

Soil microbial response to tetracycline in two different soils amended with cow manure

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1 Soil microbial response to tetracycline in two different soils amended with cow manure

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14 **Abstract**

15 High amounts of antibiotics are introduced in the soil environment by manure amendment, which is
16 the most important spreading route in soil, with a potential ecotoxicological impact on the
17 environment. The objectives of this study were a) to assess the tetracycline (Tc) bioavailability in a
18 clay and in a sandy soil, and b) to evaluate the effects of the Tc and cow manure on the structure
19 and function of soil microbial communities. Clay and sandy soils were spiked with Tc at the
20 concentrations of 100 and 500 mg Tc kg⁻¹ soil, and were amended or not with cow manure. The
21 clay soil showed greater Tc sorption capacity and bioavailable Tc was between 0.157 and 4.602 mg
22 kg⁻¹ soil. Tc-dose and time-dependent effects on soil microbial communities were investigated by
23 fluorescein diacetate activity, phospholipid fatty acids analysis, as well as by Biolog community
24 level physiological profile and microbial counts at 2, 7 and 60 days after Tc and/or manure addition.
25 The added Tc caused detrimental effect on the microbial activity and structure, particularly in the
26 short term at the highest concentrations. However, the Tc effect was transient, it decreased after 7
27 days and totally disappeared within 60 days. Cow manure shifted the bacterial structure in both
28 soils, increased the microbial activity in clay soil and contributed to recover the microbial structure
29 in Tc spiked manure treatments.

30

31 **Keywords**

32 Tetracycline; Bioavailability; Soil; Cow manure; Antibiotic ecotoxicological effect; Microbial
33 activity; Microbial fingerprint

34 **1. Introduction**

35 Antibiotics are widely used to maintain human and animal health, as well as to prevent and treat
36 infectious diseases (Sarmah et al. 2006). Although since January 1st 2006 the use of antibiotic as
37 growth promoters in animal husbandry was banned in the European Union (EC 1831/2003), it is
38 still a common practice in many countries including China (Zhu et al. 2013), USA and Canada
39 (Kim et al. 2011). Generally, from 30 to 90% of antibiotic dose administered is evacuated as
40 unchanged form (Sarmah et al. 2006) and these residues, introduced into the environment through
41 manure amendment, can affect the soil microbial communities by changing their structure and
42 function (Aminov and Mackie 2007).

43 Tetracycline (Tc) is one of the most used antibiotics in livestock practice due to its high
44 pharmacological activity and solubility in water (Chopra and Roberts 2001; Nelson and Levy 2011)
45 and each year thousands of tons of Tc are administered to animals (European Drugs Agency 2012;
46 Kümmerer 2001). Tc is an ionisable molecule which presents three acid dissociation constants
47 (Wang et al. 2010). Therefore, the pH value of the soil solution determines Tc speciation, which can
48 directly affect the Tc bioavailability in the soil environment (Sarmah et al. 2006). Studies carried
49 out on organic and inorganic soil colloids (Gu et al. 2007; Jutta et al. 2007) indicated that Tc
50 interacts strongly with clay minerals and, under acidic conditions, cation exchange is the primary
51 sorption mechanism, although its relevance decreases with increases pH (Li et al. 2010). Again, the
52 Tc sorption on soil particles influences the amount of the free Tc, namely the bioavailable form in
53 soil and, consequently, the antibiotic effects on the microbial communities (Kong et al. 2012).
54 However, whether the sorbed Tc can still maintain its antimicrobial activity or not, is a subject of
55 some debate. The Tc free concentration measured in soil is variable and depends on the soil
56 properties (Jia et al. 2008). Hamscher et al. (2002) measured Tc concentrations in topsoil ranging
57 from 0.086 to 0.171 mg kg⁻¹; moreover, Zhu et al. (2013) reported Tc values of about 0.78 mg kg⁻¹
58 and Winckler and Grafe (2000) estimated Tc concentrations from 0.45 to 0.9 mg kg⁻¹. Anyhow,
59 even low concentrations of bioavailable Tc may have a negative impact on the resident microbial
60 communities in soil (Thiele-Bruhn 2005). Several studies investigated the effects of Tc residues on
61 the soil microbial functions (Ding and He 2010; Thiele-Bruhn 2005; Wei et al. 2009; Yang et al.
62 2010), but information on the relationship among physico-chemical soil properties, Tc
63 bioavailability and Tc effects on soil microbial communities are scarce. Moreover, Tc reaches the
64 soil environment by addition of contaminated manure and most studies were focused on the effects
65 of piggery manure addition, as organic fertilisers, on the soil microbial communities. However in
66 some regions cow's breeding is more relevant than pig's one and the fertilisation with cow manure
67 is generally used.

68 In this scenario, in order to better understand the potential impact of Tc on the environment and its
69 ecotoxicological implications, it is important to evaluate the influence of manure amendment for the
70 environmental fate of antibiotics. Therefore, the present study is aimed to investigate the sorption of
71 Tc on two soils with different physical-chemical characteristics amended or not with cow manure,
72 with the goal to evaluate if the different Tc bioavailability can lead to different effects on the
73 structure and function of soil microbial communities.

74 75 **2. Materials and methods**

76 **2.1 Experimental design**

77 Two topsoils were sampled in Sardinia (Italy): the clay PU soil and the sandy SA soil. PU is a forest
78 soil and was never used for crop/pasture production or intensive farming. SA soil was collected
79 from a dry river bed in the centre of Sassari. Since several decades it was used for orchard
80 cultivation and no organic fertilisers or antibiotics were applied. However, one year before the
81 sampling SA soil was exposed to anthropogenic waste during a flooding period. The cow manure
82 used in the experiment was collected from a Sardinian (Italy) cattle farm and stored in the dark for
83 one year at room temperature. The manure was free from any antibiotics, since no antibiotics were
84 administered to the animals.

85 Triplicate samples (500 g each) of sieved (<2 mm) PU and SA soils were independently treated
86 with an aqueous Tc (Sigma-Aldrich, Milano, Italy) solution to reach a final concentration of 100
87 (Tc100) and 500 (Tc500) mg Tc kg⁻¹ soil and 50% of their maximum water-holding capacity. The
88 samples (PU/SA+Tc) were carefully mixed and incubated in the dark at 20°C until microbial and
89 biochemical analyses. Similarly, triplicate PU and SA soil samples (500 g each) were amended with
90 20 g of cow manure (4% w/w) previously contaminated with a Tc solution freshly prepared and
91 mixed for 1 h in the dark before addition to soil (PU/SA+M+Tc) whereas further samples (500 g
92 each) were solely amended with uncontaminated manure (PU/SA+M) or left untreated (PU/SA
93 soils). These untreated soils received only water before incubation at 20°C. The soil water content
94 of all samples was kept constant by water spraying on soil surface every two days. Soil aliquots
95 from each sample (a total of 50 g collected at each time-point) were used for microbiological and
96 chemical analyses, which were carried out at three time-points namely 2, 7 and 60 days after
97 incubation.

