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Ciclo XXVI

Effect of a veterinary antibiotic and manure amendment on the structure and function of soil microbial community

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Chapter 1
Introduction

1.1 Veterinary antibiotics

Antibiotics are widely used to maintain the health, and to prevent and treat infectious diseases in, humans, plants and animals, as well as for promoting growth in food-producing animals (Sarmah *et al.*, 2006). They are active against a wide range of bacteria and eukaryotes. Most of them act as bactericides. In other words they kill cells through damaging the membrane. As a result, cellular content is lost and DNA functions are inhibited. Bacteriostatic effects are produced if cellular activity or growth are inhibited. This is due to different mechanisms, such as the inhibition of the synthesis of ribosomal or the bacterial cell wall, or of folic acid, DNA and proteins (Thiele-Bruhn, 2003; Serrano, 2005).

A huge amount of veterinary antibiotics (VAs) are given to animals. In 1997 the antibiotic consumption in Europe was about 10,000 tons per year and increased by 50 tons per year from 1994 to 1998. In the same period about 5,000 tons, or half of the total amount, were prescribed by veterinary surgeons (Kummerer, 2001). Different classes of VAs are used for veterinary purposes. Tetracyclines, sulfonamides, macrolides, aminoglycosides and β-lactams antibiotic classes have been used for antibacterial, antimicrobial, antiparasitic, antiseptic, anesthetic, antacid, anthelmintic, anti-infective, anti-inflammatory, bronchodilator, diuretic, tranquilizer purposes, and even as nutritional supplements. High doses of antibiotics are given to pigs and cattle, usually orally in their feed or water, rather than by injection (Merle *et al.*, 2012). The ability of antibiotics to promote animal growth were first discovered in the mid-1950s. When they are used, livestock need less food to reach the optimal weight, and this means more profit for the farmers. These antibiotics act by selecting the intestinal microbial community, eliminating pathogens and stimulating the resident microbiota to produce vitamins. They also promote the absorption of nutrients in the animal gut (Chopra and Roberts, 2001). For this reason, significant quantities of VAs are used in the world. Use in the USA is higher than in EU countries (with the exception of UK), where the use of antibiotics as growth promoters was first banned by the EU in January 2006 (EC, 1831/2003; Kim *et al.*, 2011). Despite this ban, in recent decades the consumption of VAs has increased worldwide, so that now antibiotics make up about 70% of pharmaceuticals given to livestock (Kummerer, 2003; Sarmah *et al.*, 2006).

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Antibiotic use at sub-therapeutic levels for growth promotion is, indeed, still a common practice in many countries, including the United States (Anderson *et al.*, 2003), Canada and Australia (Johnson, 2011). In 1988 the American Institute of Medicine defined the meaning of "sub-therapeutic use" and the Food and Drug Administration stated that: "The Centre for Veterinary Medicine considers any extended use of antibiotics in feed at 200 g ton^{-1} or less beyond 2 weeks as *sub-therapeutic use*, whether it is for growth enhancement or disease prevention". Use levels are generally 200 g or less of penicillin or tetracycline per ton of feed, but dosage levels will vary by species (Mathers *et al.*, 2011). The levels approved for growth purposes and prophylaxis are usually lower than those for treating diseases. However this may not be the case for dose levels of 200 g ton^{-1} or less (Mathers *et al.*, 2011). The Food and Drug Administration only distinguishes between therapeutic and non-therapeutic use, and does not suggest any limitation on the use of antibiotics as growth promoters in livestock, which is a common practice in the United States.

VAs remain in the animal gut for a short period. Here they are poorly absorbed, and then excreted quickly (Thiele-Bruhn, 2003; Hund-Rinke *et al.*, 2004). The excretion, in faeces and urine, starts a few hours after the first administration and usually continues until 48 hours after the last application. The excretion of antibiotics continues for a long period after the last administration with the concentration gradually decreasing with time (Winckler and Grafe, 2001; Kim *et al.*, 2011). About 30-90% of total administered dose of antibiotics is evacuated, up to 75% in faeces and up to 90% in urine. They are evacuated mostly as unchanged form and are still bioactive against microorganisms (Jones *et al.*, 2005; Sarmah *et al.*, 2006; O'Connor and Aga, 2007).

Animal wastes are used as fertilizers, to restore organic matter to the soil. This common farming practice results in the soil being contaminated with antibiotics. Thiele-Bruhn (2003) reported that the antibiotic content in pig manure can reach concentrations of up to 3.5 mg kg^{-1} for sulfonamides and 4 mg kg^{-1} for tetracyclines. Excreted antibiotics may also still be bioactive and, once added to the soil, can change the activity and composition of the microbial populations and lead to them becoming resistant to antibiotics (Thiele-Bruhn, 2003).

VAs can remain bioavailable and active on different soil surfaces or be adsorbed into the soil. However, their free concentration varies greatly, depending on their affinity with particular soils. Some concentrations detected in soil are: tetracycline from 86.2 to 171.7 μg kg^{-1} (Hamscher *et al.*, 2002); oxytetracycline 27 μg kg^{-1}, tetracycline 443 μg kg^{-1}, chlorotetracycline 93 μg kg^{-1} and

sulfamethazine 4.5 μg kg^{-1} (Pawelzick *et al.*, 2004). Winckler and Grafe (2000) estimated tetracycline concentrations ranging from 450 to 900 μg kg^{-1} which are higher than those previously reported. Thiele-Bruhn (2003) also reported concentrations of macrolides ranging from 13 to 67 μg kg^{-1}, and those of fluorquinolones from 6 to 52 μg kg^{-1}.

In the soil, antibiotics are often produced by resident microorganisms to regulate and control interactions among microbial communities. The most widely used antibiotics in livestock are synthetic or semi-synthetic. This is because these are more water-soluble, are not degradable by microbial activity and are broad spectrum (Kummerer, 2004).

1.2 Most used veterinary antibiotics

Different classes of antibiotics are used in livestock. Sarmah and colleagues (2006) stated that the most used are, in decreasing order of usage, tetracyclines, sulfonamides, β-lactams, macrolides, aminoglycosides and fluoroquinolones.

Table 1. Chemical properties of selected veterinary antibiotics.

Compound class	Molar mass g mol^{-1}	Water solubility mg l^{-1}	log K_{ow}	pK_a
Tetracyclines	444.5 – 527.6	230 – 52000	-1.3 – 0.05	3.3 / 7.7 / 9.3
Sulfonamides	172.2 – 300.3	7.5 – 1500	-0.1 – 1.7	2 – 3 /4.5 / 10.6
Aminoglycosides	332.4 – 615.6	10000 – 500000	-8.1 – -0.8	6.9 – 8.5
β-Lactams	334.4 – 470.3	22 – 10100	0.9 – 2.9	2.7
Macrolides	687.9 – 916.1	0.45 – 15	1.6 – 3.1	7.7 – 8.9
Fluorquinolones	229.5 – 417.6	3.2 – 17790	-1.0 – 1.6	8.6

Tetracyclines are active against a wide range of both Gram-negative and Gram-positive microorganisms and are used to maintain the health and promote the growth of food-producing animals. They act as bacteriostatic agents, stopping protein synthesis by interacting with the 30S

ribosome subunit (Chopra and Roberts, 2001). Tetracycline, oxytetracycline and chlortetracycline are those most widely used in veterinary practice. The chemical properties of this class of antibiotics are relevant environmentally, because they can evolve and because they have different affinity toward the different substrates. They have a common napthacene structure, with four carbon rings and ionisable groups (Nelson and Levy, 2011). Tetracyclines are highly water-soluble (230 – 52,000 mg l^{-1}), with a molecular weight ranging from 444.4 to 527.6 g mol^{-1} (Table 1), and have three pK_a (3.3, 7.7, 9.6) attributable to the ionisation of tricarbonyl methane, phenolic diketone and dimethyl ammonium groups, respectively (Chopra and Roberts, 2001; Thiele-Bruhn, 2003; Sarmah *et al.*, 2006).

Sulfonamides include the main VAs sulfadiazine, sulfadimethoxine, sulfamethazine, sulfathiazole, sulfamethoxazole and sulfachloropyridazine. They are active against a broad spectrum of Gram-positive and Gram-negative bacteria and act by inhibiting the synthesis of p-aminobenzoic acid in folic acid metabolism. These antibiotics are small molecules (172.2 - 300.3 g mol^{-1}). They consist of a benzene ring provided with the *para* amine and sulfonamide substitutions necessary for the antibiotic activity. They are amphoteric and relatively insoluble in water. Their solubility ranges from 7.5 to 1,500 mg l^{-1} and the pK_a values range from 2-3 to 10.4 (Table 1). Sulfonamides are positively charged in acidic conditions, neutral over pH range from 2.5 to 6 and negatively charged in alkaline media (Thiele-Bruhn, 2003; Sarmah *et al.*, 2006).

Aminoglycosides are basic and strongly polar polycationic compounds. They are highly water-soluble, with their solubility ranging from 10 to 500 g l^{-1} (Table 1). They do not photodegrade and are able to inhibit protein synthesis. This class includes, among others, kanamycin, streptomycin and neomycin, with molecular weights ranging from 332.4 to 625.6 g mol^{-1}. Their molecular structure consists of two amino sugars linked to aminocyclitol by a glicosidic bond. This class can be divided in two groups. The first group is characterized by an aglycone of fully-substituted aminocyclitol structures such as streptomycin, fortimicin and spectinomicin. The second group is characterized by an aglycone with a 2-deoxy-streptamine structure and includes neomycin, kanamycin, gentamycin and others (Thiele-Bruhn, 2003; Nagaya *et al.*, 2005).

Macrolides act bacteriostatically, by inhibiting protein synthesis. Their structure consists of a lactone ring with at least 10 C-atoms. They are poorly soluble in water, from 0.45 to 15 mg l^{-1} (Table 1) and are unstable in acidic conditions. The macrolides include erythromycin, tylosin and oleandromycin. They have a molar mass between 687.9 and 916.1 g mol^{-1}. Tylosin has an antibacterial effect against most of the Gram-positive pathogens and some Gram-negative bacteria such as like vibrio, spirochete, coccidian and others. It is produced by fermentation of Streptomyces and is characterized by a substituted 16-member lactone ring, the aminosugar mycaminose and two neutral sugars, mycinose and mycarose. Tylosin is the most used macrolide in veterinary practice (Sarmah *et al.*, 2006), and is rather unstable in acidic and alkaline conditions, but relatively stable at the neutral pH value. Its solubility increases as solvent polarity increases (Thiele-Bruhn, 2003; Sarmah *et al.*, 2006; Ding and He, 2010).

β-Lactams can be divided into the penicillins (ampicillin, meropenem and penicillin G) and the cephalosporins (ceftiofur and cefotiam). They have a core β-lactam structure (either bicyclic or monocyciclic) and work by inhibiting cell wall biosynthesis. The water solubility of β-lactams ranges from 22 to 10,100 mg l^{-1} and their molecular weight ranges from 334.4 to 470.3 g mol^{-1} (Table 1). The best known β-lactam antibiotic is penicillin, discovered in 1928 by Fleming when he observed that *Staphylococcus aureus* growth was inhibited on an agar plate contaminated with *Penicillium notatum*, a penicillin producer. In 1948 Brotzu found penicillin N and another cephalosphorin antibiotic in *Cephalosporium acremonium*, a fungus isolated from the sea in Cagliari (Sardinia, Italy) (Brakhage, 1998; Thiele-Bruhn, 2003; Ding and He, 2010).

Fluoroquinolones are synthetic antibiotics used for treating infectious diseases in livestock. They are chemically stable molecules and can resist hydrolysis and heat, but are very sensitive to degradation by UV light. They are active against a wide range of Gram-negative and Gram-positive bacteria, through inhibiting DNA topoisomerase II and IV. This activity is related to the fluorine substitute in the C-6 position. Ciprofloxacin, enrofloxacin, flumequin, sarafloxacin and oxolinic acid are included in the fluoroquinolones class, their molecular weights ranging from 229.5 to 916.1 g mol^{-1} (Table 1) and their water solubility from 3.2 to 17,790 mg l^{-1} (Martens *et al.*, 1996; Thiele-Bruhn, 2003).

Bacitracin is a polypeptide produced by *Bacillus licheniformis.* It is used in animal feed as a growth promotion supplement, and as well as in other human and veterinary practices. Bacitracin is a mixture of more than 20 components (bacitracin A, B and C are the most important) with different antibiotic activity. However all of these components are necessary for bacteriostatic activity. This antibiotic is active mainly against Gram-positive bacteria. It inhibits the cell wall synthesis, forming a complex with C55-isoprenyl pyrophosphate, a carrier of N-acetylmuramyl peptapeptide involved in peptidoglycan synthesis (Butaye *et al.*, 2003; Thiele-Bruhn, 2003; Sarmah *et al.*, 2006).

1.3 Antibiotic adsorption on soil

Most of the antibiotics consumed by animals are excreted through faeces and urine. It is a common agricultural practice to use these animal wastes as soil fertiliser. After repeated applications of these contaminated wastes, antibiotics accumulate on the soil surface (Jones *et al.*, 2005). The antibiotics may be adsorbed on soil, depending on the physical and chemical characteristics of the specific antibiotic and the particular soil. Adsorption can be fast and complete, sometimes taking only a few hours, as in the case of sulfonamides (Jones *et al.*, 2005).
The affinity between chemicals and soil is expressed by the distribution coefficient K_d (Jones *et al.*, 2005). Thiele-Bruhn (2003) observed that K_d depends on the physical and chemical properties of the matrices. Soil organic matter (SOM) generally favours antibiotic adsorption on soil (de la Torre *et al.*, 2012). For example, sulfapyridine adsorption increases on manure with greater SOM content, because this manure contains many lipids and lignin dimers. The pH is one of the most important regulators of antibiotic adsorption on mineral and organic surfaces (Li *et al.*, 2010), because of the electrostatic forces involved in the process. Generally speaking, adsorption is greater when the soil pH is near to dissociation pK_a of the antibiotics. Cationic antibiotic molecules are attracted by negatively charged surfaces. One example is tylosin A, which is in cationic form at pH 7.8, and binds negatively charged organic compounds through electrostatic interaction (Thiele-Bruhn, 2003). Du and Liu (2012) reported a similar pH dependency for sulfathiazole and sulfamethazine adsorption on soil.
There was a decrease in the K_d of sulfachloropyridazine as the amount of manure in the soil increased (Boxall *et al.*, 2002). This finding is in contrast with those described above for SOM but confirms the importance of pH in the adsorption process. Although adding manure does not

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significantly increase the OM in soil, it increases the soil pH, reducing the extent of antibiotic adsorption. In the case of tetracyclines, adsorption in soil may occur in two steps. Tetracyclines are initially quickly adsorbed on the outer surfaces of soil particles, whereas the residual amount continues to penetrate interlayers and micropores, by binding clays and minerals (Thiele-Bruhn, 2003; Wang *et al.*, 2010; Texeido *et al.*, 2012; Zhao *et al.*, 2012).

