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

Original
The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities / Chessa, Luigi; Jechalke, Sven; Ding, Guo Chun; Pusino, Alba; Mangia, Nicoletta Pasqualina; Smalla, Kornelia. - In: BIOLOGY AND FERTILITY OF SOILS. - ISSN 0178-2762. - 52:8(2016), pp. 1121-1134. [10.1007/s00374-016-1150-4]

Availability:
This version is available at: 11388/175298 since: 2017-05-15T11:19:34Z

Publisher:

Published
DOI:10.1007/s00374-016-1150-4

Biology and Fertility of Soils

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

--Manuscript Draft--

Manuscript Number:	BFSO-D-16-00351R3
Full Title:	The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities
Article Type:	Original Paper
Keywords:	tetracycline; soil; bacterial fingerprints; antibiotic resistance genes; class 1 integron; co-selection
Corresponding Author:	Luigi Chessa, PhD University of Sassari Sassari, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Sassari
Corresponding Author's Secondary Institution:	
First Author:	Luigi Chessa, PhD
First Author Secondary Information:	
Order of Authors:	Luigi Chessa, PhD
	Sven Jechalke, PhD
	Guo-Chun Ding, PhD
	Alba Pusino, Prof.
	Nicoletta Pasqualina Mangia, PhD
	Kornelia Smalla, Prof.
Order of Authors Secondary Information:	
Funding Information:	
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. 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. 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 ARGs and MGEs 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.
Suggested Reviewers:	Martina Kyselková Biology Centre of the Czech Academy of Sciences

	martinak@upb.cas.cz She is expert and first author or co-author of several papers investigating the effect of tetracycline and Cow manure on soil microbial communities
	Heike Schmitt Utrecht University h.schmitt@uu.nl She is expert in antibiotic resistance genes and mobile genetic elements in the soil environment

September 12, 2016

Ref.: Ms. No. BFSO-D-16-00351R1
The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities
Biology and Fertility of Soils

Dear Prof. Paolo Nannipieri
BFSO Editor,

enclosed to this letter you will find the revised version of our manuscript titled "The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities".
In particular, a manuscript file with all the changes highlighted in yellow ("Revised manuscript with changes highlighted") and a clean manuscript ("Revised manuscript") were attached.
Moreover, below you will find the response to your comment and the changes which have been applied to the manuscript accordingly.

Yours sincerely,

Luigi Chessa

Luigi Chessa PhD
Department of Agriculture, University of Sassari,
Viale Italia 39, 07100 Sassari, Italy
Tel: +39 079 229289; Fax: +39 079 229370
*E-ma*il address: luigi.chessa@uniss.it

Ref.: Ms. No. BFSO-D-16-00351R2
The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities
Biology and Fertility of Soils

Dear Dr. Chessa,

I have reviewd your manuscript titled "The presence of tetracycline in cow manure changes the impact of repeated manure application on soil bacterial communities" and it is accepted for publication after revision according to the follwoing my comment:
Line 480, please add "and use of molecular techniques determining not only dominant but also rare microbial species."
Response:
the sentence was modified as suggested. Lines 480-481.

Along with your revised manuscript, you will need to supply a seperate file "author's response to the referees' comments" in which you list all the changes you have made to the manuscript and in which you detail your responses to all the comments passed by the referee(s). Should you disagree with any comment(s), please explain why. Please be sure to return the annotated copies of your manuscript.

Your revision is due by 23 September 2016.

To submit a revision, go to http://bfso.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Please make sure to submit your editable source files (i. e. Word, TeX)

Yours sincerely

Paolo Nannipieri
Editor-in-Chief
Biology and Fertility of Soils

Journal section for manuscript publication:

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

E-mail address: luigi.chessa@uniss.it

Author list

Luigi Chessa,[1,2] *, Sven Jechalke,[2#] Guo-Chun Ding,[2,3] Alba Pusino,[1] Nicoletta Pasqualina Mangia,[1] Kornelia Smalla,[2]

Addresses of the institutions

[1] Department of Agriculture, University of Sassari, Viale Italia 39, 07100 Sassari, Italy

[2] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

[3] College of Resources and Environmental Sciences, China Agricultural University, 100193, Yuanmingyuan xilu No 2, Haidian District, Beijing, China

[#] current address: Institute for Phytopathology, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32 (IFZ), 35392 Gießen, Germany

* Corresponding author. Mailing address: University of Sassari, Department of Agriculture, Viale Italia 39, 07100 Sassari, Italy. Phone: +39 079229289. FAX: +39 079229370. E-mail: luigi.chessa@uniss.it

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. 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. 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 ARGs and MGEs 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 in 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 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 (Food and Drug 2013), 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 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$_2$EDTA buffer and subsequent sonication. Tc is strongly and rapidly sorbed to soil clays and organic matter (Gu et al. 2007; Jutta et al. 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 Sardinian beef cattle farm (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 (Italy), 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 were 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-Biochemicals, 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-Biochemicals) and eluted in 50 µL final volume observing the manufacturers' protocol.

Denaturing gradient gel electrophoresis (DGGE)

Total bacterial 16S rRNA gene fragments were amplified by PCR using primers F984GC and R1378 (Heuer et al. 1997). 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^{TM} 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 µl DNA template of 1:5 diluted purified DNA solution were 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 epsilon 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 treatments analyzed 60 days after each of three amendments (Fig. S1-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+500. 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 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 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, 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 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 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 mg kg^{-1} 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 were 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. 2015; 2014a; 2014b). 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 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. 2012; 2009; 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.

Acknowledgments

Luigi Chessa gratefully acknowledges Sardinia Regional Government for the financial support of his PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of

Activity l.3.1.). Sven Jechalke was funded by the Federal Environment Agency (Umweltbundesamt) (FKZ 3713 63 402). The authors would like to thank Dr. Martina Kyselková to provide us positive controls for qPCR reactions.

References

Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, McAllister TA (2011) Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. BMC Microbiol 11:19. doi:10.1186/1471-2180-11-19

Amos GCA, Gozzard E, Carter CE, Mead A, Bowes MJ, Hawkey PM, Zhang L, Singer AC, Gaze WH, Wellington EMH (2015) Validated predictive modelling of the environmental resistome. ISME J 9:1467-1476. doi:10.1038/ismej.2014.237

Amos GCA, Hawkey PM, Gaze WH, Wellington EM (2014a) Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. J Antimicrob Chemoth 69:1785-1791. doi:10.1093/jac/dku079

Amos GCA, Zhang L, Hawkey PM, Gaze WH, Wellington EM (2014b) Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. Vet Microbiol 171:441-447. doi:10.1016/j.vetmic.2014.02.017

Bahl M, Hansen L, Goesmann A, Sørensen S (2007) The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established α, β and δ sub-groups. Plasmid 58:31-43. doi:10.1016/j.plasmid.2006.11.007

Barraud O, Baclet MC, Denis F, Ploy MC (2010) Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J Antimicrob Chemoth 65:1642-1645. doi:10.1093/jac/dkq167

Binh CT, Heuer H, Kaupenjohann M, Smalla K (2008) Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol Ecol 66:25-37. doi:10.1111/j.1574-6941.2008.00526.x

Chessa L, Pusino A, Garau G, Mangia NP, Pinna MV (2016) Soil microbial response to tetracycline in two different soils amended with cow manure. Environ Sci Pollut R 23:5807-5817. doi:10.1007/s11356-015-5789-4

Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65:232-260. doi:10.1128/MMBR.65.2.232-260.2001

Ding GC, Heuer H, Smalla K (2012) Dynamics of bacterial communities in two unpolluted soils after spiking with phenanthrene: soil type specific and common responders. Front Microbiol 3:290. doi:10.3389/fmicb.2012.00290

Ding GC, Radl V, Schloter-Hai B, Jechalke S, Heuer H, Smalla K, Schloter M (2014) Dynamics of soil bacterial communities in response to repeated application of manure containing sulfadiazine. PLoS One 9:e92958. doi:10.1371/journal.pone.0092958

Durso LM, Cook KL (2014) Impacts of antibiotic use in agriculture: what are the benefits and risks? Curr Opin Microbiol 19:37-44. doi:10.1016/j.mib.2014.05.019

EC (1831/2003) Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Official Journal of the European Union

Food and Drug (2013) Antimicrobials sold or distributed for use in food-producing animals. Administration Department of Health and Human Services,

Fulthorpe RR, McGowan C, Maltseva OV, Holben WE, Tiedje JM (1995) 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl Environ Microb 61:3274-3281.

Ghosh S, LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J 1:191-203. doi:10.1038/ismej.2007.31

Gomes NCM, Heuer H, Schönfeld J, Costa R, Mendonça-Hagler L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232:167-180. doi:10.1023/A:1010350406708

Gu C, Karthikeyan KG, Sibley SD, Pedersen JA (2007) Complexation of the antibiotic tetracycline with humic acid. Chemosphere 66:1494-1501. doi:10.1016/j.chemosphere.2006.08.028

Hammer TJ, Fierer N, Hardwick B, Simojoki A, Slade E, Taponen J, Viljanen H, Roslin T (2016) Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung beetles. Proc Biol Sci 283. doi: 10.1098/rspb.2016.0150

Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S (2008) Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. Soil Biol Biochem 40:1583-1591. doi:10.1016/j.soilbio.2008.01.010

Hamscher G, Pawelzick HT, Höper H, Nau H (2005) Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. Environ Toxicol Chem 24:861-868. doi:10.1897/04-182R.1

Hamscher G, Sczesny S, Höper H, Nau H (2002) Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Anal Chem 74:1509-1518. doi:10.1021/ac015588m

Hassan R, Ryu K-S (2012) Naturally Derived Probiotic Supplementation Effects on Physiological Properties and Manure Gas Emission of Broiler Chickens. J Agric Life Sci 46:119-127. doi:10.3382/ps.2013-03314

Heuer H, Binh CT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T, Gonzalez B, Top E, Smalla K (2012) IncP-1epsilon Plasmids are Important Vectors of Antibiotic Resistance Genes in Agricultural Systems: Diversification Driven by Class 1 Integron Gene Cassettes. Front Microbiol 3:2. doi:10.3389/fmicb.2012.00002

Heuer H, Focks A, Lamshöft M, Smalla K, Matthies M, Spiteller M (2008) Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol Biochem 40:1892-1900. doi:10.1016/j.soilbio.2008.03.014

Heuer H, Kopmann C, Binh CT, Top EM, Smalla K (2009) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. Environ Microbiol 11:937-949. doi:10.1111/j.1462-2920.2008.01819.x

Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microb 63:3233-3241.

