

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

Questa è la versione Post print del seguente articolo:

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

Terms of use:

Chiunque può accedere liberamente al full text dei lavori resi disponibili come "Open Access".

Publisher copyright

note finali coverpage

(Article begins on next page)

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:	<p>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 intl1, 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.</p>
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-mail 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 reviewed 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 following 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 separate 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

[Click here to view linked References](#)

1 **Journal section for manuscript publication:**

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3 **The presence of tetracycline in cow manure changes the impact of repeated manure**
4
5
6
7 **application on soil bacterial communities**

8
9
10 E-mail address: luigi.chessa@uniss.it

14 **Author list**

17 Luigi Chessa,^{1,2 *} Sven Jechalke,^{2#} Guo-Chun Ding,^{2,3} Alba Pusino,¹ Nicoletta Pasqualina Mangia,¹
18
19 Kornelia Smalla,²

24 **Addresses of the institutions**

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

29 ² Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for
30
31 Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

34 ³ College of Resources and Environmental Sciences, China Agricultural University, 100193,
35
36 Yuanmingyuan xilu No 2, Haidian District, Beijing, China

39 # current address: Institute for Phytopathology, Justus Liebig University Giessen, Heinrich-Buff-
40
41 Ring 26-32 (IFZ), 35392 Gießen, Germany

46 * Corresponding author. Mailing address: University of Sassari, Department of Agriculture, Viale
47
48 Italia 39, 07100 Sassari, Italy. Phone: +39 079229289. FAX: +39 079229370. E-mail:
49
50
51 luigi.chessa@uniss.it

24 **Abstract**

1
25 The effect of tetracycline (Tc) and cow manure on soil bacterial community composition and
3
4
56 antibiotic resistance gene (ARG) abundance in soil was investigated in the present microcosm
6
77 study. Repeated applications of cow manure spiked with Tc in two concentrations or without Tc on
8
9
107 28 the bacterial communities of a clayey and a sandy soil with different history of anthropogenic
11
129 pollution by sewer flooding were investigated. Soil samples were taken 60 days after each of three
13
14
1530 amendments. DGGE fingerprints of 16S rRNA gene amplicons from total community DNA
16
1731 revealed soil type-dependent changes in the bacterial community composition in response to
18
1932 manure and to Tc, which became more pronounced with repeated applications. Repeated manure
20
21
2233 amendments and Tc, in particular at high concentration, triggered the further increase of ARGs
23
2434 *tet(A)*, *tet(O)*, *tet(Q)*, *tet(W)*, *sul1*, and mobile genetic elements (MGEs) IncP-1ε plasmids and *int11*,
25
26
2735 in a soil type-dependent manner. In the clay soil with no anthropogenic history, the ARGs and
28
2936 MGEs abundances were low or not detectable while manure amendments caused pronounced
30
31
3237 increases in their relative abundance. In the sandy soil with a history of anthropogenic impact,
33
3438 ARGs and MGEs were already present at a higher level and strong increases were mainly observed
35
3639 for the relative abundances of *sul2* and MGEs. Here we show for the first time that effects of
37
38
3940 repeated cow manure applications might be dependent on soil type and foregoing anthropogenic
40
4141 soil pollution, and that the presence of Tc could further increase the abundance of ARGs and
42
43
4442 MGEs.

45
4643 **Keywords**

47
48
4944 Tetracycline, soil, bacterial fingerprints, antibiotic resistance genes, class 1 integron, co-selection.
50

5145
52
5346 **Introduction**

54
55
5647 Antibiotics are widely used in livestock to treat and prevent infectious diseases or to promote
57
5848 animal growth (Durso and Cook 2014; Sarmah et al. 2006). In EU countries since January 2006
59
60
6149 (EC 1831/2003), and in South Korea since July 2011 (Hassan and Ryu 2012), the use of antibiotics

50 as growth promoters in feed for livestock is banned, but they are still a common practice in many
1
21 countries including the USA, Canada (Kim et al. 2011) and in China (Zhu et al. 2013). About 70%
3
4
52 of total pharmaceutical products used worldwide are antibiotics (Kümmerer 2003; Sarmah et al.
6
53 2006; Thiele-Bruhn 2003), and about half of the total consumption is attributable to veterinary
8
9
54 practices (Winckler and Grafe 2001; Zhu et al. 2013). Antibiotics are normally used in livestock
10
11
155 farming to maintain animal health and productivity, but this practice has several consequences for
13
14
156 human and environmental health, such as alteration of microbiota composition in livestock and non-
15
16
157 target animals, rise of methane emission from dung (Hammer et al. 2016), and contributes to
18
19
58 spreading human and animal pathogens resistant to antibiotics, and thus poses a significant health
20
21
2259 threat (Van Boeckel et al. 2015).

2460 Among all antibiotics used in livestock, tetracyclines (Tc) are the most consumed drug
25
26
61 (Food and Drug 2013), due to their low price (Chopra and Roberts 2001) and high efficacy against
27
28
62 a wide range of bacteria, both Gram-negative and Gram-positive. Tc is able to exert a bacteriostatic
30
31
63 effect by interfering with the 30S and 70S ribosome subunits and stopping the protein synthesis
32
33
64 (Chopra and Roberts 2001; Nelson and Levy 2011). At present, 42 antibiotic resistance genes
35
36
65 (ARGs) are known coding for proteins belonging to three resistance mechanisms able to confer
37
38
66 resistance against Tc in bacteria, including efflux pumps, ribosomal protection proteins and
40
41
67 degradation enzymes (Roberts 2011), as well as five mutations of the 16S rRNA that reduce the
42
43
68 binding affinity of the drug to the ribosome (Nguyen et al. 2014). Tc resistance genes (RGs) are
45
46
69 typically associated with mobile genetic elements (MGEs) such as plasmids, transposons, and
47
48
70 associated integrons and they were detected in different environments, e.g. in manure and soil
50
51
71 (Schmitt et al. 2006). Moreover, as in the case of *tet(A)*, *tet(Q)* and *tet(W)*, they were detected in
52
53
72 both Gram-negative and Gram-positive bacteria and were often found associated with sulfonamide
54
55
73 ARG (*sulI*), as in case of *tet(A)* (Roberts 2011). Tc is highly water-soluble, poorly absorbed in the
57
58
74 animal gut and quickly excreted, mostly unchanged and still bioactive (O'Connor and Aga 2007).
59
60
75 Up to 75-90% of one administered dose can be excreted via feces or urine (Sarmah et al. 2006). For

76 this reason, high concentrations of Tc can be found in animal feces (Zhu et al. 2013) and their
1
277 extractable concentration decreases in aged manure after composting, resulting from the microbial
3
4
578 degradation or the irreversible sorption of Tc on the soil particles (Kreuzig and Hölting 2005). When
6
779 manure containing antibiotics is applied to agricultural soil as fertilizer, antibiotics are transported
8
9
1080 into the soil as well (Jechalke et al. 2014b), and this can affect the composition and function of the
11
1281 resident microbial communities, as recently shown for sulfadiazine (SDZ) introduced via manure
13
14
1582 into soil (Ding et al. 2014). Although the detection of antibiotics in soil remains experimentally
16
1783 challenging, Hamscher et al. (2002), using high-molar citric acid buffer at low pH, reported Tc
18
1984 concentrations in liquid manure fertilized topsoil ranging from 0.086 to 0.171 mg kg⁻¹, while Qiao
20
21
2285 et al. (2012) measured 0.78 mg kg⁻¹ using McIlvaine-Na₂EDTA buffer and subsequent sonication.
23
2486 Tc is strongly and rapidly sorbed to soil clays and organic matter (Gu et al. 2007; Jutta et al. 2007)
25
26
2787 but free Tc concentrations might be still bioavailable and potentially affect the microbial
28
2988 communities (Thiele-Bruhn and Beck 2005), depending on soil properties (Jia et al. 2008). In
30
31
3289 addition to antibiotic residues, manure typically contains high numbers of bacteria carrying ARGs
33
3490 on MGEs, which can further increase the abundance of antibiotic resistant bacteria in soil when
35
36
3791 manure is applied (Binh et al. 2008; Heuer et al. 2009; Jechalke et al. 2014b; Smalla et al. 2000).
38
3992 Furthermore, antibiotics such as Tc introduced via manure into soil can select the proliferation of
40
4193 resistant soil bacteria and the spread of ARGs via MGEs from manure bacteria to soil bacteria
42
43
4494 (Hammesfahr et al. 2008; Heuer et al. 2008). The application of manure several times a year, which
45
46
4795 is a typical agricultural practice (Montforts et al. 1999), might lead to an accumulation of ARGs and
48
4996 antibiotic compounds, as well as to an accumulation of effects on the soil bacterial community
50
5197 composition, as recently shown for the sulfonamide antibiotic SDZ in soil microcosm experiments
52
53
5498 (Ding et al. 2014; Heuer et al. 2011b). Moreover, manure application to soil can be important not
55
5699 only as nutrient supplement to increase crop yield but also for biocontrol of fungal pathogens by the
57
58
59100 use of manure enriched with microbial antagonists (Zhao et al. 2014), in order to stabilize, by
60
61101 integrated agricultural management, soil microbial communities important for soil health and

102 sustainability (Wu et al. 2014). Manure can also have negative effects such as the stimulation of
1
103 methane production by resident methanogenic soil bacteria, and mitigation strategies to reduce
2
3
4
104 methane emission should be considered (Ho et al. 2015).
5
6

105 Most studies on the effects of organic fertilizers on soil microbial community composition
7
8
9
106 were based on piggery manures. However, in several regions of the world fertilization with cow
10
11
107 manure is more relevant. Recently, Udikovic-Kolic et al. (2014) showed an unexpected bloom of
12
13
108 ARGs in the response to soil fertilization with cow manure free of antibiotics. Little is known so far
14
15
109 on how the presence of antibiotics might change the effects that cow manure applied to soil has on
16
17
109 the bacterial community composition and on the abundance of ARGs and MGEs (Kyselková et al.
18
19
110 2015b).
20
21
22
23

24 In the present study, we investigated how three soil amendments with cow manure, spiked
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

128 CL soil. Tc had a short-term detrimental effect and after two days reduced the microbial activity
1
129 (fluorescein diacetate hydrolysis) and shifted the microbial composition from bacteria to fungi, as
2
130 revealed by phospholipids fatty acids (PLFA) analysis, respectively. Moreover, the Tc effects were
3
4
5
6
131 transient, decreased on the seventh day and had disappeared after 60 days in the SA soil, while in
7
8
9
132 the CL soil, with no history of antibiotic pollution, the utilization of substrates in BIOLOG plates
10
11
123 still remained different among the patterns of the different treatments. Given the recent findings of
13
14
134 Chessa et al. (2016) on Tc and cow manure effects on soil microbial communities after single
15
16
135 amendment, we aimed to test the hypothesis that repeated applications of manure to soil affect the
17
18
136 soil bacterial community composition and increase the abundance of ARGs and MGEs in a soil
19
20
21
227 type-dependent manner and that these effects are more pronounced in the presence of Tc.
23

238

25

26

239

27

28

240

30

31

141

32

33

142

35

36

143

37

38

144

39

40

145

42

43

146

44

45

147

47

48

148

49

50

149

52

53

150

54

55

151

57

152

59

60

153

61

62

63

64

65

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.

154 Four soil treatments were performed with four independent replicates for each treatment. For
1
155 each replicate, 200 g of soil sieved at <2 mm were placed in a glass pot (18×10×6 cm) with the
2
3
4
156 following treatments: (i) only water was added to the soil (CL/SA); (ii) 8 g of uncontaminated aged
5
6
157 M were mixed with soil (CL/SA+M); (iii) and (iv) 8 g of aged M spiked with Tc solution were
7
8
158 added to the soil (freshly prepared by dissolving Tc in sterile deionized water and mixed by
9
10
159 agitation for 1 h in the dark before addition to soil) to reach final theoretical concentrations of 100
11
12
13
14
160 or 500 mg Tc kg⁻¹ soil (CL/SA+M+Tc100 and CL/SA+M+Tc500, respectively). Soil and M were
15
16
161 carefully mixed in pots and incubated in the dark at a constant temperature of 20°C and at 50% of
17
18
19
162 maximum water-holding capacity. Every two days, water was sprayed on the soil surface to
20
21
22
163 compensate weight loss by evaporation. Amendments were performed three times at 60-day
23
24
164 intervals and the soil was mixed after each amendment in order to observe, at microcosm scale, the
25
26
165 effect of repeated M and Tc addition to soils. After 60 days, a quadruplicate set of soil samples was
27
28
166 collected from each pot. Soil in the pot was mixed and 10 g of soil were transferred to a sterile
29
30
31
167 polypropylene jar for microbiology (Becton Dickinson International, Erembodegem, Belgium). The
32
33
168 soil in the jar was mixed again and 1 g of soil was transferred into a sterile Eppendorf tube (1.5 mL
34
35
169 volume) and stored at -20°C until total community DNA extraction (see below). The remaining soil
36
37
38
170 in the jar was put back into the pot and the next amendment was performed.
39
40

41 42 43 172 Total community DNA extraction

44
45
46
173 Total community DNA was extracted from 0.5 g of soil using the FastDNA[®] SPIN Kit for Soil (MP-
47
48
174 Biochemicals, Solon, OH, USA) with some modifications: cell lysis in soil samples by the
49
50
175 FastPrep[®] Instrument (MP Biomedicals) was performed twice for 30 s at setting 5.5. Also two
51
52
176 washing steps were performed, by addition of salt/ethanol washing solution (SEWS-M). Finally,
53
54
55
177 DNA was eluted in 100 µL of DNA elution solution (DES). Then 50 µL of DNA solution from each
56
57
178 sample was purified by the GeneClean[®] Spin Kit (Q-Biogene/MP-Biochemicals) and eluted in 50
58
59
60
179 µL final volume observing the manufacturers' protocol.
61
62
63
64
65

180
1
181
2
3
4
182
5
6
183
7
8
9
184
10
11
185
12
13
14
186
15
16
187
17
18
188
19
20
21
189
22
23
190
24
25
191
26
27
28
192
29
30
193
31
32
33
194
34
35
195
36
37
38
196
39
40
197
41
42
198
43
44
45
199
46
47
200
48
49
50
201
51
52
202
53
54
203
55
56
204
57
58
59
205
60
61
62
63
64
65

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 μ M 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

206 *rrn* copies was performed in accordance with Suzuki et al. (2000). All primers and TaqMan probes
1
207 used are listed in Table 1. Standard dilutions of PCR fragments cloned into pGEM-T vector systems
2
3
4
208 (Promega Corporation, Madison, WI, USA) were used for quantification. The PCR mastermix
5
6
209 contained 1.2 μ M forward primer Bact1369F, 1 μ M reverse primer Prok1492R, 0.5 μ M TaqMan-
7
8
210 Probe TM1389F, 0.1 mg mL⁻¹ of bovine serum albumin (BSA) and 1.25 U TrueStart Taq
9
10
211 (Fermentas, St. Leon-Rot, Germany). The PCR protocol was 5 min at 95°C, followed by 40 cycles
11
12
212 of 15 s at 95°C, 15 s at 56°C and 1 min at 60°C. A CFX96TM Real-Time PCR Detection System
13
14
213 (Bio-Rad, Hercules, CA, USA) was used. The qPCR for determination of the abundance of class 1
15
16
214 integron integrase genes *intI1* was performed using the forward primer int1-LC1, the reverse primer
17
18
215 int1-LC5 and TaqMan-Probe int1-probe previously described (Barraud et al. 2010). Total reaction
19
20
21
22
23
24
216 volume was 50 μ l. Five μ l DNA template of 1:5 diluted purified DNA solution were used and 1 \times
25
26
217 TrueStart Buffer (Fermentas), 2.5 mM MgCl₂ (Fermentas), 0.2 mM dNTPs, 0.08 mg μ L⁻¹ BSA, 0.3
27
28
218 mM of primers and probe and 1.25 U TrueStart Taq (Fermentas); 40 cycles were performed
29
30
31
219 according to the protocol described by Barraud et al. (2010). To normalize for different extraction
32
33
220 and amplification efficiencies the relative abundance of target genes was calculated by dividing the
34
35
221 copy number of each gene by the *rrn* copy number.

36
37
38
222 For *tet(Q)* and *tet(W)*, the same reaction mixture composition was used. The protocol was as
39
40
41
223 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
42
43
224 quantify *tet(Q)* and *tet(W)* were described by Smith et al. (2004). The qPCRs for *sul1* and *sul2*
44
45
225 genes were performed as described by Heuer and Smalla (2007) and Heuer et al. (2008),
46
47
226 respectively. The qPCR to measure the abundance of *trfA* genes specific for the epsilon subgroup of
48
49
50
227 IncP-1 plasmids was performed according to Heuer et al. (2012). Concentrations used in the
51
52
228 reaction mixture were the same as described for *intI1* qPCR, and amplification and detection were
53
54
229 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.
55
56
57

58
59
60
231 PCR and Southern blot hybridization of *tet(A)* and *tet(M)* genes
61
62
63
64
65

232 Primers used for the amplification of *tet(A)* and *tet(M)* were described by Lanz et al. (2003) and Ng
1
233 et al. (2001), respectively (Table 1). Digoxigenin-labeled probes were generated from PCR products
2
3
4
234 as described by Jechalke et al. (2014a) using plasmids RP4 and pAT101 as templates for *tet(A)* and
5
6
235 *tet(M)*, respectively. For both genes, 25 μ L reaction mixture composed of 1 \times TrueStart buffer
7
8
236 (Fermentas), 0.2 mM of deoxynucleoside triphosphates, 2.5 mM MgCl₂ (Fermentas), 0.1 mg mL⁻¹
9
10
11
237 bovine serum albumin, 0.5 μ M of primers, respectively, and 0.6 U TrueStart Taq (Fermentas) were
12
13
14
238 used. Amplification and detection were performed according to the following protocol: 5 min at
15
16
239 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
17
18
19
240 min step at 72°C. PCR products were loaded on an agarose gel, with size markers (DIG-labeled
20
21
241 DNA Molecular Weight Marker VI, Roche Diagnostics Deutschland GmbH, Mannheim, Germany)
22
23
242 in the borders. Southern-blotting to a Hybond-N membrane (GE Healthcare Limited, Amersham,
24
25
243 UK) and hybridization of PCR products were done as described by Sambrook et al. (1989) and
26
27
28
244 following the standard procedure of Roche Diagnostics for filter hybridization under conditions of
29
30
31
245 middle stringency (Fulthorpe et al. 1995). The exposure times were 20 min for *tet(A)* and 1 h for
32
33
246 *tet(M)*.

34
35
36
37

38 **Results**

39 **Effects of cow manure and tetracycline effects on soil bacterial communities**

40
41
42
43
250 The effects of M with or without Tc on the bacterial communities in CL and SA soils were analyzed
44
45
46
47
48
251 by DGGE fingerprinting of 16S rRNA gene fragments amplified by PCR from total community
49
50
252 DNA. Soil type-dependent treatment effects on the bacterial community composition, which
51
52
253 increased with repeated M applications, were revealed. The effects of treatments analyzed 60 days
53
54
254 after each of three amendments (Fig. S1-S3) showed that the bacterial community fingerprints of
55
56
255 the control soils (CL or SA) always formed a cluster separate from those of soils treated with M
57
58
256 unspiked and Tc-spiked, CL/SA+M and CL/SA+M+Tc, respectively (Fig. 1), except for SA soil 60
59
60
257 days after the first amendment. The amendment of soils with cow M, with or without Tc spiking
61
62
63
64
65

258 (CL/SA+M+Tc and CL/SA+M), caused a high variability of the fingerprints among replicates of
1
259 each treatment in particular after the second amendment, and a clear effect of Tc became only
3
4
260 evident 60 days after the third M amendment. Especially in CL soil the fingerprints of
5
6
261 CL+M+Tc100 and CL+M+Tc500 treated soil samples, 60 days after the third amendment, clustered
8
9
262 and were clearly separated from the M-treated soil (CL+M); in SA soil only SA+M+Tc500
10
11
263 treatment clustered separately, indicating a minor effect of SA+M+Tc100 on the soil bacterial
13
14
264 communities. In addition, 60 days after the second and third amendments, the fingerprint of the
15
16
265 bacterial community in the M was rather similar to the bacterial fingerprints of SA+M+Tc100 and
18
19
266 SA+M+500. This similarity was not observed between M and CL soils, as here the bacterial
20
21
267 community fingerprint of M was, with repeated M applications, increasingly distinct from those of
23
24
268 the M-treated soils.

269 The permutation test revealed significant differences ($P<0.05$) between the DGGE
27
28
270 fingerprints of CL control soil and the M-treated CL soil at all sampling times (Table 2). After the
30
31
271 first M amendment the bacterial community composition of CL soil was clearly more affected than
32
33
272 the community of SA soil where the differences (d-values) between control SA soil and M-treated
35
36
273 SA soils were small and not significant. However, after the second M amendment the differences
37
38
274 between control soil and the M-treated soil became also significant for SA soil. For both soils the
40
41
275 effects of M on the bacterial community composition increased with repeated M amendment and
42
43
276 the differences observed 60 days after the third M amendment were remarkably high.
44
45
277 Unexpectedly, for both soils the fingerprints of S+M+Tc100 treatments were not significantly
47
48
278 different from those of the M treatments (S+M), even after repeated application of M+Tc100. In
49
50
279 contrast, the fingerprints of the M+Tc500 treated soil were significantly different from those of the
52
53
280 M-treated soil at all time points except for the M+Tc500 treated CL soil 60 days after the second
54
55
281 treatment.