98 99 **2.2 Soil characterization and adsorption-desorption analysis**

100 Soil samples were collected at a depth of 10 cm, air-dried and sieved to <2 mm and the particle size
101 distribution was measured by the pipette method (Day 1965). The organic carbon content was

102 determined according to the modified Walkley-Black method (Jackson 1958). Soil pH was
103 determined on slurries with a soil/water ratio of 1:2.5.

104 Sorption trials were carried out using a batch equilibration technique at $25\pm 2^\circ\text{C}$. Tc sorption was
105 measured on unamended and manure-amended soils sieved through a 2 mm mesh screen. Triplicate
106 2.5 g samples were equilibrated in polyallomer centrifuge tubes with 5 mL of aqueous antibiotic
107 solution ($122.5\ \mu\text{M}$ corresponding to $100\ \text{mg Tc kg}^{-1}$ dry soil and $602.6\ \mu\text{M}$ corresponding to 500
108 mg Tc kg^{-1} dry soil respectively).

109 Preliminary batch kinetic studies indicated that the equilibrium is reached within 30 min and no
110 changes in concentration occurred after 24 h of shaking. After equilibration, the suspension was
111 centrifuged at $5,000\ g$ for 30 min, and the supernatant was pipetted off and analysed immediately.
112 The Tc amount retained by soil was calculated from the difference between the initial and final
113 concentrations of Tc in solution.

114 The concentration of Tc was determined by HPLC. All the solvents were of HPLC grade (Carlo
115 Erba Reagenti, Milan, Italy) and were used without further purification. The system was assembled
116 as follows: a Waters 1515 pump equipped with a Waters 2487 UV/VIS programmable detector
117 operating at 254 nm wavelength, a Breeze chromatography software, a $\mu\text{Bondapak C}_{18}$ analytical
118 column ($10\ \mu\text{m}$, $3.9 \times 300\ \text{mm}$). The mobile phase was a water-acetonitrile mixture (77:23, $\text{pH} = 2.5$)
119 at a flow rate of $0.7\ \text{mL min}^{-1}$. The Tc retention time under these chromatographic conditions was
120 7.98 min. The quantitative determination of Tc was performed using an external standard.
121 Calculations were based on the average peak areas of the external standard. The detection limit for
122 Tc was $0.1\ \text{mg L}^{-1}$, namely the antibiotic concentration corresponding to a detector response
123 approximately twice the background signal. Sorption data were fitted to sorption distribution
124 coefficient $K_d = C_s C_e^{-1}$ where C_s ($\mu\text{mol kg}^{-1}$) is the amount of the antibiotic adsorbed, C_e (μM) is
125 the equilibrium concentration in solution.

126

127 **2.3 Hydrolysis of fluorescein diacetate**

128 At each time point, triplicate samples from each soil (2 g fresh weight, sieved $< 2\ \text{mm}$) were added
129 with 15 mL of 60 mM potassium phosphate buffer ($\text{pH} 7.6$) and 0.2 mL of $1\ \text{mg mL}^{-1}$ of fluorescein
130 diacetate (FDA) (Sigma Aldrich, Milano, Italy) stock solution into a 50 mL conical flask. Blanks
131 were prepared without the addition of the FDA. The flasks were then incubated at 30°C for 20 min
132 at 150 rpm. The reaction was stopped by adding 15 mL of acetone and the soil suspension
133 centrifuged at 4,000 rpm for 10 min. The supernatant was then filtered (Whatman, No. 2) and the
134 optical density at 490 nm wavelength measured with a SPECTROstar^{Nano} (BMG Labtech, GmbH,
135 Offenburgh, Germany) spectrophotometer. The concentration of fluorescein released during the

136 assay was calculated using the calibration curve produced from 0 to 10 $\mu\text{g mL}^{-1}$ with Fluorescein
137 Sodium Salt (Sigma-Aldrich, Milano, Italy).

138

139 **2.4 Phospholipid fatty acids analysis**

140 The extraction, identification and quantification of phospholipid fatty acids (PLFA) from soil were
141 performed according to the method of Gutiérrez et al. (2010), while the two-step methylation was
142 carried out according to Kramer et al. (1997) and Jenkins (2010). PLFA were analysed using a gas
143 chromatograph GC Turbo 3400 CX (Varian Inc. Palo Alto, CA) equipped with a capillary column
144 (CP-select CB for Fame, 100 m \times 0.32 mm i.d., 0.25 μm film thickness; Varian Inc., Palo Alto, CA,
145 USA), a flame ionization detector and an automatic sample injector 8200 (CX Varian Inc. Palo
146 Alto, CA). The column head pressure was set at 37.00 psi. Quantification was based on the internal
147 standards method. PLFA were assigned to taxonomic groups based on recent literature (Hackl et al.
148 2005). Terminal-branched saturated PLFA a15:0, i15:0, i16:0, i17:0, and a17:0 were used as
149 markers for Gram-positive bacteria (PLFA_{g+}) while Gram-negative bacteria (PLFA_{g-}) were
150 quantified by monounsaturated PLFA (16:1 ω 7c, 18:1 ω 7c, 18:1 ω 9c) and cyclopropyl saturated
151 PLFA (cy17:0, cy19:0). The sum of signature PLFA for Gram-positive and -negative bacteria is
152 referred to as bacterial PLFA (PLFA_{bact}). The quantity of the PLFA 18:2 ω 6,9 was used as an
153 indicator of fungal biomass. Similarly to a number of recent reports, in this study we used cis-9-
154 octadecenoic acid (18:1 ω 9c) as biomarker for Gram-negative bacteria (Gutiérrez et al. 2010;
155 Hammesfahr et al. 2011a; Hund-Rinke et al. 2004; Schoug et al. 2008; Wang et al. 2015; Whalen
156 and Sampedro 2009), despite in some studies the same fatty acid was used as a fungal biomarker
157 (e.g. (Demoling et al. 2009; Dong et al. 2014).