Heuer H, Schmitt H, Smalla K (2011a) Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol 14:236-243. doi:10.1016/j.mib.2011.04.009

Heuer H, Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ Microbiol 9:657-666. doi:10.1111/j.1462-2920.2006.01185.x

Heuer H, Smalla K (2012) Plasmids foster diversification and adaptation of bacterial populations in soil. FEMS Microbiol Rev 36:1083-1104. doi:10.1111/j.1574-6976.2012.00337.x

Heuer H, Solehati Q, Zimmerling U, Kleineidam K, Schloter M, Müller T, Focks A, Thiele-Bruhn S, Smalla K (2011b) Accumulation of sulfonamide resistance genes in arable soils due to repeated application of manure containing sulfadiazine. Appl Environ Microb 77:2527-2530. doi:10.1128/AEM.02577-10

Heuer H, Weiland G, Schönfeld J, Schönwälder A, Gomes N, Smalla K (2001) Bacterial Community Profiling Using DGGE or TGGE Analysis In: Rochelle PA (Ed) Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Wymondham, UK, pp 177-190.

Ho A, El-Hawwary A, Kim SY, Meima-Franke M, Bodelier P (2015) Manure-associated stimulation of soil-borne methanogenic activity in agricultural soils. Biol Fert Soils 51:511-516. doi:10.1007/s00374-015-0995-2

Hund-Rinke R, Simon M, Lukow T (2004) Effects of Tetracycline on the Soil Microflora: Function, Diversity, Resistance. J Soil Sediment 4:11-16.

Jechalke S, Focks A, Rosendahl I, Groeneweg J, Siemens J, Heuer H, Smalla K (2014a) Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. FEMS Microbiol Ecol 87:78-88. doi:10.1111/1574-6941.12191

Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K (2014b) Fate and effects of veterinary antibiotics in soil. Trends Microbiol 22:536-545. doi:10.1016/j.tim.2014.05.005

Jia DA, Zhou DM, Wang YJ, Zhu HW, Chen JL (2008) Adsorption and cosorption of Cu(II) and tetracycline on two soils with different characteristics. Geoderma 146:224-230. doi:10.1016/j.geoderma.2008.05.023

Jindal A, Kocherginskaya S, Mehboob A, Robert M, Mackie RI, Raskin L, Zilles JL (2006) Antimicrobial use and resistance in swine waste treatment systems. Appl Environ Microb 72:7813-7820. doi:10.1128/AEM.01087-06

Jutta R, Pils V, Laird DA (2007) Sorption of Tetracycline and Chortetracycline on K- and Ca-saturated soil clays, humic Substances, and clay-humic complexes. Environ Sci Technol 41:1928-1933. doi:10.1021/es062316y

Kim KR, Owens G, Kwon SI, So KH, Lee DB, Ok YS (2011) Occurrence and Environmental Fate of Veterinary Antibiotics in the Terrestrial Environment. Water Air Soil Poll 214:163-174. doi:10.1007/s11270-010-0412-2

Kong WD, Li CG, Dolhi JM, Li SY, He JZ, Qiao M (2012) Characteristics of oxytetracycline sorption and potential bioavailability in soils with various physical-chemical properties. Chemosphere 87:542-548. doi:10.1016/j.chemosphere.2011.12.062

Kreuzig R, Höltge S (2005) Investigations on the fate of sulfadiazine in manured soil: Laboratory experiments and test plot studies. Environ Toxicol Chem 24:771-776. doi:10.1897/03-582R.1

Kropf S, Heuer H, Grüning M, Smalla K (2004) Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. J Microbiol Methods 57:187-195. doi:10.1016/j.mimet.2004.01.002

Kümmerer K (2003) Significance of antibiotics in the environment. J Antimicrob Chemother 52:5-7. doi:10.1093/jac/dkg293

Kyselková M, Jirout J, Chroňáková A, Vrchotová N, Bradley R, Schmitt H, Elhottová D (2013) Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content. Chemosphere 93:2413-2418. doi:10.1016/j.chemosphere.2013.08.058

Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015a) Spread of tetracycline resistance genes at a conventional dairy farm. Front Microbiol 6. doi: 10.3389/fmicb.2015.00536

Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015b) Spread of tetracycline resistance genes at a conventional dairy farm. Front Microbiol 6:536. doi:10.3389/fmicb.2015.00536

Lanz R, Kuhnert P, Boerlin P (2003) Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. Vet Microbiol 91:73-84.

Li Z, Chang PH, Jean JS, Jiang WT, Wang CJ (2010) Interaction between tetracycline and smectite in aqueous solution. J Colloid Interface Sci 341:311-319. doi:10.1016/j.jcis.2009.09.054

Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol Biochem 35:453-461. doi:10.1016/S0038-0717(02)00297-3

Montforts MH, Kalf DF, van Vlaardingen PL, Linders JB (1999) The exposure assessment for veterinary medicinal products. Sci Total Environ 225:119-133. doi:10.1016S0048-9697(98)00338-6

Nelson ML, Levy SB (2011) The history of the tetracyclines. Ann N Y Acad Sci 1241:17-32. doi:10.1111/j.1749-6632.2011.06354.x

Ng LK, Martin I, Alfa M, Mulvey M (2001) Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes 15:209-215. doi:10.1006/mcpr.2001.0363

Nguyen F, Starosta AL, Arenz S, Sohmen D, Donhofer A, Wilson DN (2014) Tetracycline antibiotics and resistance mechanisms. Biol Chem 395:559-575. doi:10.1515/hsz-2013-0292

O'Connor S, Aga DS (2007) Analysis of tetracycline antibiotics in soil: Advances in extraction, clean-up, and quantification. Trac-Trend Anal Chem 26:456-465. doi:10.1016/j.trac.2007.02.007

Popowska M, Rzeczycka M, Miernik A, Krawczyk-Balska A, Walsh F, Duffy B (2012) Influence of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes. Antimicrob Agents Chemother 56:1434-1443. doi:10.1128/AAC.05766-11

Qiao M, Chen W, Su J, Zhang B, Zhang C (2012) Fate of tetracyclines in swine manure of three selected swine farms in China. J Environ Sci 24:1047-1052. doi:10.1016/S1001-0742(11)60890-5

Roberts MC (2011) Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline-resistant bacteria, First Edition edn. John Wiley & Sons, Inc., Hoboken, NJ, USA

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. vol vol. 1. Cold Spring Harbor laboratory Press.

Sarmah AK, Meyer MT, Boxall AB (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65:725-759. doi:10.1016/j.chemosphere.2006.03.026

Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006) Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. Microb Ecol 51:267-276. doi:10.1007/s00248-006-9035-y

Schreiter S, Ding GC, Grosch R, Kropf S, Antweiler K, Smalla K Soil type-dependent effects of a potential biocontrol inoculant on indigenous bacterial communities in the rhizosphere of field-grown lettuce. FEMS Microbiology Ecology 90:718-730. doi:10.1111/1574-6941.12430

Selvam A, Xu D, Zhao Z, Wong JW (2012) Fate of tetracycline, sulfonamide and fluoroquinolone resistance genes and the changes in bacterial diversity during composting of swine manure. Bioresour Technol 126:383-390. doi:10.1016/j.biortech.2012.03.045

Sengeløv G, Agersø Y, Halling-Sørensen B, Baloda SB, Andersen JS, Jensen LB (2003) Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. Environ Int 28:587-595.

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000) Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl Environ Microb 66:4854-4862.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microb 67:4742-4751. doi:10.1128/aem.67.10.4742-4751.2001

Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004) Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. Appl Environ Microb 70:7372-7377. doi:10.1128/AEM.70.12.7372-7377.2004

Sun HY, Deng SP, Raun WR (2004) Bacterial community structure and diversity in a century-old manure-treated agroecosystem. Appl Environ Microb 70:5868-5874. doi:10.1128/AEM.70.10.5868-5874.2004

Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl Environ Microb 66:4605-4614. doi:

Thiele-Bruhn S (2003) Pharmaceutical antibiotic compounds in soils - A review. J Plant Nutr Soil Sci 166:145-167.

Thiele-Bruhn S, Beck IC (2005) Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. Chemosphere 59:457-465. doi:10.1016/j.chemosphere.2005.01.023

Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J (2014) Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. P Natl Acad Sci USA 111:15202-15207. doi:10.1073/pnas.1409836111

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R (2015) Global trends in antimicrobial use in food animals. P Natl Acad Sci USA 112:5649-5654. doi:10.1073/pnas.1503141112

Weinert N, Meincke R, Gottwald C, Heuer H, Gomes NC, Schloter M, Berg G, Smalla K (2009) Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their parent cultivar differ less than those of different potato cultivars. Appl Environ Microb 75:3859-3865. doi:10.1128/AEM.00414-09

Winckler C, Grafe A (2001) Use of veterinary drugs in intensive animal production. J Soil Sediment 1:66-70.

Wolters B, Ding GC, Kreuzig R, Smalla K (2016) Full-scale mesophilic biogas plants using manure as C-source: Bacterial community shifts along the process cause changes in the abundance of resistance genes and mobile genetic elements. FEMS Microbiol Ecol 92:1-17. doi:10.1093/femsec/fiv163

Wolters B, Kyselková M, Krögerrecklenfort E, Kreuzig R, Smalla K (2015) Transferable antibiotic resistance plasmids from biogas plant digestates often belong to the IncP-1ε subgroup. Front Microbiol 6. doi: 10.3389/fmicb.2014.00765

Wu K, Yuan S, Wang L, Shi J, Zhao J, Shen B, Shen Q (2014) Effects of bio-organic fertilizer plus soil amendment on the control of tobacco bacterial wilt and composition of soil bacterial communities. Biol Fert Soils 50:961-971. doi:10.1007/s00374-014-0916-9

You Y, Hilpert M, Ward MJ (2012) Detection of a common and persistent tet(L)-carrying plasmid in chicken-waste-impacted farm soil. Appl Environ Microb 78:3203-3213. doi:10.1128/AEM.07763-11

Zhang Y, Boyd SA, Teppen BJ, Tiedje JM, Li H (2014) Role of tetracycline speciation in the bioavailability to escherichia coli for uptake and expression of antibiotic resistance. Environ Sci Technol 48:4893-4900. doi:10.1021/es5003428

Zhao S, Liu D, Ling N, Chen F, Fang W, Shen Q (2014) Bio-organic fertilizer application significantly reduces the Fusarium oxysporum population and alters the composition of fungi communities of watermelon Fusarium wilt rhizosphere soil. Biol Fert Soils 50:765-774. doi:10.1007/s00374-014-0898-7

Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci U S A 110:3435-3440. doi:10.1073/pnas.1222743110

Figure captions

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

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

Figure 1 UPGMA cluster analysis of DGGE fingerprints for 16S rRNA gene amplicons from CL and SA soils 60 days after the first amendment: amplicons from (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.