57
58
282
59
60
61
62
63
64
65

283 **Treatment-dependent changes in the relative abundance of tetracycline and sulfonamide**
1
284 **resistance genes**

285 *tet(Q)*

286 The relative abundance of *tet(Q)* genes, 60 days after the first amendment, was below detection
8
287 limit in the control CL. The addition of M, which contained approximately $-5.7 \text{ Log } tet(Q)$ per *rrn*
10
288 copy numbers, significantly increased the abundance of *tet(Q)* in this soil (Fig. 2a). In contrast, 60
12
289 days after the first amendment, *tet(Q)* was detected in DNA extracted from the SA and in SA+M
14
290 soil treatments and its relative abundance was not significantly ($P<0.05$) different between these
16
291 treatments (Fig. 2b). Also the addition of M+Tc100 or M+Tc500 did not alter the abundance of
18
292 *tet(Q)* in both soils, compared to the soil amended with M alone. As the relative abundance of
20
293 *tet(Q)* in the control SA soil significantly ($P<0.05$) decreased over time, 60 days after the third
22
294 amendment, the abundance of *tet(Q)* was higher in all M treatments, both unspiked and Tc spiked.
24
295 Repeated amendments of M and M+Tc did not further increase the relative abundance of *tet(Q)* in
26
296 both soils, with the only exception of CL+M+Tc100 after the third amendment.
28
30

301 *tet(W)*

302 Similar to *tet(Q)* genes, also *tet(W)* genes were not detected in CL soil. The addition of M
304 containing $-3.3 \text{ Log } tet(W) \text{ rrn}^{-1}$ copy number increased the relative abundance of this gene in
306 CL+M, 60 days after the first amendment (Fig. 2c). In SA soil, 60 days after the first amendment,
308 *tet(W)* genes were detected also in the control SA and their relative abundance was not significantly
310 ($P<0.05$) different to SA+M (Fig. 2d). Moreover, in both soils, the first addition of M+Tc did not
312 increase the relative abundance of *tet(W)* genes, compared to M treatments. As observed for *tet(Q)*
314 gene in SA soil, also the relative abundance of *tet(W)* progressively decreased over time in SA soil.
316 However, the continuous addition of M with or without Tc spike significantly increased the relative
318 abundance of *tet(W)* in both soils.
320

309 *tet(A)* and *tet(M)*

1
310 Due to the low abundance of *tet(A)* and *tet(M)* genes it was not possible to measure their relative
3
4
311 abundance by qPCR. Therefore, these genes were amplified by PCR and detected through Southern
5
6
312 blot hybridization 60 days after the first and after the third amendments (Fig. 3). Southern blot
7
8
9
313 hybridizations revealed that cow M contained *tet(A)* carrying bacteria and, 60 days after the first
10
11
314 amendment, *tet(A)* was also detected in CL+M, CL+M+Tc100 and CL+M+Tc500, while it was not
12
13
14
315 detected in total community DNA from control CL soil (Fig. 3). At this time point, Southern blot
15
16
316 hybridization indicated that *tet(A)* abundance was low in all treatments of SA soil and was only
17
18
19
317 detected in some of the replicates. After the third amendment, the abundance of *tet(A)* decreased in
20
21
22
318 CL+M soils. Thus, *tet(A)* was detected only in three of four replicates of the CL+M+Tc100 and in
23
24
25
319 all replicates of CL+M+Tc500 whereas it was below the detection limit in CL+M. In SA soil a
26
27
28
320 remarkably increased abundance of *tet(A)* was detected in the total community DNA from all
29
30
31
322 replicates of SA+M+Tc500, indicating a selective effect of Tc (Fig. 3). The *tet(M)* was neither
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

324 *sul1* and *sul2*

325 Cow M contained -2.5 Log per *rrn* copy number of *sul1* and *sul2*. In CL soil the relative
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

334 the third amendment. Repeated addition of M+Tc caused an accumulation of *sul1* and *sul2* for both
1
335 Tc concentrations, except for the *sul1* in SA+M+Tc100 soils, while repeated additions of M did not.

336

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

338 *intI1*

339 The relative abundance of class 1 integron integrase genes (*intI1*) significantly ($P<0.05$) increased
13
340 in CL+M, compared to control CL, 60 days after the first amendment (Fig. 5a), while the relative
14
341 abundance of *intI1* gene was not significantly increased in SA+M, compared to SA soil (Fig. 5b).

342 The presence of Tc500 caused a significant increase in the relative abundance of *intI1* in CL soil at
19
20
343 all sampling times while in SA soil an increased abundance was only observed 60 days after the
21
22
344 third amendment. In the SA+M+Tc100 treatments no increase in the relative abundance of *intI1* for
23
24
25
345 SA soils was observed at all sampling times while an increase was found after the third amendment
26
27
346 in the CL+M+Tc100 treatment.

348 *IncP-1ε trfA*

349 Quantification of the *trfA* gene was used to detect and quantify the ε subgroup of IncP-1 plasmids in
36
37
38
350 response to the treatments. The abundance of IncP-1ε plasmids in CL soil was low and although the
39
40
351 M added to the soil contained a high abundance of IncP-1ε plasmids, the relative abundance of
41
42
352 these plasmids in CL+M remained low, even after repeated M additions (Fig 5c). Even the presence
43
44
45
353 of Tc did not lead to an increase in the relative abundance of IncP-1ε plasmids. In contrast, in SA
46
47
354 soil M increased the relative abundance of *trfA* in SA+M already 60 days after the first amendment,
48
49
50
355 compared to the SA soil (Fig. 5d). The presence of Tc increased the relative abundance of *trfA*,
51
52
53
356 which was significant for SA+M+Tc500 already after the first amendment while a significantly
54
55
357 increased abundance was observed for SA+M+Tc100 treatment, compared to SA+M, only after the
56
57
58
358 second amendment. After the third amendment the relative abundance of *trfA* in SA+M+Tc100 and

359 SA+M+Tc500 was even higher than in the M. An accumulation of the *trfA* gene with repeated
1
360 application was only observed in SA+M+Tc500.
2
3
4

361

362 Discussion

363 The fate of antibiotics in soil and likely also their effects, are influenced by the physicochemical
10
364 properties of the antibiotic, by soil properties (Kong et al. 2012) and soil history, intended as
11
12
13
14
365 foregoing antibiotic soil pollution due to anthropogenic activities. The present study is part of a
15
16
366 project aiming to better understand the effects of Tc and cow M on the microbial community
17
18
367 composition and on the antibiotic resistance in soil. In this study, two soils with different history of
19
20
21
368 anthropogenic pollution and physicochemical characteristics, described by Chessa et al. (2016) and
22
23
369 listed in Table S1, were used. The soils used were primarily selected for their differences in history
24
25
26
370 of anthropogenic pollution and also for differences in physicochemical composition (pH, clay, sand
27
28
371 and organic matter). The bioavailability of Tc, measured only after the first amendment by Chessa
29
30
31
372 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
32
33
373 and 0.767 and 4.468 mg kg⁻¹ in SA soil, respectively. The Tc concentrations used for spiking cow
34
35
36
374 M were higher compared to those used in other studies. However, as Tc quickly, and almost
36
37
38
375 completely, adsorbs to soil clay minerals and organic matter high amounts of Tc were applied to the
39
40
41
376 soil in order to reach soluble and potentially bioavailable concentrations, i.e. Tc available for the
42
43
44
377 bacterial uptake (Zhang et al. 2014), that were previously described for agricultural soils which are
45
46
47
378 continuously polluted (Hamscher et al. 2005; Qiao et al. 2012). In fact, the Tc concentrations not
48
49
379 adsorbed and hence potentially bioavailable found in the M+Tc100 soils were 0.155 and 0.767 mg
50
51
380 kg⁻¹ for CL and SA soil, respectively (Chessa et al. 2016). Comparable concentrations were
52
53
381 previously reported for Tc in M-treated soils and ranged between 0.15 and 0.8 mg Tc kg⁻¹ soil
54
55
382 (Hamscher et al. 2005; 2002; Qiao et al. 2012). Furthermore, we tested Tc spiked at a fivefold
56
57
383 higher concentration (M+Tc500) as a possible worst case scenario, since previous studies already
58
59
384 indicated that soil microbial populations were not affected by Tc in lower concentrations, as
60
61
62
63
64
65

385 reported by Hund-Rinke et al. (2004) where no significant effect on the bacterial composition and
1
386 Tc resistance genes were found in soil polluted with 5 and 50 mg Tc kg⁻¹, whereas Tc significantly
3
4
387 affected the microbial community composition at the spiked concentration of 500 mg kg⁻¹.
5
6

388 In the present microcosm study, we showed that the effect of cow M on the bacterial
8
9
389 community composition increased with repeated application (Table 2) while the effects of the Tc
10
11
390 were far less pronounced. After a single M or M+Tc application, significant differences to DGGE
13
14
391 fingerprints of untreated soil were only observed for CL soil. In contrast to CL soil, DGGE
15
16
392 fingerprints did not reveal such effects 60 days after the first application of M or of M+Tc to SA
18
19
393 soil. We assume that transient effects of M or M+Tc might have occurred also in SA soil but had
20
21
394 disappeared already at the time of sampling 60 days after the first amendment, as previously was
23
24
395 also observed by Selvam et al. (2012). In accordance with other studies (Ding et al. 2014;
25
26
396 Marschner et al. 2003; Sun et al. 2004) we observed that repeated application of M, with or without
28
29
397 Tc, enhanced the effects on the bacterial community composition in both soils as indicated by
30
31
398 increasing differences (d-values) between the DGGE fingerprints of untreated and M-treated soils
32
33
399 (Table 2). Chessa et al. (2016), already reported that a single cow M application changed the
35
36
400 bacterial community composition to increased PLFA ratio of Gram-positive to Gram-negative
37
38
401 bacteria in both soils, and this effect was still observed in CL soil after 60 days but not in SA soil.
40
41
402 The cow M used for the present study, the same as already used by Chessa et al. (2016), was long-
42
43
403 term stored and air-dried, and this, together with divergent soil properties, might explain differences
45
46
404 compared to the findings of other studies performed with piggery M and silt loam soil (Ding et al.
47
48
405 2014; Heuer et al. 2008). In comparison to SDZ used in these studies, the effects of Tc spiked to the
49
50
406 cow M were far less pronounced which was likely caused by the stronger sorption of Tc compared
52
53
407 to SDZ and due to different properties of soils studied: clayey and sandy soil compared to silt loam.
54
55
408 Tc100 spiked to the cow M did not cause significant shifts of the soil bacterial fingerprints,
57
58
409 compared to soil treated with unspiked M. After three amendments with M+Tc500, the fingerprints
59
60
410 significantly differed from those of the M treatments for both soils. In contrast, the presence of SDZ

411 in piggery M spiked at two concentrations (10 and 100 mg kg⁻¹) caused significant changes of the
1
412 bacterial community composition in the two soils compared to unspiked M (Ding et al. 2014; Heuer
3
413 et al. 2011a).
4
5
6

414 Although the cow M was collected from free ranged cows, which to the best of our
8
415 knowledge were not treated with antibiotics, all the ARGs and MGEs analyzed in the present study,
9
10
11
416 except *tet(M)*, were detected in the M. Thus with the cow M not only nutrients and spiked Tc were
12
13
14
417 introduced into the soils but also bacteria containing ARGs and MGEs. The abundances of ARGs
15
16
418 and MGEs were below the detection limit or at very low abundance in the CL soil, which had no
17
18
19
419 history of anthropogenic antibiotic pollution by human or animal wastes. Thus a striking increase in
20
21
420 the relative abundance of ARGs and MGEs was observed for CL soil in response to the M
22
23
421 amendment. In contrast, all ARGs except *tet(M)* and MGEs analyzed in the present study were
24
25
422 detected in the SA soil which was in the past frequently affected by flooding of a river and by
26
27
28
423 orchard farming practice. River water was previously reported as a carrier of ARGs (Amos et al.
29
30
31
424 2015; 2014a; 2014b). Thus, the differences found between the CL and SA soil likely do not only
32
33
425 result from differences in soil properties such as clay content and pH but also from their previous
34
35
426 history of anthropogenic pollutants. This pollution might have resulted in an adaptation of the
36
37
38
427 bacterial community through proliferation of resistant bacteria and horizontal gene transfer (HGT)
39
40
428 (Heuer and Smalla 2012).
41
42

429 The ARGs and MGEs analyzed in the present study were previously reported to occur in
43
44
45
430 piggery and cow M (Alexander et al. 2011; Binh et al. 2008). Recently, Kyselková et al. (2015a)
46
47
48
431 proposed that *tet(Q)* and *tet(W)* genes belong to the resistome stably associated with cow M. Here,
49
50
432 we also found that cow M from free ranged animals contained bacteria carrying the ARGs *tet(Q)*,
51
52
433 *tet(W)*, *tet(A)*, *sul1* and *sul2*. In contrast to our hypothesis, the M+Tc100 and M+Tc500 treated
53
54
55
434 soils did not show significantly increased abundance of *tet(Q)* and *tet(W)*, compared to the M-
56
57
435 treatments. However, with repeated M-applications an accumulation of *tet(Q)* and *tet(W)* could be
58
59
436 observed irrespective of the presence of Tc. Kyselková et al. (2013) also found that Tc did not show
60
61
62
63
64
65

437 additive effects on the abundance of *tet(Q)* and *tet(W)* genes, compared to unspiked M. Likely these
1
438 genes were hosted in bacteria that did not proliferate in soil, and thus neither M nor selective
2
439 pressure exerted by Tc increased their abundance. In contrast, *tet(A)* likely carried by other
3
440 bacterial hosts was clearly increased in the M+Tc500 treatments in both soils. In the control soils,
4
441 the relative abundance of *tet(Q)* and *tet(W)* tended to decrease over the time of our experiment.
5
442 Probably, the populations carrying these genes decreased in relative abundance due to the lack of
6
443 nutrient input in the untreated control soils. In cow M and in both soils the abundances of *tet(Q)*
7
444 genes were, in tendency, lower than those of *tet(W)* (Wolters et al. 2016).
8
9
10
11
12
13
14
15
16
17
18

19
20 Since *tet* genes were often reported to co-occur with *sul* genes on plasmids (Heuer et al.
21
22 2012; 2009; Roberts 2011), the relative abundances of *sul1* and *sul2* genes were also determined in
23
24 the present study. Our results showed that also the *sul1* and *sul2* genes increased in abundance with
25
26 repeated M application and that Tc500 clearly co-selected for *sul1* and *sul2* genes in CL soils.
27
28 Furthermore, our results showed that for the M+Tc500 treatments a synergistic effect of M and
29
30 Tc500 occurred, especially in CL soil, and Tc500 spiking caused an accumulation of *sul* genes as
31
32 well as of *tet(W)* genes. However, M+Tc500 did not cause a pronounced increase in the relative
33
34 abundance of *sul1* genes compared to the M-treatment in SA soil. In contrast, the relative
35
36 abundance of *sul2* genes, which are typically carried on plasmids reported from piggery M, e.g. on
37
38 LowG+C (Heuer et al. 2009) or IncQ (Smalla et al. 2000), was increased due to the repeated M
39
40 amendments in SA soil. An accumulation of *sul1* and *sul2* genes was also reported for repeated soil
41
42 applications of piggery M spiked with SDZ by Heuer et al. (2011b). In the present study, Tc co-
43
44 selected for *sul* genes likely due to the co-localization on the same MGE. An example for such a co-
45
46 localization of *tet(A)* and *sul1* was reported for IncP-1 ϵ plasmids that were recently captured by
47
48 exogenous isolation from M-treated arable soils, from M or digestates (Bahl et al. 2007; Binh et al.
49
50 2008; Heuer et al. 2012; Wolters et al. 2015), and sequencing revealed that these IncP-1 ϵ plasmids
51
52 often carried *tet(A)* upstream and *sul1* downstream of the class 1 integron.
53
54
55
56
57
58
59
60
61
62
63
64
65

462 The presence of Tc in M selected for IncP-1ε plasmids in SA soil while no such increase
1
2
463 was observed in CL soils. Probably bacterial populations carrying IncP-1ε plasmids applied with M
3
4
464 were not well adapted to conditions present in the CL soil and thus rapidly decreased in relative
5
6
465 abundance. Several previous studies showed that repeated amendments of M enhanced the HGT of
7
8
466 MGE within microbial communities (Ghosh and LaPara 2007; Heuer et al. 2011a; Jindal et al.
9
10
467 2006; Popowska et al. 2012; You et al. 2012), whereas the effect of a single M application on
11
12
468 tetracycline resistance levels in soil bacterial communities may be only transient (Chessa et al.
13
14
469 2016; Sengeløv et al. 2003).

15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 1.3, Line of

487 Activity 1.3.1.). Sven Jechalke was funded by the Federal Environment Agency
1
488 (Umweltbundesamt) (FKZ 3713 63 402). The authors would like to thank Dr. Martina Kyselková to
2
3
4
489 provide us positive controls for qPCR reactions.
5
6

490

491 **References**

- 492 Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, McAllister TA (2011)
11
12
13
14
493 Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed
15
16
494 different subtherapeutic antibiotics. *BMC Microbiol* 11:19. doi:10.1186/1471-2180-11-19
17
18
495 Amos GCA, Gozzard E, Carter CE, Mead A, Bowes MJ, Hawkey PM, Zhang L, Singer AC, Gaze
19
20
21
496 WH, Wellington EMH (2015) Validated predictive modelling of the environmental resistome.
22
23
497 *ISME J* 9:1467-1476. doi:10.1038/ismej.2014.237
24
25
498 Amos GCA, Hawkey PM, Gaze WH, Wellington EM (2014a) Waste water effluent contributes to
26
27
28
499 the dissemination of CTX-M-15 in the natural environment. *J Antimicrob Chemoth* 69:1785-1791.
29
30
500 doi:10.1093/jac/dku079
31
32
33
501 Amos GCA, Zhang L, Hawkey PM, Gaze WH, Wellington EM (2014b) Functional metagenomic
34
35
502 analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. *Vet Microbiol*
36
37
503 171:441-447. doi:10.1016/j.vetmic.2014.02.017
38
39
40
504 Bahl M, Hansen L, Goesmann A, Sørensen S (2007) The multiple antibiotic resistance IncP-1
41
42
43
505 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the
44
45
506 previously established α , β and δ sub-groups. *Plasmid* 58:31-43. doi:10.1016/j.plasmid.2006.11.007
46
47
48
507 Barraud O, Baclet MC, Denis F, Ploy MC (2010) Quantitative multiplex real-time PCR for
49
50
508 detecting class 1, 2 and 3 integrons. *J Antimicrob Chemoth* 65:1642-1645. doi:10.1093/jac/dkq167
51
52
53
509 Binh CT, Heuer H, Kaupenjohann M, Smalla K (2008) Piggery manure used for soil fertilization is
54
55
56
57
58
59
60
61
62
63
64
65

512 Chessa L, Pusino A, Garau G, Mangia NP, Pinna MV (2016) Soil microbial response to tetracycline
1
513 in two different soils amended with cow manure. *Environ Sci Pollut R* 23:5807-5817.
2
3
4
514 doi:10.1007/s11356-015-5789-4
5
6
515 Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular
7
8
9
516 biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232-260.
10
11
517 doi:10.1128/MMBR.65.2.232-260.2001
12
13
518 Ding GC, Heuer H, Smalla K (2012) Dynamics of bacterial communities in two unpolluted soils
14
15
16
519 after spiking with phenanthrene: soil type specific and common responders. *Front Microbiol* 3:290.
17
18
520 doi:10.3389/fmicb.2012.00290
19
20
521 Ding GC, Radl V, Schloter-Hai B, Jechalke S, Heuer H, Smalla K, Schloter M (2014) Dynamics of
21
22
23
522 soil bacterial communities in response to repeated application of manure containing sulfadiazine.
24
25
523 PLoS One 9:e92958. doi:10.1371/journal.pone.0092958
26
27
28
524 Durso LM, Cook KL (2014) Impacts of antibiotic use in agriculture: what are the benefits and
29
30
31
525 risks? *Curr Opin Microbiol* 19:37-44. doi:10.1016/j.mib.2014.05.019
32
33
526 EC (1831/2003) Regulation (EC) No 1831/2003 of the European Parliament and of the Council of
34
35
36
527 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union*
37
38
528 Food and Drug (2013) Antimicrobials sold or distributed for use in food-producing animals.
39
40
529 Administration Department of Health and Human Services,
41
42
43
530 Fulthorpe RR, McGowan C, Maltseva OV, Holben WE, Tiedje JM (1995) 2,4-
44
45
46
531 Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. *Appl Environ*
47
48
532 *Microb* 61:3274-3281.
49
50
533 Ghosh S, LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the
51
52
53
534 proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1:191-203.
54
55
535 doi:10.1038/ismej.2007.31
56
57
58
59
60
61
62
63
64
65