158

159 **2.5 Community level physiological profiles**

160 The community level physiological profiles (CLPPs), or the carbon source utilization pattern, was
161 determined for each microbial community extracted from treated and untreated soils using Biolog
162 EcoPlatesTM (Biolog Inc., Hayward, CA). Briefly, 20 mL aliquots of the respective 100-fold
163 dilutions prepared as for the enumeration of total fast-growing heterotrophic bacteria and fungi (see
164 paragraph 2.6) were centrifuged (2,600 g, 8 min) and supernatant was filtered with Whatman filter
165 paper 42 (Whatman, Maidstone, UK) and 120 μL of the clear supernatant were inoculated each in
166 96 microtiter wells of the Biolog EcoPlateTM. Inoculated plates were incubated in the dark at 28°C
167 for 7 days and the OD at 590 nm wavelength was measured every 24 h using a Biolog
168 MicroStationTM reader (Biolog Inc., Hayward, CA).

169

170 **2.6 Enumeration of heterotrophic bacteria, actinomycetes and fungi**

171 Total fast-growing heterotrophic bacteria, actinomycetes and fungi were enumerated using
172 conventional serial dilution and spread plate method. Solidified (15 g L⁻¹ agar) 1:10 strength TSA
173 (Tryptic Soy Agar, Microbiol, Cagliari, Italy), Actinomycetes Isolation Agar (DiFCO, Milan, Italy)
174 and GYEP pH 4.5 (Glucose Yeast Extract Peptone medium) (Garau et al. 2007) were used as the
175 growth media for counting total heterotrophic bacteria, actinomycetes and fungi, respectively.

176 At each time-point, soil samples (20 g) from treated and untreated soils were dispersed in 180 mL
177 of a sodium pyrophosphate solution (2 g L⁻¹) and shaken at 150 rpm for 30 min. Serial 10-fold
178 dilutions were then prepared using saline solution (0.89% w/v NaCl) and 150 µL aliquots of each
179 dilution (the highest used was 10⁻⁵) were spread on a quadruplicate set of plates containing the
180 respective media. Bacterial, actinomycete and fungal colonies were counted on the relevant media
181 after incubation of the plates at 28°C for 3 days and microbial counts expressed as average Log
182 Colony Forming Units (CFUs) per gram of soil dry weight.

183

184 **2.7 Statistical analysis**

185 For each soil and incubation time, mean values from FDA activity, PLFAs and microbial counts
186 were subjected to One-Way Analysis of Variance (One-Way ANOVA) to evaluate the effect of the
187 different treatments applied. Where significant *P*-values (*P*<0.05) were obtained, differences
188 between individual means were compared using the post hoc Tukey-Kramer test (*P*<0.05). The
189 results are given as the means ± standard error (SE). The One-Way Analysis of Variance (One-Way
190 ANOVA) was also used to compare mean values within the treatments comparing the mean values
191 of the tree sampling points, i.e. 2, 7 and 60 days after treatments addition to soil. The analysis of
192 CLPP data was carried out by Principal Components Analysis (PCA) from the average well colour
193 development (AWCD), based on the correlation matrix to reduce complex multidimensional data.

194

195 **3. Results and discussion**

196 **3.1 Tetracycline sorption**

197 The microbiological effect of Tc in soil depends on its bioavailability, which in turn is influenced
198 by sorption-desorption processes. In this study, the Tc affinity for two soils, i.e. an acidic clay soil
199 (PU) and an alkaline sandy soil (SA), was investigated. Furthermore, to simulate an agricultural
200 manure spreading the two soils were amended with Tc spiked cow manure at two concentrations
201 (100 and 500 mg Tc kg⁻¹ soil). The amount of manure added to soils was calculated, based on the N
202 content in manure (Table 1), within the limit of 340 kg N ha⁻¹ per year according to the Nitrate
203 Directive 91/676/EEC.

204 The Tc sorption in soils without cow manure was very fast and apparently irreversible and no
205 measurable amount of desorbed Tc was found from both unamended and amended soils under the
206 studied pH conditions suggesting that Tc remains tightly bound to soil and did not desorb readily
207 under investigated experimental conditions, according to Wan et al. (2010). The K_d parameter,
208 which expresses the Tc affinity for the soil was about 5-fold higher for the acidic clay PU soil than
209 for the alkaline sandy SA soil (Table 2). Most likely, this is due to two combined effects, i.e. the
210 lower pH value and the higher clay content of the PU soil. The findings confirm that Tc has a strong
211 tendency to bind to clay minerals (Li et al. 2010) and that Tc sorption on soil particles decreased
212 with increased pH (Zhang et al. 2014). The addition of manure did not modify substantially the Tc
213 sorption in both soils (Table 2), thus supporting the predominant role of clay, compared to the
214 organic matter, in Tc binding to soil.

215 Tc concentrations used to pollute the soils were 100 and 500 mg kg⁻¹ soil, respectively. The residual
216 amount of free Tc in solution ranged from 0.155 to 0.859 mg kg⁻¹ soil (Table 2) for the spiked Tc
217 100 mg kg⁻¹ and represents the bioavailable Tc potentially active against soil bacteria. In effect, the
218 Tc concentrations detected in topsoil and reported in literature range between 0.086 (Hamscher et
219 al. 2002) and 0.9 mg kg⁻¹ (Winckler and Grafe 2000) and are comparable to those found in the
220 present work. However, at the spiked Tc concentration of 500 mg kg⁻¹ we found bioavailable Tc
221 ranging from 1.092 to 4.602 mg kg⁻¹. These concentrations are clearly higher than those normally
222 found in the soil environment. However, according to Hund-Rinke et al. (2004), they can be useful
223 in order to highlight appreciable Tc effects on the microbial communities, otherwise not clearly
224 detectable.