The adsorption process reduces the bioavailable antibiotic fraction and thus its activity. As the adsorption does not remove totally the antibiotics from soil, the residues may still interact with microorganisms. The antibiotic affinity for soil is expressed by the K_d value. This value depends on the soil pH and on antibiotic characteristics (pK_a) (Du and Liu, 2012). Thiele-Bruhn (2003) reported the K_d values of the most commonly used antibiotics. They range from 77.6 in manure to 3020 in sewage sludge for tetracyclines, and from 0.9 (in sandy soil) to 10 (in clay soil) for sulphonamides. For tylosine, K_d ranges from 8.3 to 240 and from 260 to 5,612 for fluoroquinolones. The mineral and organic surfaces in soil (adsorbent) can adsorb organic molecules (adsorbate) and the effectiveness of this interaction depends on the strength of the adsorbate-adsorbent interaction. A strong interaction is indicative of chemical adsorption (chemisorption), in which a covalent or short-range electrostatic bond forms between the molecule and the surface. Weak adsorption is characteristic of physical adsorption, in which the bonding interaction is of low in energy.

Adsorption data are most commonly represented by an adsorption isotherm. This is a plot of the quantity of adsorbate retained by a solid, as a function of the concentration of the adsorbate in the bathing gas or solution phase which is at equilibrium with the solid. The shape of this isotherm line provides information on adsorbate-adsorbent (organic-surface) interaction. Several isotherm shapes are possible, but these can be generally classified into four diagram types (Figure 1).

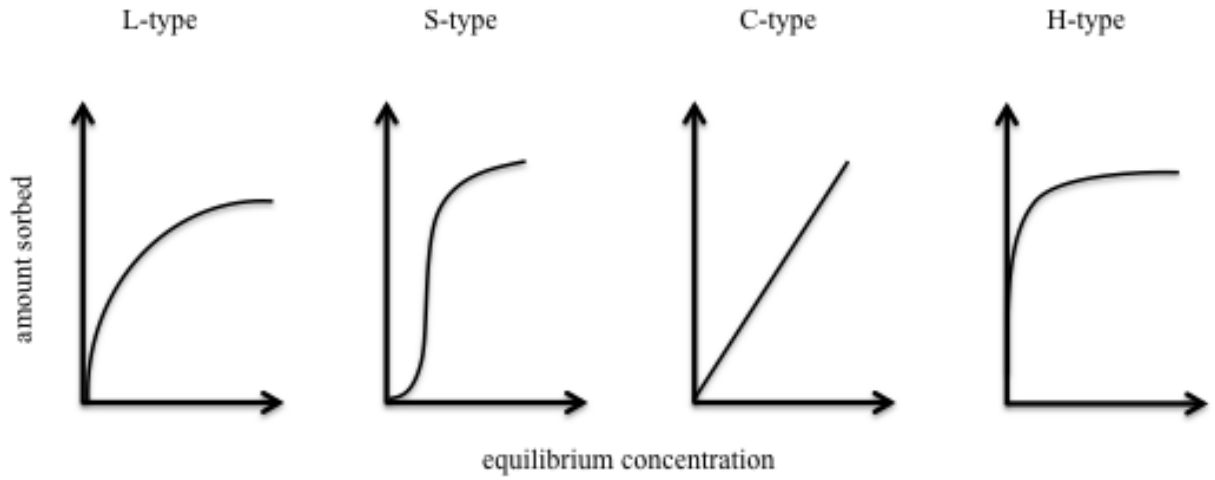

Figure 1. Classification of adsorption isotherms.

The L-type isotherm indicates that there is a relatively high affinity between the adsorbate and adsorbent, and is usually indicative of chemisorption. The S-type isotherm suggests that cooperative adsorption takes place. This happens if the adsorbate-adsorbate interaction is stronger than the adsorbate-adsorbent interaction. This condition favours the clustering of adsorbate molecules at the surface, because they bind more strongly with one another than with the surface. The C-type isotherm is a constant-partitioning, and suggests that the adsorbate molecules have a constant relative affinity for the adsorbent. It is usually only observed at low levels of adsorption. A deviation from the linear isotherm is likely at high adsorption levels. Nevertheless, because many non-polar organic compounds in soil are adsorbed at quite low concentrations, the linear C-type isotherm is often a reasonable description of adsorption behaviour. The H-type complex isotherm, indicative of a very strong adsorbate-adsorbent interaction (i.e. chemisorption), is really an extreme case of the L-type. This isotherm is not often encountered with organic molecules, because few of them form strong ionic or covalent bonds with soil colloids (McBride, 1994).

Adsorption isotherms are often fitted by the empirical Freundlich equation, formulated in 1929, as:

$$C_s = K_F C_e^{1/n} \quad (1.1)$$

Converting equation 1.1 into the logarithm form:

$$\log C_s = \log K_F + (1/n) \log C_e \quad (1.2)$$

a straight equation is obtained, where C_s (in μmol kg^{-1}) is the amount of herbicide adsorbed by soil, C_e (in μM) is the equilibrium concentration in solution, and log K_F and 1/n are empirical constants representing the intercept and the slope of the isotherm, respectively.
If n=1, the Freundlich equation becomes:

$$C_s = K_d C_e \quad (1.3)$$

where K_d represents the adsorption distribution coefficient.
The K_d coefficient describes the distribution of the soluble molecules between the soil and the solution at equilibrium (Tan, 1998; Foo and Hameed, 2010). A high K_d value corresponds to high affinity of sorbate for the soil at equilibrium (Texeido *et al.*, 2012).

1.4 Effects of antibiotics on soil microbial communities

Antibiotics can change the structure of the microbial population of the soil through positive selection of those resistant microorganisms which are able to grow in their presence. This induces an alteration in the microbial relationships between sensitive and insensitive species. Fingerprint analysis, performed by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (TRFLP), used for comparing antibiotic effects, showed changes in microbial diversity through decreasing intensity and/or the disappearance of peaks of sensitive microorganisms. However, the effects depend to a significant extent on the soil and the microbial population (Ding and He, 2010). Westergaard *et al.* (2001) reported that short-term changes in microbial diversity and composition may be evident for up to 15 days, but diversity can be recovered over time, while the microbial composition remains different from that of uncontaminated soil.
Antibacterial antibiotics can favour fungi by decreasing the bacterial population in soil. Pollutants can also significantly affect only certain bacteria, while being ineffective for others. This is due to their different composition of bacterial cell walls. This is the case for sulfadiazine, which does not modify the Gram-positive/Gram-negative bacteria ratio, while tetracycline can decrease this ratio by affecting the Gram-positive population. On the other hand, pollutants can act against microorganisms which are normally not sensitive to their activity. A mixture of the antibacterial antibiotics oxytetracycline and penicillin can reduce the length of hyphae in fungi.

In a similar way natamycin fungicides, which are normally used for stopping fungal growth, and captan can inhibit bacterial growth (Ding and He, 2010).

Antibiotics modify not only the structure of the soil microbial community but also its function. Ding and He (2010) studied the alterations in microbial activity. Several antibiotics, including ciprofloxacin, tylosin and sulfadiazine, can affect the microbial biomass and respiration, while oxytetracycline is able to reduce alkaline phosphatase activity by about 41-80% but cannot affect acidic phosphatase, urease and dehydrogenase. Thele-Bruhn (2005) reported the effect of nine antibiotics (five tetracyclines, three sulfonamides and benzimidazole) on iron(III) reduction. Microbial inhibition was dependent on the antibiotic type, pH and adsorption properties of the soil. These determine the bioavailability and power of antibiotics. The effectiveness of inhibition also mainly depends on the composition of the microbial community in the soil, which differs among soils. Thiele-Bruhn, in collaboration with Beck (2005), monitored sulfapyridine and oxytetracycline in two different soils in order to evaluate basal respiration, dehydrogenase activity, substrate-induced respiration (SIR) and iron(III) reduction. They did not find any significant variation in basal respiration and dehydrogenase activity in both soils. This was probably due to the properties of the soil and its resident microbial population, and to the concentration of antibiotics.

Several factors influence the effect of antibiotics in soil. The first is its concentration. Antibiotic bioavailability, the fraction of antibiotic that interacts directly with microorganisms, is directly dependent on its affinity with the soil, and as seen above, on soil texture and pH. In some cases, concentrations higher than those commonly found in soils can not only exert a bacteriostatic action but even kill microorganisms which are sensitive to antibiotics. By contrast, lower concentrations can have only inhibitory effects (Ding and He, 2010). The antibiotic efficacy is also dependent on the amount of time that microorganisms are exposed to these compounds. Thiele-Bruhn and Beck (2005) tested sulfapyridine and oxytetracycline, and noticed that there was a reduction in substrate-induced respiration (SIR) in the sandy soil after 24 h exposure. Incubation for another 24 hours resulted in an SIR reduction in the loamy soil. The authors attributed the reduction of SIR in the sandy soil to the decrease in the bioavailable antibiotic fraction and the microbial community's adaptation to soil spiked with antibiotics, and development of resistance.

Manure addition to the soil may also influence the microbial community. It can amplify antibiotic effects, especially of the bacteriostatic type, by stimulating certain specific microbial

groups. These are able to catabolise manure carbon sources. Manure can also carry resistance genes and, in conjunction with antibiotic action, select resistant microorganisms and modify the microbial structure (Ding and He, 2010).

1.5 Antibiotic resistance in soil

The antibiotic resistance is the capability of microorganisms to become insensitive to antibiotic activity. Acquisition of resistance can develop through chromosomal genes mutating during genome replication, but the frequency of mutation is low, ranging, in *in vitro* experiments, from 10^{-6} to 10^{-12} mutation per replication cycle (Roberts, 2011a). However, antibiotic resistance can be acquired through mobile genetic elements carrying resistance genes like plasmids, transposons and conjugative transposons. These are transmitted *via* bacterial conjugation or mediated by bacteriophage infections in different bacterial species and genera (Catry *et al.*, 2003).

The spread of antibiotics on soil can result in the microbial population developing resistance to them. Some microorganisms produce antibiotics to regulate the interactions between the microbial populations in the soil. Thus these already have antibiotic resistant genes in their genome and can grow even after antibiotics have been added to the soil, whereas sensitive microorganisms can be affected. Nonetheless, even a microorganism which is normally affected by antibiotic activity, can develop resistance through the acquisition of genes through mobile genetic elements (Roberts, 2011a). This resistance is normally introduced into the soil by manure and is then transferred to the indigenous microbial population (Thiele-Bruhn, 2003; Heuer *et al.*, 2009; Heuer *et al.*, 2011).

The recent increase in livestock has increased the consumption of VAs. For example, from 1960 to 2000 the pig production quadrupled and so did the use of VAs. In intensive farms this has led antibiotics accumulating in the soil through the manure amendments (Millet and Maertens, 2011). Manure may act as a reservoir for resistant genes (Heuer and Smalla, 2007; Heuer *et al.*, 2011). The soil environment is a "hot spot" for resistant genes, with the microbial community being subjected to selective pressure through antibiotics being spread in the soil (Smalla *et al.*, 2000; Heuer *et al.*, 2002). This can favour bacterial or fungi groups or result in specific microbial species being selected. This interferes with the natural soil network, in which resistant microorganisms are able to grow in the presence of antibiotics. The development of antibiotic

resistance in soil and on farms is dangerous not only for animals, which become insensitive to the antibiotics used to prevent infectious diseases, but also for humans, who can contract infectious diseases from zoonotic agents or possibly directly from soil and related crops. Mobile genetic elements are involved in the process of resistance development and can be transferred from non-pathogenic to pathogenic microorganisms, *via* the food chain or contact (Heuer *et al.*, 2011) through horizontal or vertical transmission (Roberts, 2011a).

The expression of resistant genes can be constitutive or inducible. For example the expression of *tet*(A) gene, which induces resistance to tetracyclines, is activated when tetracycline concentration in cytosol is higher than 0.3 mM (Roberts, 2011a). Resistant genes codes proteins involved in several mechanisms able to protect cells from antibiotic action. These mechanisms can be divided into three groups. In the first mechanism the antibiotics are modified and become inactive. The second mechanism decreases intracellular antibiotic concentration and prevents its accumulation, through the action of porin-channels and transmembrane efflux proteins. The third mechanism modifies the antibiotic binding site on its target (Catry *et al.*, 2003). For example, in fluoroquinolones resistance, the mutation in the genes which encode for DNA gyrase and topoisomerase proteins inhibits the antibiotics from binding on these targets (Catry *et al.*, 2003), while modifications in the ribosomal 30S subunit, target site of tetracycline, prevent bacteriostatic action (Roberts, 2011a).

Once mobile genetic elements, which contain resistant genes, have been acquired, bacteria can become resistant to more than one antibiotic and show multi-drug resistance. This resistance can protect the bacteria against one specific class of antibiotics, e.g. *tet*(M) confers resistance to oxytetraycline, chlortetracycline, doxycycline and minocycline, *erm*(X) to macrolides, lincosamides and B-compounds of streptogamins and *tet*(A) to tetracyclines, streptomycin, sulfamethoxazole, florfenicol and chloramphenicol), and so on (Catry *et al.*, 2003).