536 Gomes NCM, Heuer H, Schönfeld J, Costa R, Mendonça-Hagler L, Smalla K (2001) Bacterial
1
537 diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature
2
3
4
538 gradient gel electrophoresis. *Plant Soil* 232:167-180. doi:10.1023/A:1010350406708
5
6
539 Gu C, Karthikeyan KG, Sibley SD, Pedersen JA (2007) Complexation of the antibiotic tetracycline
7
8
9
540 with humic acid. *Chemosphere* 66:1494-1501. doi:10.1016/j.chemosphere.2006.08.028
10
11
541 Hammer TJ, Fierer N, Hardwick B, Simojoki A, Slade E, Taponen J, Viljanen H, Roslin T (2016)
12
13
14
542 Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung
15
16
543 beetles. *Proc Biol Sci* 283. doi: 10.1098/rspb.2016.0150
17
18
544 Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S (2008) Impact of the antibiotic
19
20
21
545 sulfadiazine and pig manure on the microbial community structure in agricultural soils. *Soil Biol*
22
23
546 *Biochem* 40:1583-1591. doi:10.1016/j.soilbio.2008.01.010
24
25
547 Hamscher G, Pawelzick HT, Höper H, Nau H (2005) Different behavior of tetracyclines and
26
27
28
548 sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem*
29
30
549 24:861-868. doi:10.1897/04-182R.1
31
32
33
550 Hamscher G, Sczesny S, Höper H, Nau H (2002) Determination of persistent tetracycline residues
34
35
36
551 in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray
37
38
552 ionization tandem mass spectrometry. *Anal Chem* 74:1509-1518. doi:10.1021/ac015588m
39
40
553 Hassan R, Ryu K-S (2012) Naturally Derived Probiotic Supplementation Effects on Physiological
41
42
43
554 Properties and Manure Gas Emission of Broiler Chickens. *J Agric Life Sci* 46:119-127.
44
45
555 doi:10.3382/ps.2013-03314
46
47
556 Heuer H, Binh CT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T,
48
49
50
557 Gonzalez B, Top E, Smalla K (2012) IncP-1epsilon Plasmids are Important Vectors of Antibiotic
51
52
53
558 Resistance Genes in Agricultural Systems: Diversification Driven by Class 1 Integron Gene
53
54
55
559 Cassettes. *Front Microbiol* 3:2. doi:10.3389/fmicb.2012.00002
56
57
58
59
60
61
62
63
64
65

560 Heuer H, Focks A, Lamshöft M, Smalla K, Matthies M, Spiteller M (2008) Fate of sulfadiazine
1
561 administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in
2
3
4
562 manure and manured soil. *Soil Biol Biochem* 40:1892-1900. doi:10.1016/j.soilbio.2008.03.014
5
6
563 Heuer H, Kopmann C, Binh CT, Top EM, Smalla K (2009) Spreading antibiotic resistance through
7
8
9
564 spread manure: characteristics of a novel plasmid type with low %G+C content. *Environ Microbiol*
10
11
565 11:937-949. doi:10.1111/j.1462-2920.2008.01819.x
12
13
566 Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete
14
15
16
567 communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic
17
18
19
568 separation in denaturing gradients. *Appl Environ Microb* 63:3233-3241.
20
21
569 Heuer H, Schmitt H, Smalla K (2011a) Antibiotic resistance gene spread due to manure application
22
23
24
570 on agricultural fields. *Curr Opin Microbiol* 14:236-243. doi:10.1016/j.mib.2011.04.009
25
26
571 Heuer H, Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic
27
28
29
572 resistance in soil over at least two months. *Environ Microbiol* 9:657-666. doi:10.1111/j.1462-
30
31
573 2920.2006.01185.x
32
33
574 Heuer H, Smalla K (2012) Plasmids foster diversification and adaptation of bacterial populations in
34
35
36
575 soil. *FEMS Microbiol Rev* 36:1083-1104. doi:10.1111/j.1574-6976.2012.00337.x
37
38
576 Heuer H, Solehati Q, Zimmerling U, Kleineidam K, Schloter M, Müller T, Focks A, Thiele-Bruhn
39
40
41
577 S, Smalla K (2011b) Accumulation of sulfonamide resistance genes in arable soils due to repeated
42
43
44
578 application of manure containing sulfadiazine. *Appl Environ Microb* 77:2527-2530.
45
46
579 doi:10.1128/AEM.02577-10
47
48
580 Heuer H, Weiland G, Schönfeld J, Schönwälder A, Gomes N, Smalla K (2001) Bacterial
49
50
581 Community Profiling Using DGGE or TGGE Analysis In: Rochelle PA (Ed) *Environmental*
51
52
53
582 *Molecular Microbiology: Protocols and Applications*. Horizon Scientific Press, Wymondham, UK,
53
54
55
583 pp 177-190.
56
57
58
59
60
61
62
63
64
65

584 Ho A, El-Hawwary A, Kim SY, Meima-Franke M, Bodelier P (2015) Manure-associated
1 stimulation of soil-borne methanogenic activity in agricultural soils. *Biol Fert Soils* 51:511-516.
585 doi:10.1007/s00374-015-0995-2
2
3
4
586
5
6
587 Hund-Rinke R, Simon M, Lukow T (2004) Effects of Tetracycline on the Soil Microflora: Function,
8
9
588 Diversity, Resistance. *J Soil Sediment* 4:11-16.
10
11
589 Jechalke S, Focks A, Rosendahl I, Groeneweg J, Siemens J, Heuer H, Smalla K (2014a) Structural
13
14
590 and functional response of the soil bacterial community to application of manure from difloxacin-
15
16
591 treated pigs. *FEMS Microbiol Ecol* 87:78-88. doi:10.1111/1574-6941.12191
18
19
592 Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K (2014b) Fate and effects of veterinary
20
21
593 antibiotics in soil. *Trends Microbiol* 22:536-545. doi:10.1016/j.tim.2014.05.005
22
23
594 Jia DA, Zhou DM, Wang YJ, Zhu HW, Chen JL (2008) Adsorption and cosorption of Cu(II) and
25
26
595 tetracycline on two soils with different characteristics. *Geoderma* 146:224-230.
27
28
596 doi:10.1016/j.geoderma.2008.05.023
30
31
597 Jindal A, Kocherginskaya S, Mehboob A, Robert M, Mackie RI, Raskin L, Zilles JL (2006)
32
33
598 Antimicrobial use and resistance in swine waste treatment systems. *Appl Environ Microb* 72:7813-
35
36
599 7820. doi:10.1128/AEM.01087-06
37
38
600 Jutta R, Pils V, Laird DA (2007) Sorption of Tetracycline and Chortetracycline on K- and Ca-
40
41
601 saturated soil clays, humic Substances, and clay-humic complexes. *Environ Sci Technol* 41:1928-
42
43
602 1933. doi:10.1021/es062316y
44
45
603 Kim KR, Owens G, Kwon SI, So KH, Lee DB, Ok YS (2011) Occurrence and Environmental Fate
47
48
604 of Veterinary Antibiotics in the Terrestrial Environment. *Water Air Soil Poll* 214:163-174.
49
50
605 doi:10.1007/s11270-010-0412-2
52
53
606 Kong WD, Li CG, Dolhi JM, Li SY, He JZ, Qiao M (2012) Characteristics of oxytetracycline
54
55
607 sorption and potential bioavailability in soils with various physical-chemical properties.
57
58
608 *Chemosphere* 87:542-548. doi:10.1016/j.chemosphere.2011.12.062
59
60
61
62
63
64
65

609 Kreuzig R, Höltge S (2005) Investigations on the fate of sulfadiazine in manured soil: Laboratory
1
610 experiments and test plot studies. *Environ Toxicol Chem* 24:771-776. doi:10.1897/03-582R.1
3
4
611 Kropf S, Heuer H, Grüning M, Smalla K (2004) Significance test for comparing complex microbial
6
612 community fingerprints using pairwise similarity measures. *J Microbiol Methods* 57:187-195.
8
9
613 doi:10.1016/j.mimet.2004.01.002
10
11
614 Kümmerer K (2003) Significance of antibiotics in the environment. *J Antimicrob Chemother* 52:5-
13
14
615 7. doi:10.1093/jac/dkg293
15
16
616 Kyselková M, Jirout J, Chroňáková A, Vrchotová N, Bradley R, Schmitt H, Elhottová D (2013)
18
617 Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their
20
21
618 oxytetracycline content. *Chemosphere* 93:2413-2418. doi:10.1016/j.chemosphere.2013.08.058
23
24
619 Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015a) Spread of tetracycline
25
26
620 resistance genes at a conventional dairy farm. *Front Microbiol* 6. doi: 10.3389/fmicb.2015.00536
27
28
621 Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015b) Spread of tetracycline
30
31
622 resistance genes at a conventional dairy farm. *Front Microbiol* 6:536.
32
33
623 doi:10.3389/fmicb.2015.00536
35
36
624 Lanz R, Kuhnert P, Boerlin P (2003) Antimicrobial resistance and resistance gene determinants in
37
38
625 clinical *Escherichia coli* from different animal species in Switzerland. *Vet Microbiol* 91:73-84.
40
41
626 Li Z, Chang PH, Jean JS, Jiang WT, Wang CJ (2010) Interaction between tetracycline and smectite
42
43
627 in aqueous solution. *J Colloid Interface Sci* 341:311-319. doi:10.1016/j.jcis.2009.09.054
44
45
628 Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial
47
48
629 community in a long-term fertilizer experiment. *Soil Biol Biochem* 35:453-461.
49
50
630 doi:10.1016/S0038-0717(02)00297-3
52
53
631 Montforts MH, Kalf DF, van Vlaardingen PL, Linders JB (1999) The exposure assessment for
54
55
632 veterinary medicinal products. *Sci Total Environ* 225:119-133. doi:10.1016/S0048-9697(98)00338-6
57
58
633 Nelson ML, Levy SB (2011) The history of the tetracyclines. *Ann N Y Acad Sci* 1241:17-32.
59
60
634 doi:10.1111/j.1749-6632.2011.06354.x
61
62
63
64
65

635 Ng LK, Martin I, Alfa M, Mulvey M (2001) Multiplex PCR for the detection of tetracycline
1
636 resistant genes. *Mol Cell Probes* 15:209-215. doi:10.1006/mcpr.2001.0363
3
4
637 Nguyen F, Starosta AL, Arenz S, Sohmen D, Donhofer A, Wilson DN (2014) Tetracycline
5
6
638 antibiotics and resistance mechanisms. *Biol Chem* 395:559-575. doi:10.1515/hsz-2013-0292
8
9
639 O'Connor S, Aga DS (2007) Analysis of tetracycline antibiotics in soil: Advances in extraction,
10
11
640 clean-up, and quantification. *Trac-Trend Anal Chem* 26:456-465. doi:10.1016/j.trac.2007.02.007
13
14
641 Popowska M, Rzeczycka M, Miernik A, Krawczyk-Balska A, Walsh F, Duffy B (2012) Influence
15
16
642 of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated
18
19
643 resistance genes. *Antimicrob Agents Chemother* 56:1434-1443. doi:10.1128/AAC.05766-11
20
21
644 Qiao M, Chen W, Su J, Zhang B, Zhang C (2012) Fate of tetracyclines in swine manure of three
22
23
645 selected swine farms in China. *J Environ Sci* 24:1047-1052. doi:10.1016/S1001-0742(11)60890-5
25
26
646 Roberts MC (2011) Mechanisms of bacterial antibiotic resistance and lessons learned from
27
28
647 environmental tetracycline-resistant bacteria, First Edition edn. John Wiley & Sons, Inc., Hoboken,
30
31
648 NJ, USA
32
33
649 Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. vol vol. 1.
35
36
650 Cold Spring Harbor laboratory Press.
37
38
651 Sarmah AK, Meyer MT, Boxall AB (2006) A global perspective on the use, sales, exposure
40
41
652 pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment.
42
43
653 *Chemosphere* 65:725-759. doi:10.1016/j.chemosphere.2006.03.026
44
45
654 Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006) Tetracyclines and tetracycline
47
48
655 resistance in agricultural soils: microcosm and field studies. *Microb Ecol* 51:267-276.
49
50
656 doi:10.1007/s00248-006-9035-y
52
53
657 Schreiter S, Ding GC, Grosch R, Kropf S, Antweiler K, Smalla K Soil type-dependent effects of a
54
55
658 potential biocontrol inoculant on indigenous bacterial communities in the rhizosphere of field-
57
58
659 grown lettuce. *FEMS Microbiology Ecology* 90:718-730. doi:10.1111/1574-6941.12430
59
60
61
62
63
64
65

660 Selvam A, Xu D, Zhao Z, Wong JW (2012) Fate of tetracycline, sulfonamide and fluoroquinolone
1
661 resistance genes and the changes in bacterial diversity during composting of swine manure.
2
3
4
662 *Bioresour Technol* 126:383-390. doi:10.1016/j.biortech.2012.03.045
5
6
663 Sengeløv G, Agersø Y, Halling-Sørensen B, Baloda SB, Andersen JS, Jensen LB (2003) Bacterial
7
8
9
664 antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry.
10
11
665 *Environ Int* 28:587-595.
12
13
666 Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000) Exogenous
14
15
667 isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence
16
17
18
668 and diversity of IncQ-like plasmids. *Appl Environ Microb* 66:4854-4862.
19
20
21
669 Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001)
22
23
670 Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis:
24
25
671 plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microb* 67:4742-4751.
26
27
28
672 doi:10.1128/aem.67.10.4742-4751.2001
29
30
31
673 Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004)
32
33
674 Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl Environ*
34
35
675 *Microb* 70:7372-7377. doi:10.1128/AEM.70.12.7372-7377.2004
36
37
38
676 Sun HY, Deng SP, Raun WR (2004) Bacterial community structure and diversity in a century-old
39
40
677 manure-treated agroecosystem. *Appl Environ Microb* 70:5868-5874.
41
42
43
678 doi:10.1128/AEM.70.10.5868-5874.2004
44
45
679 Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in
46
47
48
680 mixed microbial populations via 5'-nuclease assays. *Appl Environ Microb* 66:4605-4614. doi:
49
50
681 Thiele-Bruhn S (2003) Pharmaceutical antibiotic compounds in soils - A review. *J Plant Nutr Soil*
51
52
682 *Sci* 166:145-167.
53
54
55
683 Thiele-Bruhn S, Beck IC (2005) Effects of sulfonamide and tetracycline antibiotics on soil
56
57
684 microbial activity and microbial biomass. *Chemosphere* 59:457-465.
58
59
60
685 doi:10.1016/j.chemosphere.2005.01.023
61
62
63
64
65

686 Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J (2014) Bloom of resident antibiotic-
1
687 resistant bacteria in soil following manure fertilization. P Natl Acad Sci USA 111:15202-15207.
2
3
4
688 doi:10.1073/pnas.1409836111
5
6
689 Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A,
7
8
9
690 Laxminarayan R (2015) Global trends in antimicrobial use in food animals. P Natl Acad Sci USA
10
11
691 112:5649-5654. doi:10.1073/pnas.1503141112
12
13
692 Weinert N, Meincke R, Gottwald C, Heuer H, Gomes NC, Schloter M, Berg G, Smalla K (2009)
14
15
693 Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their
16
17
18
694 parent cultivar differ less than those of different potato cultivars. Appl Environ Microb 75:3859-
19
20
21
695 3865. doi:10.1128/AEM.00414-09
22
23
696 Winckler C, Grafe A (2001) Use of veterinary drugs in intensive animal production. J Soil
24
25
26
697 Sediment 1:66-70.
27
28
698 Wolters B, Ding GC, Kreuzig R, Smalla K (2016) Full-scale mesophilic biogas plants using manure
29
30
31
699 as C-source: Bacterial community shifts along the process cause changes in the abundance of
32
33
34
700 resistance genes and mobile genetic elements. FEMS Microbiol Ecol 92:1-17.
35
36
701 doi:10.1093/femsec/fiv163
37
38
702 Wolters B, Kyselková M, Krögerrecklenfort E, Kreuzig R, Smalla K (2015) Transferable antibiotic
39
40
41
703 resistance plasmids from biogas plant digestates often belong to the IncP-1ε subgroup. Front
42
43
704 Microbiol 6. doi: 10.3389/fmicb.2014.00765
44
45
705 Wu K, Yuan S, Wang L, Shi J, Zhao J, Shen B, Shen Q (2014) Effects of bio-organic fertilizer plus
46
47
48
706 soil amendment on the control of tobacco bacterial wilt and composition of soil bacterial
49
50
51
707 communities. Biol Fert Soils 50:961-971. doi:10.1007/s00374-014-0916-9
52
53
708 You Y, Hilpert M, Ward MJ (2012) Detection of a common and persistent tet(L)-carrying plasmid
54
55
56
709 in chicken-waste-impacted farm soil. Appl Environ Microb 78:3203-3213.
57
58
710 doi:10.1128/AEM.07763-11
59
60
61
62
63
64
65

711 Zhang Y, Boyd SA, Teppen BJ, Tiedje JM, Li H (2014) Role of tetracycline speciation in the
1
712 bioavailability to escherichia coli for uptake and expression of antibiotic resistance. Environ Sci
2
713 Technol 48:4893-4900. doi:10.1021/es5003428
3
4
5
6
714 Zhao S, Liu D, Ling N, Chen F, Fang W, Shen Q (2014) Bio-organic fertilizer application
7
8
9
715 significantly reduces the Fusarium oxysporum population and alters the composition of fungi
10
11
716 communities of watermelon Fusarium wilt rhizosphere soil. Biol Fert Soils 50:765-774.
12
13
14
717 doi:10.1007/s00374-014-0898-7
15
16
718 Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM (2013)
17
18
719 Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci U S A
19
20
720 110:3435-3440. doi:10.1073/pnas.1222743110
21
22
23

24
25

26 27 **Figure captions**

28
29
30

31
32 **Table 1** Sequences of primers and TaqMan probes used for the detection of ARGs and MGEs.
33

34
35

36
37 **Table 2** Percent difference (d-values) of soil bacterial community composition based on Pearson
38
39 correlations of background-subtracted densitometric curves from DGGE analysis (60 days after the
40
41 first, second and third amendments). Significance is indicated by the respective *P*-value.
42

43
44 S: soil CL or SA; M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil
45
46 dry weight.
47

48
49
50

51
52 **Figure 1** UPGMA cluster analysis of DGGE fingerprints for 16S rRNA gene amplicons from CL
53
54 and SA soils 60 days after the first amendment: amplicons from (a) and (d), respectively; 60 days
55
56 after the second amendment: (b) and (e), respectively; 60 days after the third amendment: (c) and
57
58 (f), respectively. Horizontal axis indicates the % of similarity. UPGMA cluster analysis was based
59
60 on Pearson correlation indices to compare the effect of treatments in each soil at a specific time
61

62
63
64
65

737 point. S: soil CL or SA; M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹
1
738 soil dry weight.

739
4
740 **Figure 2** Relative abundances of *tet(Q)* and *tet(W)* genes in CL (a, c) and SA (b, d) soils,
8
741 respectively, were measured by qPCR 60 days after the first, second and third amendments. Tukey-
10
742 Kramer post-hoc test ($P<0.05$) was used to compare the relative abundances of the target genes in
13
743 total community-DNA at the same time point or within the treatment after repeated amendments.
14
744 M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight; *:
16
745 below the detection limit. For each time-point, average values which share the same white capital
20
746 letter within columns do not differ significantly at the 5% level. For each treatment, average values
21
747 which share the same letters above columns do not significantly differ at the 5% level. Error bars
25
748 indicate the standard deviation of four replicates.

26
749
30
31
750 **Figure 3** PCR Southern blot hybridization of the *tet(A)* gene in CL and SA soils 60 days after the
32
33
751 first and third amendments. Four replicates for each treatment are shown. The lanes at the utmost
35
752 right and left are size markers. The figure is composed of six hybridized membranes (three
37
753 membranes per soil) which were reassembled by photoshop.