225

226 **3.2 Hydrolysis of fluorescein diacetate**

227 The FDA hydrolysis has been utilised to estimate the overall microbial activity in soil (Sánchez-
228 Monedero et al. 2008; van Elsas et al. 2007), i.e. the activity of lipases, proteases and unspecific
229 esterases (Adam and Duncan 2001). The concentration of fluorescein was much higher in SA than
230 PU soil and revealed a clear influence of Tc on the microbial activity of the whole microbial
231 community of both soils (Fig. 1). This is similar to what reported by Thiele-Bruhn and Beck (2005)
232 about the effect of antibiotics on soil microbial activities at concentrations comparable to those
233 tested in this work. In particular, after 2 days microbial activity of PU soil was significantly
234 ($P<0.05$) reduced by Tc presence, although it did not appear strictly dependent to Tc bioavailability.
235 Indeed, no significant differences were observed between PU+Tc 0.157 mg Tc kg⁻¹ and PU+Tc
236 1.201 mg Tc kg⁻¹ soil. The addition of manure in PU+M stimulated microbial activity, compared to
237 control PU, and nullified the effect of Tc in spiked manure treatments, which did not significantly

238 differ from control PU. More likely, manure addition revitalised and stimulated PU soil microbial
239 population (Das and Adhya 2014; Lundquist et al. 1999) by supplying easily metabolisable carbon
240 sources (Parham et al. 2003). After 7 days, the effect of Tc and manure definitely disappeared in PU
241 soil, and the microbial activity was completely restored. This trend was confirmed after 60 days.
242 In SA soil after 2 days, Tc caused negative effects and the decrease in FDA hydrolysis was related
243 to Tc bioavailability. In fact, compared to control, the microbial activity was reduced in SA+Tc
244 0.859 mg kg⁻¹, but the greatest effect was observed in the presence of 4.602 mg Tc kg⁻¹ soil (Fig.
245 1b). Contrary to PU soil, the addition of cow manure did not stimulate FDA hydrolysis, although
246 restored the microbial activity in SA+M+Tc 0.767 mg kg⁻¹ soil. After 7 days the Tc effect seemed
247 to be reduced and the detrimental impact of the antibiotic was detected only at the highest Tc
248 concentration 4.602 mg kg⁻¹ soil, compared to control SA. In SA soil the Tc perturbation was a bit
249 longer than in PU soil, probably due to the highest Tc bioavailability. Moreover, within 60 days in
250 both PU and SA control soils the FDA hydrolysis significantly ($P<0.05$) decreased over time,
251 owing to a plausible reduction of available carbon sources for soil microbial communities (Schnürer
252 and Rosswall 1982).

253

254 **3.3 Phospholipid fatty acids analysis**

255 The PLFA analysis is a suitable tool for monitoring shifts in the structure of overall microbial
256 communities in soil (Ding and He 2010; Frostegård et al. 2011). It is based on fingerprint referred
257 to all living cells at the specific time of extraction and involves the most part of the bacterial and
258 fungal communities in soil (van Elsas et al. 2007).

259 After 2 days, in both PU and SA soils we observed a significant ($P<0.05$) increase in the
260 fungi/bacteria ratio (F/B) after addition of the sole Tc (Fig. 2a, b). This finding suggests that Tc had
261 unfavourable impact on soil bacterial communities of both soils. In PU soil, this increase was
262 proportional to bioavailable Tc whereas in SA soil the F/B seemed unrelated from the bioavailable
263 Tc concentration. Likely in SA soil the bioavailable Tc concentration 0.859 mg kg⁻¹ was sufficient
264 to induce the greatest effect and no additional Tc effects on the F/B were observed at higher Tc
265 bioavailable concentrations. The influence of Tc was strong in the early stage (2 days) but missed
266 after 7 days incubation in all soil treatments suggesting a short term Tc perturbation. Indeed the
267 effect was only transient and the whole microbial communities restored their initial equilibrium. On
268 the contrary, some authors (Hund-Rinke et al. 2004; Thiele-Bruhn and Beck 2005) observed
269 significant effects on the F/B up to 2 months after the amendment.

270 The addition of unspiked cow manure was ineffective and, compared to the respective controls, did
271 not change the F/B in both soils; conversely, other authors (Hammesfahr et al. 2008; Marschner et

272 al. 2003) reported significant increases in the F/B after manure addition. In the present work,
273 probably, the manure equally stimulated bacteria and fungi in soil and for this reason the F/B was
274 not affected. The role of manure was also important in the Tc spiked manure treatments of PU soil
275 where the F/B did not statistically ($P<0.05$) differ from control, thus suggesting a role of cow
276 manure on nullifying Tc effect, probably by carrying Tc resistant bacteria (Binh et al. 2008;
277 Kyselková et al. 2015). In fact in Tc spiked manure treatments the bioavailable Tc fraction was not
278 so different from Tc spiked soils (Table 2). Conversely, in SA soil the cow manure only reduced the
279 impact of the antibiotic in Tc spiked manure treatments, likely due to very high Tc bioavailability
280 especially in SA+M+Tc 4.468 mg kg⁻¹. According to Hammesfahr et al. (2011b), the manure plays
281 an important role in nullifying or mitigating the Tc impact in soils. However, the mitigating effect
282 of manure in SA soil was remarkable and reflected the trend observed for FDA hydrolysis in this
283 soil. The increase of the F/B was the consequence of bacterial population decrease, due to a
284 bacteriostatic effect exerted by Tc. Moreover, within the 60 days of incubation the F/B was reduced
285 in all treatments spiked with Tc, with and without manure, suggesting a loss of the biological
286 activity of Tc.

287 Since Tc affects the bacterial population, the antibiotic effect on Gram-positive and Gram-negative
288 bacteria (Fig. 2c, d) was monitored and expressed as Gram-positive/Gram-negative PLFA ratio
289 (G^+/G^-). Two days after Tc addition the antibiotic was effective, only on PU soil at the highest
290 bioavailable concentration (1.201 mg Tc kg⁻¹ soil), at increasing the G^+/G^- , probably reducing the
291 Gram-negative bacteria. This effect was transient and disappeared after 7 days. Although in SA soil
292 the Tc bioavailability was higher than PU soil, Tc did not affect the G^+/G^- suggesting that probably
293 Gram-positive and Gram-negative bacteria in SA soil could be equally susceptible to Tc. The
294 addition of manure to PU soil increased in long term the G^+/G^- (up to 60 days) in agreement with
295 other authors (Ai et al. 2012; Marschner et al. 2003), which tested the effect of manure amendment
296 on microbial communities in bulk soil. This G^+/G^- increases in PU soil was attributable to Gram-
297 positive bacteria supplied with cow manure, which were also responsible for the increase of G^+/G^-
298 in Tc spiked manure treatments, compared to control. In SA soil the effect of unspiked manure was
299 shorter than in PU soil and was detected up to 7 days. In this soil, the effect of cow manure on
300 carrying Gram-positive bacteria was clear, but any Tc effect was observed (Fig. 2d).