Figure 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. 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.

Figure 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.

Figure 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. 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.

Figure 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.

Journal section for manuscript publication:

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

E-mail address: luigi.chessa@uniss.it

Author list

Luigi Chessa,[1,2] *, Sven Jechalke,[2#] Guo-Chun Ding,[2,3] Alba Pusino,[1] Nicoletta Pasqualina Mangia,[1] Kornelia Smalla,[2]

Addresses of the institutions

[1] Department of Agriculture, University of Sassari, Viale Italia 39, 07100 Sassari, Italy

[2] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

[3] College of Resources and Environmental Sciences, China Agricultural University, 100193, Yuanmingyuan xilu No 2, Haidian District, Beijing, China

[#] current address: Institute for Phytopathology, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32 (IFZ), 35392 Gießen, Germany

* Corresponding author. Mailing address: University of Sassari, Department of Agriculture, Viale Italia 39, 07100 Sassari, Italy. Phone: +39 079229289. FAX: +39 079229370. E-mail: luigi.chessa@uniss.it

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. 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. 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 ARGs and MGEs 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 in 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 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 (Food and Drug 2013), 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 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; Jutta et al. 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 Sardinian beef cattle farm (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 (Italy), 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 were 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-Biochemicals, 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-Biochemicals) and eluted in 50 μL final volume observing the manufacturers' protocol.

Denaturing gradient gel electrophoresis (DGGE)

Total bacterial 16S rRNA gene fragments were amplified by PCR using primers F984GC and R1378 (Heuer et al. 1997). 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 µl DNA template of 1:5 diluted purified DNA solution were 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 epsilon 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 treatments analyzed 60 days after each of three amendments (Fig. S1-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+500. 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 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 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, 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 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 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 mg kg^{-1} 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 were 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. 2015; 2014a; 2014b). 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 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. 2012; 2009; 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.

Acknowledgments

Luigi Chessa gratefully acknowledges Sardinia Regional Government for the financial support of his PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of

Activity l.3.1.). Sven Jechalke was funded by the Federal Environment Agency (Umweltbundesamt) (FKZ 3713 63 402). The authors would like to thank Dr. Martina Kyselková to provide us positive controls for qPCR reactions.

References

Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, McAllister TA (2011) Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed different subtherapeutic antibiotics. BMC Microbiol 11:19. doi:10.1186/1471-2180-11-19

Amos GCA, Gozzard E, Carter CE, Mead A, Bowes MJ, Hawkey PM, Zhang L, Singer AC, Gaze WH, Wellington EMH (2015) Validated predictive modelling of the environmental resistome. ISME J 9:1467-1476. doi:10.1038/ismej.2014.237

Amos GCA, Hawkey PM, Gaze WH, Wellington EM (2014a) Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. J Antimicrob Chemoth 69:1785-1791. doi:10.1093/jac/dku079

Amos GCA, Zhang L, Hawkey PM, Gaze WH, Wellington EM (2014b) Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. Vet Microbiol 171:441-447. doi:10.1016/j.vetmic.2014.02.017

Bahl M, Hansen L, Goesmann A, Sørensen S (2007) The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established α, β and δ sub-groups. Plasmid 58:31-43. doi:10.1016/j.plasmid.2006.11.007

Barraud O, Baclet MC, Denis F, Ploy MC (2010) Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. J Antimicrob Chemoth 65:1642-1645. doi:10.1093/jac/dkq167

Binh CT, Heuer H, Kaupenjohann M, Smalla K (2008) Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol Ecol 66:25-37. doi:10.1111/j.1574-6941.2008.00526.x

Chessa L, Pusino A, Garau G, Mangia NP, Pinna MV (2016) Soil microbial response to tetracycline in two different soils amended with cow manure. Environ Sci Pollut R 23:5807-5817. doi:10.1007/s11356-015-5789-4

Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev 65:232-260. doi:10.1128/MMBR.65.2.232-260.2001

Ding GC, Heuer H, Smalla K (2012) Dynamics of bacterial communities in two unpolluted soils after spiking with phenanthrene: soil type specific and common responders. Front Microbiol 3:290. doi:10.3389/fmicb.2012.00290

Ding GC, Radl V, Schloter-Hai B, Jechalke S, Heuer H, Smalla K, Schloter M (2014) Dynamics of soil bacterial communities in response to repeated application of manure containing sulfadiazine. PLoS One 9:e92958. doi:10.1371/journal.pone.0092958

Durso LM, Cook KL (2014) Impacts of antibiotic use in agriculture: what are the benefits and risks? Curr Opin Microbiol 19:37-44. doi:10.1016/j.mib.2014.05.019

EC (1831/2003) Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Official Journal of the European Union

Food and Drug (2013) Antimicrobials sold or distributed for use in food-producing animals. Administration Department of Health and Human Services,

Fulthorpe RR, McGowan C, Maltseva OV, Holben WE, Tiedje JM (1995) 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl Environ Microb 61:3274-3281.

Ghosh S, LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J 1:191-203. doi:10.1038/ismej.2007.31

Gomes NCM, Heuer H, Schönfeld J, Costa R, Mendonça-Hagler L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232:167-180. doi:10.1023/A:1010350406708

Gu C, Karthikeyan KG, Sibley SD, Pedersen JA (2007) Complexation of the antibiotic tetracycline with humic acid. Chemosphere 66:1494-1501. doi:10.1016/j.chemosphere.2006.08.028

Hammer TJ, Fierer N, Hardwick B, Simojoki A, Slade E, Taponen J, Viljanen H, Roslin T (2016) Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung beetles. Proc Biol Sci 283. doi: 10.1098/rspb.2016.0150

Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S (2008) Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. Soil Biol Biochem 40:1583-1591. doi:10.1016/j.soilbio.2008.01.010

Hamscher G, Pawelzick HT, Höper H, Nau H (2005) Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. Environ Toxicol Chem 24:861-868. doi:10.1897/04-182R.1

Hamscher G, Sczesny S, Höper H, Nau H (2002) Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Anal Chem 74:1509-1518. doi:10.1021/ac015588m

Hassan R, Ryu K-S (2012) Naturally Derived Probiotic Supplementation Effects on Physiological Properties and Manure Gas Emission of Broiler Chickens. J Agric Life Sci 46:119-127. doi:10.3382/ps.2013-03314

Heuer H, Binh CT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T, Gonzalez B, Top E, Smalla K (2012) IncP-1epsilon Plasmids are Important Vectors of Antibiotic Resistance Genes in Agricultural Systems: Diversification Driven by Class 1 Integron Gene Cassettes. Front Microbiol 3:2. doi:10.3389/fmicb.2012.00002

Heuer H, Focks A, Lamshöft M, Smalla K, Matthies M, Spiteller M (2008) Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biol Biochem 40:1892-1900. doi:10.1016/j.soilbio.2008.03.014

Heuer H, Kopmann C, Binh CT, Top EM, Smalla K (2009) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. Environ Microbiol 11:937-949. doi:10.1111/j.1462-2920.2008.01819.x

Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microb 63:3233-3241.

Heuer H, Schmitt H, Smalla K (2011a) Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol 14:236-243. doi:10.1016/j.mib.2011.04.009

Heuer H, Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ Microbiol 9:657-666. doi:10.1111/j.1462-2920.2006.01185.x

Heuer H, Smalla K (2012) Plasmids foster diversification and adaptation of bacterial populations in soil. FEMS Microbiol Rev 36:1083-1104. doi:10.1111/j.1574-6976.2012.00337.x

Heuer H, Solehati Q, Zimmerling U, Kleineidam K, Schloter M, Müller T, Focks A, Thiele-Bruhn S, Smalla K (2011b) Accumulation of sulfonamide resistance genes in arable soils due to repeated application of manure containing sulfadiazine. Appl Environ Microb 77:2527-2530. doi:10.1128/AEM.02577-10

Heuer H, Weiland G, Schönfeld J, Schönwälder A, Gomes N, Smalla K (2001) Bacterial Community Profiling Using DGGE or TGGE Analysis In: Rochelle PA (Ed) Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Wymondham, UK, pp 177-190.

Ho A, El-Hawwary A, Kim SY, Meima-Franke M, Bodelier P (2015) Manure-associated stimulation of soil-borne methanogenic activity in agricultural soils. Biol Fert Soils 51:511-516. doi:10.1007/s00374-015-0995-2

Hund-Rinke R, Simon M, Lukow T (2004) Effects of Tetracycline on the Soil Microflora: Function, Diversity, Resistance. J Soil Sediment 4:11-16.

Jechalke S, Focks A, Rosendahl I, Groeneweg J, Siemens J, Heuer H, Smalla K (2014a) Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. FEMS Microbiol Ecol 87:78-88. doi:10.1111/1574-6941.12191

Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K (2014b) Fate and effects of veterinary antibiotics in soil. Trends Microbiol 22:536-545. doi:10.1016/j.tim.2014.05.005

Jia DA, Zhou DM, Wang YJ, Zhu HW, Chen JL (2008) Adsorption and cosorption of Cu(II) and tetracycline on two soils with different characteristics. Geoderma 146:224-230. doi:10.1016/j.geoderma.2008.05.023

Jindal A, Kocherginskaya S, Mehboob A, Robert M, Mackie RI, Raskin L, Zilles JL (2006) Antimicrobial use and resistance in swine waste treatment systems. Appl Environ Microb 72:7813-7820. doi:10.1128/AEM.01087-06

Jutta R, Pils V, Laird DA (2007) Sorption of Tetracycline and Chortetracycline on K- and Ca-saturated soil clays, humic Substances, and clay-humic complexes. Environ Sci Technol 41:1928-1933. doi:10.1021/es062316y

Kim KR, Owens G, Kwon SI, So KH, Lee DB, Ok YS (2011) Occurrence and Environmental Fate of Veterinary Antibiotics in the Terrestrial Environment. Water Air Soil Poll 214:163-174. doi:10.1007/s11270-010-0412-2

Kong WD, Li CG, Dolhi JM, Li SY, He JZ, Qiao M (2012) Characteristics of oxytetracycline sorption and potential bioavailability in soils with various physical-chemical properties. Chemosphere 87:542-548. doi:10.1016/j.chemosphere.2011.12.062

Kreuzig R, Höltge S (2005) Investigations on the fate of sulfadiazine in manured soil: Laboratory experiments and test plot studies. Environ Toxicol Chem 24:771-776. doi:10.1897/03-582R.1

Kropf S, Heuer H, Grüning M, Smalla K (2004) Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. J Microbiol Methods 57:187-195. doi:10.1016/j.mimet.2004.01.002

Kümmerer K (2003) Significance of antibiotics in the environment. J Antimicrob Chemother 52:5-7. doi:10.1093/jac/dkg293

Kyselková M, Jirout J, Chroňáková A, Vrchotová N, Bradley R, Schmitt H, Elhottová D (2013) Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content. Chemosphere 93:2413-2418. doi:10.1016/j.chemosphere.2013.08.058

Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015a) Spread of tetracycline resistance genes at a conventional dairy farm. Front Microbiol 6. doi: 10.3389/fmicb.2015.00536

Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015b) Spread of tetracycline resistance genes at a conventional dairy farm. Front Microbiol 6:536. doi:10.3389/fmicb.2015.00536

Lanz R, Kuhnert P, Boerlin P (2003) Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. Vet Microbiol 91:73-84.