40
754
42
43
755 **Figure 4** Relative abundances of *sul1* and *sul2* genes in CL (a, c) and SA (b, d) soils, respectively,
45
756 measured by qPCR 60 days after the first, second and third amendments. The Tukey-Kramer post-
47
757 hoc test ($P<0.05$) was used to compare the relative abundances of the target genes in the total
48
758 community-DNA at the same time point or within the treatment after repeated amendments. M:
50
759 manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight; *: under
52
760 the detection limit. For each time-point, average values which share the same white capital letter
54
761 within columns do not differ significantly at the 5% level. For each treatment, average values which
55
56
57
58
59
60
61
62
63
64
65

762 share the same letters above columns do not differ significantly at the 5% level. Error bars indicate
1
763 the standard deviation of four replicates.
2
3
4
764
5
6
765 **Figure 5** Relative abundances of *intI1* and *trfA* genes in CL (a, c) and SA (b, d) soils, respectively,
7
8
9
766 measured by qPCR 60 days after the first, second and third amendments. The Tukey-Kramer post-
10
11
767 hoc test ($P<0.05$) was used to compare the relative abundances of the target genes in the total
12
13
768 community-DNA at the same time point or within the treatment after repeated amendments. M:
14
15
769 manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight. For each
16
17
770 time-point, average values which share the same white capital letter within columns do not differ
18
19
20
771 significantly at the 5% level. For each treatment, average values which share the same letters above
21
22
23
772 columns do not differ significantly at the 5% level. Error bars indicate the standard deviation of four
24
25
773 replicates.
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

[Click here to view linked References](#)

1 **Journal section for manuscript publication:**

1
2
3

4 **The presence of tetracycline in cow manure changes the impact of repeated manure**

5
6
7

8 **application on soil bacterial communities**

9
10

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

12
13

14 **Author list**

15
16

17 Luigi Chessa,^{1,2 *} Sven Jechalke,^{2#} Guo-Chun Ding,^{2,3} Alba Pusino,¹ Nicoletta Pasqualina Mangia,¹

18
19

20 Kornelia Smalla,²

21
22

23 **Addresses of the institutions**

24
25

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

27
28

29 ² Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for

30
31

32 Epidemiology and Pathogen Diagnostics, Messeweg 11-12, 38104 Braunschweig, Germany

33
34

35 ³ College of Resources and Environmental Sciences, China Agricultural University, 100193,

36
37

38 Yuanmingyuan xilu No 2, Haidian District, Beijing, China

39
40

41 [#] current address: Institute for Phytopathology, Justus Liebig University Giessen, Heinrich-Buff-

42
43

44 Ring 26-32 (IFZ), 35392 Gießen, Germany

45
46

47 * Corresponding author. Mailing address: University of Sassari, Department of Agriculture, Viale

48
49

50 Italia 39, 07100 Sassari, Italy. Phone: +39 079229289. FAX: +39 079229370. E-mail:

51
52

53 luigi.chessa@uniss.it

54
55

56
57

58
59

60
61

62
63
64
65

24 **Abstract**

1
25 The effect of tetracycline (Tc) and cow manure on soil bacterial community composition and
3
4
56 antibiotic resistance gene (ARG) abundance in soil was investigated in the present microcosm
6
77 study. Repeated applications of cow manure spiked with Tc in two concentrations or without Tc on
8
9
107 28 the bacterial communities of a clayey and a sandy soil with different history of anthropogenic
11
129 pollution by sewer flooding were investigated. Soil samples were taken 60 days after each of three
13
14
1530 amendments. DGGE fingerprints of 16S rRNA gene amplicons from total community DNA
16
1731 revealed soil type-dependent changes in the bacterial community composition in response to
18
1932 manure and to Tc, which became more pronounced with repeated applications. Repeated manure
20
21
2233 amendments and Tc, in particular at high concentration, triggered the further increase of ARGs
23
2434 *tet(A)*, *tet(O)*, *tet(Q)*, *tet(W)*, *sul1*, and mobile genetic elements (MGEs) IncP-1ε plasmids and *int11*,
25
26
2735 in a soil type-dependent manner. In the clay soil with no anthropogenic history, the ARGs and
28
2936 MGEs abundances were low or not detectable while manure amendments caused pronounced
30
31
3237 increases in their relative abundance. In the sandy soil with a history of anthropogenic impact,
33
3438 ARGs and MGEs were already present at a higher level and strong increases were mainly observed
35
3639 for the relative abundances of *sul2* and MGEs. Here we show for the first time that effects of
37
38
3940 repeated cow manure applications might be dependent on soil type and foregoing anthropogenic
40
4141 soil pollution, and that the presence of Tc could further increase the abundance of ARGs and
42
43
4442 MGEs.

45
4643 **Keywords**

47
48
4944 Tetracycline, soil, bacterial fingerprints, antibiotic resistance genes, class 1 integron, co-selection.
50

5145
52
5346 **Introduction**

54
55
5647 Antibiotics are widely used in livestock to treat and prevent infectious diseases or to promote
57
5848 animal growth (Durso and Cook 2014; Sarmah et al. 2006). In EU countries since January 2006
59
60
6149 (EC 1831/2003), and in South Korea since July 2011 (Hassan and Ryu 2012), the use of antibiotics

50 as growth promoters in feed for livestock is banned, but they are still a common practice in many
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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).

60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100

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 (*sulI*), 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

76 this reason, high concentrations of Tc can be found in animal feces (Zhu et al. 2013) and their
1
277 extractable concentration decreases in aged manure after composting, resulting from the microbial
3
4
578 degradation or the irreversible sorption of Tc on the soil particles (Kreuzig and Hölting 2005). When
6
779 manure containing antibiotics is applied to agricultural soil as fertilizer, antibiotics are transported
8
9
1080 into the soil as well (Jechalke et al. 2014b), and this can affect the composition and function of the
11
1281 resident microbial communities, as recently shown for sulfadiazine (SDZ) introduced via manure
13
14
1582 into soil (Ding et al. 2014). Although the detection of antibiotics in soil remains experimentally
16
1783 challenging, Hamscher et al. (2002), using high-molar citric acid buffer at low pH, reported Tc
18
1984 concentrations in liquid manure fertilized topsoil ranging from 0.086 to 0.171 mg kg⁻¹, while Qiao
20
21
2285 et al. (2012) measured 0.78 mg kg⁻¹ using McIlvaine-Na₂EDTA buffer and subsequent sonication.
23
2486 Tc is strongly and rapidly sorbed to soil clays and organic matter (Gu et al. 2007; Jutta et al. 2007)
25
26
2787 but free Tc concentrations might be still bioavailable and potentially affect the microbial
28
2988 communities (Thiele-Bruhn and Beck 2005), depending on soil properties (Jia et al. 2008). In
30
31
3289 addition to antibiotic residues, manure typically contains high numbers of bacteria carrying ARGs
33
3490 on MGEs, which can further increase the abundance of antibiotic resistant bacteria in soil when
35
36
3791 manure is applied (Binh et al. 2008; Heuer et al. 2009; Jechalke et al. 2014b; Smalla et al. 2000).
38
3992 Furthermore, antibiotics such as Tc introduced via manure into soil can select the proliferation of
40
4193 resistant soil bacteria and the spread of ARGs via MGEs from manure bacteria to soil bacteria
42
43
4494 (Hammesfahr et al. 2008; Heuer et al. 2008). The application of manure several times a year, which
45
46
4795 is a typical agricultural practice (Montforts et al. 1999), might lead to an accumulation of ARGs and
48
4996 antibiotic compounds, as well as to an accumulation of effects on the soil bacterial community
50
5197 composition, as recently shown for the sulfonamide antibiotic SDZ in soil microcosm experiments
52
53
5498 (Ding et al. 2014; Heuer et al. 2011b). Moreover, manure application to soil can be important not
55
5699 only as nutrient supplement to increase crop yield but also for biocontrol of fungal pathogens by the
57
58
59100 use of manure enriched with microbial antagonists (Zhao et al. 2014), in order to stabilize, by
60
61101 integrated agricultural management, soil microbial communities important for soil health and

102 sustainability (Wu et al. 2014). Manure can also have negative effects such as the stimulation of
1
103 methane production by resident methanogenic soil bacteria, and mitigation strategies to reduce
2
3
4
104 methane emission should be considered (Ho et al. 2015).
5
6

105 Most studies on the effects of organic fertilizers on soil microbial community composition
7
8
9
106 were based on piggery manures. However, in several regions of the world fertilization with cow
10
11
107 manure is more relevant. Recently, Udikovic-Kolic et al. (2014) showed an unexpected bloom of
12
13
108 ARGs in the response to soil fertilization with cow manure free of antibiotics. Little is known so far
14
15
109 on how the presence of antibiotics might change the effects that cow manure applied to soil has on
16
17
109 the bacterial community composition and on the abundance of ARGs and MGEs (Kyselková et al.
18
19
110 2015b).
20
21
22
23

24 In the present study, we investigated how three soil amendments with cow manure, spiked
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

128 CL soil. Tc had a short-term detrimental effect and after two days reduced the microbial activity
1
129 (fluorescein diacetate hydrolysis) and shifted the microbial composition from bacteria to fungi, as
2
130 revealed by phospholipids fatty acids (PLFA) analysis, respectively. Moreover, the Tc effects were
3
4
5
6
131 transient, decreased on the seventh day and had disappeared after 60 days in the SA soil, while in
7
8
9
132 the CL soil, with no history of antibiotic pollution, the utilization of substrates in BIOLOG plates
10
11
123 still remained different among the patterns of the different treatments. Given the recent findings of
13
14
134 Chessa et al. (2016) on Tc and cow manure effects on soil microbial communities after single
15
16
135 amendment, we aimed to test the hypothesis that repeated applications of manure to soil affect the
17
18
136 soil bacterial community composition and increase the abundance of ARGs and MGEs in a soil
19
20
21
227 type-dependent manner and that these effects are more pronounced in the presence of Tc.
23

238

25

26

239

27

28

240

30

31

141

32

33

142

35

36

143

37

38

144

39

40

145

42

43

146

44

45

147

47

48

148

49

50

149

52

53

150

54

55

151

57

152

59

60

153

61

62

63

64

65

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.

154 Four soil treatments were performed with four independent replicates for each treatment. For
1
155 each replicate, 200 g of soil sieved at <2 mm were placed in a glass pot (18×10×6 cm) with the
3
156 following treatments: (i) only water was added to the soil (CL/SA); (ii) 8 g of uncontaminated aged
4
6 M were mixed with soil (CL/SA+M); (iii) and (iv) 8 g of aged M spiked with Tc solution were
157 added to the soil (freshly prepared by dissolving Tc in sterile deionized water and mixed by
8
158 agitation for 1 h in the dark before addition to soil) to reach final theoretical concentrations of 100
10
159 or 500 mg Tc kg⁻¹ soil (CL/SA+M+Tc100 and CL/SA+M+Tc500, respectively). Soil and M were
11
160 carefully mixed in pots and incubated in the dark at a constant temperature of 20°C and at 50% of
12
161 maximum water-holding capacity. Every two days, water was sprayed on the soil surface to
13
162 compensate weight loss by evaporation. Amendments were performed three times at 60-day
14
163 intervals and the soil was mixed after each amendment in order to observe, at microcosm scale, the
15
164 effect of repeated M and Tc addition to soils. After 60 days, a quadruplicate set of soil samples was
16
165 collected from each pot. Soil in the pot was mixed and 10 g of soil were transferred to a sterile
17
166 polypropylene jar for microbiology (Becton Dickinson International, Erembodegem, Belgium). The
18
167 soil in the jar was mixed again and 1 g of soil was transferred into a sterile Eppendorf tube (1.5 mL
19
168 volume) and stored at -20°C until total community DNA extraction (see below). The remaining soil
20
169 in the jar was put back into the pot and the next amendment was performed.
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40

41 42 43 44 Total community DNA extraction

45
46
47 Total community DNA was extracted from 0.5 g of soil using the FastDNA[®] SPIN Kit for Soil (MP-
48
49 Biochemicals, Solon, OH, USA) with some modifications: cell lysis in soil samples by the
50
51 FastPrep[®] Instrument (MP Biomedicals) was performed twice for 30 s at setting 5.5. Also two
52
53 washing steps were performed, by addition of salt/ethanol washing solution (SEWS-M). Finally,
54
55 DNA was eluted in 100 µL of DNA elution solution (DES). Then 50 µL of DNA solution from each
56
57 sample was purified by the GeneClean[®] Spin Kit (Q-Biogene/MP-Biochemicals) and eluted in 50
58
59 µL final volume observing the manufacturers' protocol.
60
61
62
63
64
65

180
1
181
2
3
4
182
5
6
183
7
8
9
184
10
11
185
12
13
186
14
15
187
16
17
188
18
19
20
21
189
22
23
190
24
25
191
26
27
28
192
29
30
193
31
32
33
194
34
35
195
36
37
38
196
39
40
197
41
42
198
43
44
45
199
46
47
200
48
49
50
201
51
52
202
53
54
203
55
56
204
57
58
59
205
60
61
62
63
64
65

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 μ M 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

206 *rrn* copies was performed in accordance with Suzuki et al. (2000). All primers and TaqMan probes
1
207 used are listed in Table 1. Standard dilutions of PCR fragments cloned into pGEM-T vector systems
2
3
4
208 (Promega Corporation, Madison, WI, USA) were used for quantification. The PCR mastermix
5
6
209 contained 1.2 μ M forward primer Bact1369F, 1 μ M reverse primer Prok1492R, 0.5 μ M TaqMan-
7
8
9
210 Probe TM1389F, 0.1 mg mL⁻¹ of bovine serum albumin (BSA) and 1.25 U TrueStart Taq
10
11
211 (Fermentas, St. Leon-Rot, Germany). The PCR protocol was 5 min at 95°C, followed by 40 cycles
12
13
14
212 of 15 s at 95°C, 15 s at 56°C and 1 min at 60°C. A CFX96TM Real-Time PCR Detection System
15
16
213 (Bio-Rad, Hercules, CA, USA) was used. The qPCR for determination of the abundance of class 1
17
18
19
214 integron integrase genes *intI1* was performed using the forward primer int1-LC1, the reverse primer
20
21
22
215 int1-LC5 and TaqMan-Probe int1-probe previously described (Barraud et al. 2010). Total reaction
23
24
216 volume was 50 μ l. Five μ l DNA template of 1:5 diluted purified DNA solution were used and 1 \times
25
26
217 TrueStart Buffer (Fermentas), 2.5 mM MgCl₂ (Fermentas), 0.2 mM dNTPs, 0.08 mg μ L⁻¹ BSA, 0.3
27
28
218 mM of primers and probe and 1.25 U TrueStart Taq (Fermentas); 40 cycles were performed
29
30
31
219 according to the protocol described by Barraud et al. (2010). To normalize for different extraction
32
33
34
220 and amplification efficiencies the relative abundance of target genes was calculated by dividing the
35
36
221 copy number of each gene by the *rrn* copy number.
37

38
39
40
41
222 For *tet(Q)* and *tet(W)*, the same reaction mixture composition was used. The protocol was as
42
43
44
223 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
45
46
47
224 quantify *tet(Q)* and *tet(W)* were described by Smith et al. (2004). The qPCRs for *sull* and *sul2*
48
49
50
225 genes were performed as described by Heuer and Smalla (2007) and Heuer et al. (2008),
51
52
53
226 respectively. The qPCR to measure the abundance of *trfA* genes specific for the epsilon subgroup of
54
55
56
227 IncP-1 plasmids was performed according to Heuer et al. (2012). Concentrations used in the
57
58
59
228 reaction mixture were the same as described for *intI1* qPCR, and amplification and detection were
60
61
62
229 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.
63

64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

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

232 Primers used for the amplification of *tet(A)* and *tet(M)* were described by Lanz et al. (2003) and Ng
1
233 et al. (2001), respectively (Table 1). Digoxigenin-labeled probes were generated from PCR products
2
3
4
234 as described by Jechalke et al. (2014a) using plasmids RP4 and pAT101 as templates for *tet(A)* and
5
6
235 *tet(M)*, respectively. For both genes, 25 μ L reaction mixture composed of 1 \times TrueStart buffer
7
8
236 (Fermentas), 0.2 mM of deoxynucleoside triphosphates, 2.5 mM MgCl₂ (Fermentas), 0.1 mg mL⁻¹
9
10
11
1237 bovine serum albumin, 0.5 μ M of primers, respectively, and 0.6 U TrueStart Taq (Fermentas) were
12
13
14
15
16
1738 used. Amplification and detection were performed according to the following protocol: 5 min at
14
15
16
1739 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
16
17
18
19
20
21
2240 min step at 72°C. PCR products were loaded on an agarose gel, with size markers (DIG-labeled
19
20
21
2241 DNA Molecular Weight Marker VI, Roche Diagnostics Deutschland GmbH, Mannheim, Germany)
21
22
23
2442 in the borders. Southern-blotting to a Hybond-N membrane (GE Healthcare Limited, Amersham,
22
23
24
25
26
2743 UK) and hybridization of PCR products were done as described by Sambrook et al. (1989) and
24
25
26
27
28
2944 following the standard procedure of Roche Diagnostics for filter hybridization under conditions of
26
27
28
29
30
31
3245 middle stringency (Fulthorpe et al. 1995). The exposure times were 20 min for *tet(A)* and 1 h for
29
30
31
32
33
3446 *tet(M)*.
31
32
33
34
35

36
37

38 **Results**

39 **Effects of cow manure and tetracycline effects on soil bacterial communities**

40
4149 The effects of M with or without Tc on the bacterial communities in CL and SA soils were analyzed
41
42
43
44
250 by DGGE fingerprinting of 16S rRNA gene fragments amplified by PCR from total community
42
43
44
45
4651 DNA. Soil type-dependent treatment effects on the bacterial community composition, which
43
44
45
46
47
48
49
252 increased with repeated M applications, were revealed. The effects of treatments analyzed 60 days
45
46
47
48
49
50
253 after each of three amendments (Fig. S1-S3) showed that the bacterial community fingerprints of
46
47
48
49
50
51
254 the control soils (CL or SA) always formed a cluster separate from those of soils treated with M
47
48
49
50
51
52
255 unspiked and Tc-spiked, CL/SA+M and CL/SA+M+Tc, respectively (Fig. 1), except for SA soil 60
48
49
50
51
52
53
256 days after the first amendment. The amendment of soils with cow M, with or without Tc spiking
49
50
51
52
53
54
55
56
57
58
59
257
54
55
56
57
58
59
60
61
62
63
64
65

258 (CL/SA+M+Tc and CL/SA+M), caused a high variability of the fingerprints among replicates of
1
259 each treatment in particular after the second amendment, and a clear effect of Tc became only
2
3
4
260 evident 60 days after the third M amendment. Especially in CL soil the fingerprints of
5
6
261 CL+M+Tc100 and CL+M+Tc500 treated soil samples, 60 days after the third amendment, clustered
7
8
9
262 and were clearly separated from the M-treated soil (CL+M); in SA soil only SA+M+Tc500
10
11
263 treatment clustered separately, indicating a minor effect of SA+M+Tc100 on the soil bacterial
12
13
14
264 communities. In addition, 60 days after the second and third amendments, the fingerprint of the
15
16
265 bacterial community in the M was rather similar to the bacterial fingerprints of SA+M+Tc100 and
17
18
19
266 SA+M+500. This similarity was not observed between M and CL soils, as here the bacterial
20
21
267 community fingerprint of M was, with repeated M applications, increasingly distinct from those of
22
23
268 the M-treated soils.
24
25

269 The permutation test revealed significant differences ($P<0.05$) between the DGGE
26
27
28
290 fingerprints of CL control soil and the M-treated CL soil at all sampling times (Table 2). After the
29
30
31
271 first M amendment the bacterial community composition of CL soil was clearly more affected than
32
33
272 the community of SA soil where the differences (d-values) between control SA soil and M-treated
34
35
36
273 SA soils were small and not significant. However, after the second M amendment the differences
37
38
39
274 between control soil and the M-treated soil became also significant for SA soil. For both soils the
39
40
41
275 effects of M on the bacterial community composition increased with repeated M amendment and
42
43
44
276 the differences observed 60 days after the third M amendment were remarkably high.
45
46
277 Unexpectedly, for both soils the fingerprints of S+M+Tc100 treatments were not significantly
47
48
278 different from those of the M treatments (S+M), even after repeated application of M+Tc100. In
49
50
279 contrast, the fingerprints of the M+Tc500 treated soil were significantly different from those of the
51
52
53
280 M-treated soil at all time points except for the M+Tc500 treated CL soil 60 days after the second
54
55
281 treatment.
56
57

282
58
59

60
61
62
63
64
65

283 **Treatment-dependent changes in the relative abundance of tetracycline and sulfonamide**
1
284 **resistance genes**

285 *tet(Q)*

286 The relative abundance of *tet(Q)* genes, 60 days after the first amendment, was below detection
8
287 limit in the control CL. The addition of M, which contained approximately -5.7 Log *tet(Q)* per *rrn*
10
288 copy numbers, significantly increased the abundance of *tet(Q)* in this soil (Fig. 2a). In contrast, 60
12
289 days after the first amendment, *tet(Q)* was detected in DNA extracted from the SA and in SA+M
14
290 soil treatments and its relative abundance was not significantly ($P<0.05$) different between these
16
291 treatments (Fig. 2b). Also the addition of M+Tc100 or M+Tc500 did not alter the abundance of
18
292 *tet(Q)* in both soils, compared to the soil amended with M alone. As the relative abundance of
20
293 *tet(Q)* in the control SA soil significantly ($P<0.05$) decreased over time, 60 days after the third
22
294 amendment, the abundance of *tet(Q)* was higher in all M treatments, both unspiked and Tc spiked.
24
295 Repeated amendments of M and M+Tc did not further increase the relative abundance of *tet(Q)* in
26
296 both soils, with the only exception of CL+M+Tc100 after the third amendment.
28
30
31
32