301 The effect of manure and Tc was tested on the whole actinomycete population by PLFA analysis to
302 better understand the effect of treatments on these bacteria, which are antibiotic producers.
303 Compared to control soils, the addition of Tc at the highest concentrations and of manure
304 significantly ($P<0.05$) increased the abundance of actinomycetes in both soils (Fig. 2e, f). When
305 added together, Tc likely selected for resistant actinomycetes while manure stimulated their growth

306 (Dong et al. 2014) resulting in a synergic effect of both Tc and manure on actinomycete bacteria.
307 The Tc and manure capacity of increase actinomycetes was the greatest in the short period (2 days)
308 then progressively decreased ($P<0.05$) in both soils and after 60 days no significant differences
309 were observed among treatments.

310 In general, PLFA data are in agreement with those obtained by FDA hydrolysis. In particular, the
311 antibiotic effect was more evident after 2 days at the higher Tc concentration, in both soils, and
312 decreased over time until disappear completely after 60 days.

313

314 **3.4 Community level physiological profiles**

315 In PU soil after 2 days, Principal Components Analysis (PCA) of carbon source utilization data
316 underlined a cluster effect of the highest Tc concentration and of manure (Fig. 3). Control PU soil
317 clustered with PU+Tc100, PU+M+Tc100 and PU+M+Tc500 treatments, suggesting a similar
318 carbon source utilization pattern, but after 7 days the effect of Tc disappeared and the functional
319 potential of the microbial community was comparable to control PU soil. On the contrary, PU+M
320 still showed a distinct metabolic pattern from control soil. Moreover, all manure treatments formed
321 single clusters according to Tc spiking. At the following time-point (60 days) the microbial
322 consortia of PU soil progressively differentiated their catabolic profiles in four well-defined clusters
323 depending on Tc dose supplied and manure addition. In SA soil after 2 days of incubation the
324 treatments with the Tc500 treatments clustered highlighting a strong Tc effect at the highest dose.
325 The addition of manure did not reveal changes in microbial communities of SA soil and neither
326 reduced the Tc effect in spiked manure. After 7 days the scenario was comparable to the previous
327 time point. The PCA analysis evidenced a more obvious separation between the treatments with
328 higher Tc dose (Tc500 and M+Tc500) but after 60 days all effects disappeared in SA soil.

329 On the whole, CLPPs data suggest that microbial communities from the two soils were both
330 susceptible, in the short term, to Tc action at the highest concentration (Tc500 mg kg⁻¹). In SA soil
331 the effect was more evident and persisted up to 7 days, according to results of FDA hydrolysis assay
332 (Fig. 1b), probably due to higher Tc bioavailability in this soil.

333 As far as the manure is concerned, both unspiked and Tc spiked manure differentiated in long term
334 (60 days) the CLPPs of PU soil. A similar trend was observed for the PLFA G⁺/G⁻ ratio where
335 manure, both Tc unspiked and Tc spiked, shifted the structure of the bacterial population for up to
336 60 days. On the contrary, in SA soil manure did not influence the microbial metabolic pattern.
337 CLPP analysis through Biolog EcoplatesTM is able to distinguish the physiological pattern among
338 complex heterotrophic bacterial communities in soil. Although CLPP investigates only the
339 culturable portion of the total bacteria in soil, which is estimated to contribute in about 0.01 – 1% of

340 total bacteria (Bakken 1997; Richaume et al. 1993; Torsvik et al. 1990), it allows to observe the
341 effect of treatments on the soil bacterial communities based on their catabolic potential (Garland
342 1997; Insam et al. 1996). Therefore, the investigation of changes on the bacterial structure assessed
343 by both CLPPs and PLFA gives similar information about the impact of Tc and manure on soil and,
344 if used together, they could be helpful to understand shifts in complex microbial communities.

345

346 **3.5 Microbial counts**

347 The effect of Tc and manure on the culturable portion of fast growing bacteria, fungi and
348 actinomycetes of PU (Table 3) and SA (Table 4) soils was investigated. After 2 days, in PU soil the
349 bacterial number significantly ($P<0.05$) decreased after the addition of solely Tc at the
350 concentration 500 mg kg^{-1} soil. At the same time point, the addition of manure stimulated bacterial
351 growth in PU+M and nullified the antibiotic effect in PU+M+Tc500 treatment, which was
352 significantly lower than PU+M and comparable to control soil. After 7 days the antibiotic effect
353 completely disappeared in PU+M+Tc500, which did not significantly differ from PU+M.
354 Moreover, the stimulatory effect of manure was still operating in contaminated manure treatments
355 where the bacterial number was higher than control PU. Actually, manure stimulated culturable
356 bacteria up to 60 days both in unspiked and Tc spiked manure treatments. The fungal population in
357 PU soil was never influenced by Tc and/or manure and no significant differences were found
358 among treatments at each time point. Finally, as expected on the basis of PLFA analysis, the
359 addition of Tc spiked manure induced increase in the number of actinomycetes in PU soil at all the
360 time points.

361 In SA soil after 2 days from the Tc addition, only the highest Tc concentration (SA+Tc500) reduced
362 the bacterial number (Table 4). On the other hand, the added manure stimulated the bacterial
363 population and completely nullified the Tc effect in contaminated manure treatments. Both effects
364 were transient and by the 7 days time point onwards they were no longer detectable. In SA soil after
365 2 days since Tc addition, fungi were not influenced by the treatments. However, after 7 days in
366 SA+Tc500 and SA+M+Tc500 fungi increased likely as the result of easily metabolisable carbon
367 sources released from dead bacterial cells sensitive to Tc (Vaclavik et al. 2004). The actinomycetes
368 in SA soil were stimulated by both Tc and manure by synergic and additive effect. However, the Tc
369 activity rapidly disappeared and after 7 days only manure effect was observed. After 60 days no Tc
370 or manure effect were detectable.

371 Tc is a bacteriostatic antibiotic and exerts its effect on bacteria by interfering with the 30S ribosome
372 subunit (Nelson and Levy 2011). The bacterial counts, together with other culturable and
373 unculturable-dependent methods assessed in this work confirmed a short term effect of Tc in both

374 soils especially at higher doses of the antibiotic. As expected, Tc did not influence fungal
375 population but clearly stimulated actinomycete bacteria, antibiotic-producers. In fact, about two-
376 thirds of the antibiotics isolated from the environment are produced by culturable actinomycetes
377 (Basil et al. 2004). More specifically, since actinomycetes are able to determine the composition of
378 microbial populations (Bull et al. 1992; Du and Liu 2012; Heuer et al. 1997), due their antibiotic
379 production, they could be used as indicators of Tc effect in soil. Indeed we observed that Tc
380 stimulated the resistant actinomycetes and manure was able to boost the actinomycete population by
381 supplying easily metabolisable carbon sources, according to what reported by other authors (Gong
382 et al. 2009; Mokni-Tlili et al. 2009; Yu et al. 2010).