Genes able to confer resistance to antibiotics are located in chromosomes and can be transferred vertically to daughter cells during the cellular cycle, or horizontally by mobile genetic elements. The latter can be easily transferred through conjugation or diffusion from non-pathogenic to pathogenic microorganisms, including commensal ones, and thus become a risk for human health (Roberts, 2011a). Even low antibiotic concentrations are able to select resistant bacteria (Gullberg *et al.*, 2011) and thus allow resistant genes to spread to other bacteria living in other environments, such as water, drinking water and hospitals (Kummerer, 2003; Kummerer, 2004).

1.6 Health risks linked to antibiotic spread in the environment

The use of VAs in animal husbandry leads to resistance development in the microbial community. Sub-inhibitory levels of these molecules increase the spread of resistant genes in the environment and this resistance can be transferred to pathogenic bacteria, even commensal ones, and thus be a risk for human health, due to antibiotics becoming ineffective. Resistant genes can be spread by direct contact or through the food chain, with commensal microorganisms such as *Salmonella* and *Campylobacter* becoming resistant to antibiotics, and thus a major human health problem (Catry *et al.*, 2003). Anderson *et al.* (2003) studied *Campylobacter jejuni* in Minnesota, which had been responsible for severe infectious diseases in infants and immuno-compromised people. They found that the resistance to *C. jejuni* in humans treated with fluoroquinolones increased from 1% in 1992 to 10% in 1998. They also found that the number of fluoroquinolone resistant genes in poultry treated in 1995 increased between 1996 and 1998. A significant correlation was also observed between human and poultry *C. jejuni* strains, with resistant strains increasing by up to 18%. *Salmonella enterica* Serotype Typhimurium type 104 was able to resist antibiotics such as ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline, used together in tandem. It increased multidrug resistance patterns from 0.6% in 1979 to 34% in 1996 and was able to spread antibiotic resistance among animals and humans. Other commensal bacteria, such as *Shigella* spp., *Escherichia coli*, and *Enterococcus fecium*, which inhabit the intestines of both humans and animals, were able to transfer resistant genes through mobile genetic elements to other organisms, and even to pathogens.

The transmission of resistant genes in soil is promoted by manure and zoonotic bacteria. These bacteria may be in contact with human skin or may be inhaled, and may cause severe infectious diseases. Soil plays an important role in the transmission of resistant genes to humans (Figure 2) (Heuer *et al.*, 2011).

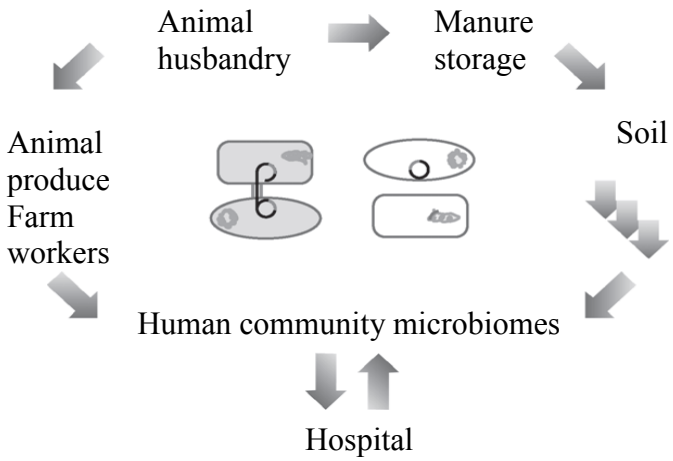

Figure 2. Antibiotic resistance genes transmission via mobile genetic elements and its route through the environments. From Heuer *et al.*, 2001.

Barza (2002) reported that five mechanisms are involved in the transmission of antibiotic resistance to pathogen or commensal bacteria, responsible of human health risks. The first is that human microbiota lose some of its protective efficacy when antibiotics are taken. This can alter the microbial composition in the gut, so that the hosts become sensitive to microbial pathogens. The second mechanism is the transfer of resistant genes, associated to virulence factors, to human-associated microorganisms. The third mechanism is the acquisition of resistant genes which may reduce the effectiveness of pharmacological treatments in humans. This means that increased doses of antibiotics become necessary in order to obtain the same effect. In this case, human health may be threatened because of humans absorbing therapeutic or sub-therapeutic levels of antibiotics that have been administered to the animals that they eat in their feed, and thus developing resistance. Fourthly, resistance developed in animals to pathogenic bacteria harmful for humans can be transferred to them via food chain, as can, fifthly, commensal bacteria which are common to humans and animals. These last two mechanisms are important in soil environment. Pathogens such as *Escherichia coli*, enterococci, *Bacteroides*, *Klebsiella* and *Enterobacter* species can use the manure amendment as a way for reaching humans, either via the food chain or through direct contact, and thus cause health problems.

1.7 Tetracyclines

Tetracyclines were first discovered in the early 1940s by Duggar at the University of Wisconsin, when he extracted and isolated a yellow substance from the bacterium *Streptomyces aureofaciens*. This substance, which was called aureomycin because of its gold colour, was able to inhibit the growth of Gram-negative and Gram-positive bacteria. In 1948 Duggar (Duggar, 1948) published his discovery in the Annals of the New York Academy of Sciences and on the 1st December 1948 the Food and Drug Administration approved the use of Aureomycin™ to treat infectious diseases, due to its broad spectrum activity. In the following years research on antibiotic compounds developed, and in 1950 Finlay and his colleagues isolated terramycin, which was similar in colour to aureomycin from *Streptomyces rimosus*. This had greater antibiotic activity than aureomycin, and became a business competitor of aureomycin, after it had been approved by the Food and Drug Administration in 1950. Pfizer and Woodward studied the structure of these antibiotics at Harvard University, and found that a DCBA naphthacene core is present in both aureomycin and terramycin (Figure 3).

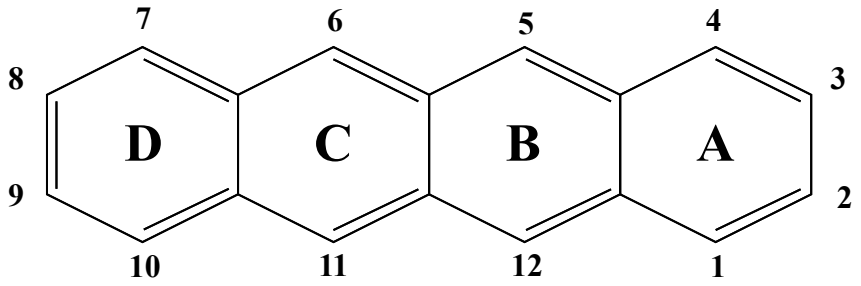

Figure 3. Naphthacene chemical structure.

Since the structure is made up of four carbon rings, and the researchers decided to group them into a new class of antibiotics, the tetracyclines. Aureomycin and terramycin differ for 2 substituent groups on the naphthacene core. Auromycin has, indeed, a chlorine atom in the C7 position and thus is also called chlortetracycline, while terramycin has a hydroxyl group in C5 and may be called oxytetracycline. Lloyd Conover, a contributor of Pfizer, hypothesised that modifying the chemical structure of both auromycin and terramycin could improve their microbial activity. Indeed, Conover removed the chlorine group on C7 from aureomycin and obtained a more water soluble and effective compound. To this compound was given the name

tetracycline (Tc). Tetracyclines can inhibit the growth of a wide range of both Gram-negative and Gram-positive bacteria, by binding to the S4, S7 and S18 proteins of the 30S ribosomal subunit and then exerting a strong bacteriostatic effect (Chopra and Roberts, 2001; Nelson and Levy, 2011).

Today about 52-65% of the antibiotics are tetracyclines and 78% of these are administered to pigs in doses ranging from 0.75 to 1.25 kg t^{-1} (Winckler and Grafe, 2001). Tetracyclines remain in the animal body for quite a short period of time. Most are excreted in the urine and faeces after from 24-48 h to up to several days after administration (Winckler and Grafe, 2001), and can reach soil in animal manure. Their antibiotic use for veterinary purposes modifies the structure and function of the resident microbial community and resistance development in the soil environment. The continuous and indiscriminate use of antibiotics increases the antimicrobial resistance in commensal pathogen microorganisms (Bahl *et al.*, 2009), with repercussions on the treatment of infectious diseases in humans (Johnson, 2011).

Chapter 2
Aim of the PhD work

Tc is one of the oldest and most widely used tetracycline antibiotics given to livestock. It can reach the soil environment through the spreading of contaminated manure on agricultural land. There is still little, and certainly not exhaustive, information available in the literature on the effects of Tc on soil microbiota. Thus the aims of this work were:

- to study Tc adsorption-desorption behaviour in two soils;
- to clarify the influence of Tc and manure on the structure and function of the microbial communities in the two soils;
- to assess whether adding manure contaminated with Tc to the soil influences the antibiotic resistance of the resident microbial populations.

In order to achieve these objectives, we evaluated, at different times and at microcosm scale, the effects of two Tc concentrations, 100 and 500 mg kg^{-1} soil (both administered in a single dose) and manure, on the structure of microbial communities. This was done using culture dependent methods i.e. microbial counts, Biolog® community level physiological profiles (CLPP) and phospholipid fatty acids (PLFA) analysis. The function of the soil, after the addition of Tc and/or manure, was evaluated through quantification of fluorescein diacetate (FDA) hydrolysis.

The effect of repeated amendments on the structure of the soil microbial community was also assessed by fingerprinting the genes encoding 16S rRNA, amplified by polymerase chain reaction (PCR) and subsequent separation in denaturing gradient gel electrophoresis (DGGE) analysis (16S rRNA-PCR DGGE). The spread of Tc resistant genes and mobile genetic elements within the microbial community was assessed by real-time quantitative PCR (rt-qPCR).

Experimental design

Experiments were performed in microcosms. Two soils were used: PU and SA from Putifigari and Sassari (Sardinia, Italy) respectively. Two different Tc concentrations of 100 mg kg^{-1} soil (Tc100) and 500 mg kg^{-1} soil (Tc500) were added to soil alone and to soil added with cow manure, free from antibiotic.

The Tc affinity for soils, amended or not with manure, was studied by adsorption-desorption experiments.

The investigation of manure and Tc effect on soil microbial communities was divided in two experiments. In the first, we observed the Tc and manure impact on the structure and function of microbial soil community in the short, medium and long time (2, 7 and 60 days respectively) after one single addition of Tc and/or manure on soil. Since Tc normally reaches soil through contaminated manure, which is involved in antibiotic resistance spreading, in the second experiment, we tested the effects of manure, either unspiked or spiked with Tc, on the bacterial community structure and resistance development. In this experiment, treatments were performed three times to soil. Bacterial fingerprinting and quantification of resistance genes were performed 60 days after each amendment.

Influence of single Tc and manure administration on the soil microbial community structure and function

Six different treatments were considered for each soil (with three replicates for each treatment). The effect were evaluated at 2, 7 and 60 days after single amendment:

- PU/SA soils;
- PU/SA soils + manure (M);
- PU/SA soils + Tc100;
- PU/SA soils + Tc500;
- PU/SA soils + M + Tc100;
- PU/SA soils + M + Tc500.

Fresh soils were used. Microcosms of both soils were kept at 50% of their respective maximum water holding capacity, incubated in the dark at 20°C and humidity loss was periodically restored by adding sterile water to soil surface to recover the weight lost by evaporation. The soil microbial community structure was monitored at 2, 7 and 60 days after Tc addition through microbial counts, Biolog® CLPP and PLFA profiling. The soil microbial activity was monitored FDA hydrolysis.

Influence of repeated manure and Tc amendments on the structure and resistance development of bacterial community

Since manure is commonly spread into soil several times per year to restore the level of SOM content (Paul, 2007), we investigated if repeated amendments can influence the structure

of soil bacterial community and the spread of antibiotic resistance genes. Treatments used for this experiment (four replicates for each treatment) were:

- PU/SA soils;
- PU/SA soils + M;
- PU/SA soils + M + Tc100;
- PU/SA soils + M + Tc500.

Manure, spiked or not with Tc, was applied three times with two-months intervals, and 60 days after each amendment soil samples were collected, i.e. at 60, 120 and 180 days. Hence effects on the whole bacterial community structure were analysed by denaturing gradient gel electrophoresis (DGGE) fingerprinting of 16S rRNA gene freagments amplified by polymerase chain reaction (PCR) from total community DNA.

The development of antibiotic resistance was monitored by detection and quantification of tetracycline resistance genes (*tet*), *sul* genes and genes related to their transfer on microbial population (*intI1* and *trfA*) through PCR-Southern Blot hybridization or real-time quantitative PCR (rt-qPCR) analyses.

Chapter 3

Materials and methods

3.1 Soils, manure and chemicals

Tetracycline hydrochloride was supplied by Sigma-Aldrich (Milano, Italy). Its water solubility is 50 g L^{-1}, pK_{a1} = 3.30, pK_{a2} = 7.68 and pK_{a3}= 9.68.

Fluorescein diacetate, fluorescein sodium salt bioreagent, FAME 37 Supelco Mix, and Methyl nonadecanoate 98% GC were supplied from Sigma-Aldrich (Milano, Italy).

CP-Mix Bacterial acid methyl esters was supplied by Biotrend Chemikalien GmbH (Köln, Germany).

All the solvents were of HPLC grade (Carlo Erba Reagenti, Milan, Italy) and were used without further purification.

Two Sardinian soils (Italy), a clay soil from Putifigari (PU) and a sandy soil from Sassari (SA) were used. Soil samples were air-dried for 15 days and sieved to <2 mm. The particle size distribution was measured by Purdue University Soil Testing Laboratory using the pipette method (Day, 1965). The organic carbon content was determined according to the modified Walkley-Black method (Jackson, 1958). Soil pH was determined on slurries with a soil/water ratio of 1:2.5. The cow manure used in the experiment was collected from a Sardinian cattle farm near Tula 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.

Selected physico-chemical properties of SA and PU soils and manure are listed in Table 2.

Table 2. Selected physical and chemical properties of the soils and manure investigated*.

	pH	OM (%)	OC (%)	Sand (%)	Clay (%)	Silt (%)
PU soil	5.77	6.90	4.00	39.40	41.37	19.21
SA soil	7.60	4.87	2.83	72.71	16.63	10.61
Manure	7.81	52.72	30.58	-	-	-

* OM, Organic Matter; OC, Organic Carbon.