297
33
34
35
298 *tet(W)*

299 Similar to *tet(Q)* genes, also *tet(W)* genes were not detected in CL soil. The addition of M
40
300 containing -3.3 Log *tet(W)* *rrn*⁻¹ copy number increased the relative abundance of this gene in
42
301 CL+M, 60 days after the first amendment (Fig. 2c). In SA soil, 60 days after the first amendment,
44
302 *tet(W)* genes were detected also in the control SA and their relative abundance was not significantly
46
303 ($P<0.05$) different to SA+M (Fig. 2d). Moreover, in both soils, the first addition of M+Tc did not
48
304 increase the relative abundance of *tet(W)* genes, compared to M treatments. As observed for *tet(Q)*
50
305 gene in SA soil, also the relative abundance of *tet(W)* progressively decreased over time in SA soil.
52
306 However, the continuous addition of M with or without Tc spike significantly increased the relative
54
307 abundance of *tet(W)* in both soils.
56
57
58
59
60
61

308

309 *tet(A)* and *tet(M)*

1
310 Due to the low abundance of *tet(A)* and *tet(M)* genes it was not possible to measure their relative
3
4
311 abundance by qPCR. Therefore, these genes were amplified by PCR and detected through Southern
5
6
312 blot hybridization 60 days after the first and after the third amendments (Fig. 3). Southern blot
7
8
313 hybridizations revealed that cow M contained *tet(A)* carrying bacteria and, 60 days after the first
9
10
314 amendment, *tet(A)* was also detected in CL+M, CL+M+Tc100 and CL+M+Tc500, while it was not
11
12
315 detected in total community DNA from control CL soil (Fig. 3). At this time point, Southern blot
13
14
316 hybridization indicated that *tet(A)* abundance was low in all treatments of SA soil and was only
15
16
317 detected in some of the replicates. After the third amendment, the abundance of *tet(A)* decreased in
17
18
318 CL+M soils. Thus, *tet(A)* was detected only in three of four replicates of the CL+M+Tc100 and in
19
20
319 all replicates of CL+M+Tc500 whereas it was below the detection limit in CL+M. In SA soil a
21
22
320 remarkably increased abundance of *tet(A)* was detected in the total community DNA from all
23
24
321 replicates of SA+M+Tc500, indicating a selective effect of Tc (Fig. 3). The *tet(M)* was neither
25
26
322 detected in soil, treated soil, nor in M (data not shown).
27
28
29
30
31
32
33

34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

324 *sul1* and *sul2*

325 Cow M contained -2.5 Log per *rrn* copy number of *sul1* and *sul2*. In CL soil the relative
326 abundances of *sul1* and *sul2* genes were, respectively, -4.6 and -6.3 Log per *rrn* copy number and
327 60 days after the first amendment, the relative abundance of *sul1* and *sul2* genes significantly
328 increased after application of M, compared to control CL (Fig. 4a, c). In contrast, 60 days after M
329 addition to SA soil, an increased relative abundance was only observed for *sul2* but not for *sul1*
330 (Fig. 4b, d). In CL+M+Tc100 and CL+M+Tc500 a significantly increased relative abundance of
331 *sul1* was observed, compared to CL+M, while *sul2* was only increased in the CL+M+Tc500
332 treatments. The effect of the presence of Tc was even less pronounced in SA soil. Here, only the
333 relative abundance of *sul1* was significantly increased in SA+M+Tc500, compared to SA+M after

334 the third amendment. Repeated addition of M+Tc caused an accumulation of *sul1* and *sul2* for both
1
335 Tc concentrations, except for the *sul1* in SA+M+Tc100 soils, while repeated additions of M did not.

336

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

338 *intI1*

339 The relative abundance of class 1 integron integrase genes (*intI1*) significantly ($P<0.05$) increased
13
340 in CL+M, compared to control CL, 60 days after the first amendment (Fig. 5a), while the relative
14
341 abundance of *intI1* gene was not significantly increased in SA+M, compared to SA soil (Fig. 5b).

342 The presence of Tc500 caused a significant increase in the relative abundance of *intI1* in CL soil at
19
20
343 all sampling times while in SA soil an increased abundance was only observed 60 days after the
21
22
344 third amendment. In the SA+M+Tc100 treatments no increase in the relative abundance of *intI1* for
23
24
25
345 SA soils was observed at all sampling times while an increase was found after the third amendment
26
27
346 in the CL+M+Tc100 treatment.

348 *IncP-1ε trfA*

349 Quantification of the *trfA* gene was used to detect and quantify the ε subgroup of IncP-1 plasmids in
36
37
38
350 response to the treatments. The abundance of IncP-1ε plasmids in CL soil was low and although the
39
40
351 M added to the soil contained a high abundance of IncP-1ε plasmids, the relative abundance of
41
42
352 these plasmids in CL+M remained low, even after repeated M additions (Fig 5c). Even the presence
43
44
45
353 of Tc did not lead to an increase in the relative abundance of IncP-1ε plasmids. In contrast, in SA
46
47
354 soil M increased the relative abundance of *trfA* in SA+M already 60 days after the first amendment,
48
49
50
355 compared to the SA soil (Fig. 5d). The presence of Tc increased the relative abundance of *trfA*,
51
52
53
356 which was significant for SA+M+Tc500 already after the first amendment while a significantly
54
55
357 increased abundance was observed for SA+M+Tc100 treatment, compared to SA+M, only after the
56
57
358 second amendment. After the third amendment the relative abundance of *trfA* in SA+M+Tc100 and

359 SA+M+Tc500 was even higher than in the M. An accumulation of the *trfA* gene with repeated
1
360 application was only observed in SA+M+Tc500.
3
4

361

362 Discussion

363 The fate of antibiotics in soil and likely also their effects, are influenced by the physicochemical
10
364 properties of the antibiotic, by soil properties (Kong et al. 2012) and soil history, intended as
11
13
14
365 foregoing antibiotic soil pollution due to anthropogenic activities. The present study is part of a
15
16
366 project aiming to better understand the effects of Tc and cow M on the microbial community
18
19
367 composition and on the antibiotic resistance in soil. In this study, two soils with different history of
20
21
22
368 anthropogenic pollution and physicochemical characteristics, described by Chessa et al. (2016) and
23
24
369 listed in Table S1, were used. The soils used were primarily selected for their differences in history
25
26
370 of anthropogenic pollution and also for differences in physicochemical composition (pH, clay, sand
27
28
371 and organic matter). The bioavailability of Tc, measured only after the first amendment by Chessa
30
31
372 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
32
33
373 and 0.767 and 4.468 mg kg⁻¹ in SA soil, respectively. The Tc concentrations used for spiking cow
35
36
374 M were higher compared to those used in other studies. However, as Tc quickly, and almost
37
38
375 completely, adsorbs to soil clay minerals and organic matter high amounts of Tc were applied to the
40
41
376 soil in order to reach soluble and potentially bioavailable concentrations, i.e. Tc available for the
42
43
377 bacterial uptake (Zhang et al. 2014), that were previously described for agricultural soils which are
44
45
378 continuously polluted (Hamscher et al. 2005; Qiao et al. 2012). In fact, the Tc concentrations not
47
48
379 adsorbed and hence potentially bioavailable found in the M+Tc100 soils were 0.155 and 0.767 mg
49
50
380 kg⁻¹ for CL and SA soil, respectively (Chessa et al. 2016). Comparable concentrations were
52
53
381 previously reported for Tc in M-treated soils and ranged between 0.15 and 0.8 mg Tc kg⁻¹ soil
54
55
382 (Hamscher et al. 2005; 2002; Qiao et al. 2012). Furthermore, we tested Tc spiked at a fivefold
57
58
383 higher concentration (M+Tc500) as a possible worst case scenario, since previous studies already
59
60
384 indicated that soil microbial populations were not affected by Tc in lower concentrations, as
62
63
64
65

385 reported by Hund-Rinke et al. (2004) where no significant effect on the bacterial composition and
1
386 Tc resistance genes were found in soil polluted with 5 and 50 mg Tc kg⁻¹, whereas Tc significantly
3
4
387 affected the microbial community composition at the spiked concentration of 500 mg kg⁻¹.
5
6

388 In the present microcosm study, we showed that the effect of cow M on the bacterial
8
9
389 community composition increased with repeated application (Table 2) while the effects of the Tc
10
11
390 were far less pronounced. After a single M or M+Tc application, significant differences to DGGE
13
14
391 fingerprints of untreated soil were only observed for CL soil. In contrast to CL soil, DGGE
15
16
392 fingerprints did not reveal such effects 60 days after the first application of M or of M+Tc to SA
18
19
393 soil. We assume that transient effects of M or M+Tc might have occurred also in SA soil but had
20
21
394 disappeared already at the time of sampling 60 days after the first amendment, as previously was
23
24
395 also observed by Selvam et al. (2012). In accordance with other studies (Ding et al. 2014;
25
26
396 Marschner et al. 2003; Sun et al. 2004) we observed that repeated application of M, with or without
28
29
397 Tc, enhanced the effects on the bacterial community composition in both soils as indicated by
30
31
398 increasing differences (d-values) between the DGGE fingerprints of untreated and M-treated soils
32
33
399 (Table 2). Chessa et al. (2016), already reported that a single cow M application changed the
35
36
400 bacterial community composition to increased PLFA ratio of Gram-positive to Gram-negative
37
38
401 bacteria in both soils, and this effect was still observed in CL soil after 60 days but not in SA soil.
40
41
402 The cow M used for the present study, the same as already used by Chessa et al. (2016), was long-
42
43
403 term stored and air-dried, and this, together with divergent soil properties, might explain differences
45
46
404 compared to the findings of other studies performed with piggery M and silt loam soil (Ding et al.
47
48
405 2014; Heuer et al. 2008). In comparison to SDZ used in these studies, the effects of Tc spiked to the
49
50
406 cow M were far less pronounced which was likely caused by the stronger sorption of Tc compared
52
53
407 to SDZ and due to different properties of soils studied: clayey and sandy soil compared to silt loam.
54
55
408 Tc100 spiked to the cow M did not cause significant shifts of the soil bacterial fingerprints,
57
58
409 compared to soil treated with unspiked M. After three amendments with M+Tc500, the fingerprints
59
60
410 significantly differed from those of the M treatments for both soils. In contrast, the presence of SDZ

411 in piggery M spiked at two concentrations (10 and 100 mg kg⁻¹) caused significant changes of the
1
412 bacterial community composition in the two soils compared to unspiked M (Ding et al. 2014; Heuer
3
413 et al. 2011a).
4
5
6

414 Although the cow M was collected from free ranged cows, which to the best of our
8
415 knowledge were not treated with antibiotics, all the ARGs and MGEs analyzed in the present study,
10
416 except *tet(M)*, were detected in the M. Thus with the cow M not only nutrients and spiked Tc were
13
417 introduced into the soils but also bacteria containing ARGs and MGEs. The abundances of ARGs
15
418 and MGEs were below the detection limit or at very low abundance in the CL soil, which had no
18
419 history of anthropogenic antibiotic pollution by human or animal wastes. Thus a striking increase in
20
420 the relative abundance of ARGs and MGEs was observed for CL soil in response to the M
23
421 amendment. In contrast, all ARGs except *tet(M)* and MGEs analyzed in the present study were
25
422 detected in the SA soil which was in the past frequently affected by flooding of a river and by
27
423 orchard farming practice. River water was previously reported as a carrier of ARGs (Amos et al.
30
424 2015; 2014a; 2014b). Thus, the differences found between the CL and SA soil likely do not only
32
425 result from differences in soil properties such as clay content and pH but also from their previous
35
426 history of anthropogenic pollutants. This pollution might have resulted in an adaptation of the
37
427 bacterial community through proliferation of resistant bacteria and horizontal gene transfer (HGT)
40
428 (Heuer and Smalla 2012).
42

429 The ARGs and MGEs analyzed in the present study were previously reported to occur in
45
430 piggery and cow M (Alexander et al. 2011; Binh et al. 2008). Recently, Kyselková et al. (2015a)
47
431 proposed that *tet(Q)* and *tet(W)* genes belong to the resistome stably associated with cow M. Here,
49
50
432 we also found that cow M from free ranged animals contained bacteria carrying the ARGs *tet(Q)*,
52
433 *tet(W)*, *tet(A)*, *sul1* and *sul2*. In contrast to our hypothesis, the M+Tc100 and M+Tc500 treated
54
434 soils did not show significantly increased abundance of *tet(Q)* and *tet(W)*, compared to the M-
57
435 treatments. However, with repeated M-applications an accumulation of *tet(Q)* and *tet(W)* could be
59
436 observed irrespective of the presence of Tc. Kyselková et al. (2013) also found that Tc did not show
61

437 additive effects on the abundance of *tet(Q)* and *tet(W)* genes, compared to unspiked M. Likely these
1
438 genes were hosted in bacteria that did not proliferate in soil, and thus neither M nor selective
2
3
439 pressure exerted by Tc increased their abundance. In contrast, *tet(A)* likely carried by other
4
5
6
440 bacterial hosts was clearly increased in the M+Tc500 treatments in both soils. In the control soils,
7
8
9
441 the relative abundance of *tet(Q)* and *tet(W)* tended to decrease over the time of our experiment.
10
11
442 Probably, the populations carrying these genes decreased in relative abundance due to the lack of
12
13
14
443 nutrient input in the untreated control soils. In cow M and in both soils the abundances of *tet(Q)*
15
16
444 genes were, in tendency, lower than those of *tet(W)* (Wolters et al. 2016).
17
18

19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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-1 ϵ 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-1 ϵ plasmids often carried *tet(A)* upstream and *sul1* downstream of the class 1 integron.

462 The presence of Tc in M selected for IncP-1ε plasmids in SA soil while no such increase
1
2
463 was observed in CL soils. Probably bacterial populations carrying IncP-1ε plasmids applied with M
3
4
464 were not well adapted to conditions present in the CL soil and thus rapidly decreased in relative
5
6
465 abundance. Several previous studies showed that repeated amendments of M enhanced the HGT of
7
8
466 MGE within microbial communities (Ghosh and LaPara 2007; Heuer et al. 2011a; Jindal et al.
9
10
467 2006; Popowska et al. 2012; You et al. 2012), whereas the effect of a single M application on
11
12
468 tetracycline resistance levels in soil bacterial communities may be only transient (Chessa et al.
13
14
469 2016; Sengeløv et al. 2003).

15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 1.3, Line of

487 Activity 1.3.1.). Sven Jechalke was funded by the Federal Environment Agency
1
488 (Umweltbundesamt) (FKZ 3713 63 402). The authors would like to thank Dr. Martina Kyselková to
2
3
4
489 provide us positive controls for qPCR reactions.
5
6

490

491 **References**

- 11
492 Alexander TW, Yanke JL, Reuter T, Topp E, Read RR, Selinger BL, McAllister TA (2011)
12
13
493 Longitudinal characterization of antimicrobial resistance genes in feces shed from cattle fed
14
15
494 different subtherapeutic antibiotics. *BMC Microbiol* 11:19. doi:10.1186/1471-2180-11-19
16
17
495 Amos GCA, Gozzard E, Carter CE, Mead A, Bowes MJ, Hawkey PM, Zhang L, Singer AC, Gaze
18
19
20
496 WH, Wellington EMH (2015) Validated predictive modelling of the environmental resistome.
21
22
23
497 *ISME J* 9:1467-1476. doi:10.1038/ismej.2014.237
24
25
498 Amos GCA, Hawkey PM, Gaze WH, Wellington EM (2014a) Waste water effluent contributes to
26
27
28
499 the dissemination of CTX-M-15 in the natural environment. *J Antimicrob Chemoth* 69:1785-1791.
29
30
500 doi:10.1093/jac/dku079
31
32
33
501 Amos GCA, Zhang L, Hawkey PM, Gaze WH, Wellington EM (2014b) Functional metagenomic
34
35
502 analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. *Vet Microbiol*
36
37
503 171:441-447. doi:10.1016/j.vetmic.2014.02.017
38
39
40
504 Bahl M, Hansen L, Goesmann A, Sørensen S (2007) The multiple antibiotic resistance IncP-1
41
42
505 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the
43
44
45
506 previously established α , β and δ sub-groups. *Plasmid* 58:31-43. doi:10.1016/j.plasmid.2006.11.007
46
47
48
507 Barraud O, Baclet MC, Denis F, Ploy MC (2010) Quantitative multiplex real-time PCR for
49
50
508 detecting class 1, 2 and 3 integrons. *J Antimicrob Chemoth* 65:1642-1645. doi:10.1093/jac/dkq167
51
52
53
509 Binh CT, Heuer H, Kaupenjohann M, Smalla K (2008) Piggery manure used for soil fertilization is
54
55
56
57
58
59
60
61
62
63
64
65

512 Chessa L, Pusino A, Garau G, Mangia NP, Pinna MV (2016) Soil microbial response to tetracycline
1
513 in two different soils amended with cow manure. *Environ Sci Pollut R* 23:5807-5817.
2
3
4
514 doi:10.1007/s11356-015-5789-4
5
6
515 Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular
7
8
9
516 biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232-260.
10
11
517 doi:10.1128/MMBR.65.2.232-260.2001
12
13
518 Ding GC, Heuer H, Smalla K (2012) Dynamics of bacterial communities in two unpolluted soils
14
15
16
519 after spiking with phenanthrene: soil type specific and common responders. *Front Microbiol* 3:290.
17
18
520 doi:10.3389/fmicb.2012.00290
19
20
521 Ding GC, Radl V, Schloter-Hai B, Jechalke S, Heuer H, Smalla K, Schloter M (2014) Dynamics of
21
22
23
522 soil bacterial communities in response to repeated application of manure containing sulfadiazine.
24
25
523 PLoS One 9:e92958. doi:10.1371/journal.pone.0092958
26
27
28
524 Durso LM, Cook KL (2014) Impacts of antibiotic use in agriculture: what are the benefits and
29
30
31
525 risks? *Curr Opin Microbiol* 19:37-44. doi:10.1016/j.mib.2014.05.019
32
33
526 EC (1831/2003) Regulation (EC) No 1831/2003 of the European Parliament and of the Council of
34
35
36
527 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union*
37
38
528 Food and Drug (2013) Antimicrobials sold or distributed for use in food-producing animals.
39
40
529 Administration Department of Health and Human Services,
41
42
43
530 Fulthorpe RR, McGowan C, Maltseva OV, Holben WE, Tiedje JM (1995) 2,4-
44
45
46
531 Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. *Appl Environ*
47
48
532 *Microb* 61:3274-3281.
49
50
533 Ghosh S, LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the
51
52
53
534 proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* 1:191-203.
54
55
535 doi:10.1038/ismej.2007.31
56
57
58
59
60
61
62
63
64
65

536 Gomes NCM, Heuer H, Schönfeld J, Costa R, Mendonça-Hagler L, Smalla K (2001) Bacterial
1
537 diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature
2
3
4
538 gradient gel electrophoresis. *Plant Soil* 232:167-180. doi:10.1023/A:1010350406708
5
6
539 Gu C, Karthikeyan KG, Sibley SD, Pedersen JA (2007) Complexation of the antibiotic tetracycline
7
8
9
540 with humic acid. *Chemosphere* 66:1494-1501. doi:10.1016/j.chemosphere.2006.08.028
10
11
541 Hammer TJ, Fierer N, Hardwick B, Simojoki A, Slade E, Taponen J, Viljanen H, Roslin T (2016)
12
13
14
542 Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung
15
16
543 beetles. *Proc Biol Sci* 283. doi: 10.1098/rspb.2016.0150
17
18
544 Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S (2008) Impact of the antibiotic
19
20
21
545 sulfadiazine and pig manure on the microbial community structure in agricultural soils. *Soil Biol*
22
23
24
546 *Biochem* 40:1583-1591. doi:10.1016/j.soilbio.2008.01.010
25
26
547 Hamscher G, Pawelzick HT, Höper H, Nau H (2005) Different behavior of tetracyclines and
27
28
548 sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environ Toxicol Chem*
29
30
31
549 24:861-868. doi:10.1897/04-182R.1
32
33
550 Hamscher G, Sczesny S, Höper H, Nau H (2002) Determination of persistent tetracycline residues
34
35
36
551 in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray
37
38
552 ionization tandem mass spectrometry. *Anal Chem* 74:1509-1518. doi:10.1021/ac015588m
39
40
553 Hassan R, Ryu K-S (2012) Naturally Derived Probiotic Supplementation Effects on Physiological
41
42
43
554 Properties and Manure Gas Emission of Broiler Chickens. *J Agric Life Sci* 46:119-127.
44
45
555 doi:10.3382/ps.2013-03314
46
47
556 Heuer H, Binh CT, Jechalke S, Kopmann C, Zimmerling U, Krögerrecklenfort E, Ledger T,
48
49
50
557 Gonzalez B, Top E, Smalla K (2012) IncP-1epsilon Plasmids are Important Vectors of Antibiotic
51
52
53
558 Resistance Genes in Agricultural Systems: Diversification Driven by Class 1 Integron Gene
53
54
55
559 Cassettes. *Front Microbiol* 3:2. doi:10.3389/fmicb.2012.00002
56
57
58
59
60
61
62
63
64
65