Li Z, Chang PH, Jean JS, Jiang WT, Wang CJ (2010) Interaction between tetracycline and smectite in aqueous solution. J Colloid Interface Sci 341:311-319. doi:10.1016/j.jcis.2009.09.054

Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol Biochem 35:453-461. doi:10.1016/S0038-0717(02)00297-3

Montforts MH, Kalf DF, van Vlaardingen PL, Linders JB (1999) The exposure assessment for veterinary medicinal products. Sci Total Environ 225:119-133. doi:10.1016S0048-9697(98)00338-6

Nelson ML, Levy SB (2011) The history of the tetracyclines. Ann N Y Acad Sci 1241:17-32. doi:10.1111/j.1749-6632.2011.06354.x

Ng LK, Martin I, Alfa M, Mulvey M (2001) Multiplex PCR for the detection of tetracycline resistant genes. Mol Cell Probes 15:209-215. doi:10.1006/mcpr.2001.0363

Nguyen F, Starosta AL, Arenz S, Sohmen D, Donhofer A, Wilson DN (2014) Tetracycline antibiotics and resistance mechanisms. Biol Chem 395:559-575. doi:10.1515/hsz-2013-0292

O'Connor S, Aga DS (2007) Analysis of tetracycline antibiotics in soil: Advances in extraction, clean-up, and quantification. Trac-Trend Anal Chem 26:456-465. doi:10.1016/j.trac.2007.02.007

Popowska M, Rzeczycka M, Miernik A, Krawczyk-Balska A, Walsh F, Duffy B (2012) Influence of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes. Antimicrob Agents Chemother 56:1434-1443. doi:10.1128/AAC.05766-11

Qiao M, Chen W, Su J, Zhang B, Zhang C (2012) Fate of tetracyclines in swine manure of three selected swine farms in China. J Environ Sci 24:1047-1052. doi:10.1016/S1001-0742(11)60890-5

Roberts MC (2011) Mechanisms of bacterial antibiotic resistance and lessons learned from environmental tetracycline-resistant bacteria, First Edition edn. John Wiley & Sons, Inc., Hoboken, NJ, USA

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. vol vol. 1. Cold Spring Harbor laboratory Press.

Sarmah AK, Meyer MT, Boxall AB (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65:725-759. doi:10.1016/j.chemosphere.2006.03.026

Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006) Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. Microb Ecol 51:267-276. doi:10.1007/s00248-006-9035-y

Schreiter S, Ding GC, Grosch R, Kropf S, Antweiler K, Smalla K Soil type-dependent effects of a potential biocontrol inoculant on indigenous bacterial communities in the rhizosphere of field-grown lettuce. FEMS Microbiology Ecology 90:718-730. doi:10.1111/1574-6941.12430

Selvam A, Xu D, Zhao Z, Wong JW (2012) Fate of tetracycline, sulfonamide and fluoroquinolone resistance genes and the changes in bacterial diversity during composting of swine manure. Bioresour Technol 126:383-390. doi:10.1016/j.biortech.2012.03.045

Sengeløv G, Agersø Y, Halling-Sørensen B, Baloda SB, Andersen JS, Jensen LB (2003) Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. Environ Int 28:587-595.

Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000) Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl Environ Microb 66:4854-4862.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microb 67:4742-4751. doi:10.1128/aem.67.10.4742-4751.2001

Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004) Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. Appl Environ Microb 70:7372-7377. doi:10.1128/AEM.70.12.7372-7377.2004

Sun HY, Deng SP, Raun WR (2004) Bacterial community structure and diversity in a century-old manure-treated agroecosystem. Appl Environ Microb 70:5868-5874. doi:10.1128/AEM.70.10.5868-5874.2004

Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl Environ Microb 66:4605-4614. doi:

Thiele-Bruhn S (2003) Pharmaceutical antibiotic compounds in soils - A review. J Plant Nutr Soil Sci 166:145-167.

Thiele-Bruhn S, Beck IC (2005) Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. Chemosphere 59:457-465. doi:10.1016/j.chemosphere.2005.01.023

Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J (2014) Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. P Natl Acad Sci USA 111:15202-15207. doi:10.1073/pnas.1409836111

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R (2015) Global trends in antimicrobial use in food animals. P Natl Acad Sci USA 112:5649-5654. doi:10.1073/pnas.1503141112

Weinert N, Meincke R, Gottwald C, Heuer H, Gomes NC, Schloter M, Berg G, Smalla K (2009) Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their parent cultivar differ less than those of different potato cultivars. Appl Environ Microb 75:3859-3865. doi:10.1128/AEM.00414-09

Winckler C, Grafe A (2001) Use of veterinary drugs in intensive animal production. J Soil Sediment 1:66-70.

Wolters B, Ding GC, Kreuzig R, Smalla K (2016) Full-scale mesophilic biogas plants using manure as C-source: Bacterial community shifts along the process cause changes in the abundance of resistance genes and mobile genetic elements. FEMS Microbiol Ecol 92:1-17. doi:10.1093/femsec/fiv163

Wolters B, Kyselková M, Krögerrecklenfort E, Kreuzig R, Smalla K (2015) Transferable antibiotic resistance plasmids from biogas plant digestates often belong to the IncP-1ε subgroup. Front Microbiol 6. doi: 10.3389/fmicb.2014.00765

Wu K, Yuan S, Wang L, Shi J, Zhao J, Shen B, Shen Q (2014) Effects of bio-organic fertilizer plus soil amendment on the control of tobacco bacterial wilt and composition of soil bacterial communities. Biol Fert Soils 50:961-971. doi:10.1007/s00374-014-0916-9

You Y, Hilpert M, Ward MJ (2012) Detection of a common and persistent tet(L)-carrying plasmid in chicken-waste-impacted farm soil. Appl Environ Microb 78:3203-3213. doi:10.1128/AEM.07763-11

Zhang Y, Boyd SA, Teppen BJ, Tiedje JM, Li H (2014) Role of tetracycline speciation in the bioavailability to escherichia coli for uptake and expression of antibiotic resistance. Environ Sci Technol 48:4893-4900. doi:10.1021/es5003428

Zhao S, Liu D, Ling N, Chen F, Fang W, Shen Q (2014) Bio-organic fertilizer application significantly reduces the Fusarium oxysporum population and alters the composition of fungi communities of watermelon Fusarium wilt rhizosphere soil. Biol Fert Soils 50:765-774. doi:10.1007/s00374-014-0898-7

Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci U S A 110:3435-3440. doi:10.1073/pnas.1222743110

Figure captions

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

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

Figure 1 UPGMA cluster analysis of DGGE fingerprints for 16S rRNA gene amplicons from CL and SA soils 60 days after the first amendment: amplicons from (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.

Figure 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. 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.

Figure 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.

Figure 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. 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.

Figure 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.

Supporting Information

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

Luigi Chessa[1,2] *, Sven Jechalke[2#], Guo-Chun Ding[2,3], Alba Pusino[1], Nicoletta Pasqualina Mangia[1], Kornelia Smalla[2],

[1] Department of Agriculture, University of Sassari, Viale Italia 39, 07100 Sassari, Italy;

[2] Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany;

[3] College of Resources and Environmental Sciences, China Agricultural University, 100193, Yuanmingyuan xilu No 2, Haidian District, Beijing, China;

[#] current address: Institute for Phytopathology, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32 (IFZ), 35392 Gießen, Germany;

*for correspondence: Email: luigi.chessa@uniss.it; Tel.: +39 079229289;

Fax: +39 079229370

Table S1 Selected physical and chemical properties of the soils and manure investigated. From Chessa et al. (2016).

Treatment	pH	OM (%)	OC (%)	Sand (%)	Clay (%)	Silt (%)
CL soil	5.8	6.9	4.0	39.4	41.4	19.2
SA soil	7.6	4.9	2.8	72.7	16.7	10.6
Manure	7.8	52.7	30.6	-	-	-

OM: Organic Matter; OC: Organic Carbon; % of dry weight

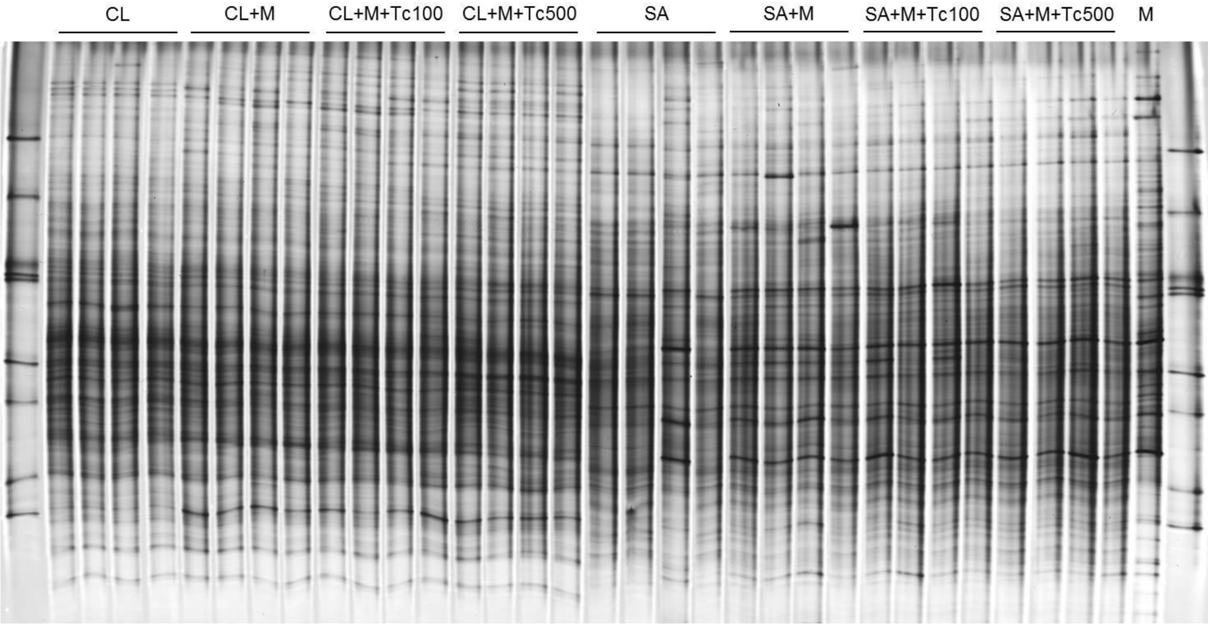


Figure S1 Bacterial 16S rRNA gene amplicons of CL and SA soil 60 days after the first amendment. Four replicates for each soil treatment were performed. M: manure; Tc100: 100 mg Tc kg^{-1} soil dry weight; Tc500: 500 mg Tc kg^{-1} soil dry weight.