383 Overall, microbial counts are in good agreement with the results of the different analyses carried out
384 in the present work. Since the culturable microorganisms just represent a small portion of total
385 microbial soil community, the deviations observed from this general trend, for instance PLFA
386 analysis, could be due to contribution of the whole microbial community. Moreover, the culturable
387 bacteria in PU soil were influenced in the long term by manure but not in the SA soil, where manure
388 effect was only detectable up to 2 days, and this was in general agreement with CLPPs data. Since
389 the culturable portion of bacteria is more influenced by exogenous input (Bulluck III et al. 2002)
390 than unculturable bacteria (Stewart 2012), the carbon sources added through manure most likely
391 stimulated in the long term the culturable bacteria in PU soil, probably due to a priming effect. The
392 PLFA data obtained for PU soil, instead, seem to suggest the absence of any stimulatory effect of
393 manure on bacteria (fungi/bacteria ratio), although it should be noted that PLFA analysis concerns
394 the behaviour of total microbial community while bacterial counts regard only the culturable
395 fraction.

396

397 **4. Conclusions**

398 The results of the present study suggest that the Tc bioavailability is influenced by soil chemical-
399 physical properties. Tc only transiently influences the structure and function of the microbial
400 communities. In particular, the Tc effect was evident in the short time then progressively
401 disappeared within two months. Interestingly, the addition of cow manure increases the microbial
402 activity and changes the structure of the bacterial communities in soils. Moreover, cow manure
403 seems to reduce the antibiotic inhibitory effects and the shifts in the composition of soil microbial
404 communities, while contributing to the environmental soil recovery.

405

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410 Activity I.3.1.).

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412 **References**

413

- 414 Adam G, Duncan H (2001) Development of a sensitive and rapid method for the measurement of total microbial
415 activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biol Biochem* 33:943-951
416 doi:10.1016/S0038-0717(00)00244-3
- 417 Ai C, Liang GQ, Sun JW, Wang XB, Zhou W (2012) Responses of extracellular enzyme activities and microbial
418 community in both the rhizosphere and bulk soil to long-term fertilization practices in a fluvo-aquic soil.
419 *Geoderma* 173:330-338 doi:10.1016/j.geoderma.2011.07.020
- 420 Aminov RI, Mackie RI (2007) Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol Lett* 271:147-161
421 doi:10.1111/j.1574-6968.2007.00757.x
- 422 Bakken LR (1997) Culturable and nonculturable bacteria in soil. In: van Elsas JD, Trevors JT (eds) *Modern soil*
423 *microbiology*. Wellington, E.M.H. (Eds.), Marcek Dekker, New York, N.Y., pp 47-61
- 424 Basil AJ, Strap JL, Knotek-Smith HM, Crawford DL (2004) Studies on the microbial populations of the rhizosphere of
425 big sagebrush (*Artemisia tridentata*). *J Ind Microbiol Biotechnol* 31:278-288 doi:10.1007/s10295-004-0140-y
- 426 Binh CT, Heuer H, Kaupenjohann M, Smalla K (2008) Piggery manure used for soil fertilization is a reservoir for
427 transferable antibiotic resistance plasmids. *Fems Microbiol Ecol* 66:25-37 doi:10.1111/j.1574-
428 6941.2008.00526.x
- 429 Bull AT, Goodfellow M, Slater JH (1992) Biodiversity as a Source of Innovation in Biotechnology. *Annu Rev*
430 *Microbiol* 46:219-252
- 431 Bulluck III LR, Brosius M, Evanylo GK, Ristaino JB (2002) Organic and synthetic fertility amendments influence soil
432 microbial, physical and chemical properties on organic and conventional farms *Applied Soil Ecology* 19:147-
433 160 doi:doi:10.1016/S0929-1393(01)00187-1
- 434 Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and
435 epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232-260 doi:10.1128/MMBR.65.2.232-
436 260.2001
- 437 Das S, Adhya TK (2014) Effect of combine application of organic manure and inorganic fertilizer on methane and
438 nitrous oxide emissions from a tropical flooded soil planted to rice. *Geoderma* 213:185-192
439 doi:10.1016/j.geoderma.2013.08.011
- 440 Day PR (1965) Particle fractionation and particle-size analysis. In: Black CA (ed) *Methods of soil analysis, Part 1:*
441 *Physical and mineralogical methods*, vol American Society of Agronomy. Madison, WI, pp 545-566
- 442 Demoling LA, Bååth E, Greve G, Wouterse M, Schmitt H (2009) Effects of sulfamethoxazole on soil microbial
443 communities after adding substrate. *Soil Biol Biochem* 41:840-848 doi:10.1016/j.soilbio.2009.02.001
- 444 Ding C, He J (2010) Effect of antibiotics in the environment on microbial populations. *Appl Microbiol Biotechnol*
445 87:925-941 doi:10.1007/s00253-010-2649-5
- 446 Dong W-Y et al. (2014) Changes in soil microbial community composition in response to fertilization of paddy soils in
447 subtropical China. *Appl Soil Ecol* 84:140-147 doi:10.1016/j.apsoil.2014.06.007
- 448 Du LF, Liu WK (2012) Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agron Sustain*
449 *Dev* 32:309-327 doi:10.1007/s13593-011-0062-9
- 450 EC (1831/2003) Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003
451 on additives for use in animal nutrition. *Official Journal of the European Union*
- 452 European Drugs Agency (2012) Sales of veterinary antimicrobial agents in 19 EU/EEA countries in 2010.
- 453 Frostegård Å, Tunlid A, Bååth E (2011) Use and misuse of PLFA measurements in soils. *Soil Biol Biochem* 43:1621-
454 1625 doi:10.1016/j.soilbio.2010.11.021
- 455 Garau G, Castaldi P, Santona L, Deiana P, Melis P (2007) Influence of red mud, zeolite and lime on heavy metal
456 immobilization, culturable heterotrophic microbial populations and enzyme activities in a contaminated soil.
457 *Geoderma* 142:47-57 doi:10.1016/j.geoderma.2007.07.011
- 458 Garland JL (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. *Fems*
459 *Microbiol Ecol* 24:289-300 doi:10.1111/j.1574-6941.1997.tb00446.x