560 Heuer H, Focks A, Lamshöft M, Smalla K, Matthies M, Spiteller M (2008) Fate of sulfadiazine
1
561 administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in
2
3
4
562 manure and manured soil. *Soil Biol Biochem* 40:1892-1900. doi:10.1016/j.soilbio.2008.03.014
5
6
563 Heuer H, Kopmann C, Binh CT, Top EM, Smalla K (2009) Spreading antibiotic resistance through
7
8
9
564 spread manure: characteristics of a novel plasmid type with low %G+C content. *Environ Microbiol*
10
11
565 11:937-949. doi:10.1111/j.1462-2920.2008.01819.x
12
13
566 Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete
14
15
16
567 communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic
17
18
19
568 separation in denaturing gradients. *Appl Environ Microb* 63:3233-3241.
20
21
569 Heuer H, Schmitt H, Smalla K (2011a) Antibiotic resistance gene spread due to manure application
22
23
24
570 on agricultural fields. *Curr Opin Microbiol* 14:236-243. doi:10.1016/j.mib.2011.04.009
25
26
571 Heuer H, Smalla K (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic
27
28
29
572 resistance in soil over at least two months. *Environ Microbiol* 9:657-666. doi:10.1111/j.1462-
30
31
573 2920.2006.01185.x
32
33
574 Heuer H, Smalla K (2012) Plasmids foster diversification and adaptation of bacterial populations in
34
35
36
575 soil. *FEMS Microbiol Rev* 36:1083-1104. doi:10.1111/j.1574-6976.2012.00337.x
37
38
576 Heuer H, Solehati Q, Zimmerling U, Kleineidam K, Schloter M, Müller T, Focks A, Thiele-Bruhn
39
40
41
577 S, Smalla K (2011b) Accumulation of sulfonamide resistance genes in arable soils due to repeated
42
43
44
578 application of manure containing sulfadiazine. *Appl Environ Microb* 77:2527-2530.
45
46
579 doi:10.1128/AEM.02577-10
47
48
580 Heuer H, Weiland G, Schönfeld J, Schönwälder A, Gomes N, Smalla K (2001) Bacterial
49
50
581 Community Profiling Using DGGE or TGGE Analysis In: Rochelle PA (Ed) *Environmental*
51
52
53
582 *Molecular Microbiology: Protocols and Applications*. Horizon Scientific Press, Wymondham, UK,
54
55
583 pp 177-190.
56
57
58
59
60
61
62
63
64
65

584 Ho A, El-Hawwary A, Kim SY, Meima-Franke M, Bodelier P (2015) Manure-associated
1 stimulation of soil-borne methanogenic activity in agricultural soils. *Biol Fert Soils* 51:511-516.
585 doi:10.1007/s00374-015-0995-2
2
3
4
586
5
6
587 Hund-Rinke R, Simon M, Lukow T (2004) Effects of Tetracycline on the Soil Microflora: Function,
8
9
588 Diversity, Resistance. *J Soil Sediment* 4:11-16.
10
11
589 Jechalke S, Focks A, Rosendahl I, Groeneweg J, Siemens J, Heuer H, Smalla K (2014a) Structural
13
14
590 and functional response of the soil bacterial community to application of manure from difloxacin-
15
16
591 treated pigs. *FEMS Microbiol Ecol* 87:78-88. doi:10.1111/1574-6941.12191
18
19
592 Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K (2014b) Fate and effects of veterinary
20
21
593 antibiotics in soil. *Trends Microbiol* 22:536-545. doi:10.1016/j.tim.2014.05.005
22
23
594 Jia DA, Zhou DM, Wang YJ, Zhu HW, Chen JL (2008) Adsorption and cosorption of Cu(II) and
25
26
595 tetracycline on two soils with different characteristics. *Geoderma* 146:224-230.
27
28
596 doi:10.1016/j.geoderma.2008.05.023
30
31
597 Jindal A, Kocherginskaya S, Mehboob A, Robert M, Mackie RI, Raskin L, Zilles JL (2006)
32
33
598 Antimicrobial use and resistance in swine waste treatment systems. *Appl Environ Microb* 72:7813-
35
36
599 7820. doi:10.1128/AEM.01087-06
37
38
600 Jutta R, Pils V, Laird DA (2007) Sorption of Tetracycline and Chortetracycline on K- and Ca-
40
41
601 saturated soil clays, humic Substances, and clay-humic complexes. *Environ Sci Technol* 41:1928-
42
43
602 1933. doi:10.1021/es062316y
44
45
603 Kim KR, Owens G, Kwon SI, So KH, Lee DB, Ok YS (2011) Occurrence and Environmental Fate
47
48
604 of Veterinary Antibiotics in the Terrestrial Environment. *Water Air Soil Poll* 214:163-174.
49
50
605 doi:10.1007/s11270-010-0412-2
52
53
606 Kong WD, Li CG, Dolhi JM, Li SY, He JZ, Qiao M (2012) Characteristics of oxytetracycline
54
55
607 sorption and potential bioavailability in soils with various physical-chemical properties.
57
58
608 *Chemosphere* 87:542-548. doi:10.1016/j.chemosphere.2011.12.062
59
60
61
62
63
64
65

609 Kreuzig R, Höltge S (2005) Investigations on the fate of sulfadiazine in manured soil: Laboratory
1
610 experiments and test plot studies. *Environ Toxicol Chem* 24:771-776. doi:10.1897/03-582R.1
3
4
611 Kropf S, Heuer H, Grüning M, Smalla K (2004) Significance test for comparing complex microbial
6
612 community fingerprints using pairwise similarity measures. *J Microbiol Methods* 57:187-195.
8
9
613 doi:10.1016/j.mimet.2004.01.002
10
11
614 Kümmerer K (2003) Significance of antibiotics in the environment. *J Antimicrob Chemother* 52:5-
13
615 7. doi:10.1093/jac/dkg293
14
15
616 Kyselková M, Jirout J, Chroňáková A, Vrchotová N, Bradley R, Schmitt H, Elhottová D (2013)
18
617 Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their
20
618 oxytetracycline content. *Chemosphere* 93:2413-2418. doi:10.1016/j.chemosphere.2013.08.058
21
22
619 Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015a) Spread of tetracycline
25
620 resistance genes at a conventional dairy farm. *Front Microbiol* 6. doi: 10.3389/fmicb.2015.00536
26
27
621 Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015b) Spread of tetracycline
30
622 resistance genes at a conventional dairy farm. *Front Microbiol* 6:536.
31
32
623 doi:10.3389/fmicb.2015.00536
33
35
624 Lanz R, Kuhnert P, Boerlin P (2003) Antimicrobial resistance and resistance gene determinants in
37
625 clinical *Escherichia coli* from different animal species in Switzerland. *Vet Microbiol* 91:73-84.
38
40
626 Li Z, Chang PH, Jean JS, Jiang WT, Wang CJ (2010) Interaction between tetracycline and smectite
42
627 in aqueous solution. *J Colloid Interface Sci* 341:311-319. doi:10.1016/j.jcis.2009.09.054
43
44
45
628 Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial
47
629 community in a long-term fertilizer experiment. *Soil Biol Biochem* 35:453-461.
48
49
50
630 doi:10.1016/S0038-0717(02)00297-3
52
53
631 Montforts MH, Kalf DF, van Vlaardingen PL, Linders JB (1999) The exposure assessment for
54
55
632 veterinary medicinal products. *Sci Total Environ* 225:119-133. doi:10.1016/S0048-9697(98)00338-6
57
58
633 Nelson ML, Levy SB (2011) The history of the tetracyclines. *Ann N Y Acad Sci* 1241:17-32.
59
60
634 doi:10.1111/j.1749-6632.2011.06354.x
61
62
63
64
65

635 Ng LK, Martin I, Alfa M, Mulvey M (2001) Multiplex PCR for the detection of tetracycline
1
636 resistant genes. *Mol Cell Probes* 15:209-215. doi:10.1006/mcpr.2001.0363
2
3
4
637 Nguyen F, Starosta AL, Arenz S, Sohmen D, Donhofer A, Wilson DN (2014) Tetracycline
5
6
638 antibiotics and resistance mechanisms. *Biol Chem* 395:559-575. doi:10.1515/hsz-2013-0292
7
8
639 O'Connor S, Aga DS (2007) Analysis of tetracycline antibiotics in soil: Advances in extraction,
9
10
640 clean-up, and quantification. *Trac-Trend Anal Chem* 26:456-465. doi:10.1016/j.trac.2007.02.007
11
12
641 Popowska M, Rzeczycka M, Miernik A, Krawczyk-Balska A, Walsh F, Duffy B (2012) Influence
13
14
642 of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated
15
16
643 resistance genes. *Antimicrob Agents Chemother* 56:1434-1443. doi:10.1128/AAC.05766-11
17
18
644 Qiao M, Chen W, Su J, Zhang B, Zhang C (2012) Fate of tetracyclines in swine manure of three
19
20
645 selected swine farms in China. *J Environ Sci* 24:1047-1052. doi:10.1016/S1001-0742(11)60890-5
21
22
646 Roberts MC (2011) Mechanisms of bacterial antibiotic resistance and lessons learned from
23
24
647 environmental tetracycline-resistant bacteria, First Edition edn. John Wiley & Sons, Inc., Hoboken,
25
26
648 NJ, USA
27
28
649 Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. vol vol. 1.
29
30
650 Cold Spring Harbor laboratory Press.
31
32
33
651 Sarmah AK, Meyer MT, Boxall AB (2006) A global perspective on the use, sales, exposure
34
35
652 pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment.
36
37
653 *Chemosphere* 65:725-759. doi:10.1016/j.chemosphere.2006.03.026
38
39
654 Schmitt H, Stoob K, Hamscher G, Smit E, Seinen W (2006) Tetracyclines and tetracycline
40
41
655 resistance in agricultural soils: microcosm and field studies. *Microb Ecol* 51:267-276.
42
43
656 doi:10.1007/s00248-006-9035-y
44
45
657 Schreiter S, Ding GC, Grosch R, Kropf S, Antweiler K, Smalla K Soil type-dependent effects of a
46
47
658 potential biocontrol inoculant on indigenous bacterial communities in the rhizosphere of field-
48
49
659 grown lettuce. *FEMS Microbiology Ecology* 90:718-730. doi:10.1111/1574-6941.12430
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

660 Selvam A, Xu D, Zhao Z, Wong JW (2012) Fate of tetracycline, sulfonamide and fluoroquinolone
1
661 resistance genes and the changes in bacterial diversity during composting of swine manure.
2
3
4
662 *Bioresour Technol* 126:383-390. doi:10.1016/j.biortech.2012.03.045
5
6
663 Sengeløv G, Agersø Y, Halling-Sørensen B, Baloda SB, Andersen JS, Jensen LB (2003) Bacterial
7
8
664 antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry.
9
10
665 *Environ Int* 28:587-595.
11
12
666 Smalla K, Heuer H, Götz A, Niemeyer D, Krögerrecklenfort E, Tietze E (2000) Exogenous
13
14
667 isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence
15
16
668 and diversity of IncQ-like plasmids. *Appl Environ Microb* 66:4854-4862.
17
18
669 Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001)
19
20
670 Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis:
21
22
671 plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microb* 67:4742-4751.
23
24
672 doi:10.1128/aem.67.10.4742-4751.2001
25
26
673 Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, Galland JC, Graham DW (2004)
27
28
674 Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl Environ*
29
30
675 *Microb* 70:7372-7377. doi:10.1128/AEM.70.12.7372-7377.2004
31
32
676 Sun HY, Deng SP, Raun WR (2004) Bacterial community structure and diversity in a century-old
33
34
677 manure-treated agroecosystem. *Appl Environ Microb* 70:5868-5874.
35
36
678 doi:10.1128/AEM.70.10.5868-5874.2004
37
38
679 Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in
39
40
680 mixed microbial populations via 5'-nuclease assays. *Appl Environ Microb* 66:4605-4614. doi:
41
42
681 Thiele-Bruhn S (2003) Pharmaceutical antibiotic compounds in soils - A review. *J Plant Nutr Soil*
43
44
682 *Sci* 166:145-167.
45
46
683 Thiele-Bruhn S, Beck IC (2005) Effects of sulfonamide and tetracycline antibiotics on soil
47
48
684 microbial activity and microbial biomass. *Chemosphere* 59:457-465.
49
50
685 doi:10.1016/j.chemosphere.2005.01.023
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

686 Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J (2014) Bloom of resident antibiotic-
1
687 resistant bacteria in soil following manure fertilization. P Natl Acad Sci USA 111:15202-15207.
2
3
4
688 doi:10.1073/pnas.1409836111
5
6
689 Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A,
7
8
9
690 Laxminarayan R (2015) Global trends in antimicrobial use in food animals. P Natl Acad Sci USA
10
11
691 112:5649-5654. doi:10.1073/pnas.1503141112
12
13
692 Weinert N, Meincke R, Gottwald C, Heuer H, Gomes NC, Schloter M, Berg G, Smalla K (2009)
14
15
693 Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their
16
17
18
694 parent cultivar differ less than those of different potato cultivars. Appl Environ Microb 75:3859-
19
20
21
695 3865. doi:10.1128/AEM.00414-09
22
23
696 Winckler C, Grafe A (2001) Use of veterinary drugs in intensive animal production. J Soil
24
25
26
697 Sediment 1:66-70.
27
28
698 Wolters B, Ding GC, Kreuzig R, Smalla K (2016) Full-scale mesophilic biogas plants using manure
29
30
31
699 as C-source: Bacterial community shifts along the process cause changes in the abundance of
32
33
34
700 resistance genes and mobile genetic elements. FEMS Microbiol Ecol 92:1-17.
35
36
701 doi:10.1093/femsec/fiv163
37
38
702 Wolters B, Kyselková M, Krögerrecklenfort E, Kreuzig R, Smalla K (2015) Transferable antibiotic
39
40
41
703 resistance plasmids from biogas plant digestates often belong to the IncP-1ε subgroup. Front
42
43
704 Microbiol 6. doi: 10.3389/fmicb.2014.00765
44
45
705 Wu K, Yuan S, Wang L, Shi J, Zhao J, Shen B, Shen Q (2014) Effects of bio-organic fertilizer plus
46
47
48
706 soil amendment on the control of tobacco bacterial wilt and composition of soil bacterial
49
50
51
707 communities. Biol Fert Soils 50:961-971. doi:10.1007/s00374-014-0916-9
52
53
708 You Y, Hilpert M, Ward MJ (2012) Detection of a common and persistent tet(L)-carrying plasmid
54
55
56
709 in chicken-waste-impacted farm soil. Appl Environ Microb 78:3203-3213.
57
58
710 doi:10.1128/AEM.07763-11
59
60
61
62
63
64
65

711 Zhang Y, Boyd SA, Teppen BJ, Tiedje JM, Li H (2014) Role of tetracycline speciation in the
 1
 712 bioavailability to escherichia coli for uptake and expression of antibiotic resistance. Environ Sci
 2
 713 Technol 48:4893-4900. doi:10.1021/es5003428
 3
 4
 5
 6
 714 Zhao S, Liu D, Ling N, Chen F, Fang W, Shen Q (2014) Bio-organic fertilizer application
 7
 715 significantly reduces the Fusarium oxysporum population and alters the composition of fungi
 8
 716 communities of watermelon Fusarium wilt rhizosphere soil. Biol Fert Soils 50:765-774.
 9
 717 doi:10.1007/s00374-014-0898-7
 10
 11
 12
 13
 14
 15
 16
 718 Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM (2013)
 17
 719 Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc Natl Acad Sci U S A
 18
 720 110:3435-3440. doi:10.1073/pnas.1222743110
 19
 20
 21
 22
 23

24
 25

26
 27 **Figure captions**

28
 29
 30

31
 32 **Table 1** Sequences of primers and TaqMan probes used for the detection of ARGs and MGEs.
 33

34
 35

36
 37 **Table 2** Percent difference (d-values) of soil bacterial community composition based on Pearson
 38
 39 correlations of background-subtracted densitometric curves from DGGE analysis (60 days after the
 40
 41 first, second and third amendments). Significance is indicated by the respective *P*-value.
 42

43
 44 S: soil CL or SA; M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil
 45
 46 dry weight.
 47

48
 49

50
 51 **Figure 1** UPGMA cluster analysis of DGGE fingerprints for 16S rRNA gene amplicons from CL
 52
 53 and SA soils 60 days after the first amendment: amplicons from (a) and (d), respectively; 60 days
 54
 55 after the second amendment: (b) and (e), respectively; 60 days after the third amendment: (c) and
 56
 57 (f), respectively. Horizontal axis indicates the % of similarity. UPGMA cluster analysis was based
 58
 59 on Pearson correlation indices to compare the effect of treatments in each soil at a specific time
 60
 61

62
 63
 64
 65

737 point. S: soil CL or SA; M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹
1
738 soil dry weight.

739
4
740 **Figure 2** Relative abundances of *tet(Q)* and *tet(W)* genes in CL (a, c) and SA (b, d) soils,
8
741 respectively, were measured by qPCR 60 days after the first, second and third amendments. Tukey-
10
742 Kramer post-hoc test ($P<0.05$) was used to compare the relative abundances of the target genes in
13
743 total community-DNA at the same time point or within the treatment after repeated amendments.
14
744 M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight; *:
16
745 below the detection limit. For each time-point, average values which share the same white capital
20
746 letter within columns do not differ significantly at the 5% level. For each treatment, average values
21
747 which share the same letters above columns do not significantly differ at the 5% level. Error bars
25
748 indicate the standard deviation of four replicates.

28
749
30
31
750 **Figure 3** PCR Southern blot hybridization of the *tet(A)* gene in CL and SA soils 60 days after the
32
33
751 first and third amendments. Four replicates for each treatment are shown. The lanes at the utmost
35
36
752 right and left are size markers. The figure is composed of six hybridized membranes (three
37
38
753 membranes per soil) which were reassembled by photoshop.

40
754
42
43
755 **Figure 4** Relative abundances of *sul1* and *sul2* genes in CL (a, c) and SA (b, d) soils, respectively,
45
756 measured by qPCR 60 days after the first, second and third amendments. The Tukey-Kramer post-
47
48
757 hoc test ($P<0.05$) was used to compare the relative abundances of the target genes in the total
49
50
758 community-DNA at the same time point or within the treatment after repeated amendments. M:
52
759 manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight; *: under
54
55
760 the detection limit. For each time-point, average values which share the same white capital letter
57
761 within columns do not differ significantly at the 5% level. For each treatment, average values which

762 share the same letters above columns do not differ significantly at the 5% level. Error bars indicate
1
763 the standard deviation of four replicates.

764
6
765 **Figure 5** Relative abundances of *intI1* and *trfA* genes in CL (a, c) and SA (b, d) soils, respectively,
8
9
766 measured by qPCR 60 days after the first, second and third amendments. The Tukey-Kramer post-
10
11
767 hoc test ($P<0.05$) was used to compare the relative abundances of the target genes in the total
13
14
768 community-DNA at the same time point or within the treatment after repeated amendments. M:
15
16
769 manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight. For each
18
19
770 time-point, average values which share the same white capital letter within columns do not differ
20
21
771 significantly at the 5% level. For each treatment, average values which share the same letters above
23
24
772 columns do not differ significantly at the 5% level. Error bars indicate the standard deviation of four
25
26
773 replicates.