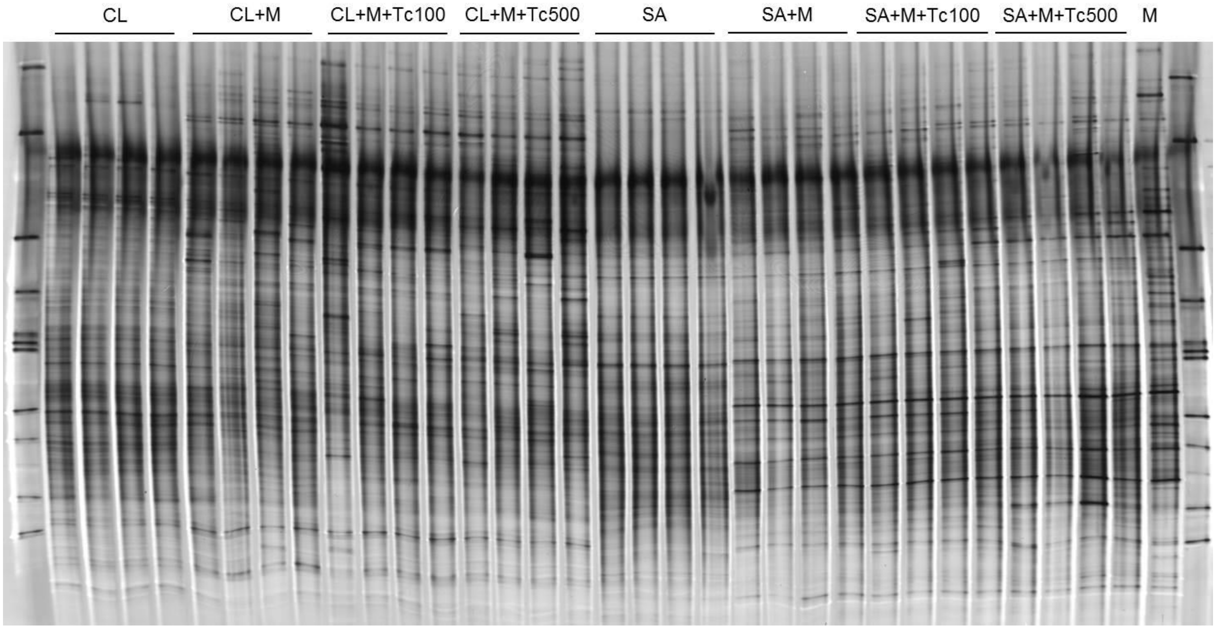


Figure S2 Bacterial 16S rRNA gene amplicons of CL and SA soil 60 days after the second amendment. Four replicates for each soil treatment were performed. M: manure; Tc100: 100 mg Tc kg^{-1} soil dry weight; Tc500: 500 mg Tc kg^{-1} soil dry weight.

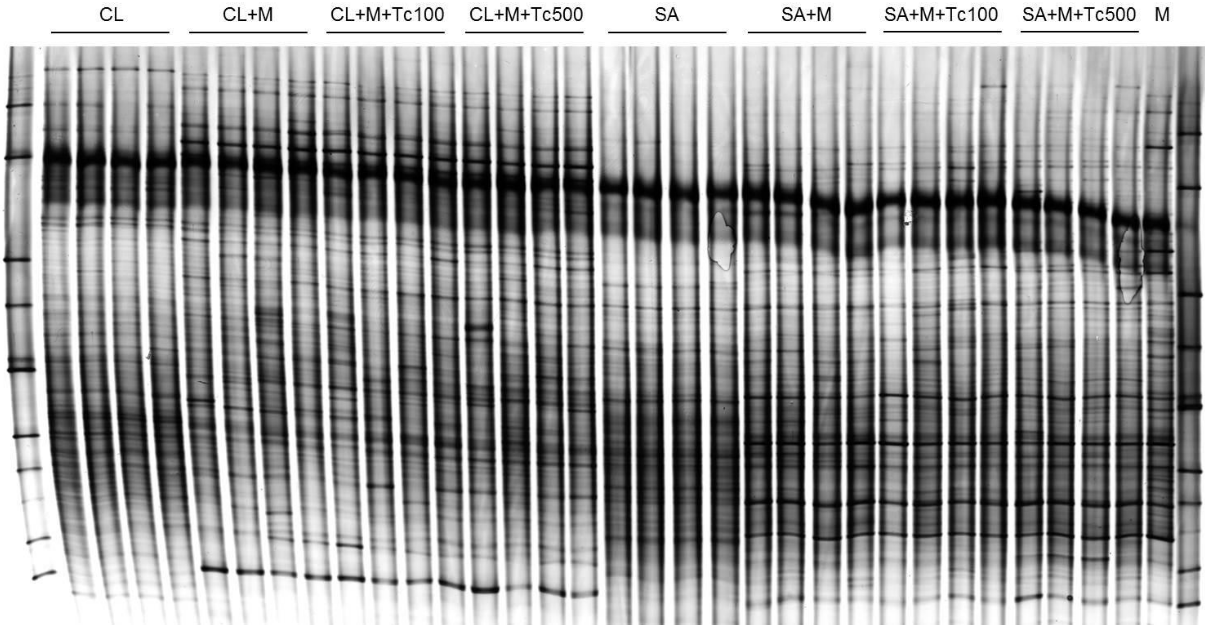


Figure S3 Bacterial 16S rRNA gene amplicons of CL and SA soil 60 days after the third amendment. Four replicates for each soil treatment were performed. M: manure; Tc100: 100 mg Tc kg^{-1} soil dry weight; Tc500: 500 mg Tc kg^{-1} soil dry weight.

Figure 1

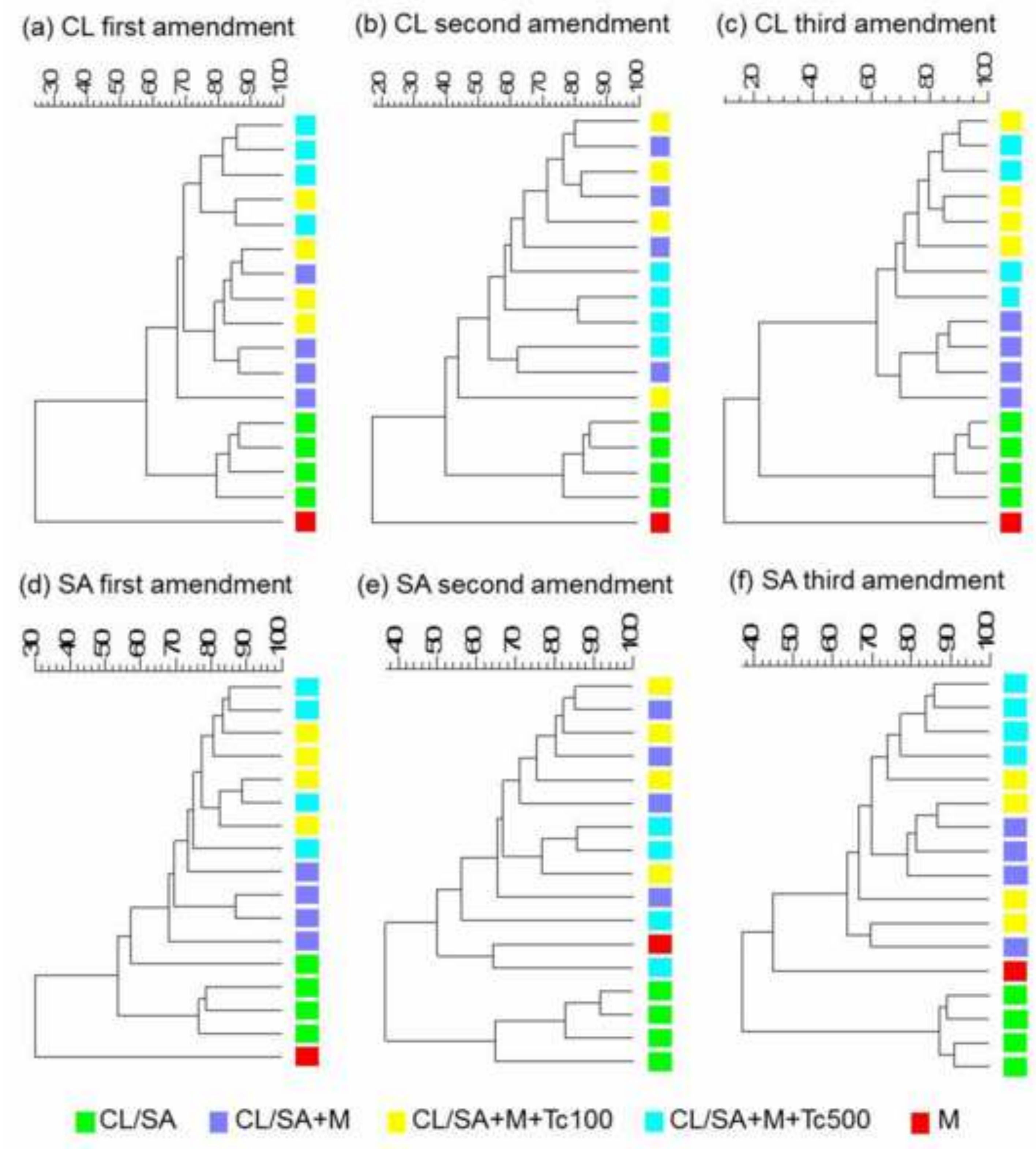

(a) CL first amendment
30 40 50 60 70 80 90 100
(b) CL second amendment
20 30 40 50 60 70 80 90 100
(c) CL third amendment
20 40 60 80 100
(d) SA first amendment
30 40 50 60 70 80 90 100
(e) SA second amendment
40 50 60 70 80 90 100
(f) SA third amendment
40 50 60 70 80 90 100
CL/SA
CL/SA+M
CL/SA+M+Tc100
CL/SA+M+Tc500
M