Luigi Chessa, Effect of a veterinary antibiotic and manure amendment on the structure and function of soil microbial community, Tesi di dottorato in Scienze e biotecnologie dei Sistemi Agrari e Forestali e delle Produzioni alimentari, indirizzo in Biotecnologie Microbiche Agroalimentari, Università degli Studi di Sassari

3.2 Sorption on soils and manure-amended soils

Sorption trials were carried out using a batch equilibration technique at 25±2°C. Tc sorption was measured on unamended and manure-amended soils sieved through a 2 mm mesh screen. Triplicate 2.5 g samples of both unamended and amended soils were equilibrated in polyallomer centrifuge tubes with 5 ml of aqueous antibiotic solution (122.5 μM corresponding to a Tc soil concentration of 100 mg kg^{-1} and 602.6 μM corresponding to a concentration of 500 mg kg^{-1}). The tubes were shaken (end over end) for 30 min. After equilibration, the suspension was centrifuged at 5000 *g* for 30 min, and the supernatant was pipetted off and analysed immediately. The Tc amount adsorbed by soil was calculated from the difference between the initial and final concentrations of Tc in solution. The concentration of Tc was determined by high performance liquid chromatography (HPLC). The system was assembled as follows: a Waters 1515 pump equipped with a Waters 2487 UV/VIS programmable detector (Waters, Mildford, MA) operating at 254 nm, a Breeze chromatography software, a μBondapak C_{18} analytical column (10 μ, 3.9x300 mm) (Waters, Mildford, MA). The mobile phase was acetonitrile plus water (23+77 by volume, pH = 2.5) at a flow rate of 0.7 ml min^{-1}. The Tc retention time under these chromatographic conditions was 7.985 min. The quantitative determination of Tc was performed by using an external standard. Calculations were based on the average peak areas of the external standard.

3.3 Set up of soil microcosms

Soils were air-dried for 2 days before microbial analyses. Soil samples (500 g each) of sieved (< 2 mm) PU and SA soils were independently treated with an aqueous Tc 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, PU and SA soil samples (500 g each) were amended with 20 g of cow manure previously contaminated with Tc solution freshly prepared (Tc100 and Tc500) 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 and SA). These latter soils received only water before mixing and incubating. Every two days, water was sprayed on the soil surface

to compensate the weight loss due to evaporation. Soil aliquots from each sample were used for microbiological and biochemical analyses which were carried out at three time-points, namely after 2, 7 and 60 days incubation. Before sampling, soil was mixed and the pots further incubated.

3.4 Enumeration of fast-growing heterotrophic bacteria and fungi in soils

Total fast-growing heterotrophic bacteria and fungi were enumerated on treated and untreated soils using conventional serial dilution and spread plate method. Solidified (15 g l^{-1} agar) 1:10 strength TSA (Tryptic Soy Agar, Microbiol, Cagliari, Italy), GYEP pH 4.5 (Glucose Yeast Extract Peptone medium) (Garau *et al.*, 2007) and Actinomycetes Isolation Agar (DiFCO, Milan, Italy) were used as the growth media for counting total heterotrophic bacteria, fungi and actinomycetes respectively.

At each time-point triplicate samples (20 g) from treated and untreated soil were dispersed in 180 ml of a pyrophosphate (2 g l^{-1}) solution and shaken at 150 rpm for 30 min. Serial 10-fold dilutions were then prepared using saline solution (0.89% w/v NaCl) and 150 µl aliquots of each dilution were used to inoculate a quadruplicate set of plates containing the respective media. Bacterial and fungal colonies were counted on the relevant media after incubation of the plates at 28°C for 3 days and microbial counts expressed as average Log CFUs (Colony Forming Units) ± standard deviation per gram of soil dry weight.

3.5 Phospholipid fatty acids (PLFA) extraction from soils and analysis

Phospholipids extraction from soil, identification and quantification was essentially done according to Gutiérrez *et al.* (2010). Twenty grams of soil were mixed with 70 ml of extraction solution (1:2:0.8 chloroform:methanol:citrate buffer (citrate buffer: 6.3 g citric acid monohydrate in 200 mL of deionized water, adjusted to pH 4.0 with KOH pellets). The mixture was shaken for 2 h at 225 rpm on a horizontal rotary shaker and subsequently centrifuged (4,000 rpm for 30 min). The supernatant was transferred to a separation funnel and the residue was extracted a second time (20 ml extraction solution, 1 h shaking). To the extract, 25 ml of chloroform and 25 ml of citrate buffer were added in the separation funnel which was shaken vigorously by hand

and subsequently in a horizontal rotary shaker (225 rpm) for 10 min. After phase separation (overnight), the chloroform phase containing lipids was transferred into 25 ml conical flasks and dried. Afterwards, the samples were subjected to a methylation by two step methylation method reported by Kramer *et al.* (1997) and modified by Jenkins (2010). Initially, 2 ml of sodium methoxide 0.5 M and, after 10 min, 3 ml of freshly prepared 10% methanolic hydrochloric acid were added to the samples at 50°C. Subsequently, 1 ml of hexane containing C:19 fatty acid standard and 7.5 ml of 6% potassium carbonate were added and samples were centrifuged for 7 min at 2,000 rpm. The upper organic phase was transferred to a GC vial and analysed.

Separation and quantification of the fatty acid methyl esters were carried out 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 8,200 (CX Varian Inc. Palo Alto, CA). The column head pressure set at 37.00 psi. Helium was used as carrier gas with a flow rate of 1 ml min^{-1}. The initial oven temperature was 75 °C for 1 min, it gradually increased for 5 °C each min until 148 °C and further increased to 165 °C at 8 °C min^{-1} and held for 35 min. Temperature ramped again to 210 °C at 5.5 °C min^{-1} and finally to 230 °C at 3 °C min^{-1} and held for 14.7 min.

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, 18:1ω 9c) and cyclopropyl saturated PLFA (cy17:0, cy19:0). The sum of signature PLFA for Gram-positive and Gram-negative bacteria is referred to as bacterial PLFA ($PLFA_{bact}$). The quantity of the PLFA 18:2 ω 6,9 was used as an indicator of fungal biomass.

3.6 Fluorescein diacetate (FDA) hydrolysis

Hydrolysis of fluorescein diacetate (FDA) was used for microbial soil activity measurement. In a 50 ml conical flask, triplicate samples from each soil (2 g fresh weight, sieved < 2 mm) were added with 15 ml of 60 mM potassium phosphate buffer (pH 7.6) and 0.2 ml of 1 mg FDA ml^{-1} solution in ethanol. Blanks were prepared without the addition of the FDA. The

flasks were then incubated at 30 °C for 20 min at 150 rpm. After incubation, the reaction was stopped by adding acetone (15 ml) and the soil suspension was centrifuged at 4,000 rpm for 10 min. The supernatant was then filtered (Whatman, no. 2) and the optical density at 490 nm was measured with a SPECTROstar^{Nano} (BMG LABTECH, GmbH, Offenburgh, Germany) spectrophotometer. The concentration of fluorescein released during the assay was calculated using the calibration graph obtained from a solution of fluorescein sodium salt in the concentration range 0 - 10 μg mL^{-1}.

3.7 Biolog® Community Level Physiological Profile (CLPP)

The Biolog® Community Level Physiological Profile (CLPP), 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 10-fold dilutions used for the enumeration of total fast-growing heterotrophic bacteria and fungi were centrifuged (4,600 rpm, 8 min) and 120 μl of the clear supernatant were used to inoculate each of the 96 microtiter wells of the Biolog plate. Every well of the plate contains a single carbon compound and a redox dye which reveals oxidative catabolism. A triplicate set of 31 carbon sources is present within each plate (eight carbohydrates, eight carboxylic acids, four polymers, eight amino acids and three miscellaneous compounds) as well as three control wells with no-carbon. Inoculated plates were incubated in the dark at 28 °C for 7 days and the evolution of the carbon source utilization within each well was quantified by measuring the optical density values at 590 nm (OD_{590}) every 24 hours using a Biolog MicroStation^{TM} reader (Biolog Inc., Hayward, CA). The analysis of CLPP data (i.e. the carbon source utilization data or the OD_{590} values) was carried out by Principal Components Analysis (PCA) to reduce complex multidimensional data and allow for a more straightforward interpretation of the results, i.e. the treatment effect on the physiological profile of each microbial community.

3.8 Total community DNA extraction and PCR-amplification of bacterial 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE) analysis

The DNA Bio101 was extracted from 0.5 g of frozen soil samples using the FastDNA®SPIN Kit for Soil (Bio101 Q-Biogene/MP-Biochemicals. Solon, OH, USA) with some modifications: cell lyses in soil samples by the FastPrep® Instrument was performed for 30 sec at 5.5 speeds and repeated two times. Also the washing step, by addition of SEWS-M solution, was repeated two times instead one. Finally DNA was eluted in final 100 µl solution. Then, 50 µl of Bio101 DNA from each sample were purified by Geneclean® Spin Kit (Q-Biogene/MP-Biochemicals, Solon, OH, USA) and suspended in 50 µl final volume following the manufacturers' protocol. Amplification of bacterial 16S rRNA gene fragments (positions 984 to 1378) was performed using primers F948GC and R1378 (Heuer *et al.*, 1997). Reaction mixture, in total 25 µl was: 1 µl DNA template, 0.6 U TrueStart Taq DNA polymerase and 3.75 mM $MgCl_2$ and buffer (Fermentas, St. Leon-rot, Germany), 0.2 mM of each deoxynucleotide triphosphate, 4% acetamid and 0.2 mM of each primer. Template DNA samples were diluted five times in 10% TE buffer 10 mM and 90% Tris-HCl buffer pH 8.5 solution prior amplification. PCR products have a GC clamp to prevent the complete melting during separation in denaturation gradient gel. After 5 min denaturation at 94 °C, 35 thermal cycles of 1 min at 94 °C, 1 min at 53 °C and 2 min at 72 °C were performed in according to (Heuer *et al.*, 1997; Gomes *et al.*, 2001), followed by a final extension step at 72 °C for 10 min.

The DGGE fingerprinting of 16S rRNA gene fragments was performed by the Ingeny PhorU system (Ingeny, Goes, The Netherlands) according to Weinert *et al.* (2009). The 16S rRNA gene fragment was separated in a double denaturing gradient from 46.5 to 65% of denaturant (100% denaturant was defined as 7 M urea and 40% formamide) and from 6.2 to 9% acrylamide. On the top of the acrylamide gel, a 15% acrylamide staking gel was pipetted. About 5 µl of each DNA product were loaded flanked to each other on the gel. Bacterial markers, composed by a mixture of 11 bacterial GC-clamp fragments with different electrophoretic mobility (Heuer *et al.*, 2002), were placed twice on the gel (in some gels three times) flanking the DNA samples. Gels run in 1 × Tris-acetate-EDTA buffer for 17 h on a constant voltage of 140 V and at the constant temperature of 58 °C. Polyacrylamide gels were stained on silver nitrate (0.2% v/v) by the method described by Heuer *et al.* (2001) then digitalized and pairwise analysis was performed by

using the software GelCompar II® (version 6.5, Applied Maths, Austin, TX) (Smalla *et al.*, 2001) to calculate Pearson correlation indices through unweighted pair group method using arithmetic averages (UPGMA). Statistical analysis was carried out using Pearson correlation indices for significance calculation through Permutation test described by Kropf *et al.* (2004). Permutation test, based on random pairwise comparison (10^4) between lanes and groups of lanes, was used to calculate effect of treatments in one soil (PU/SA) at specific time-point and also in one soil at two different time points (60 and 180 days). Distance (d-value) between treatments was calculated by the average correlation of coefficients within treatments minus correlation coefficients between treatments.

3.9 Real-time quantitative PCR (rt-qPCR) for tetracycline resistance genes and mobile genetic elements

The copy numbers of target genes (*tet*(Q), *tet*(W), *intI1*, *trfA*, *sul1* and *sul2*) were quantified by real time quantitative PCR (rt-qPCR) using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The copy number of 16S rRNA genes (*rrn*) was used to normalize the calculation of the relative abundance of gene targets with respect to bacterial population (target gene copies/*rrn* copies) and also to compensate for differences in DNA extraction and amplification of soil samples.

The rt-qPCR of *rrn* genes were performed according to Heuer *et al.* (2008). For quantification of *tet*(Q) and *tet*(W) genes the same reaction mixture composition was used but with different primers and probes. In 50 µl total volume reaction 5 µl of DNA template, 1.25 U TrueStart Taq DNA polymerase and 2.5 mM $MgCl_2$ and buffer (Fermentas, St. Leon-rot, Germany), 0.2 mM of each deoxynucleotide triphosphate, 2 µl of bovine serum albumin (BSA) (2 mg ml^{-1}) and 0.3 mM of primers and probe were used. The reaction protocol was: 10 min at 95 °C followed 40 cycles by 15 sec at 95 °C and 45 sec at 60 °C. Primers and probes for *tet*(Q) (GeneBank accession no. X58717) and *tet*(W) (GeneBank accession no. AJ222769) were described by Smith *et al.* (2004). The relative abundance of class 1 integrons integrase gene (*intI1*), product size 196 bp, was measured using primers LC1 and LC5 and the *intI1*-probe described by Barraud *et al.* (2010). The reaction mixture was the same used for quantification of *tet* genes. The programme, in agreement with Barraud *et al.* (2010), consisted of 40 cycles. The quantitative PCR to measure

the abundance of plasmid IncP-1ε plasmids by *trfA* gene fragments (281 bp) was performed in 50 µl of reaction mixture. Concentrations used in the reaction mixture were the same described for *tet* genes rt-qPCR. Forward and reverse primers and probe used were in according to Bahl *et al.* (2009). Thermal cycles were 10 min at 95 °C followed by 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C. Quantification of Genes of *sul1* and *sul2* genes abundance was in agreement with Heuer and Smalla (2007) and Heuer *et al.* (2008) respectively.

