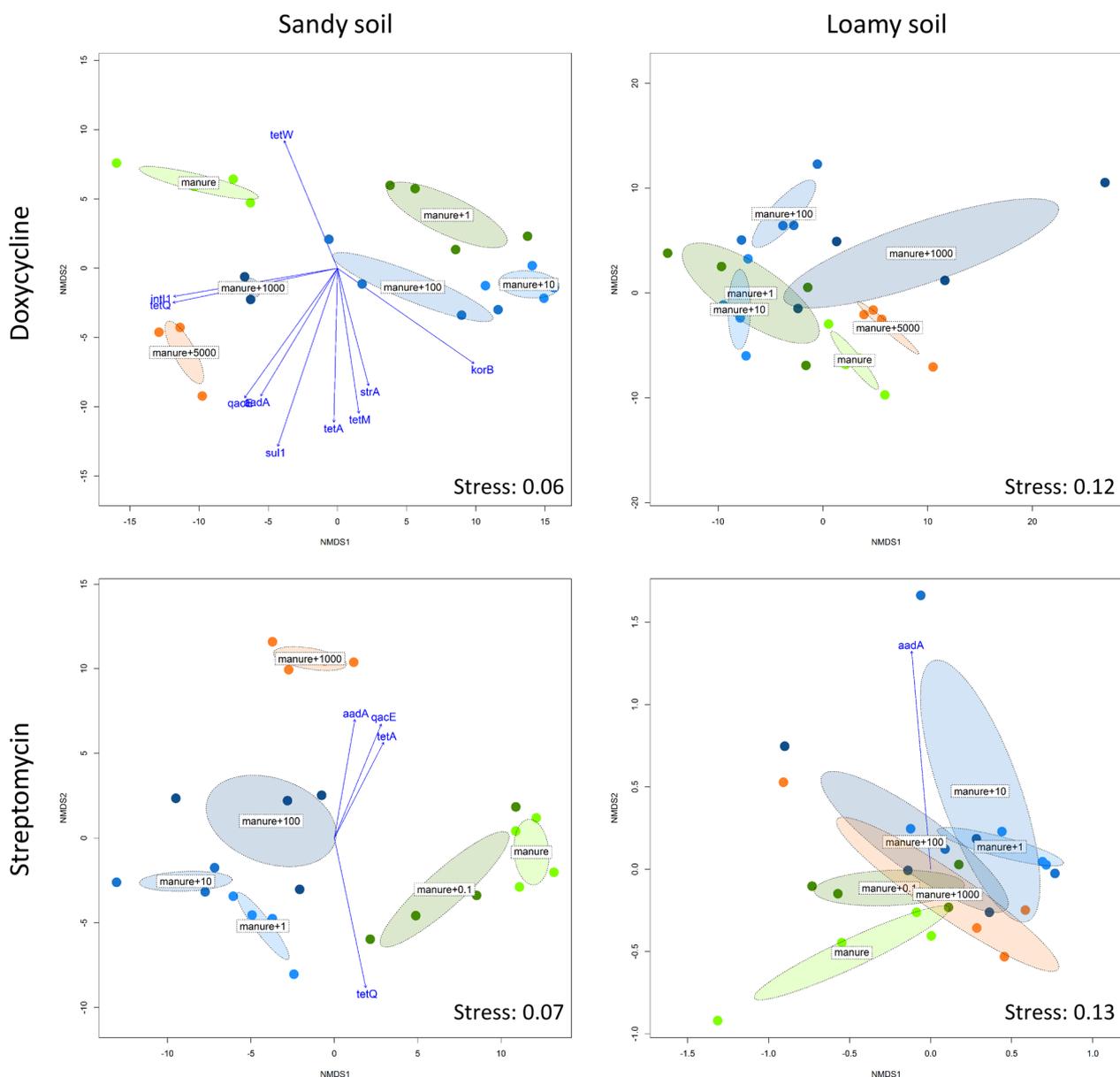
Significant associations ( $P < 0.05$ ) between bacterial community composition and relative abundance of target genes were observed in doxycycline-treated sandy but not in loamy soil. On day 28, all quantifiable genes were significantly associated with the community composition while only for *intI1*, *qacE/qacEΔ1*, *aadA*, *tet(M)*, *tet(Q)*, *tet(A)* and *sul1* the direction of the vectors indicated a correlation with antibiotic concentration (Fig. 3). On day 92 in doxycycline-treated sandy soil, only genes *sul1*, *tet(M)*, *tet(Q)* and *tet(W)* were significantly associated with the community composition (Fig. 4).