Figure 2

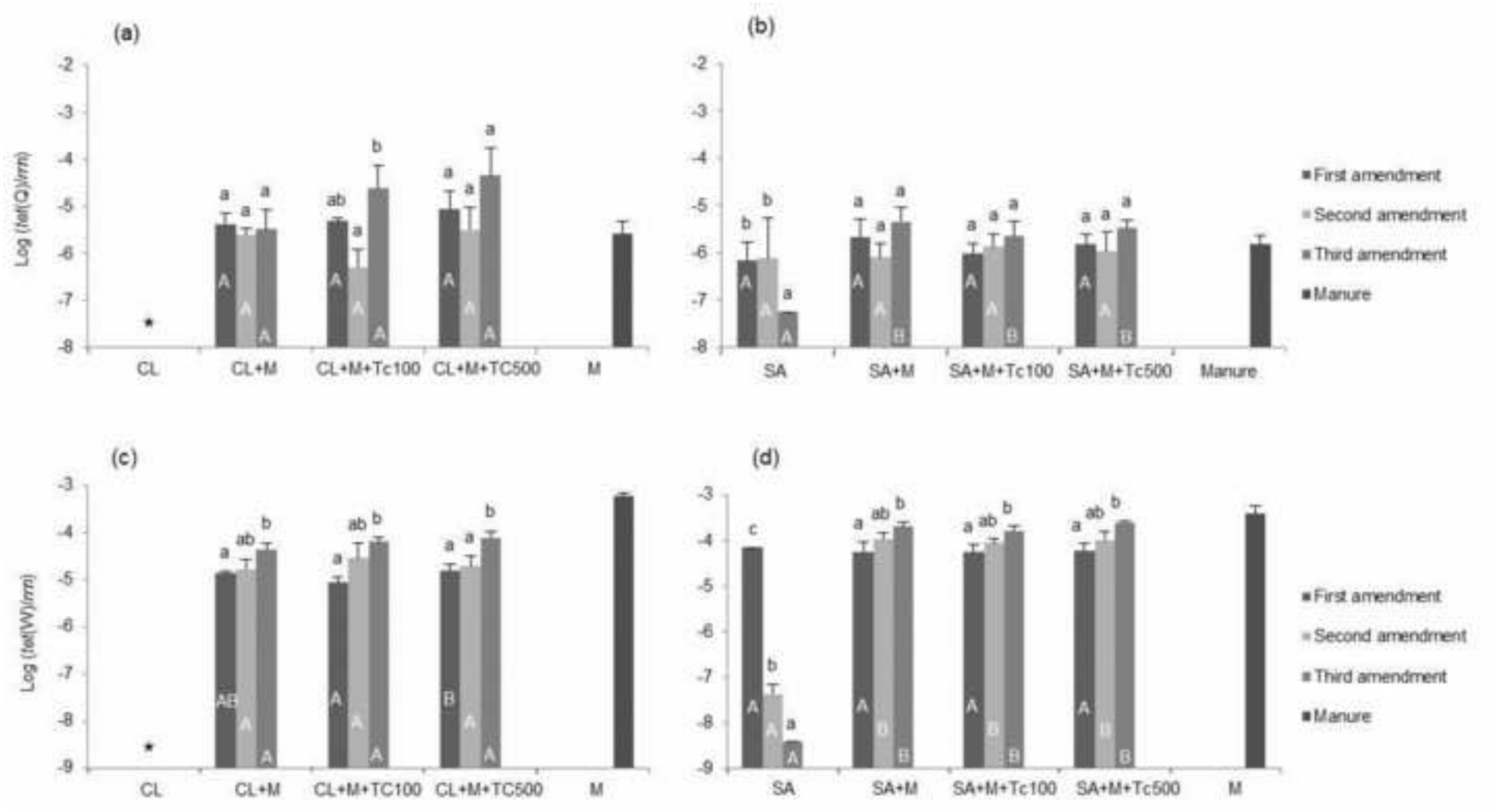

(a)
Log (tet(Q)/rrn)
-2
-3
-4
-5
-6
-7
-8
CL
CL+M
CL+M+Tc100
CL+M+TC500
M
(b)
SA
SA+M
SA+M+Tc100
SA+M+Tc500
Manure
First amendment
Second amendment
Third amendment
Manure
(c)
Log (tet(W)/rrn)
-3
-4
-5
-6
-7
-8
-9
CL
CL+M
CL+M+TC100
CL+M+TC500
M
(d)
SA
SA+M
SA+M+Tc100
SA+M+Tc500
M
First amendment
Second amendment
Third amendment
Manure

Figure 3

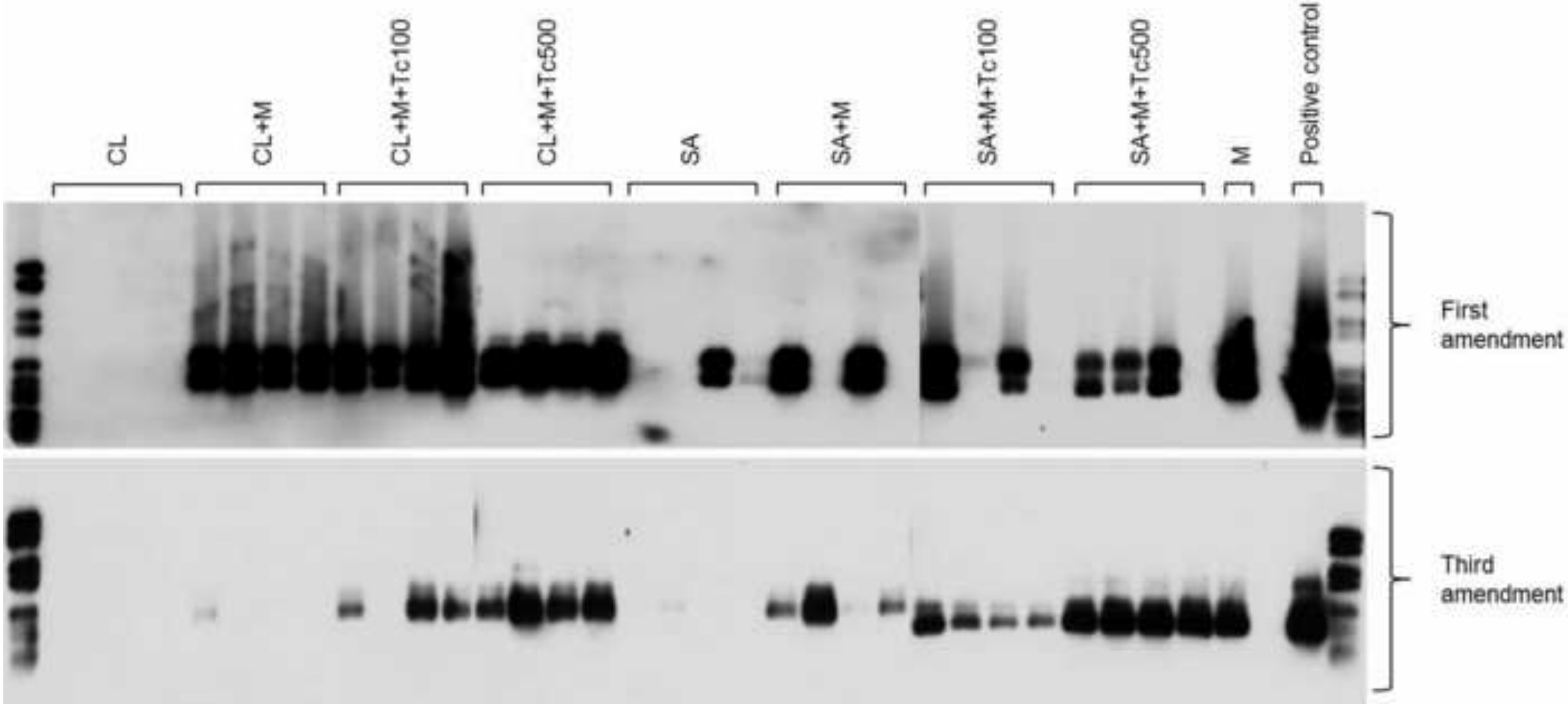
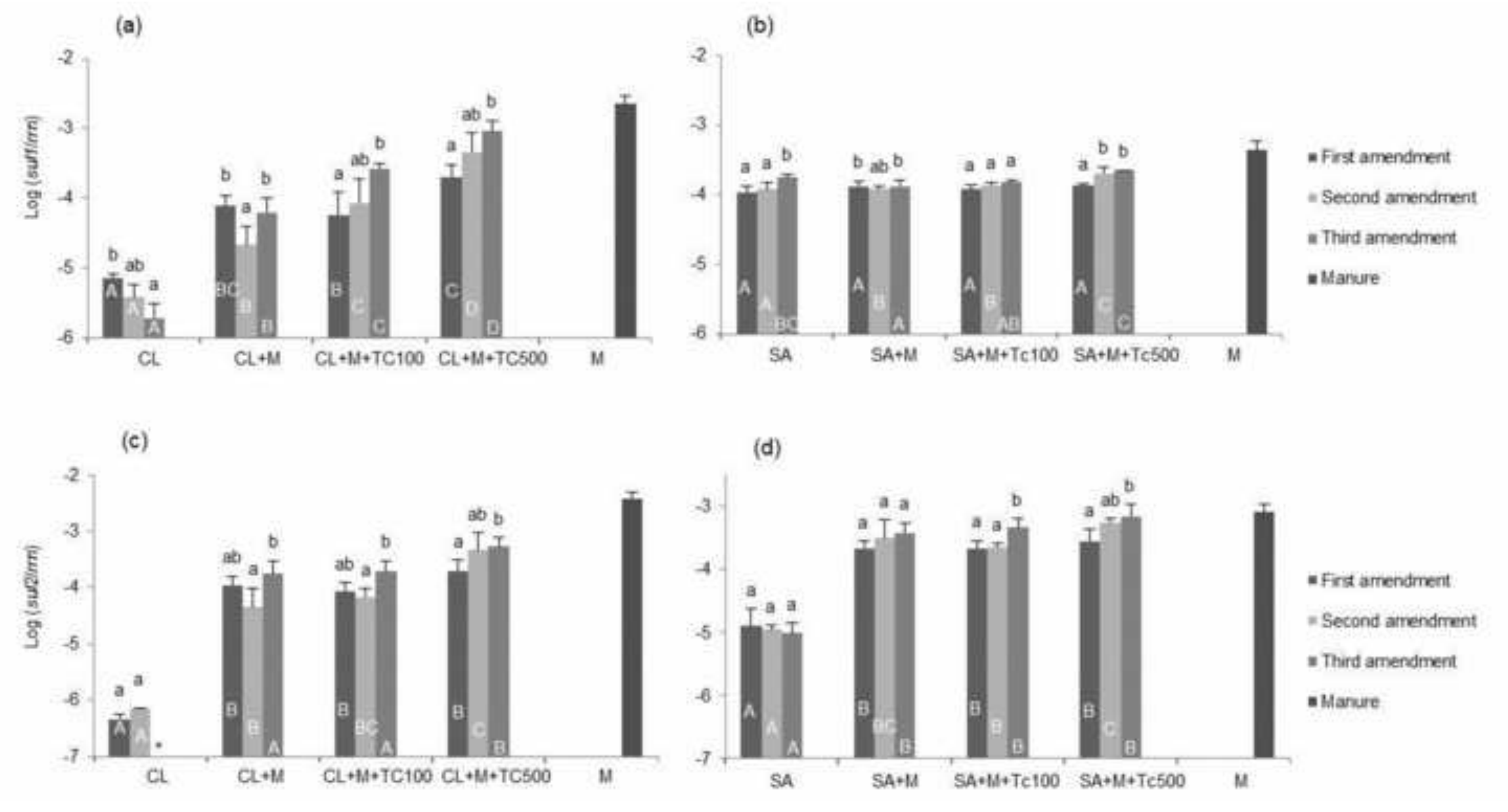