3.10 Southern blot hybridization for tetracycline resistance genes detection

The occurrence of *tet*(A) and *tet*(M) genes was detected by PCR amplification according to Lanz *et al.* (2003) and Ng *et al.* (2001). The reaction mixture, in total 25 µl, identical in composition for both genes, was composed of 0.6 U TrueStart Taq DNA polymerase and 2.5 mM $MgCl_2$ and buffer (Fermentas, St. Leon-rot, Germany), 0.1 mg ml^{-1} BSA (2 mg mL^{-1}), 0.2 mM of each deoxynucleotide triphosphate and 0.5 µM of each primer. The PCR protocol was performed, for *tet*(A) by an initial denaturation for 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C and a final 7 min step at 72 °C. The protocol for *tet*(M) was: 5 min at 94 °C, 30 sec at 94 °C, 30 sec at 55 °C, 1 min at 72 °C and final 5 min at 72 °C.
PCR products were subsequently hybridised by Southern blot hybridisation (Sambrook *et al.*, 1989). Probes were obtained by PCR products from plasmids pGEM-tetA for *tet*(A) and from pAT101 for *tet*(M) and then excised from the agarose gel after electrophoresis and labelled with digoxigenin according to manifacturer's instructions (Roche, Applied Science, Mannheim, Germany).

3.11 Statistical analysis

For each soil and incubation time, mean values from microbial counts, PLFA, enzyme activities and relative abundance of genes target were subjected to One-Way Analysis of Variance (One-Way ANOVA) to evaluate the effect of the different treatments. Where significant *P*-values were obtained ($P<0.05$), differences between individual means were compared using the post hoc Tukey-Kramer test ($P<0.05$). Pearson correlation was used to assess the concordance of treatments' effect among these different analyses (e.g. microbial counts,

PLFA and FDA). In the CLPP data analysis, the carbon sources utilization data were normalised by dividing each OD590 for the Average Well Colour Development (AWCD, a measure of the potential metabolic activity of the microbial community) of the plate. Then, a Principal Components Analysis (PCA) was used to reduce complex multidimensional data and allow for a more straightforward interpretation of results. For Permutation test was used One-Way ANOVA to compare the differences between treatments at specific time-points and Two-Ways ANOVA to compare the effects of treatment and time (repeated amendments). ANOVA test was also used to check the effects of treatments on resistance gene abundance in soil.

Chapter 4
Results and discussion

4.1 Tetracycline adsorption-desorption

The biological effect of Tc in soil depends on its bioavailability, which in turn is influenced by adsorption-desorption processes. Therefore, the first goal of this study was to assess the Tc affinity for the two soils tested. Some chemical and physical properties of soils and manure are shown in Table 2. PU, a clay soil, is more acid than SA, a sandy soil. The organic carbon (OC) content was greater in PU (4.00 %) than in SA soil (2.83 %). Manure pH was 7.81 and OC content 30.58 % (Table 2).

The adsorption on both manure-amended and unamended soils was very fast and the equilibrium was reached within 30 min. In all cases, only minor amounts of Tc were detected in equilibrium solution. Namely, in PU soil the equilibrium Tc concentration was 0.157 and 1.201 mg kg^{-1} for Tc spiked applications of 100 and 500 mg kg^{-1}, respectively. These concentrations were up to five times lower than those measured in SA soil (0.859 and 4.602 mg kg^{-1}). In the presence of manure, Tc was adsorbed to a slightly higher extent, on both soils (Table 3). No Tc degradation was observed in both unamended and amended soils.

Table 3. K_d values and bioavailable Tc (mg Tc kg^{-1} soil) in PU and SA soils unamended and amended with Tc contaminated manure.

	Soil		**Soil + Manure**	
	mg Tc kg^{-1}	**K_d**	**mg Tc kg^{-1}**	**K_d**
PU 100 mg kg^{-1}	0.157	1495	0.155	1400
PU 500 mg kg^{-1}	1.201	979	1.092	993
SA 100 mg kg^{-1}	0.859	272	0.767	281
SA 500 mg kg^{-1}	4.602	254	4.468	241

A slight decrease of Tc in equilibrium solution was detected in PU+M soil (0.155 and 1.092 mg kg^{-1}) and SA+M soil (0.767 and 4.468 mg kg^{-1}), most probably because of the greater content of organic matter. According to the K_d parameter, the adsorption occurred to a higher extent in PU than in SA soil (Table 3). The K_d values reported by Thiele-Bruhn (2003) confirm the strong

affinity of Tc for soil (Table 1). The adsorption, generally, depends on soil properties like SOM content, texture and pH value, the last parameter being the most effective in the case of ionisable molecules. Indeed; Tc exhibits three dissociation constants, pK_{a1}= 3.30, pK_{a2}= 7.68 and pK_{a3}= 9.68, respectively (Figure 4).

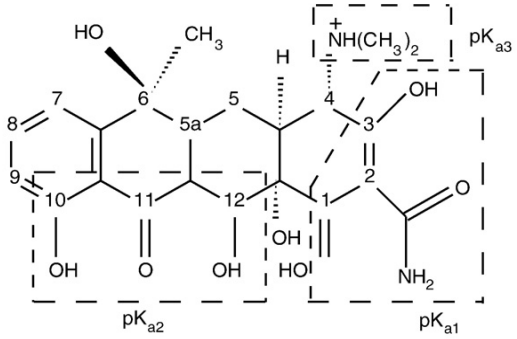

Figure 4. Tc chemical structure and pK_a values.

Therefore, with varying pH value, Tc can exhibit cationic, zwitterionic or anionic form (Figure 5). At pH value <2 Tc is cationic (Tc+00), from 3.5 to 7.5 Tc tends to assume zwitterion form (Tc+-0) and at pH 5.5 is totally neutral. Above pH 7.5 Tc is in two anionic forms (Tc+-- and Tc0--) (Wang *et al.*, 2010; Zhao *et al.*, 2012).

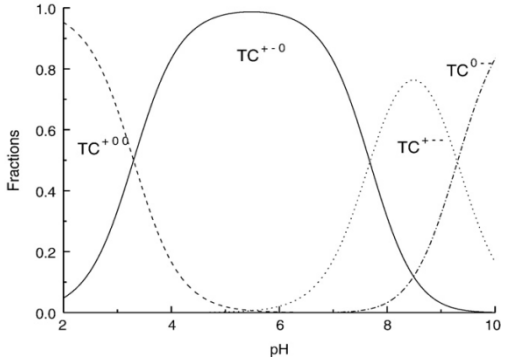

Figure 5. Tc speciation as a function of pH. From Whang *et al.*, 2010.

The extent of Tc adsorption observed on PU soil is most likely due to the combined effects of lower pH value and higher clay content. These findings are consistent with the study carried out by Zhao *et al.* (2012) and Li *et al.* (2010), confirming that Tc has a strong tendency to bind clay minerals by surface complexation (binding) of the zwitterion form, involving cation exchange. This is the major mechanism of Tc retention in soil and the extent of adsorption decreases with

increasing pH. The addition of manure did not affect significantly the Tc adsorption in both soils (Table 3), substantiating a minor role of organic matter, compared to clay.

Manure is spread on soil, as a common agricultural practice, to restore the SOM content and maintain soil productivity. Usually the manure derives from animals treated with VAs. Depending on the therapy (Tolls, 2001), the Tc content in manure can reach a concentration up to 4 mg kg^{-1} (Thiele-Bruhn, 2003). Tetracycline concentration in soil ranges from 12-100 μg kg^{-1} (Hamscher *et al.*, 2002) to 450-900 μg kg^{-1} (Winckler and Grafe, 2000). Tc is quite persistent and controversial information about its half-life is available. Thiele-Bruhn (2003) reported that Tc remains stable in soil until 180 days, whereas Winckler and Grafe (2001), after storing contaminated manure in the dark to prevent photodegradation, measured a half-life of 55-105 days, contrarily to Kühne *et al.* (2000), who reported a shorter half-life of 4.5-9 days. Moreover, Tc can accumulate in soil with repeated manure applications (Jacobsen *et al.*, 2004), e. g., after spreading pig slurries on field for two consecutive years, Tc content in soil was up to 1.691 mg kg^{-1} (Kay *et al.*, 2004). In agreement with previous reports (Wan *et al.*, 2010), Tc adsorption on soil was rather irreversible, suggesting that a significant amount of Tc remained tightly bound to soil and did not desorb readily under our experimental conditions. The amounts of Tc in solution at the equilibrium are shown in Table 3 and these may be assumed as the Tc fraction available for soil microbial community.

The effects of antibiotics are highly dependent on their bioavailability (Kong *et al.*, 2012). Nevertheless, the mechanism of antimicrobial activity of TC, once bound to soil particles, is not well known. Parolo *et al.* (2008) reported that the antimicrobial activity of Tc was maintained after adsorption on montmorillonite clay. On the other hand, a significant decrease of Tc activity, as a result of its strong adsorption on montmorillonite clay, was recently suggested (Lv *et al.*, 2013).

We amended soil microcosms with a manure concentration comparable to that used in agricultural practice (Heuer *et al.*, 2009). Two Tc concentrations, 100 and 500 mg kg^{-1}, higher than those commonly measured in soil (Hamscher *et al.*, 2002; Sarmah *et al.*, 2006), were selected in order to have a measurable antibiotic effect on the microbial community. Otherwise the effect could be not detectable (Hund-Rinke *et al.*, 2004), also because of the high Tc affinity for colloidal soil components (Lv *et al.*, 2013). Moreover, it can be noted that the bioavailable Tc concentration, remaining at starting Tc concentration of 100 mg kg^{-1}, was comparable to that usually found in soil (Kummerer, 2003; Sarmah *et al.*, 2006; Andreu *et al.*, 2009; Arikan *et al.*,

2009; Karci and Balcioğlu, 2009). Furthermore, at a concentration of 100 mg kg^{-1}, Tc did not show to have great effects on the bacterial structure respect to free-antibiotic manure treatment. Indeed, when the concentration of 500 mg kg^{-1} was used, the bioavailable fraction was about five times greater than above.

4.2 Study of tetracycline and manure effects on the structure and function of soil microbial community after single amendment

The impact of Tc and manure addition on soil microbial structure and activity was evaluated 2, 7 and 60 days after a single amendment by several analyses:

- counts of culturable fast-growing heterotrophic bacteria, fungi and actinomycetes;
- phospholipid fatty acids (PLFA) fingerprinting on total bacteria, fungi and actinomycetes;
- fluorescein diacetate (FDA) hydrolysis test;
- Biolog® Community Level Physiological Profile (CLPP).

4.2.1 Microbial counts

The effects of Tc and manure were observed on the culturable portion of fast-growing heterotropic bacteria, fungi and actinomycetes.

Bacteria. The population of total culturable bacterial changed significantly after Tc and manure addition. Both soils, 2 days after a single amendment, manure and Tc, at the highest concentrations, showed opposite effects. Tc 500 mg kg^{-1} (PU/SA+Tc500) reduced the bacterial population while manure stimulated its growth. Bacterial population was 7.27 Log CFU g^{-1} soil in PU+Tc500, significantly (Tukey-Kramer test, $P<0.05$) lower than 7.62 CFU g^{-1} in the untreated PU (Table 4). Similarly, in SA+Tc500 (7.54 Log CFU g^{-1}) bacterial counts were significantly ($P<0.05$) lower than in untreated SA soil (7.81 Log CFU g^{-1}) (Table 5). The concentration Tc100 did not affect bacterial population in both soils, compared to untreated controls, 2 days after amendment.

Conversely, manure mitigated Tc effects in PU soil and completely nullified them in SA soil. Bacterial population in PU+M+Tc500 (7.72 Log CFU g^{-1}) was statistically (P<0.05) lower than in PU+M (7.89 Log CFU g^{-1}) but not statistically (P<0.05) different from untreated PU. In SA+M and SA+M+Tc500 bacterial counts were not statistically different (8.43 and 8.23 CFU g^{-1} respectively).

Table 4. Total culturable bacterial, fungi and actinomycete counts in PU soil (Log CFU g^{-1} soil dry weight)*.

Time	PU soil	PU+M	PU+Tc100	PU+Tc500	PU+M+Tc100	PU+M+Tc500
			bacteria			
2 days	7.62 ^{b}	7.89 ^{c}	7.64 ^{b}	7.27 ^{a}	7.89 ^{c}	7.72 ^{b}
7 days	7.82 ^{a}	7.80 ^{a}	7.90 ^{ab}	7.92 ^{ab}	7.95 ^{b}	8.03 ^{b}
60 days	8.36 ^{ab}	8.61 ^{c}	8.30 ^{a}	8.35 ^{ab}	8.53 ^{c}	8.49 ^{b}
			fungi			
2 days	6.35 ^{a}	6.40 ^{a}	6.36 ^{a}	6.45 ^{a}	6.41 ^{a}	6.45 ^{a}
7 days	5.57 ^{a}	5.63 ^{a}	5.66 ^{a}	5.67 ^{a}	5.71 ^{a}	5.77 ^{a}
60 days	5.92 ^{a}	5.95 ^{a}	6.06 ^{a}	6.04 ^{a}	6.10 ^{a}	6.02 ^{a}
			actinomycetes			
2 days	6.74 ^{a}	7.09 ^{b}	6.85 ^{a}	6.98 ^{b}	7.23 ^{c}	7.32 ^{c}
7 days	6.87 ^{a}	6.96 ^{b}	7.02 ^{ab}	7.09 ^{b}	7.06 ^{ab}	7.10 ^{b}
60 days	7.94 ^{a}	8.28 ^{b}	7.92 ^{a}	7.98 ^{a}	8.43 ^{c}	8.63 ^{d}

* M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter, do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test.

Table 5. Total culturable bacterial, fungi and actinomycetes counts in SA soil (Log CFU g^{-1} soil dry weight)*.

