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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

2. Materials and methods

2.1 Experimental design

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

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

2.2 Soil characterization and adsorption-desorption analysis

Soil samples were collected at a depth of 10 cm, air-dried and sieved to <2 mm and the particle size 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±2°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 μM corresponding to 100 mg Tc kg^{-1} dry soil and 602.6 μM 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 μBondapak C_{18} analytical
118 column (10 μ, 3.9 × 300 mm). The mobile phase was a water-acetonitrile mixture (77:23, pH= 2.5)
119 at a flow rate of 0.7 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 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 $K_d = Cs\ Ce^{-1}$ where Cs (μmol kg^{-1}) is the amount of the antibiotic adsorbed, Ce (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 mm) were added
129 with 15 mL of 60 mM potassium phosphate buffer (pH 7.6) and 0.2 mL of 1 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

assay was calculated using the calibration curve produced from 0 to 10 μg mL^{-1} with Fluorescein Sodium Salt (Sigma-Aldrich, Milano, Italy).

2.4 Phospholipid fatty acids analysis

The extraction, identification and quantification of phospholipid fatty acids (PLFA) from soil were performed according to the method of Gutiérrez et al. (2010), while the two-step methylation was carried out according to Kramer et al. (1997) and Jenkins (2010). PLFA were analysed using a gas chromatograph GC Turbo 3400 CX (Varian Inc. Palo Alto, CA) equipped with a capillary column (CP-select CB for Fame, 100 m × 0.32 mm i.d., 0.25 μm film thickness; Varian Inc., Palo Alto, CA, USA), a flame ionization detector and an automatic sample injector 8200 (CX Varian Inc. Palo Alto, CA). The column head pressure was set at 37.00 psi. Quantification was based on the internal standards method. PLFA were assigned to taxonomic groups based on recent literature (Hackl et al. 2005). Terminal-branched saturated PLFA a15:0, i15:0, i16:0, i17:0, and a17:0 were used as markers for Gram-positive bacteria ($PLFA_{g+}$) while Gram-negative bacteria ($PLFA_{g-}$) were quantified by monounsaturated PLFA (16:1ω7c, 18:1ω7c, 18:1ω9c) and cyclopropyl saturated PLFA (cy17:0, cy19:0). The sum of signature PLFA for Gram-positive and -negative bacteria is referred to as bacterial PLFA ($PLFA_{bact}$). The quantity of the PLFA 18:2ω6,9 was used as an indicator of fungal biomass. Similarly to a number of recent reports, in this study we used cis-9-octadecenoic acid (18:1ω9c) as biomarker for Gram-negative bacteria (Gutiérrez et al. 2010; Hammesfahr et al. 2011a; Hund-Rinke et al. 2004; Schoug et al. 2008; Wang et al. 2015; Whalen and Sampedro 2009), despite in some studies the same fatty acid was used as a fungal biomarker ~~(e.g.~~ (Demoling et al. 2009; Dong et al. 2014).

2.5 Community level physiological profiles

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

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.

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

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

3.2 Hydrolysis of fluorescein diacetate

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

differ from control PU. More likely, manure addition revitalised and stimulated PU soil microbial population (Das and Adhya 2014; Lundquist et al. 1999) by supplying easily metabolisable carbon sources (Parham et al. 2003). After 7 days, the effect of Tc and manure definitely disappeared in PU soil, and the microbial activity was completely restored. This trend was confirmed after 60 days.

In SA soil after 2 days, Tc caused negative effects and the decrease in FDA hydrolysis was related to Tc bioavailability. In fact, compared to control, the microbial activity was reduced in SA+Tc 0.859 mg kg^{-1}, but the greatest effect was observed in the presence of 4.602 mg Tc kg^{-1} soil (Fig. 1b). Contrary to PU soil, the addition of cow manure did not stimulate FDA hydrolysis, although restored the microbial activity in SA+M+Tc 0.767 mg kg^{-1} soil. After 7 days the Tc effect seemed to be reduced and the detrimental impact of the antibiotic was detected only at the highest Tc concentration 4.602 mg kg^{-1} soil, compared to control SA. In SA soil the Tc perturbation was a bit longer than in PU soil, probably due to the highest Tc bioavailability. Moreover, within 60 days in both PU and SA control soils the FDA hydrolysis significantly (P<0.05) decreased over time, owing to a plausible reduction of available carbon sources for soil microbial communities (Schnürer and Rosswall 1982).

3.3 Phospholipid fatty acids analysis

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

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

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

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

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

The effect of manure and Tc was tested on the whole actinomycete population by PLFA analysis to better understand the effect of treatments on these bacteria, which are antibiotic producers. Compared to control soils, the addition of Tc at the highest concentrations and of manure significantly (P<0.05) increased the abundance of actinomycetes in both soils (Fig. 2e, f). When 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 Ecoplates^{TM} 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

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

3.5 Microbial counts

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

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

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

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

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

4. Conclusions

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

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Tables and figure captions

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

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

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

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

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

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

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

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

Fig.1 FDA activity in PU (a) and SA (b) soils after 2, 7 and 60 days. The horizontal axes indicate bioavailable Tc (mg kg^{-1} soil); M: manure. For each time-point, average values which shared the same white capital letters within columns do not significantly differ at the 5% level (P<0.05). For each treatment, average values, which shared the same letter above columns, do not significantly differ at the 5% level (P<0.05), according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=12 replicates

Fig. 2 PLFA analysis. Fungi/bacteria and $Gram^+/Gram^-$ ratio, actinomycetes in PU (a, c, e, respectively) and SA (b, d, f respectively) soils after 2, 7 and 60 days. The horizontal axes indicate bioavailable Tc (mg kg^{-1} soil); M, manure. For each time-point, average values which shared the same white capital letters within columns do not significantly differ at the 5% level (P<0.05). For each treatment, average values which shared the same letter above columns do not significantly at the 5% level (P<0.05), according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=12 replicates

Fig. 3 Principal Component Analysis (PCA) applied to Biolog® CLPP data in PU (a, b, c, respectively) and SA (d, e, f, respectively) soils after 2, 7 and 60 days. S: PU or SA soil; M: manure; Tc 100: spiked Tc at 100 mg kg^{-1} soil; Tc 500: Tc spiked at 500 mg kg^{-1} soil