CL
CL+M
CL+M+Tc100
CL+M+Tc500
SA
SA+M
SA+M+Tc100
SA+M+Tc500
M
Positive control
First amendment
Third amendment

Figure 4


(a)
Log (sul1/rrn)
-2
-3
-4
-5
-6
CL
CL+M
CL+M+TC100
CL+M+TC500
M
b
ab
a
A
A
A
b
a
b
BC
B
B
a
ab
b
B
C
C
a
ab
b
C
D
D
(b)
-2
-3
-4
-5
-6
SA
SA+M
SA+M+Tc100
SA+M+Tc500
M
a
a
b
A
A
BC
b
ab
b
A
B
A
a
a
a
A
B
AB
a
b
b
A
C
C
■ First amendment
■ Second amendment
■ Third amendment
■ Manure
(c)
Log (sul2/rrn)
-2
-3
-4
-5
-6
-7
CL
CL+M
CL+M+TC100
CL+M+TC500
M
a
a
A
A
*
ab
a
b
B
B
A
ab
a
b
B
BC
A
a
ab
b
B
C
B
(d)
-3
-4
-5
-6
-7
SA
SA+M
SA+M+Tc100
SA+M+Tc500
M
a
a
a
A
A
A
a
a
a
B
BC
B
a
a
b
B
B
B
a
ab
b
B
C
B
■ First amendment
■ Second amendment
■ Third amendment
■ Manure

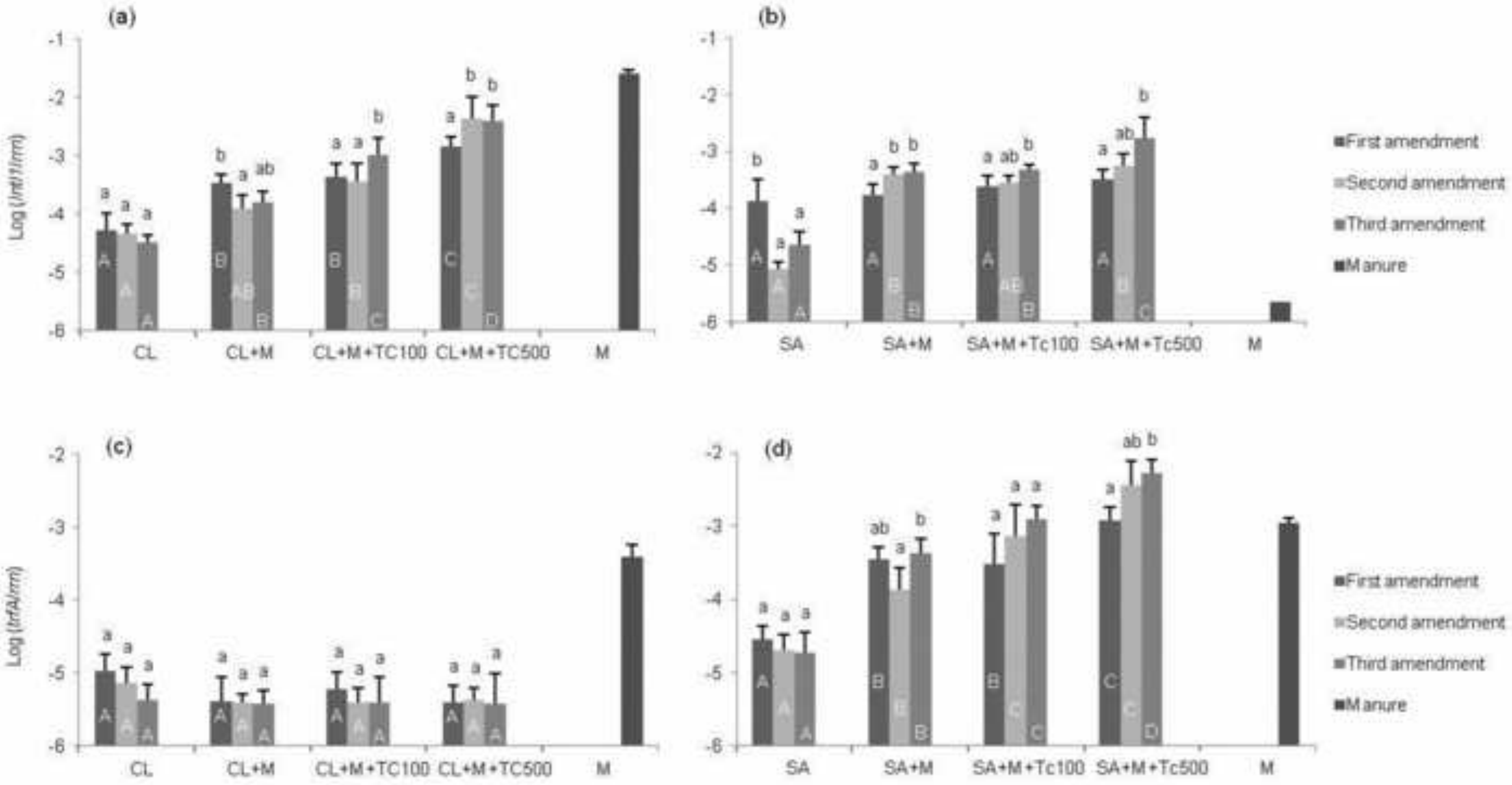

(a)
Log (intI1/rrn)
-1
-2
-3
-4
-5
-6
a a a
A A A
b a ab
B AB B
a a b
B B C
a b b
C C D
CL
CL+M
CL+M+TC100
CL+M+TC500
M
(b)
-1
-2
-3
-4
-5
-6
b a a
A A A
a b b
A B B
a ab b
A AB B
a ab b
A B C
SA
SA+M
SA+M+Tc100
SA+M+Tc500
M
First amendment
Second amendment
Third amendment
Manure
(c)
Log (trfA/rrn)
-2
-3
-4
-5
-6
a a a
A A A
a a a
A A A
a a a
A A A
a a a
A A A
CL
CL+M
CL+M+TC100
CL+M+TC500
M
(d)
-2
-3
-4
-5
-6
a a a
A A A
ab a b
B B B
a a a
B C C
a ab b
C C D
SA
SA+M
SA+M+Tc100
SA+M+Tc500
M
First amendment
Second amendment
Third amendment
Manure

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

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

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). Significance is indicated by the respective *P*-value.