28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 Pusino¹, Nicoletta Pasqualina Mangia¹, Kornelia Smalla²,

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;

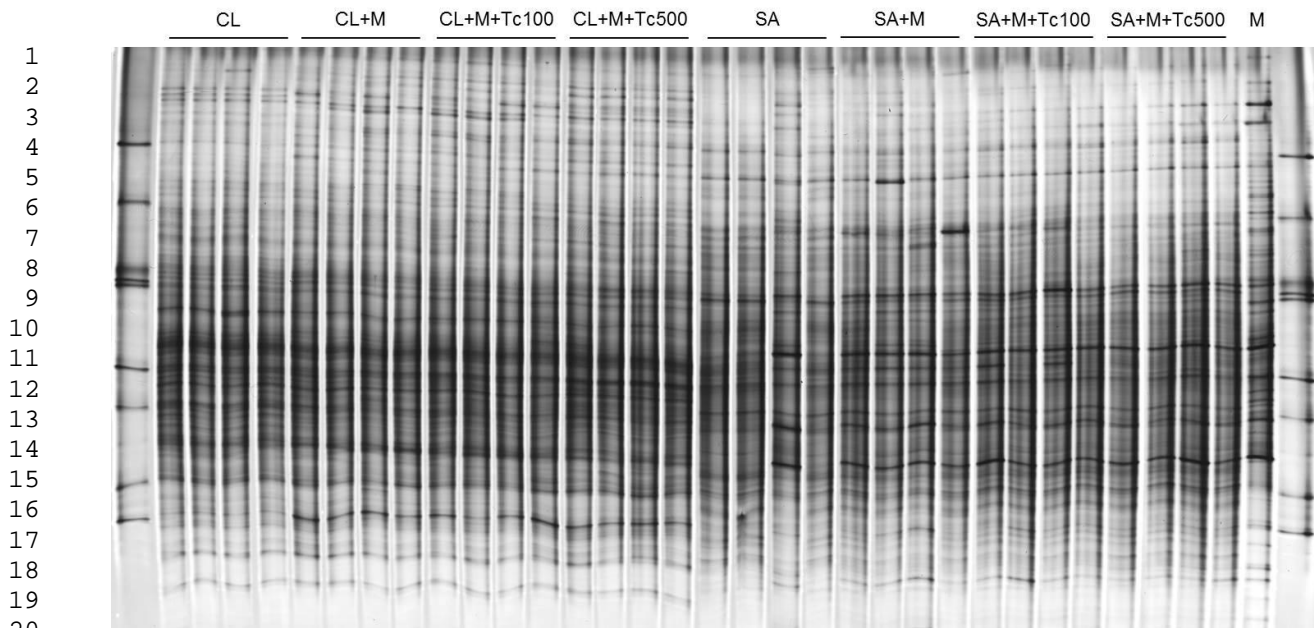
Fax: +39 079229370

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65



21
22 **Figure S1** Bacterial 16S rRNA gene amplicons of CL and SA soil 60 days after the first
23 amendment. Four replicates for each soil treatment were performed. M: manure; Tc100: 100 mg Tc
24 kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight.
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

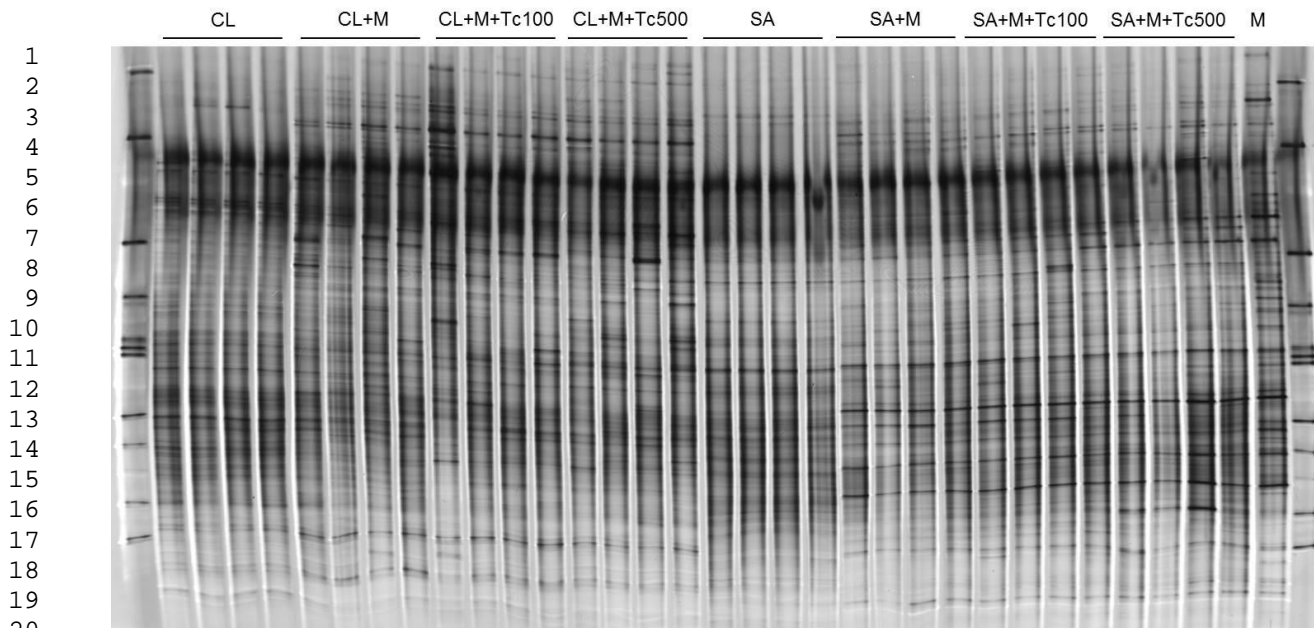


Figure S2 Bacterial 16S rRNA gene amplicons of CL and SA soil 60 days after the second amendment. Four replicates for each soil treatment were performed. M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

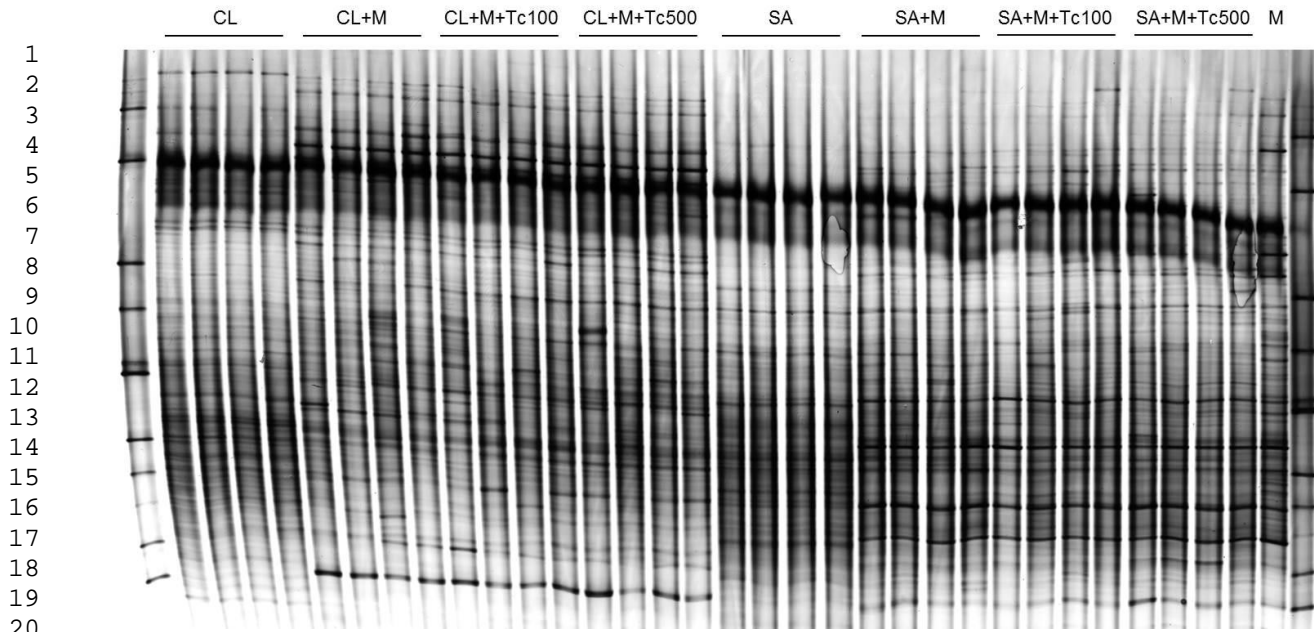
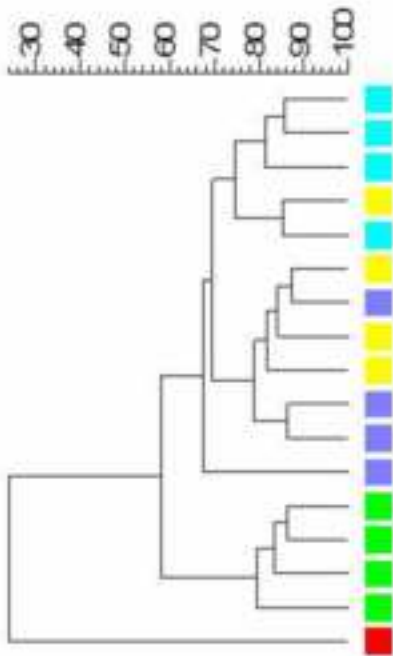


Figure S3 Bacterial 16S rRNA gene amplicons of CL and SA soil 60 days after the third amendment. Four replicates for each soil treatment were performed. M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight.

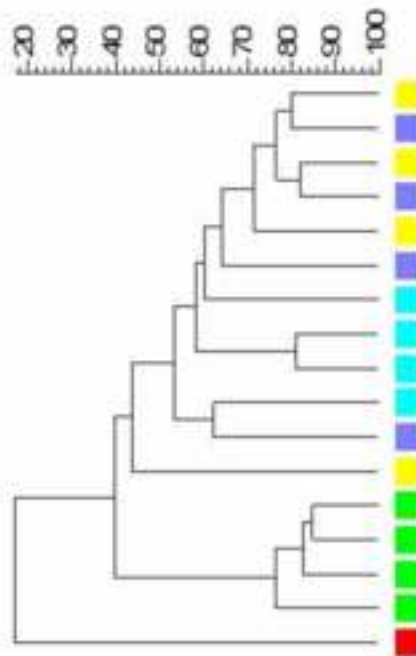
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

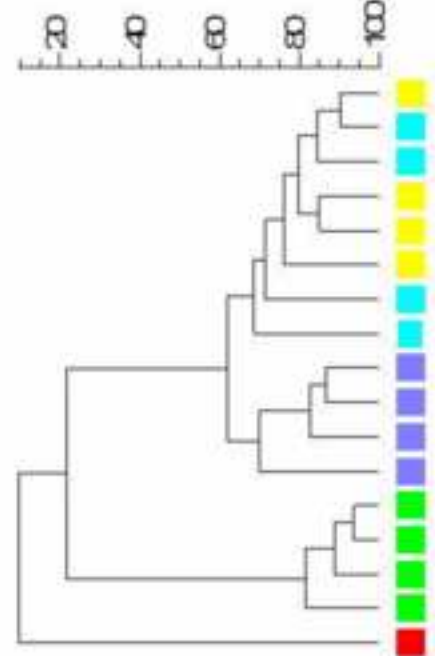
(a) CL first amendment



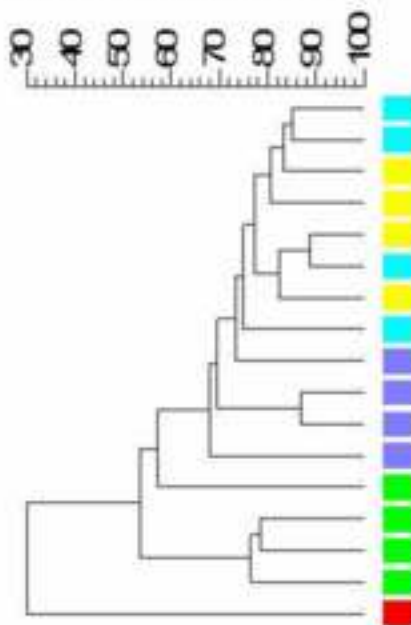
(b) CL second amendment



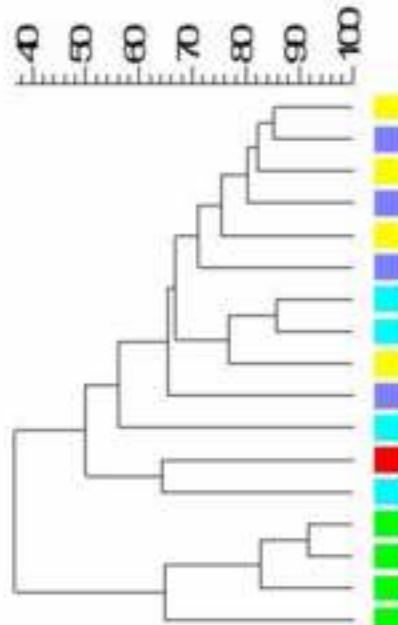
(c) CL third amendment



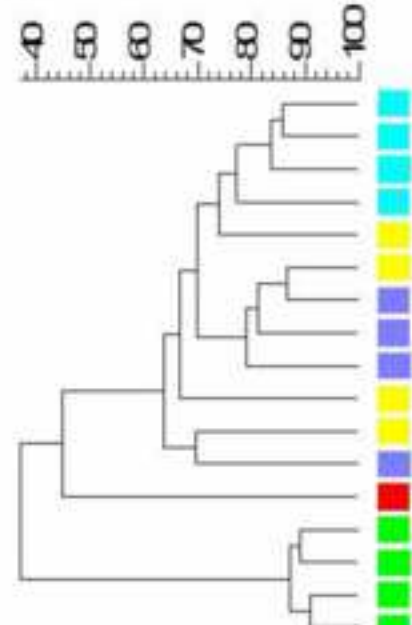
(d) SA first amendment



(e) SA second amendment



(f) SA third amendment



■ CL/SA ■ CL/SA+M ■ CL/SA+M+Tc100 ■ CL/SA+M+Tc500 ■ M

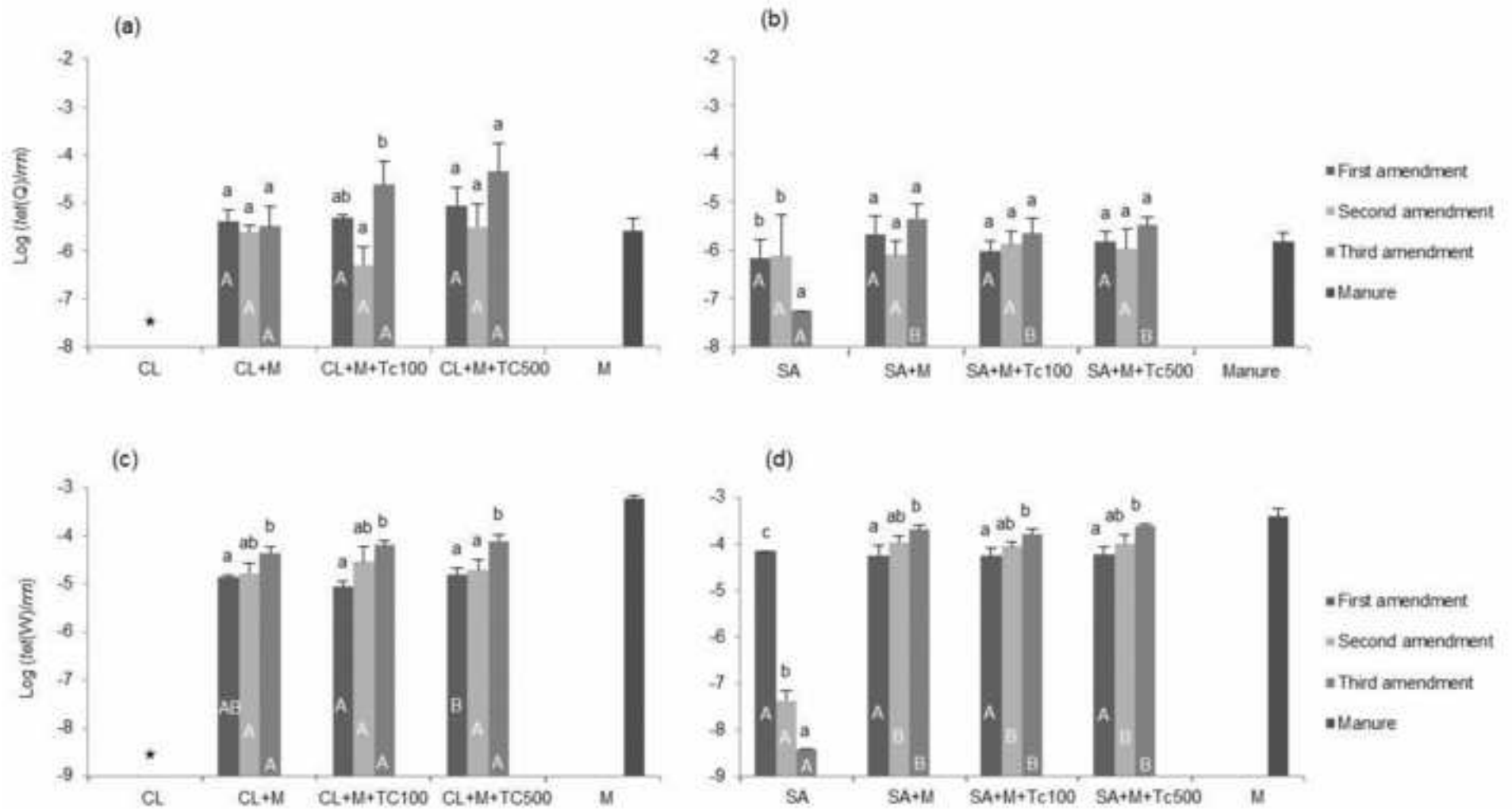
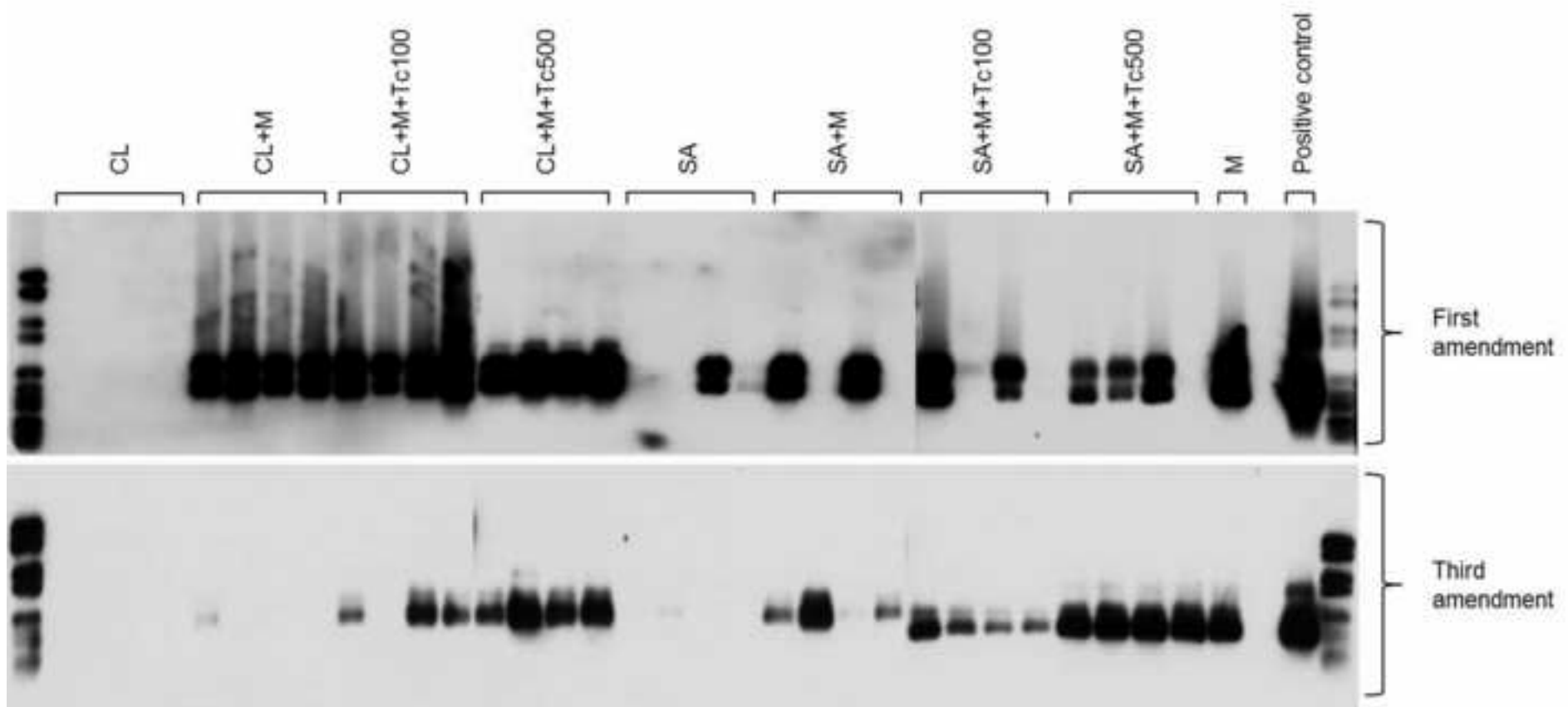
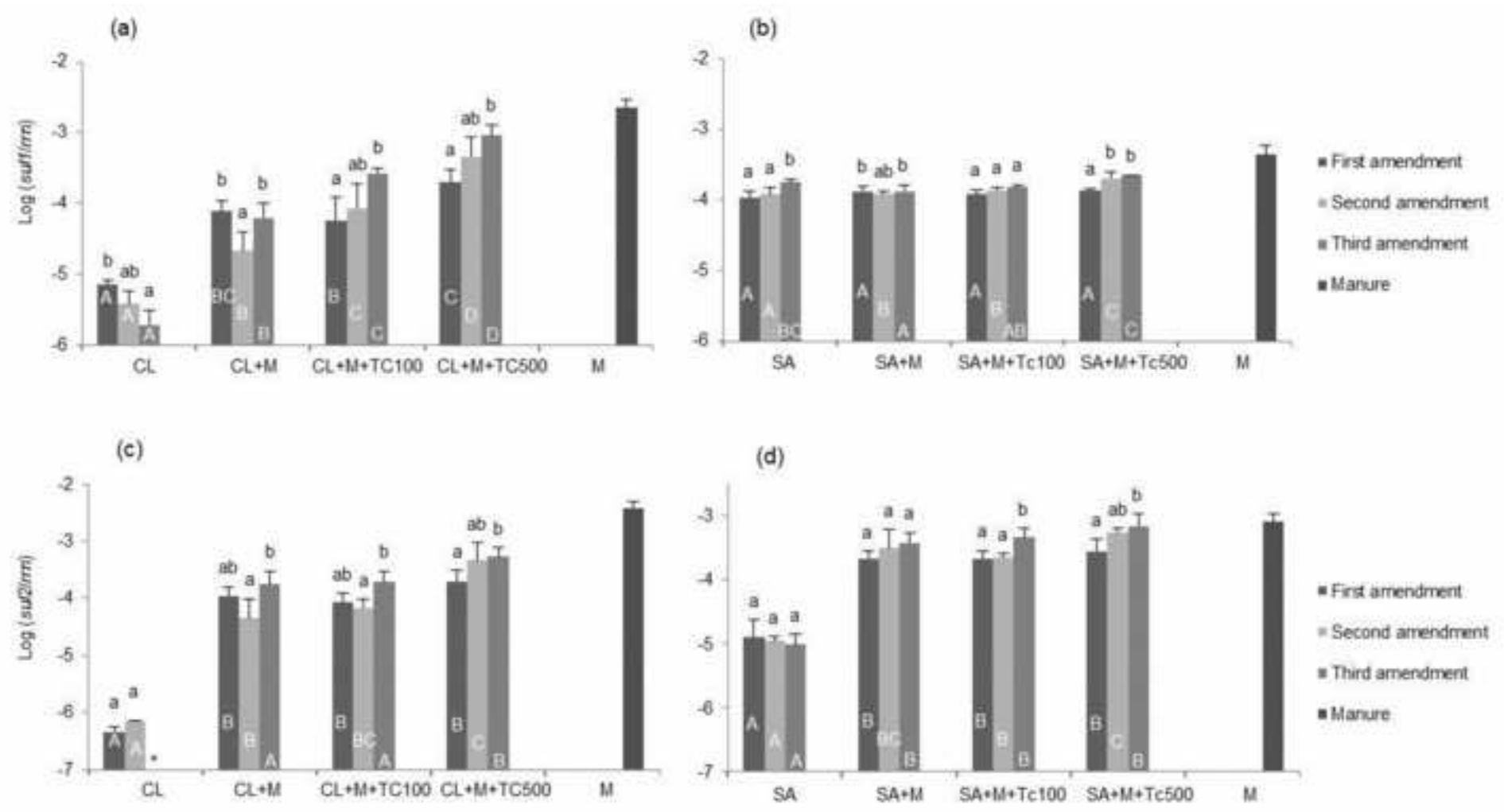