For streptomycin on day 28, clear and significant effects of spiked manure on the bacterial community composition compared to control manure were observed in the sandy soil beginning at 1 mg streptomycin kg<sup>-1</sup> manure (Table 3). In the loamy soil, the effects of streptomycin were negligible. On day 92, the effect of streptomycin was less pronounced in the sandy soil at low concentrations while the differences slightly increased

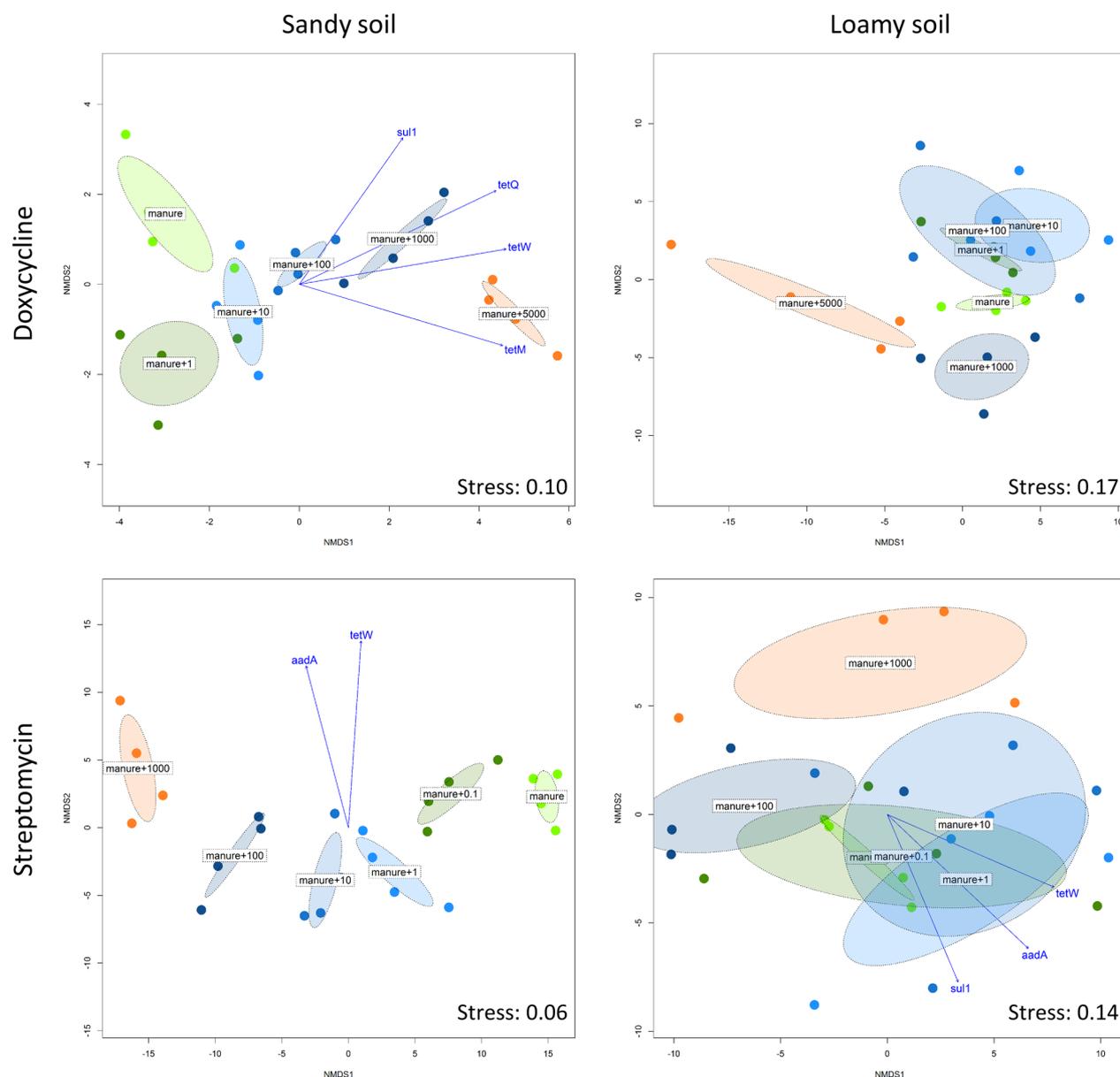
**Table 3.** Percent difference (d-values) of soil bacterial community composition based on Pearson correlations of background-subtracted densitometric curves from DGGE analyses (days 28 and 92).