Time	SA soil	SA+M	SA+Tc100	SA+Tc500	SA+M+Tc100	SA+M+Tc500
			bacteria			
2 days	7.81 [b]	8.43 [c]	7.69 [ab]	7.54 [a]	7.38 [c]	8.23 [c]
7 days	7.97 [a]	7.97 [a]	7.96 [a]	8.07 [a]	8.00 [a]	8.11 [a]
60 days	7.66 [a]	7.94 [b]	7.54 [a]	7.58 [a]	8.06 [b]	8.11 [b]
			fungi			
2 days	5.40 [a]	5.32 [a]	5.50 [a]	5.48 [a]	5.41 [a]	5.51 [a]
7 days	6.07 [a]	6.54 [b]	6.29 [ab]	6.52 [b]	6.50 [b]	6.44 [b]
60 days	4.43 [a]	5.27 [b]	4.92 [ab]	4.54 [a]	5.18 [b]	5.27 [b]
			actinomycetes			
2 days	7.46 [a]	7.88 [ab]	7.64 [ab]	7.90 [ab]	8.03 [b]	8.07 [b]
7 days	7.54 [a]	7.98 [b]	7.52 [a]	7.71 [ab]	8.07 [b]	8.01 [b]
60 days	7.01 [a]	7.45 [b]	7.11 [a]	7.16 [a]	6.92 [a]	6.98 [a]

* M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter, do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test.

Seven days after amendment, the effects of Tc and manure completely disappeared in SA soil (Table 5) while statistical differences were still observed in PU soil. In PU+M+Tc100 and PU+M+Tc500, bacterial counts were higher than in PU+M and PU (Table 4). Sixty days after amendment, soils treated with only Tc (PU/SA+Tc100 and PU/SA+Tc500) exhibited no significant (P<0.05) difference from their respective controls, PU and SA. Conversely, all manure treatments, in both soils (8.61 Log CFU g^{-1} for PU+M, 8.53 for PU+M+Tc100, 8.49 for PU+M+Tc500, 7.94 for SA+M, 8.06 for SA+M+Tc100 and 8.11 for SA+M+Tc500), were statistically higher than the untreated PU (8.36 Log CFU g^{-1}) and SA soils (7.66 Log CFU g^{-1}), respectively (Table 4-5).

Fungi. The fungal population in PU soil was not influenced by Tc and manure addition at all time points. Fungal counts, 2 days after the amendment, were 6.35 Log CFU g^{-1} in untreated PU, 6.40 in PU+M and 6.45 in PU+Tc500 and PU+M+Tc500 and decreased in all treatments over time (Table 4). Similarly, in SA soil, 2 days after amendment, not statistical (P<0.05) differences were observed among treatments (Table 5). Instead, 7 days after amendment, the readily culturable fungi statistically (P<0.05) increase in all treatments (they ranged between

6.44 and 6.54 Log CFU g^{-1}) respect to control SA (6.07 Log CFU g^{-1}), with the exception of SA+Tc100. Sixty days after amendment, only in manured soils (5.27 Log CFU g^{-1} in SA+M, 5.18 in SA+M+Tc100 and 5.27 in SA+M+Tc500) the fungal component was statistically (P<0.05) higher than in control SA (4.43 Log CFU g^{-1}) (Table 5).

Actinomycetes. The effects of Tc and manure on readily culturable soil actinomycetes were also studied. In PU soil a meaningful synergistic effect of manure and Tc was observed, which determined a significant (P<0.05) increase of actinomycete population (Table 4). Such a result was quite evident 2 and 60 days after amendment, when actinomycete counts in PU+M+Tc500 reached values up to 7.32 and 8.63 Log CFU g^{-1} soil, respectively. Instead, in manure treatment (PU+M), they were 7.09 and 8.28 Log CFU g^{-1} soil and in untreated soil 6.74 and 7.94 Log CFU g^{-1} soil. In SA soil, actinomycete population was 7.46 Log CFU g^{-1}, increased significantly (P<0.05) only in treatments SA+M+Tc100 (8.03 Log CFU g^{-1}) and SA+M+Tc500 (8.07 Log CFU g^{-1}) respectively, 2 days after amendment (Table 5). Moreover, a significant effect (P<0.05) of Tc500 was detected in PU but not in SA soil (Table 4-5). Seven days after amendment, in PU soil, the actinomycete population was higher than control only in treatments spiked with the highest Tc concentration and/or manure (PU+M, PU+Tc500 and PU+M+Tc500). In SA soil, all manured soils (SA+M, SA+M+Tc100 and SA+M+Tc500) were significantly (P<0.05) higher than control. Sixty days since treatment addition, only in SA+M actinomycete counts were higher than in untreated SA (Table 5). In PU+M, PU+M+Tc100 and PU+M+Tc500, actinomycete counts were statistically higher (P<0.05) than in untreated PU and increased with increasing Tc concentration (Table 4).

To test the influence of single Tc (and/or manure) administration on soil microbial community, a number of readily culturable microbial groups were monitored in PU and SA soils at different time-points, i.e. 2, 7 and 60 days after amendment. Culturable microorganisms, like total heterotrophic fungi and bacteria represent only a small fraction with about 1% of the total soil microbial community (Kummerer, 2004). Nevertheless, it has been suggested that they could play a major role in soil functionality (van Elsas *et al.*, 1997). Accordingly, Bakken and Olsen (1987) underlined that culturable soil bacteria represent the majority of the bacterial biovolume, i.e. about 80-90%, and that they manage the major part of energy flow in soil. Consequently, the number of culturable microorganisms is often used (together with culture independent methods) to assess the impact of management and/or xenobiotics on soil microbial community structure

(Zhang *et al.*, 2010; Nelson *et al.*, 2011; Pinna *et al.*, 2012; Ding *et al.*, 2013; Mukherjee *et al.*, 2013; Rodrigues *et al.*, 2013; Shen *et al.*, 2013).

As mentioned above, Tc works by interacting with the bacterial 30S ribosomal subunit. Therefore, fungi should not be sensitive to Tc action. Indeed, fungal population was not affected by Tc treatments, but bacteria were highly susceptible to Tc in a short-term effect (up to 2 days after amendment). Manure treatments provided organic compounds easily catabolisable by soil microorganisms, in particular bacteria, which increased in number. In the present study manure treatments, in contrast to Nelson *et al.* (2011), but in agreement with Parham *et al.* (2002), increased the bacterial population likely by stimulating their ability to use soil's C-compounds faster than fungi. Manure did not impact on fungal population in PU soil, and as expected, neither Tc (Table 4). Conversely, in SA soil, manure affected fungal population in the medium-long term (7-60 days) and also Tc treatments did, 7 days after treatment addition, probably since the reduction of the bacterial population due to antagonism effects. However Tc effect disappeared 7 days after its addition to soil. Moreover only the higher Tc concentration (500 mg kg^{-1}) was effective on bacterial population, substantiating the importance of the bioavailable fraction of Tc after its addition to soil. Indeed it probably decreased, consequently to biotic degradation, or maybe the sensitive fraction of culturable bacteria defeated. The addition of manure did not affect bacteria and fungi in PU soil since the poor contribution of manure amendment on overall SOM content in PU microcosms, formerly high. Conversely in SA, the sandy soil, manure was steadily effective since manure's OM supply.

Actinomycetes are antibiotic-producer bacteria able to regulate interactions among different microbial population in soil (Bull *et al.*, 1992; Heuer *et al.*, 1997; Du and Liu, 2012). About two-thirds of antibiotics isolated from the environment are produced by culturable actinomycetes (Basil *et al.*, 2004). The increase of culturable actinomycete population of PU soil, treated with manure and Tc, is likely due to C-sources introduced with manure (Gong *et al.*, 2009; Mokni-Tlili *et al.*, 2009; Yu *et al.*, 2010) and to the synergistic behaviour of Tc that stimulated and selected resistant actinomycetes. An external Tc input, therefore, should advantage these microorganisms which benefit of supplementary C-sources released by Tc sensitive microorganisms. On the other hand, Tc had no effect on the actinomycete population of SA soil indeed they were affected only by manure addition. This suggests the presence of a foregoing antiobiotic resistance of actinomycetes in SA soil and the addition of Tc and manure positively selected resistant actinomycetes and stimulated their growth. These results also confirm, as

already discussed for fungi and bacteria, the importance of manure providing on stimulate and increase the culturable portion of soil's microorganisms.

4.2.2 Phospholipid fatty acids (PLFA)

The analysis of phospholipid fatty acids (PLFA) is a well established tool suitable to monitor changes in the structure of all microbial community. PLFA are called "markers of life" because the resulting fingerprinting is referred to all living cells at the specific time of extraction. Besides, it allows to depict a larger picture involving the most part of the bacterial and fungal community in soil (van Elsas *et al.*, 2007). The effects of Tc and manure were investigated by observing changes in the ratios fungi/bacteria and Gram-positive/Gram negative and in the size of the actinomycete population.

Fungi/bacteria ratio. The addition of Tc increased significantly (Tukey-Kramer test, $P<0.05$) the fungi/bacteria ratio in both soils, compared to untreated soils, showing a strong Tc effect 2 days after a single amendment. In PU soil a Tc concentration-dependent effect, not observed in SA soil, was also observed. Manure amendment did not significantly ($P<0.05$) affect this ratio but reduced Tc action in SA soil and completely hide the antibiotic effect in PU soil (Figure 6). Seven days after single amendment, Tc effect was reduced in PU soil, in PU+Tc500 was still effective. In PU+M+Tc100 and PU+M+Tc500 the fungi/bacteria ratio became significantly ($P<0.05$) higher than in untreated PU. Also, in SA soil, Tc effect was reduced and only in SA+Tc500 the fungi/bacteria ratio was still significantly ($P<0.05$) higher than in untreated SA soil (Figure 6). Similarly to PU soil, also in SA soil treated with manure spiked with Tc the ratio was statistically higher than in control soil. Sixty days after amendment, all effects disappeared in both soils, and no statistical ($P<0.05$) difference was observed among treatments (Figure 6).

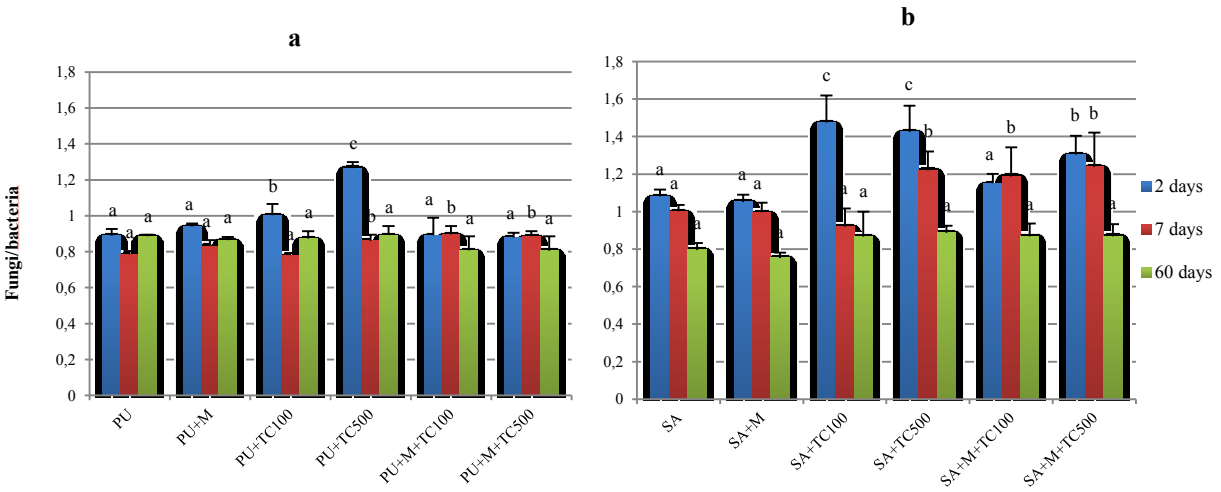

Figure 6. PLFA fungi/bacteria ratio in PU soil (a) and SA soil (b) M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

Gram-positive/Gram-negative (G+/G-) ratio. Two days after amendment, Tc showed a strong effect, only at concentration 500 mg kg^{-1}, in PU soil but not in SA soil, by increasing the G+/G- ratio in treatment PU+Tc500 and apparently favouring Gram-positive bacteria. The concentration 100 mg kg^{-1} (PU/SA+Tc100) did not statistically (P<0.05) influence this ratio, compared to PU or SA controls (Figure 7).

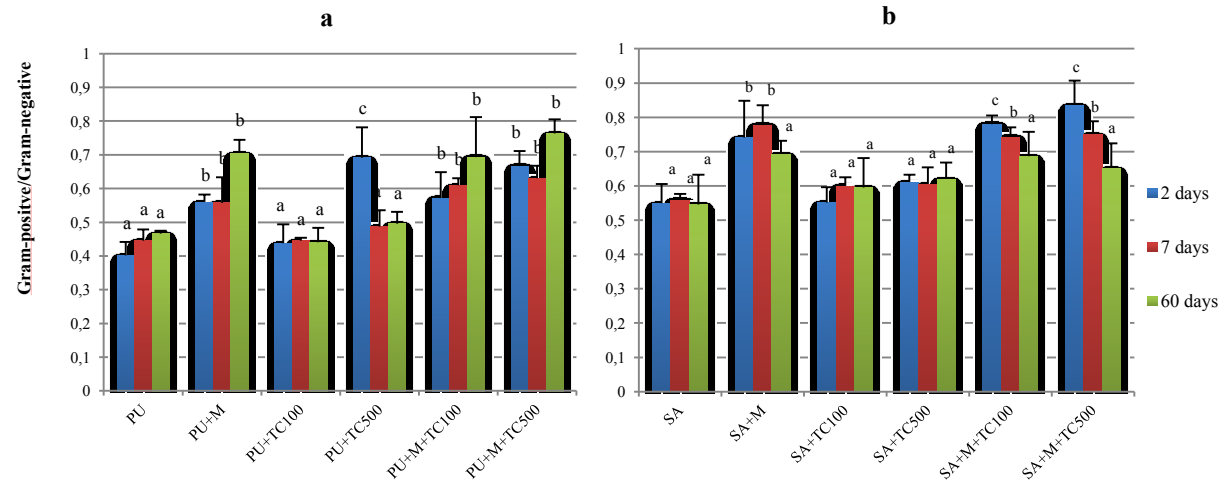

Figure 7. PLFA Gram-positive/Gram-negative ratio in PU soil (a) and SA soil (b). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

In contrast, Tc treatments were always effective if added in combination with manure (Figure 7). Seven days after amendment, in both soils, Tc effect disappeared and only in manure treatments, unspiked and spiked with Tc (PU/SA+M, PU/SA+M+Tc100 and PU/SA+M+Tc500), the G+/G- ratio was significantly (P<0.05) higher than control (PU/SA soil). Interestingly, 60 days after amendment, manure treatments (PU+M, PU+M+Tc100 and PU+M+Tc500) were still effective in PU soil, with respect to untreated PU, whereas in SA soil differences completely disappeared among all treatments (Figure 7).