Figure 3

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

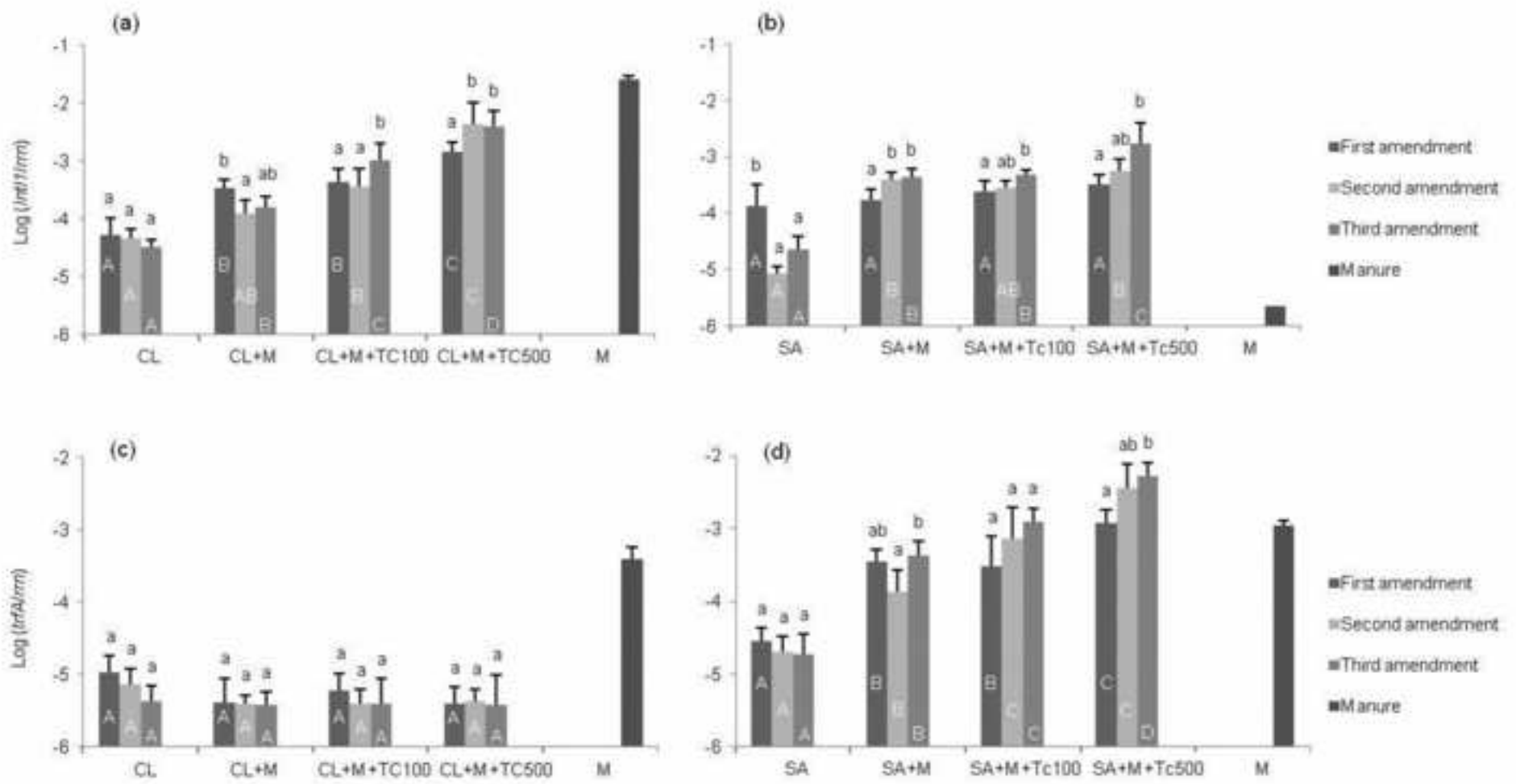


Table 1 Sequences of primers and TaqMan probes used for the detection of ARGs and MGEs

Gene target	Forward primer	Reverse primer	Probe	Annealing temp. (°C)	Reference
16S rRNA	5'CGGTGAATACGTTCTY CGG	5'GGWTACCTTGTTAC GACTT	5'CTTGACACACCGCC CGTC	56	Suzuki et al. (2000)
<i>tet(Q)</i>	5'AGGTGCTGAACCTTG TTTGATTCT	5'GGCCGGACGGAGGAT TT	5'FAM- TCGCATCAGCATCCCG CTC-TAMRA	60	Smith et al. (2004)
<i>tet(W)</i>	5'GCAGAGCGTGGTTCA GTCT	5'GACACCGTCTGCTTG ATGATAAT	5'FAM- TTCGGGATAAGCTCTCC GCCGA-TAMRA	60	Smith et al. (2004)
<i>tet(A)</i>	5'GGCGGTCTTCTTCATC ATGC	5'CGGCAGGCAGAGCAA GTAGA		64	Lanz et al. (2003)
<i>tet(M)</i>	5'GTGGACAAAGGTACA ACGAG	5'CGGTAAAGTTCGTCA CACAC		64	Ng et al. (2001)
<i>sul1</i>	5'CTGAACGATATCCAA GGATTYCC	5'CTCAATGATATTCGCG GTTYCC	5'FAM- CAGCGAGCCTTGCGGC GG-TAMRA	60	Heuer and Smalla (2007)
<i>sul2</i>	5'CGGCTGCGCTTCGATT	5'CGCGCGCAGAAAGGA TT	5'FAM- CGGTGCTTCTGTCTGTT TCGCGC-TAMRA	53	Heuer et al. (2008)
<i>intI1</i>	5'GCCTTGATGTTACCCG AGAG	5'GATCGGTCTGAATGCG TGT	5'FAM- ATTCCTGGCCGTGGTTC TGGGTTTT-TAMRA	60	Barraud et al. (2010)
<i>incP-1ε/trfA</i>	5'ACGAAGAAATGGTTG TCCTGTTCT	5'CGTCAGCTTGCGGTA CTTCTC	5'FAM- CCGGCGACCATTACAG CAAGTTCATTT-TAMRA	60	Heuer et al. (2012)

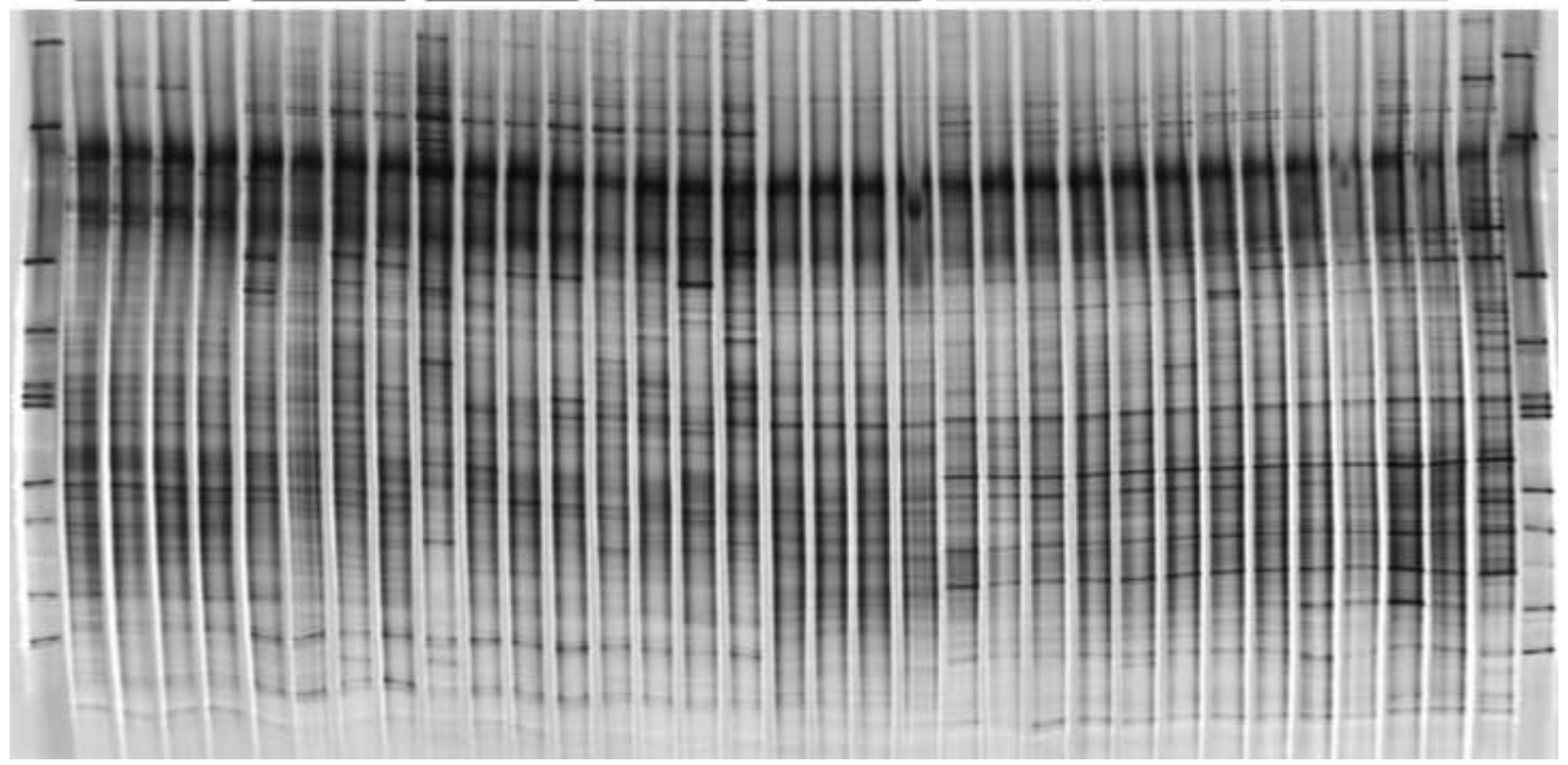
Table 2 Percent difference (d-values) of soil bacterial community structure based on Pearson correlations of background-subtracted densitometric curves from DGGE analysis (60 days after the first, second and third amendment). Significance is indicated by the respective *P*-value.

		CL soil		SA soil	
		d-value	<i>P</i>	d-value	<i>P</i>
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

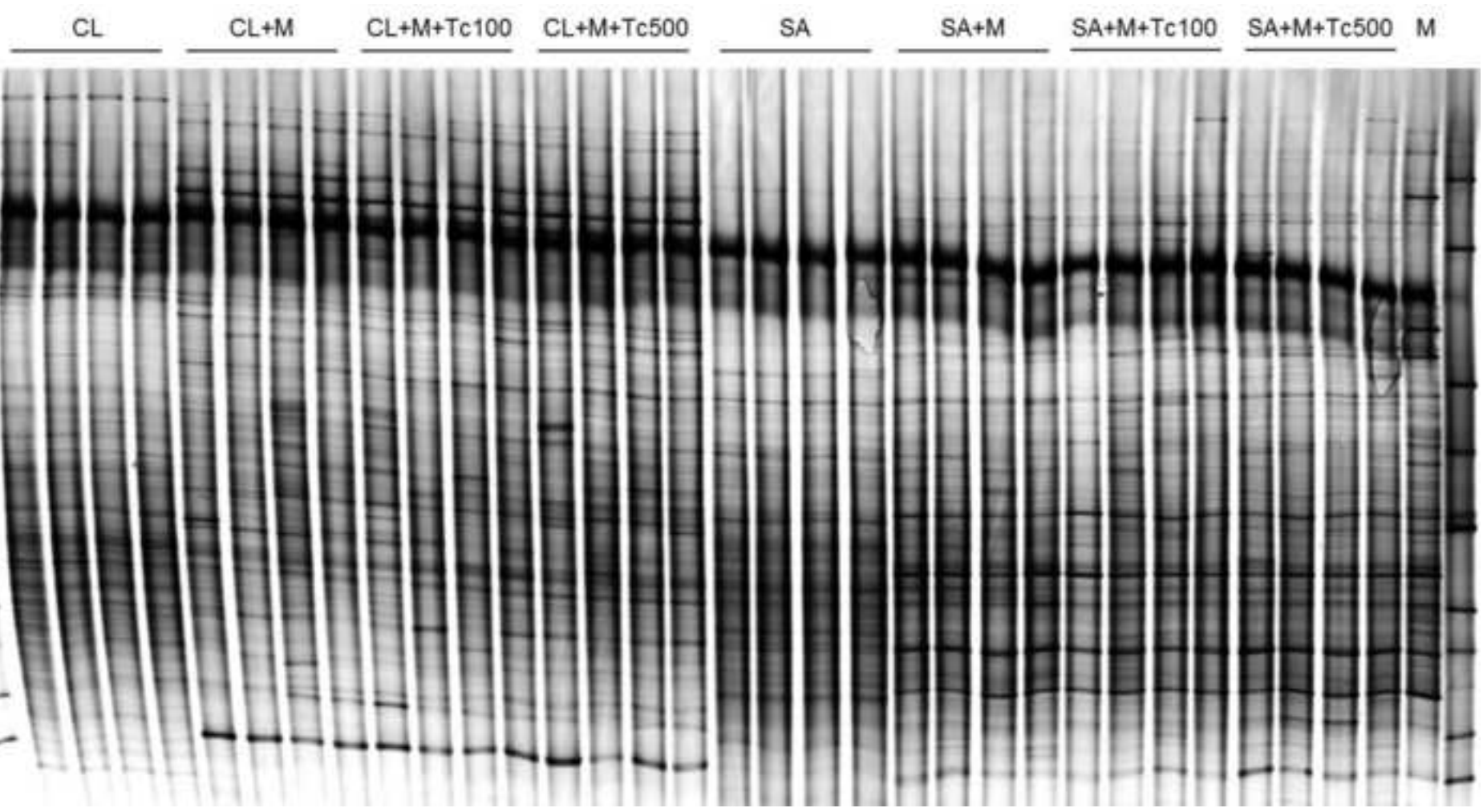
S: soil CL or SA; M: manure; Tc100: 100 mg Tc kg⁻¹ soil dry weight; Tc500: 500 mg Tc kg⁻¹ soil dry weight

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

CL CL+M CL+M+Tc100 CL+M+Tc500 SA SA+M SA+M+Tc100 SA+M+Tc500 M



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49

CL CL+M CL+M+Tc100 CL+M+Tc500 SA SA+M SA+M+Tc100 SA+M+Tc500 M

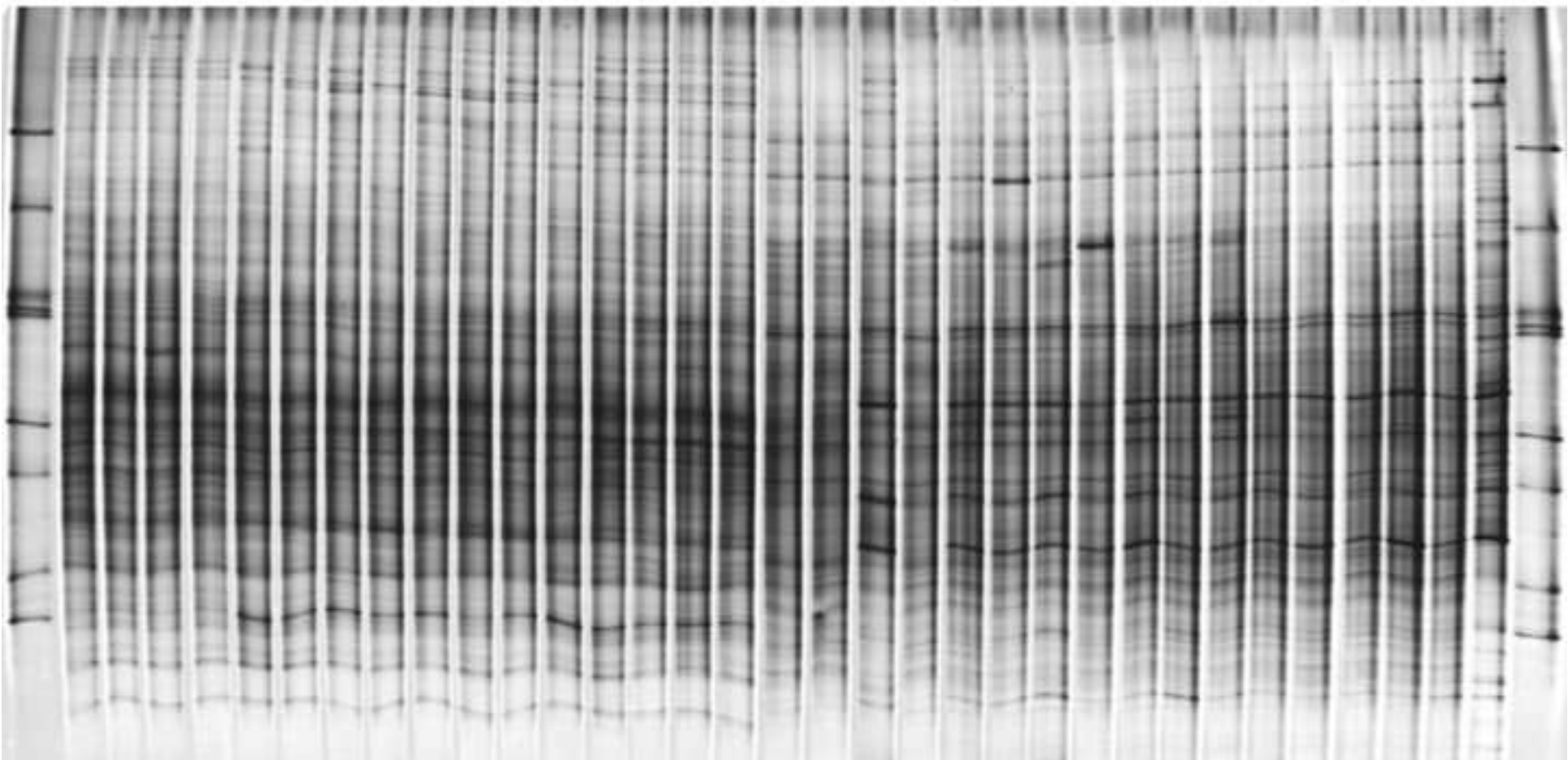


Table S1 Selected physical and chemical properties of the soils and manure investigated. From Chessa et al. (2016).

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65