	Day 28		Day 92	
	Sandy soil	Loamy soil	Sandy soil	Loamy soil
Soil/soil + manure	32.5*	0.0	22.9*	1.4
Soil + manure/soil + manure + S 0.1	1.8	0.0	2.6*	-0.4
Soil + manure/soil + manure + S 1	10.8*	0.4	6.6*	1.8*
Soil + manure/soil + manure + S 10	15.7*	0.4*	11.7*	1.5
Soil + manure/soil + manure + S 100	10.0*	0.2*	16.9*	2.2
Soil + manure/soil + manure + S 1000	11.2*	0.2	25.9*	2.1*

Differences are shown between untreated and manure-treated soils as well as between soils treated with unspiked manure and manure spiked with different concentrations of streptomycin (S, mg kg<sup>-1</sup> manure). P-values below 0.05 are indicated by asterisk.



**Figure 3.** Non-metric multidimensional scaling ordinations of DGGE banding patterns of bacterial communities on day 28 in sandy and loamy soil. Results from the first two ordination axes are given. Different manure treatments with or without doxycycline and streptomycin (mg antibiotic per kg manure wet weight) are indicated by colors and labels. Groupings are indicated by circles showing 40% confidence limits. Gene relative abundance data with significant associations ( $P < 0.05$ ) are indicated as vectors.



**Figure 4.** Non-metric multidimensional scaling ordinations of DGGE banding patterns of bacterial communities on day 92 in sandy and loamy soil. Results from the first two ordination axes are given. Different manure treatments with or without doxycycline and streptomycin (mg antibiotic per kg manure wet weight) are indicated by colors and labels. Groupings are indicated by circles showing 40% confidence limits. Gene relative abundance data with significant associations ( $P < 0.05$ ) are indicated as vectors.

at higher concentrations. In the loamy soil on day 92, no clear effects of streptomycin on differences between bacterial fingerprints between soil treated with spiked and control manure were observed. In the streptomycin-treated sandy soil on day 28, significant associations (10 000 random permutations,  $P < 0.05$ ) between bacterial community composition and relative abundance of target genes were observed for *aadA*, *qacE/qacEΔ1*, *tet(A)* and *tet(Q)*, while the direction of the *tet(Q)* vector indicates a negative correlation with streptomycin concentration (Fig. 3). In loamy soil on day 28, only *aadA* was significantly associated with the bacterial community composition. On day 92, the genes *aadA* and *tet(W)* were significantly associated with the community composition in sandy soil while *aadA*, *tet(W)* and *sul1* were significantly associated with the community composition in loamy soil (Fig. 4).

The original DGGE gels are shown in Figures S1–S8 (Supporting Information).

## DISCUSSION

### Manure application strongly affected the relative abundance of ARG and MGE genes in soil

The application of manure to soil increased the abundance of tested ARGs and integrase genes to detectable levels (except *korB* in the loamy soil and *intI2*). This was as expected since nutrients but also substantial numbers of bacteria carrying resistance genes associated with MGEs are typically applied to soil with manure (Ding et al. 2014; Udikovic-Kolic et al. 2014; Jechalke et al. 2014a; Wolters et al. 2016b). Already before spiking, the manure

used in this study had a high relative abundance of all ARGs and most MGEs. Likely, these were selected not only by antibiotic residues but also by Zn and Cu compounds. Furthermore, soils used in this study were not treated with organic manure for at least 5 years which might explain that in both untreated soils the relative abundances of the tested genes were below the detection limit of the qPCR method. Although the relative abundance of ARGs was below the detection limit in both soils, *tet(Q)* and *tet(M)* were higher in manure-treated loamy than in sandy soil, which might be associated with anaerobic conditions in the loamy soil favoring bacteria such as *Clostridium* and *Bacteroides* that were reported to be associated with these resistance genes (Lépine et al. 1993; Wolters et al. 2016a).