Actinomycetes. The addition of Tc and manure, in both soils, significantly (P<0.05) increased the actinomicete population 2 days after single amendment, with exception for PU/SA+Tc100 treatment (Figure 8). Although the Tc concentration of 100 mg kg^{-1} was not effective, the concentration 500 mg kg^{-1} significantly (P<0.05) increased the actinomycete population in both soils (PU/SA+Tc500). Also manure addition (PU/SA+M) increased whole actinomycetes. Moreover, when manure was added to soil in combination with Tc, actinomycetes raised more showing also a Tc dose-related effect in SA but not in PU soil (Figure 8).

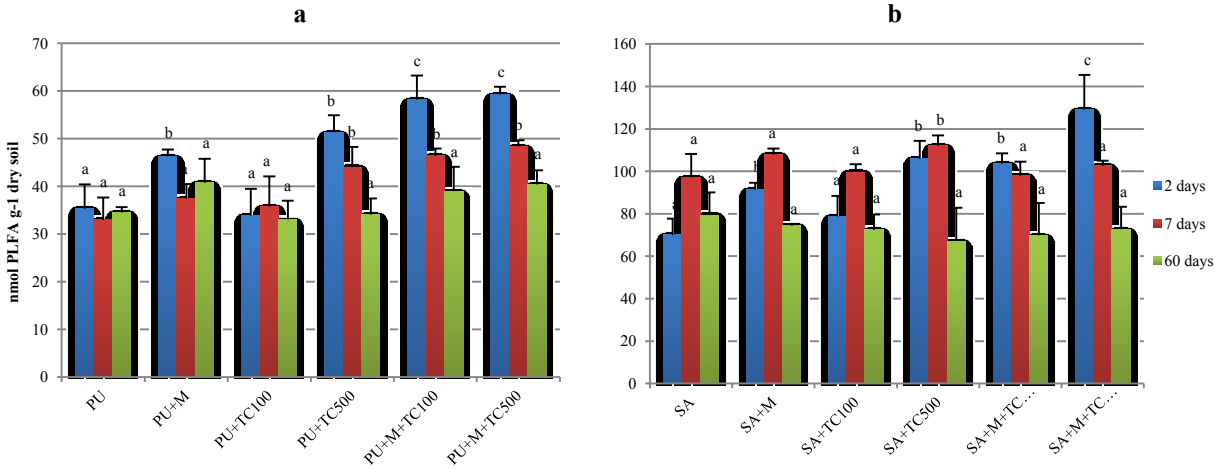

Figure 8. PLFA actinomycete population in PU soil (a) and SA soil (b). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

Seven days after amendment, PU/SA+M did not differ significantly (P<0.05) from controls and Tc500 was still effective (PU/SA+Tc500). In manure contaminated treatments, in PU soil (PU+M+Tc100 and PU+M+Tc500), the actinomycete population was higher than in untreated PU. Conversely in SA soil these treatments not statistically (P<0.05) differed from untreated SA.

Sixty days after amendment, actinomycete community of both soils restored and not differences were observed among all treatments (Figure 8).

The fungi/bacteria ratio is commonly used to monitor the effect of antibiotic treatments on soil microbial community (Ding and He, 2010; Frostegård *et al.*, 2011). The PLFA analysis carried out in this study evidenced a marked Tc effect in all treatments. In fact, increasing of the fungi/bacteria ratio, 2 days after amendment, indicated a reduction of bacterial population and likely growth of fungal community, in agreement with other reports (Hammesfahr *et al.*, 2011a). The addition of Tc to soil likely promoted competition between bacteria and fungi for ecological niches inhabited from Tc-sensitive bacteria and, due to prolonged Tc activity, probably dead bacteria constitute a useful C-source for other microorganisms (Vaclavik *et al.*, 2004) able to growth in presence of Tc. Hund-Rinke *et al.* (2004) studied the effect of Tc on the microbial community of sandy soil spiked with Tc at 500 mg kg^{-1}, the same concentration used in our study. They observed a significant (P<0.05) Tc effect which raised the fungi/bacteria ratio on long term, up to 2 months after the amendment. Also Thiele-Bruhn and Beck (2005) argue that Tc effects on the structure of soil microbial community can persist for a long time (e.g. several weeks), even if Tc could be undetectable anymore. In our work, Tc effect was limited to short-medium term (2-7 days) and, apparently, no manure effect was observed on the fungi/bacteria ratio, when added to soil. In contrast, other authors observed increasing on the fungi/bacteria ratio after manure addition to soil (Bossio and Scow, 1998; Marschnera *et al.*, 2003; Hammesfahr *et al.*, 2008; Hammesfahr *et al.*, 2011a). Nevertheless, manure, when added together with Tc reduced the impact of antibiotic on soil microbial structure, according to Hammesfahr *et al.* (2011b). Over time, the effect of manure and Tc steadily disappeared till 60 days after the amendment (Figure 6). In all microcosms, manure was added to soil to reach the final concentration of 4% in weight. Unexpectedly, manure amendment did not alter the fungi/bacteria ratio in this case. This was probably as the further addition of organic matter to soil PU, through manure amendment, did not substantially increase the already high OM content of this soil. Also in SA, a sandy soil which contains lower OM than PU soil, the addition of manure was not effective on the fungi/bacteria ratio probably since manure stimulated both fungi and bacteria. However, as supposed, manure alleviated Tc effects in soils by reducing its bioavailable fraction and also stimulating growth of resistant bacteria. Although manure addition was effective in PU soil and masked Tc effect, in SA soil the effect of manure was less clear since the higher Tc bioavailability in this soil. In fact in PU soil, Tc altered the fungi/bacteria

ratio but after 7 days the effect disappeared. Indeed, in SA soil, the Tc effect, at the highest concentration, was still observed 7 days after the amendment, since its poor affinity for the sandy soil, but anyway disappeared 60 days after the amendment. The ratio fungi/bacteria is useful to investigate the effects of treatments on the whole microbial structure. Since Tc is a bacteriostatic antibiotic and is effective mostly against bacteria, its impact was evaluated on the bacterial structure, particularly on the Gram-positive/Gram-negative (G+/G-) ratio. This is normally assumed, like the fungi/bacteria ratio, as an indicator for changes on soil bacterial structure (Ding and He, 2010). In our case, Tc increased the G+/G- ratio and the trend was quite similar in both PU and SA soils suggesting higher susceptibility of Gram-negative bacteria to Tc. This finding is in contrast with the reports of Hund-Rinke *et al.* (2004) and Ding and He (2010). Also manure addition increased the ratio G+/G-, in agreement with other authors (Marschner *et al.*, 2003; Ai *et al.*, 2012), and a long-term effect occurs in PU soil 60 days after single addition, but not in SA soil. However, the impact of manure, not observed on the fungi/bacteria ratio, revealed its effectiveness on bacterial structure by promoting growth in Gram-positive bacteria. The effect of manure, unspiked and spiked with Tc, disappeared in SA soil 60 days after amendment. Most likely, the OM added to sandy soil through the treatment, was metabolised completely and rapidly by soil bacteria. Otherwise in SA soil manure induced priming effect which persisted up to long-term detection (60 days). Actinomycetes, typical soil inhabitants, are bacteria very effective in producing antibiotics (Heuer *et al.*, 1997; Čermák *et al.*, 2008). We observed an increase of actinomycete population after single addition of Tc or manure, in contrast to (Zhang *et al.*, 2012). Moreover, Tc synergistically increased actinomycetes if added with manure. We observed the same Tc effect, with PU soil, on the culturable fraction (microbial counts of aerobic fast-growing heterotropic, Table 4-5) and on the whole actinomycetes (PLFA fingerprints, Figure 8). Instead, in SA soil the effect of manure spiked with Tc at concentration 500 mg kg^{-1}, was more evident on culturable actinomycetes than on whole actinomycete population. Probably, the culturable portion was easily inducible by manure, as observed for culturable bacteria (Table 4-5) and Tc positively selected resistant actinomycetes able to growth in its presence. Because of the synergistic action of manure and Tc, probably resident actinomycetes of PU and SA soils were largely resistant to Tc. However, 60 days after amendment the whole actinomycete population, monitored by PLFA fingerprinting, was restored in both soils. On the contrary, in PU soil the culturable fraction of actinomycetes was still affected by manure, either unspiked or spiked with Tc. Most likely, this was the result of a priming effect and, because of the higher

SOM in PU, they continue to metabolise soil's carbon sources until 60 days after the amendment. Moreover, in culturable actinomycetes a Tc concentration effect was also observed in spiked manure at long-term, which is not evident in total actinomycete population.
The PLFA analysis confirmed that the effects on microbial soil structure are strictly dependent to Tc concentration. Thus the addition 100 mg kg^{-1} to soil was not effective on microbial population substantiating the decision to test also the concentration 500 mg kg^{-1} to observe effects on microcosms experiments. Moreover, total community structure substantially restored after long-term (60 days after amendment) whilst culturable fraction was still affected.

4.2.3 Hydrolysis of fluorescein diacetate (FDA)

The hydrolysis of fluorescein diacetate is recognised as a suitable tool for overall estimation of microbial activity in soil since it is positively correlated to microbial biomass in soil (van Elsas *et al.*, 2007; Sánchez-Monedero *et al.*, 2008). The 3',6'-Diacetyl-fluorescein is fluorescein diacetate conjugated with two acetate radicals that can be hydrolysed by free cytosolic and membrane-related enzymes like lipases, unspecific esterases and proteases released by a number of microbial environmental decomposers. Since more than 90% of energy flow in soil involves microbial decomposers, the assessment of FDA hydrolysis gives a good estimation of total microbial activity (Adam and Duncan, 2001). In 1980, Swisher and Carrol found also a correlation between FDA hydrolysis and the size of the microbial population.
The FDA hydrolytic activity assay indicated a detrimental effect of Tc, both in SA+Tc100 and SA+Tc500, 2 days after amendment, suggesting a Tc dose-related effect in this soil (Figure 9b). In PU soil, Tc addition did not significantly (Tukey-Kramer test, $P<0.05$) influence the FDA activity, compared to the control, at all time points (Figure 9a). Conversely, manure addition sorely increased the FDA activity in PU but not in SA soil. Moreover, manure addition completely nullified Tc effect in SA+M+Tc100 and only reduced it in SA+M+Tc500 (Figure 9b). In PU+M+Tc100 and PU+M+Tc500, FDA did not statistically ($P<0.05$) differ from the untreated PU (Figure 9a). Seven days after amendment, the manure effect definitely disappeared in PU+M, whilst temporally decreased in SA+M. Moreover, after 7 days, only SA+Tc500 was statistically ($P<0.05$) lower than control SA (Fig 9b). In untreated SA soil, FDA activity progressively decreased within 60 days after amendment and other treatments were statistically higher ($P<0.05$) (Figure 9b).

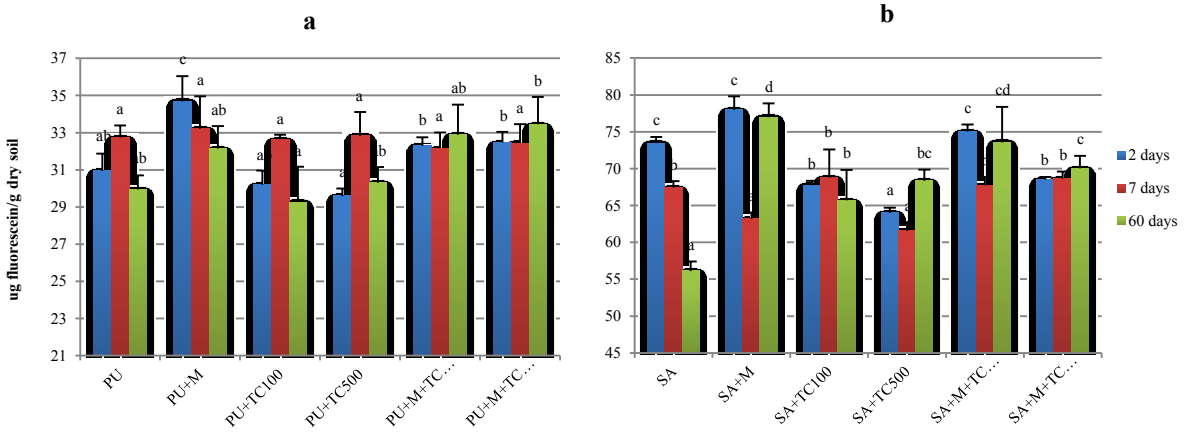

Figure 9. Fluorescein diacetate (FDA) activity in PU (a) and SA (b) soils. Tukey-Kramer statistic test (P<0.05). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=3 replicates.