- 460 Gong W, Yan XY, Wang JY, Hu TX, Gong YB (2009) Long-term manure and fertilizer effects on soil organic matter
461 fractions and microbes under a wheat-maize cropping system in northern China. *Geoderma* 149:318-324
462 doi:DOI 10.1016/j.geoderma.2008.12.010
- 463 Gu C, Karthikeyan KG, Sibley SD, Pedersen JA (2007) Complexation of the antibiotic tetracycline with humic acid.
464 *Chemosphere* 66:1494-1501 doi:10.1016/j.chemosphere.2006.08.028
- 465 Gutiérrez IR, Watanabe N, Harter T, Glaser B, Radke M (2010) Effect of sulfonamide antibiotics on microbial diversity
466 and activity in a Californian Mollic Haploxeralf. *J Soils Sediments* 10:537-544 doi:10.1007/s11368-009-0168-
467 8
- 468 Hackl E, Pfeffer M, Donat C, Bachmann G, Zechmeister-Boltenstern S (2005) Composition of the microbial
469 communities in the mineral soil under different types of natural forest. *Soil Biol Biochem* 37:661-671
470 doi:10.1016/j.soilbio.2004.08.023
- 471 Hammesfahr U, Bierl R, Thiele-Bruhn S (2011a) Combined effects of the antibiotic sulfadiazine and liquid manure on
472 the soil microbial-community structure and functions. *J Plant Nutr Soil Sc* 174:614-623
473 doi:10.1002/jpln.201000322
- 474 Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Bruhn S (2008) Impact of the antibiotic sulfadiazine and pig
475 manure on the microbial community structure in agricultural soils. *Soil Biol Biochem* 40:1583-1591
476 doi:10.1016/j.soilbio.2008.01.010
- 477 Hammesfahr U, Kotzerke A, Lamshöft M, Wilke BM, Kandeler E, Thiele-Bruhn S (2011b) Effects of sulfadiazine-
478 contaminated fresh and stored manure on a soil microbial community. *Eur J Soil Biol* 47:61-68
479 doi:10.1016/j.ejsobi.2010.10.004
- 480 Hamscher G, Sczesny S, Höper H, Nau H (2002) Determination of persistent tetracycline residues in soil fertilized with
481 liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass
482 spectrometry. *Anal Chem* 74:1509-1518
- 483 Heuer H, Krsek M, Baker P, Smalla K, Wellington EM (1997) Analysis of actinomycete communities by specific
484 amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl*
485 *Environ Microbiol* 63:3233-3241
- 486 Hund-Rinke R, Simon M, Lukow T (2004) Effects of Tetracycline on the Soil Microflora: Function, Diversity,
487 Resistance. *J Soils Sediments* 4:11-16
- 488 Insam H, Amor K, Renner M, Crepez C (1996) Changes in functional abilities of the microbial community during
489 composting of manure. *Microb Ecol* 31:77-87 doi:10.1007/BF00175077
- 490 Jackson ML (1958) *Soil Chemical Analysis*. Prentice-Hall, Englewood Cliffs, NJ.
- 491 Jenkins TC (2010) Technical note: Common analytical errors yielding inaccurate results during analysis of fatty acids in
492 feed and digesta samples. *J Dairy Sci* 93:1170-1174
- 493 Jia DA, Zhou DM, Wang YJ, Zhu HW, Chen JL (2008) Adsorption and cosorption of Cu(II) and tetracycline on two
494 soils with different characteristics. *Geoderma* 146:224-230 doi:10.1016/j.geoderma.2008.05.023
- 495 Jutta R, Pils V, Laird DA (2007) Sorption of Tetracycline and Chortetracycline on K- and Ca-saturated soil clays,
496 humic Substances, and clay-humic complexes. *Environ Sci Technol* 41:1928-1933
- 497 Kim KR, Owens G, Kwon SI, So KH, Lee DB, Ok YS (2011) Occurrence and Environmental Fate of Veterinary
498 Antibiotics in the Terrestrial Environment *Water Air and Soil Pollution* 214:163-174 doi:DOI 10.1007/s11270-
499 010-0412-2
- 500 Kong WD, Li CG, Dolhi JM, Li SY, He JZ, Qiao M (2012) Characteristics of oxytetracycline sorption and potential
501 bioavailability in soils with various physical-chemical properties. *Chemosphere* 87:542-548
502 doi:10.1016/j.chemosphere.2011.12.062
- 503 Kramer JK, Fellner V, Dugan ME, Sauer FD, Mossoba MM, Yurawecz MP (1997) Evaluating acid and base catalysts in
504 the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty
505 acids. *Lipids* 32:1219-1228
- 506 Kümmerer K (2001) Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by
507 hospitals in relation to other sources - A review. *Chemosphere* 45:957-969 doi:10.1016/S0045-6535(01)00144-
508 8
- 509 Kyselková M, Jirout J, Vrchotová N, Schmitt H, Elhottová D (2015) Spread of tetracycline resistance genes at a
510 conventional dairy farm. *Front Microbiol* 6:536 doi:10.3389/fmicb.2015.00536
- 511 Li Z, Chang PH, Jean JS, Jiang WT, Wang CJ (2010) Interaction between tetracycline and smectite in aqueous solution.
512 *J Colloid Interface Sci* 341:311-319 doi:10.1016/j.jcis.2009.09.054
- 513 Lundquist EJ, Jackson LE, Scow KM, Hsu C (1999) Changes in microbial biomass and community composition, and
514 soil carbon and nitrogen pools after incorporation of rye into three California agricultural soils. *Soil Biol*
515 *Biochem* 31:221-236 doi:10.1016/S0038-0717(98)00093-5
- 516 Marschner P, Kandeler E, Marschner B (2003) Structure and function of the soil microbial community in a long-term
517 fertilizer experiment. *Soil Biol Biochem* 35:453-461 doi:10.1016/S0038-0717(02)00297-3
- 518 Mokni-Thili S, Jaoua L, Murano F, Jedidi N, Hassen A (2009) Study of the effects of urban organic residues on the
519 distribution of culturable actinomycetes in a Tunisian agricultural soil. *Waste Manag Res* 27:224-232
520 doi:10.1177/0734242X08090405

521 Nelson ML, Levy SB (2011) The history of the tetracyclines. *Ann N Y Acad Sci* 1241:17-32 doi:10.1111/j.1749-
522 6632.2011.06354.x

523 Parham JA, Deng SP, Da HN, Sun HY, Raun WR (2003) Long-term cattle manure application in soil. II. Effect on soil
524 microbial populations and community structure. *Biol Fert Soils* 38:209-215 doi:DOI 10.1007/s00374-003-
525 0657-7