Over time, the majority of target genes quantified decreased but were still detectable on day 92. This is in agreement with previous studies suggesting that changes due to resistance gene carrying bacteria and nutrients applied with manure are rather transient because manure bacteria might not be well adapted to soil conditions and decrease in abundance over time (Hammesfahr et al. 2008; Heuer et al. 2008; Jechalke et al. 2013c; Ding et al. 2014; Sandberg and LaPara 2016).

### Correlations between doxycycline and streptomycin concentrations and relative abundance of ARGs and MGEs in sandy and loamy soil

Compared to the effect of manure on the relative abundance of ARGs and MGEs in soil, the effects of spiked antibiotics were rather minor. On day 28, significant positive correlations between concentrations of the spiked antibiotics and relative abundance of tested genes were only detected in the manure-treated sandy but not in the loamy soil. It was demonstrated that sorption of tetracycline and streptomycin to soil is strong and influenced by soil physicochemical properties (Thiele-Bruhn 2003; Teixido et al. 2012). Therefore, reduced effects of manure spiked with antibiotics on the abundance of ARGs and MGEs in loamy soil might be related to differences in soil properties that might have increased the sorption of the antibiotics while reducing their bioaccessibility and bioavailability.

In sandy soil on day 28, concentrations of applied doxycycline were positively correlated with the relative abundance of three tetracycline resistance genes. This is in agreement with previous studies finding strong correlations between the abundance of antibiotic resistance genes and tetracycline concentrations in manured soil (Zhu et al. 2013; Chessa et al. 2016a). Additionally in sandy soil, the *qacEΔ1*, *sul1* and *aadA* genes typically associated with class 1 integrons were positively correlated with the amount of doxycycline spiked. This indicates that the tetracycline resistance genes might be located on or in genetic association with class 1 integrons as previously described for *tet(A)* that was reported to occur on *IncP-1<sub>ε</sub>* plasmids and transposons (Heuer et al. 2012; Popowska and Krawczyk-Balska 2013; Wolters et al. 2015). Furthermore, the results support the hypothesis and findings that the widespread class 1 integrons might be used as an alternative or additional marker for anthropogenic impacts such as soil pollution with manure containing antibiotics and resistance gene carrying bacteria (Gaze et al. 2011; Gillings et al. 2014), but also highlight the importance of co-selection processes.

Similar to doxycycline, positive correlations between streptomycin concentrations and tested target genes were observed in the sandy but not in the loamy soil. On day 28, the concentration of applied streptomycin was positively correlated with *aadA*, *tet(A)* and *qacE/qacEΔ1* but not with *strA* or *int11* genes.

This did not confirm a close association previously reported between *aadA* genes and class 1 integrons (Sunde and Norström 2005; Binh et al. 2009) but still highlights the importance of co-selection processes.

### Long-term effects of manure spiked with antibiotics on the abundance of tested genes

On day 92, a number of genes were still significantly enriched in relative abundance in the doxycycline manure-treated compared to manure-treated sandy soil but only at the highest concentration, indicating a prolonged effect of doxycycline only when applied at a concentration of 200 mg kg<sup>-1</sup> soil. A similar result was observed in recent studies for tetracycline repeatedly applied in two concentrations with cow manure to a clayey and a sandy soil (Chessa et al. 2016a,b). The authors found that the effects of tetracycline and manure on ARGs and MGEs were more pronounced with a high amount of tetracycline applied, particularly in the short term. Furthermore, it must be considered that the two soils used in the study of Chessa et al. (2016a) differed in soil texture but also in their history of anthropogenic pollution.

After 92 days of incubation, the concentration of applied streptomycin was still positively correlated with streptomycin resistance gene *aadA* in sandy soil. However, the relative abundance of *aadA* was not significantly higher in the different streptomycin treatments compared to manured soil without streptomycin. It was reported that streptomycin strongly adsorbs to soil but can also be microbiologically degraded in soil (Pramer 1958), leading to a rapid decrease to undetectable levels (Gavalchin and Katz 1994), which might have reduced the long-term effects of streptomycin on levels of ARGs and MGEs. However, it should be considered that compared to doxycycline the highest concentration of streptomycin was five times lower.