		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

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

Figure S2

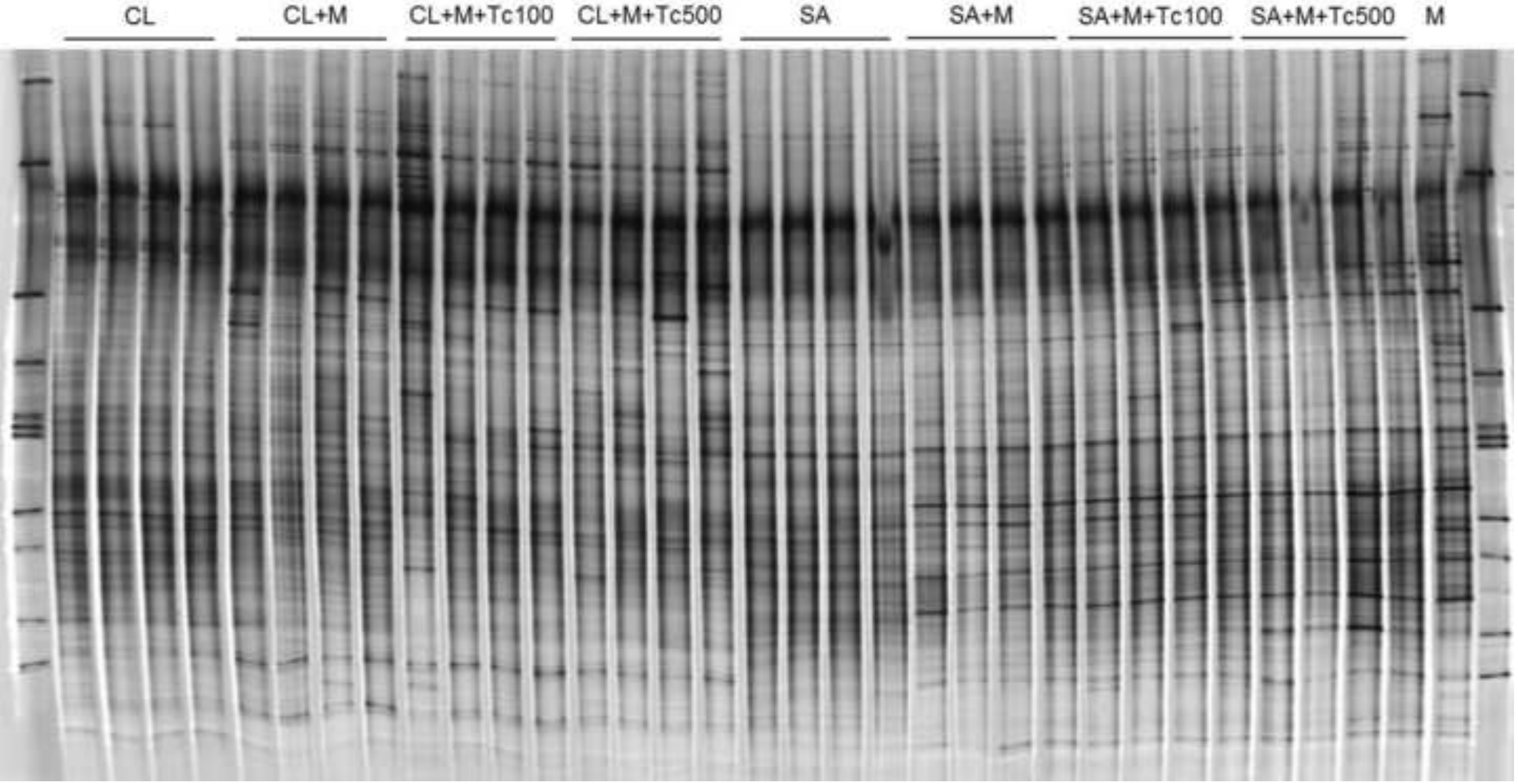


Figure S3

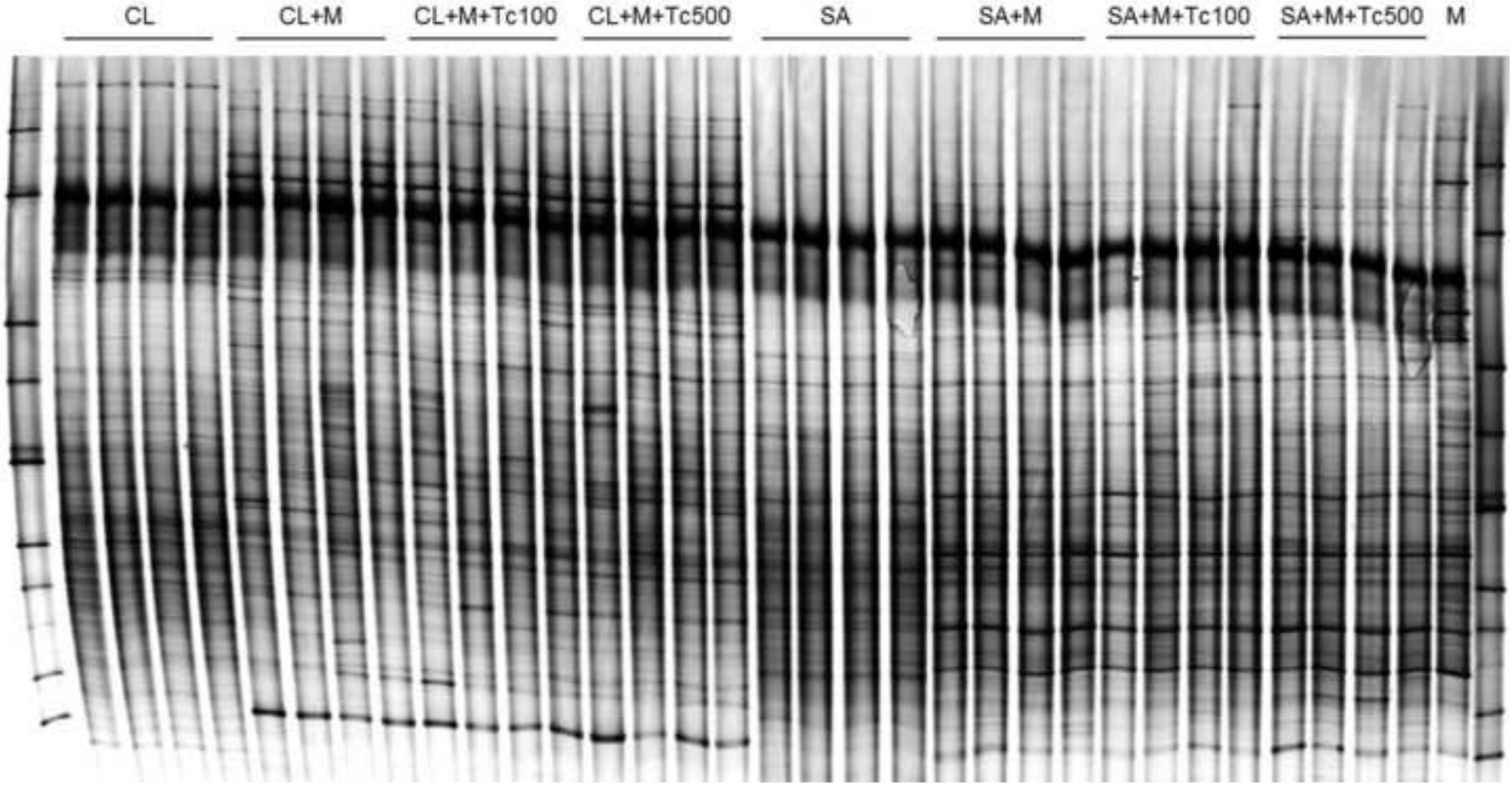


Figure S1

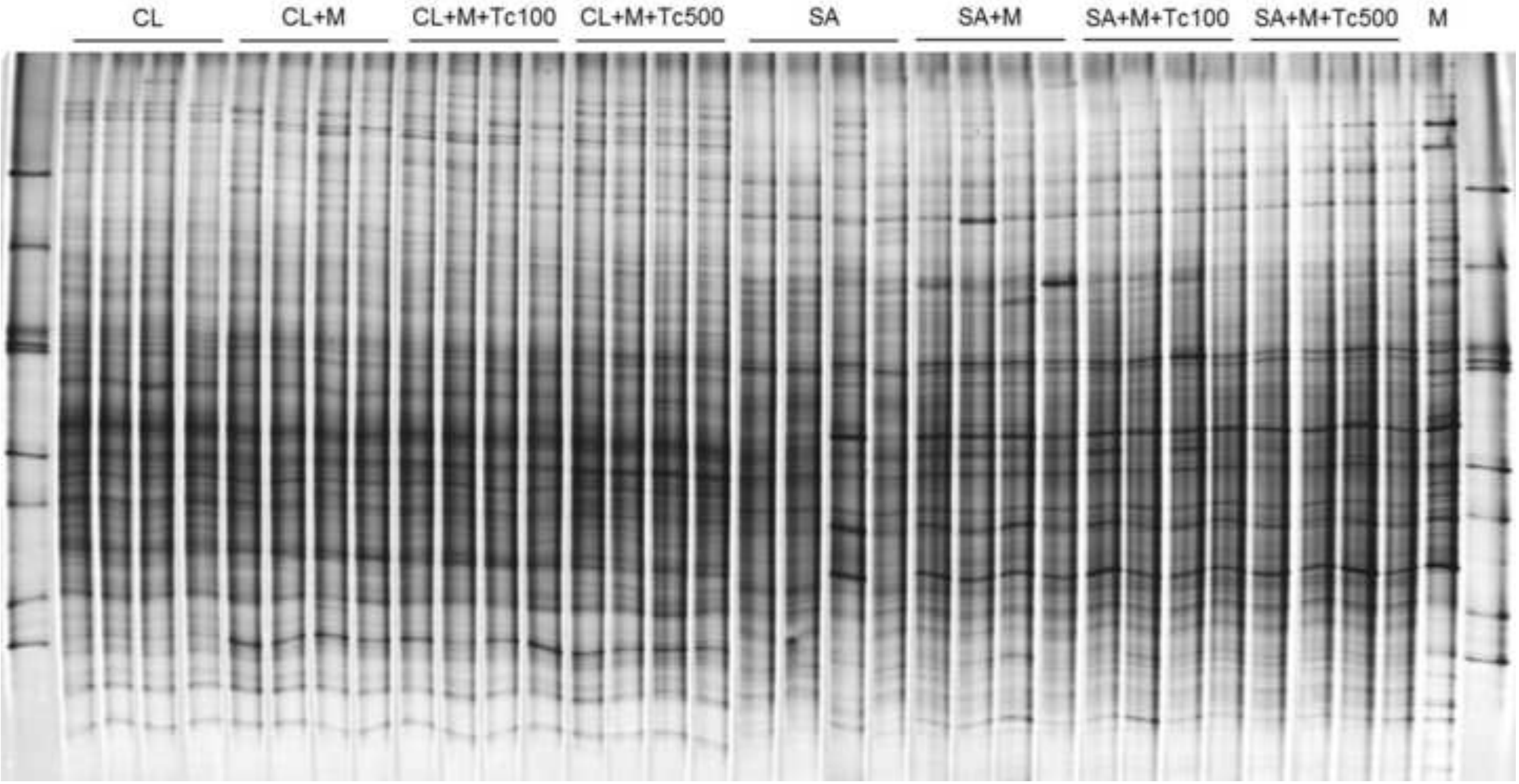


Table S1 Selected physical and chemical properties of the soils and manure investigated. From Chessa et al. (2016).

Treatment	pH	OM (%)	OC (%)	Sand (%)	Clay (%)	Silt (%)
CL soil	5.8	6.9	4.0	39.4	41.4	19.2
SA soil	7.6	4.9	2.8	72.7	16.7	10.6
Manure	7.8	52.7	30.6	-	-	-

OM: Organic Matter; OC: Organic Carbon; % of dry weight