526 Richaume A, Steinberg C, Jocteurmonrozier L, Faurie G (1993) Differences between Direct and Indirect Enumeration
527 of Soil Bacteria - the Influence of Soil-Structure and Cell Location. *Soil Biol Biochem* 25:641-643 doi:Doi
528 10.1016/0038-0717(93)90206-Q

529 Sánchez-Monedero MA, Mondini C, Cayuela ML, Roig A, Contin M, De Nobili M (2008) Fluorescein diacetate
530 hydrolysis, respiration and microbial biomass in freshly amended soils. *Biol Fert Soils* 44:885-890
531 doi:10.1007/s00374-007-0263-1

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

535 Schnürer J, Rosswall T (1982) Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil litter.
536 *Appl Environ Microbiol* 43:1256-1261

537 Schoug Å, Fischer J, Heipieper HJ, Schnürer J, Håkansson S (2008) Impact of fermentation pH and temperature on
538 freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* S13 *Journal of Industrial*
539 *Microbiology and Biotechnology* 35:175-181 doi:10.1007/s10295-007-0281-x

540 Stewart EJ (2012) Growing unculturable bacteria. *Journal of bacteriology* 194:4151-4160 doi:10.1128/JB.00345-12

541 Thiele-Bruhn S (2005) Microbial inhibition by pharmaceutical antibiotics in different soil-dose-response relations
542 determined with the iron(III) reduction test. *Environ Toxicol Chem* 24:869-876

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

545 Torsvik V, Goksøyr J, Daae FL (1990) High diversity in DNA of soil bacteria. *Appl Environ Microbiol* 56:782-787

546 Vaclavik E, Halling-Sørensen B, Ingerslev F (2004) Evaluation of manometric respiration tests to assess the effects of
547 veterinary antibiotics in soil. *Chemosphere* 56:667-676 doi:10.1016/j.chemosphere.2004.02.018

548 van Elsas J, Jansson J, Trevors J (eds) (2007) *Modern soil microbiology - 2nd ed.* CRC Press, Broken Sound Parkway
549 NW

550 Wan Y, Bao Y, Zhou Q (2010) Simultaneous adsorption and desorption of cadmium and tetracycline on cinnamon soil.
551 *Chemosphere* 80:807-812

552 Wang C, Wang G, Wang Y, Rafique R, Ma L, Hu L, Luo Y (2015) Urea addition and litter manipulation alter plant
553 community and soil microbial community composition in a *Kobresia humilis* meadow *European Journal of*
554 *Soil Biology* 70:7-14 doi:<http://dx.doi.org/10.1016/j.ejsobi.2015.06.003>

555 Wang YJ, Sun RJ, Xiao AY, Wang SQ, Zhou DM (2010) Phosphate affects the adsorption of tetracycline on two soils
556 with different characteristics. *Geoderma* 156:237-242 doi:DOI 10.1016/j.geoderma.2010.02.022

557 Wei X, Wu SC, Nie XP, Yediler A, Wong MH (2009) The effects of residual tetracycline on soil enzymatic activities
558 and plant growth. *J Environ Sci Health B* 44:461-471 doi:10.1080/03601230902935139

559 Whalen JK, Sampedro L (2009) *Soil Ecology and Managem.* CABI,

560 Winckler C, Grafe A (2000) Stoffeintrag durch Tierarzneimittel und pharmakologisch wirksame Futterzusatzstoffe
561 unter besonderer Berücksichtigung von Tetrazyklinen. Paper presented at the UBA-Texte 44/00, Berlin,

562 Yang Q, Zhang J, Zhang W, Wang Z, Xie Y, Zhang H (2010) *Journal of Environmental Science and Health, Part B:*
563 *Pesticides, Food Contaminants, and Agricultural Wastes.* *J Environ Sci Heal B* 45:190-197

564 Yu FB, Luo XP, Song CF, Shan SD (2010) Concentrated biogas slurry enhanced soil fertility and tomato quality *Acta*
565 *Agriculturae Scandinavica Section B: Soil and Plant Science* 60:262-268 doi:10.1080/09064710902893385

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

569 Zhu YG et al. (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Natl Acad Sci U S*
570 *A* 110:3435-3440 doi:10.1073/pnas.1222743110

571

572

573 **Tables and figure captions**

574

575 **Table 1** Selected physical and chemical properties of the soils and manure investigated

576 OM: Organic Matter; OC: Organic Carbon; N: Nitrogen; M: manure

577

578 **Table 2** K_d values and Tc free residues in PU and SA soil

579 K_d : repartition constant; 100 and 500: Tc spiked on soil (mg kg^{-1}); M: manure

580

581 **Table 3** Bacterial counts in PU soil (Log CFU/g soil dry weight)

582 M: manure; Tukey-Kramer statistical analysis ($P < 0.05$)

583

584 **Table 4** Bacterial counts in SA soil (Log CFU/g soil dry weight)

585 M: manure; Tukey-Kramer statistical analysis ($P < 0.05$)

586

587 **Fig.1** FDA activity in PU (a) and SA (b) soils after 2, 7 and 60 days. The horizontal axes indicate

588 bioavailable Tc (mg kg^{-1} soil); M: manure. For each time-point, average values which shared the

589 same white capital letters within columns do not significantly differ at the 5% level ($P < 0.05$). For

590 each treatment, average values, which shared the same letter above columns, do not significantly

591 differ at the 5% level ($P < 0.05$), according to the Tukey-Kramer multiple comparison test. Error bars

592 indicate the average of $n=12$ replicates

593

594 **Fig. 2** PLFA analysis. Fungi/bacteria and Gram⁺/Gram⁻ ratio, actinomycetes in PU (a, c, e,

595 respectively) and SA (b, d, f respectively) soils after 2, 7 and 60 days. The horizontal axes indicate

596 bioavailable Tc (mg kg^{-1} soil); M, manure. For each time-point, average values which shared the

597 same white capital letters within columns do not significantly differ at the 5% level ($P < 0.05$). For

598 each treatment, average values which shared the same letter above columns do not significantly at

599 the 5% level ($P < 0.05$), according to the Tukey-Kramer multiple comparison test. Error bars indicate

600 the average of $n=12$ replicates

601

602 **Fig. 3** Principal Component Analysis (PCA) applied to Biolog® CLPP data in PU (a, b, c,

603 respectively) and SA (d, e, f, respectively) soils after 2, 7 and 60 days. S: PU or SA soil; M:

604 manure; Tc 100: spiked Tc at 100 mg kg^{-1} soil; Tc 500: Tc spiked at 500 mg kg^{-1} soil