### Effects of manure and antibiotic application on the soil bacterial community composition

The effects of manure application on the bacterial DGGE fingerprints tended to be higher in the sandy than in the loamy soil, which corresponds to the study of Abubaker et al. (2013) who also observed the highest effects of biogas residues and cattle slurry on the bacterial community structure in sandy soil, indicating that bacterial communities in low organic matter content soils are more sensitive to organic inputs. Over time, the differences between DGGE fingerprints of manure-treated and untreated sandy soils decreased but were still detectable on day 92. As discussed above, this is likely due to transient effects of applied nutrients and manure bacteria that might not be well adapted to soil conditions and decrease in abundance over time.

Significant differences between bacterial DGGE fingerprints were observed for soil treated with manure and manure spiked with doxycycline and streptomycin. This is in agreement with previous studies showing the effects of these antibiotics on soil bacterial community composition and functions (Ingham and Coleman 1984; Halling-Sørensen 2001; Szatmari et al. 2012). These differences were still observed on day 92 but were less pronounced in the loamy soil, which might be again related to differences in soil properties that might have differently affected the bioaccessibility and bioavailability of the antibiotics.

Interestingly, significant associations between bacterial community composition and relative abundance of ARGs were

observed in doxycycline or streptomycin-treated sandy but not in loamy soil. This might indicate that hosts carrying different resistance genes were differently affected by doxycycline and streptomycin availability in the two soils. For example, Leclercq et al. (2016) found that gut-associated species of *Clostridium* as well as environmental *Acinetobacter* and *Pseudomonas* are responsible for the persistence of ARGs in manure-amended soils. Peng et al. (2016) further reported that also native soil microbial communities could play a crucial role in the accumulation of tetracycline resistance genes in manure-amended soil.

### Potential effects of MSCs of applied antibiotics

The range of concentrations and amounts of antibiotics applied in this study are covering the range of concentrations typically found in manure or soil (Chen et al. 2012; Szatmari et al. 2012). The concentrations exceeding those by at least one order of magnitude (up to 200 and 40 mg kg<sup>-1</sup> soil for doxycycline and streptomycin, respectively) might simulate effects of worst-case scenarios of potential contamination 'hot spots' and repeated applications of contaminated manure. The lowest amounts of applied doxycycline and streptomycin (40 and 4 µg kg<sup>-1</sup> soil, respectively) were in the range of PNECs for resistance selection of 2 and 16 µg L<sup>-1</sup>, respectively (Bengtsson-Palme and Larsson 2016). Although rather low when compared to the effects of manure application, additional effects of antibiotics on the bacterial community composition in sandy soil were already observed at the lowest concentrations applied, further challenging the concept of defining threshold concentrations for antibiotics in manure. However, it cannot be excluded that background concentrations of doxycycline and potentially other antibiotics in the manure used in this study might have influenced the effects of additionally spiked doxycycline and streptomycin on the soil bacterial communities.

### CONCLUSIONS

Manure application to soil affected the bacterial community composition and caused a strong increase in relative abundance of most of the genes analyzed. Spiking with doxycycline or streptomycin caused further enrichment in gene abundances, although almost exclusively at high concentrations. This effect was strongly dependent on the soil texture. While in the sandy soil considerable effects on the bacterial community composition and relative abundance of ARGs, class 1 integrons and associated genes were observed, the loamy soil bacterial community was less impacted. These effects tended to decrease with increasing incubation time but were still detectable on day 92 in the sandy soil. This implies that for the prediction of effects of antibiotic concentrations applied with manure to the complex soil environment, threshold concentrations for antibiotics in manure are likely to be much higher than can be expected from PNEC values for resistance selection and should consider the specific soil properties and the physicochemical characteristics of each antibiotic. Furthermore, different host bacteria carrying resistance genes and MGEs might be very differently affected and influenced by the interaction with plants, making the definition of no-effect concentrations for antibiotics very difficult or even impossible.

In future, tools such as next generation sequencing or omics technologies will likely provide further insights into the complex interactions between soil bacterial populations and antibiotics and antibiotic-resistant bacteria applied with manure.

### SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://femsec.org) online.

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