Tc has a detrimental effect on FDA hydrolysis on medium-term (7 days) in SA soil. Bioavailable Tc concentrations in our microcosms ranged from 0.155 to 1.201 mg kg^{-1} in PU soil and from 0.767 to 4.602 mg kg^{-1} in SA soil (Table 2). According to Thiele-Bruhn and Beck (2005), who reported a significant effect of antibiotics in soil microbial activity at concentrations between 0.003 and 7.35 mg kg^{-1}, we observed a stronger Tc detrimental effect on SA soil, where the Tc bioavailability was highest (Figure 9b). Lacking of Tc effect in PU soil may be attributable to lower bioavailable Tc concentration. Also in the work of Toth *et al.* (2011), the antibiotic, at the concentration 100 mg kg^{-1}, did not affect the soil microbial activity. Moreover, detrimental activity persisted in SA soil 7 days after amendment at the antibiotic concentration of 500 mg kg^{-1}, likely a plenty Tc fraction was still bioavailable. As described for microbial counts, manure showed opposite effect than Tc, increasing the microbial activity which is stimulated by easily catabolisable C-sources added to soil by manure amendment (Parham *et al.*, 2002). Thiele-Bruhn and Beck (2005) tested also sulfapyridine and oxytetracycline antibiotics on microbial substrate-induced respiration (SIR). Since they did not observe changes in SIR after 4 hours, decided to extend the time of exposure up to 24 hours but they noticed reduction in respiration only in the sandy soil. Therefore, they prolonged the time of incubation later 24 hours and observed SIR reduction only in the loamy soil. The authors attributed SIR reduction in the sandy soil, to decreasing on antibiotic bioavailablity and adaptation and resistance development on soil

microbial community. Analogously, our results could suggest that Tc had a stronger impact on the microbial activity of the SA soil since its higher bioavailablity than PU soil (0.859-4.602 mg Tc kg^{-1} in SA and 0.157-1.201 mg Tc kg^{-1} in PU soil respectively) (Table 3). On the other hand, manure addition stimulated microbial activity and also alleviated Tc detrimental effect in SA soil by reducing, although slightly, the bioavailable Tc fraction. Therefore, the manure effect was stronger in SA soil and persisted until 60 days after amendment due to the larger impact of SOM addition to this soil, which contains less SOM than PU soil. Again, in SA soil the FDA activity decreased over time in the untreated soil owing to a plausible decrease in C-sources available for resident microbial communities (Schnürer and Rosswall, 1982).

4.2.4 Statistical correlation of microbial counts, PLFA and FDA activity

Reports of bacterial and actinomycete PLFA, microbial counts and FDA activity were analysed by Pearson's correlation test (Row-Wise Deletion, $P<0.05$) to detect possible relationships among the microbial features analysed. Correlation matrices were obtained for each soil and time points (2, 7 and 60 days). In PU soil, a positive and significant ($P<0.05$) correlation was observed between FDA activity and bacterial and actinomycete agar plate counts (0.478, $P<0.05$) as well as bacterial PLFA (0.626, $P<0.01$), 2 days after amendment (Table 6). In addition, 60 days after amendment, FDA was positively correlated also to actinomycete PLFA (0.592, $P<0.05$) (Table 6). This statistical test confirmed the results of microbial analyses previously described. The detrimental Tc effect observed in FDA hydrolysis test is strictly correlated to a decrease in bacterial population (Table 4) and actinomycete (Table 4) counts and bacterial PLFA fingerprints (data not shown). FDA activity is directly dependent to esterase enzymes, also bacterial, and increasing of metabolically active bacterial populations enhance FDA activity in soil (Table 6).

Table 6. Pearson's correlation matrix (Row-Wise Deletion, $P<0.05$) of microbial features detected in PU soil 2, 7 and 60 days after amendment.

PU soil	Time	fungal counts	actinomycete counts	bacterial PLFA	actinomycete PLFA	FDA
bacterial counts	2 days	ns	ns	0.514 *	ns	0.594 **
	7 days	0.615 **	ns	ns	0.707 **	ns
	60 days	ns	0.586 *	ns	0.520 *	0.662 **
fungal counts	2 days		ns	ns	ns	ns
	7 days		0.566 *	ns	0.621 **	ns
	60 days		ns	ns	ns	ns
actinomycete counts	2 days			ns	0.844 ***	0.478 *
	7 days			ns	ns	ns
	60 days			0.550 *	0.558 *	0.818 ***
bacterial PLFA	2 days				ns	0.626 *
	7 days				ns	ns
	60 days				ns	0.525 *
actinomycete PLFA	2 days					ns
	7 days					ns
	60 days					0.592 *

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant.

Instead, 7 days since treatment addition, no significant correlation is observed between FDA and other tests, probably due to a stabilization of soil microbial activity at this time point (Table 6 and Figure 9a). Moreover, a significant correlation is evident between microbial counts and PLFA analysis suggesting that, in this study, culturable bacteria are representative for the whole microbial community and agar plate counts could be a useful indicator of microbial fluctuations in soil after xenobiotic addition. The same analysis revealed a positive correlation in SA soil between FDA activity and microbial counts and bacterial PLFA. Positive correlation is evident 2 days since amendment between bacterial PLFA and bacterial counts whilst, as expected, it was negative between bacterial PLFA and fungal counts, likely to higher Tc bioavailability in SA than in PU soil (Table 7).

Table 7. Pearson's correlation matrix (Row-Wise Deletion, $P<0.05$) of microbial features detected in SA soil at 2, 7 and 60 days after amendment.

SA soil	Time	fungal counts	actinomycete counts	bacterial PLFA	actinomycete PLFA	FDA
bacterial counts	2 days	ns	ns	0.704 **	0.468 *	0.685 **
	7 days	ns	ns	ns	ns	ns
	60 days	0.517 *	ns	ns	ns	0.520 *
fungal counts	2 days		ns	-0.522 *	ns	-0.648 **
	7 days		0,496 *	ns	ns	ns
	60 days		ns	ns	ns	ns
actinomycete counts	2 days			ns	0.530 *	ns
	7 days			ns	ns	ns
	60 days			ns	ns	ns
bacterial PLFA	2 days				ns	0.870 ***
	7 days				ns	ns
	60 days				ns	0.444 *
actinomycete PLFA	2 days					ns
	7 days					-0.691 **
	60 days					ns

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant.

4.2.5 Biolog® Community Level Physiological Profile (CLPP)

Biolog® Community Level Physiological Profile (CLPP) can be a useful approach to detect structural changes on environmental bacterial populations based on their catabolic potential (Insam *et al.*, 1996; Garland, 1997; van Elsas *et al.*, 2007). However, it should be highlighted that the picture provided by the Biolog® CLPP is not exhaustive since only a fraction of the soil microbial population is responsible for colour development in Biolog® plates (i.e. fast-growing Proteobacteria). Instead, the contribution of other microbial groups is quite negligible, e.g. Actinobacteria and/or fungi, (Smalla *et al.*, 1998; Ros *et al.*, 2008). Despite these accepted limitations, Biolog® EcoPlates are commonly and successfully used to detect changes in soil microbial communities (Westergaard *et al.*, 2001; Garau *et al.*, 2007; Pinna *et al.*, 2012; Lorenzo *et al.*, 2013).

The Principal Components Analysis (PCA) of carbon source utilisation data suggested that microbial communities from the two soils were differently affected by manure and Tc addition. In particular, 2 days since treatment, the microbial communities of PU+M and PU+Tc500 were clustered together (i.e. showed a similar carbon source utilisation pattern) and well apart from all the other communities which could be arranged in a different group (Figure 10). On the contrary, at the same time-point Tc 500 mg kg^{-1} had a more clear and consistent effect on the microbial communities of the SA soil where the community extracted from the Tc500 and Tc500+M soils were separately clustered with respect to the other communities (Figure 10).

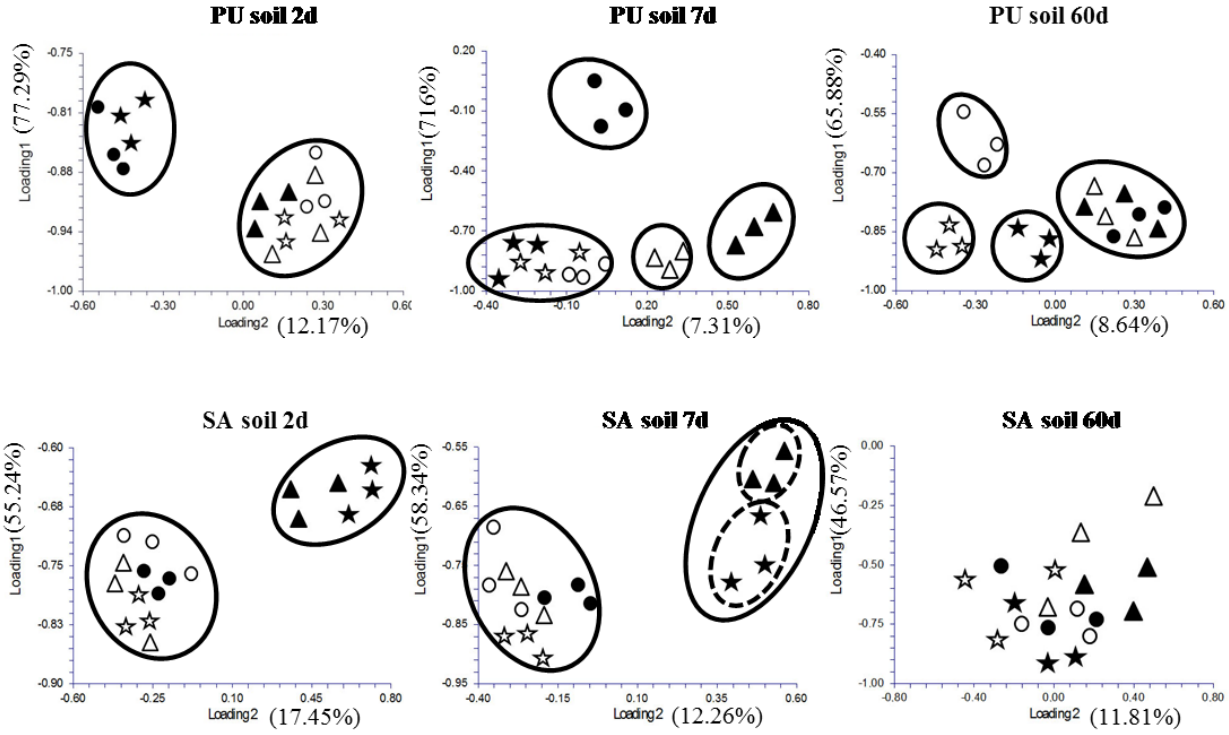

Figure 10. Results of Principal Component Analysis (PCA) applied to Biolog data (carbon source utilization) after 96 h incubation of Biolog EcoPlates. Symbols refer to microbial communities extracted from different soil samples: ○, PU and SA soils; ●, PU and SA+M; ☆, PU and SA+Tc100; ★, PU and SA+Tc500; △, PU and SA+M+Tc100; ▲, PU and SA+M+Tc500.

At the following time-points, microbial consortia of the two soils showed opposite trends: the PU communities progressively differentiated their catabolic profiles and, 60 days after amendment, could be grouped in four well-defined clusters according to manure addition and Tc dose

supplied (Figure 10). On the contrary, the differences highlighted, at 2 days time indicate that the SA communities progressively disappeared and 60 days after amendment no clear cluster could be identified.

Taken together, our results suggest that Tc had strongest short-term impact on the community structure of the SA soil which however showed a greater resilience compared to PU soil. On the contrary, it seems that Tc has a more long lasting effect on the microbial community of PU soil which did not extinguished after 60 days. According to Pinna *et al.* (2012), the addition of antibiotic and manure, spiked with Tc, changes the structure of soil microbial community in medium-long term, i.e. 60 days after single amendment in PU soil. Moreover, a high antibiotic concentration, i.e. 500 mg kg^{-1}, according to Demoling *et al.* (2009), clearly changed the bacterial community structure. The effect of Tc500, observed in both soils 2 days after amendment, disappeared in SA soil 7 days since addition, most likely due to a decrease of the concentration of antibiotic, which perhaps is mineralised (Demoling *et al.*, 2009) or inactivated by resistant microorganisms. Manure, both unspiked and spiked with Tc, produced a strong effect in PU soil. Also a Tc effect was evident in PU soil and persisted 60 days after amendment. Probably, the addition of manure plays a priming effect stimulating the bacterial population of PU soil to use soil's and manure's C-sources. Conversely, in SA soil, because of its low SOM content, manure C-sources were exhausted within 60 days, therefore, the resident bacteria missed essential energy sources for their metabolism

4.3 Study of tetracycline and manure effect on bacterial community structure and resistance development in the soil environment after three repeated amendments

After a single amendment, Tc and manure modify the structure and the function of soil microbial communities, then it was though interesting to study how repeated amendments influence the bacterial structure and the distribution of antibiotic resistance genes in soil. Manure is effective in spreading antibiotic resistance in the soil environment by addition of resistant bacteria. These bacteria are selected in the animal gut by antibiotic administration (Heuer and Smalla, 2007). Antibiotic resistance genes can be closely affiliated to mobile genetic elements (Chopra and Roberts, 2001; Roberts, 2005) which disseminate resistance genes through

microbial populations by vertical or horizontal transfer (van Elsas *et al.*, 2007), also to humans (Anderson *et al.*, 2003). In total 43 different genes are known to encode as many proteins involved in tetracycline resistance in bacteria. The *tet* and *otr* genes are located on mobile genetic elements like plasmids, conjugative transposons and integrons (Roberts, 2011b), which are spread to soil by manure amendment (Heuer and Smalla, 2007; Heuer *et al.*, 2008; Heuer *et al.*, 2009; Heuer *et al.*, 2011).

4.3.1 Denaturing gradient gel electrophoresis (DGGE)

Effects due to manure and Tc on total bacterial community structure in PU and SA soils were observed through denaturing gradient gel electrophoresis (DGGE) fingerprinting of bacterial 16S rRNA gene fragments. These contained the three variable regions V6, V7 and V8, amplified by PCR (16S rRNA-PCR DGGE) and including a GC clamp to obtain a circular DNA loop (Nübel *et al.*, 1996; Heuer *et al.*, 1997). DGGE is a technique introduced in 1980 (Woese *et al.*, 1980) and applied to environmental microbiology since 1993 (G Muyzer *et al.*, 1993). The method is based on the separation of nucleotide fragments of the same length (433 bp in our study) on polyacrylamide gel according to their melting temperatures (T_m), assumed as the temperature at which 50% of the double strand fragment is denatured (van Elsas *et al.*, 2007). The 16S rRNA gene fragments migrate, driven by an electric current, through a chemical denaturant gradient. The velocity of migration decreases with increasing T_m, which results in opening of DNA rings and decrease of mobility on gel (van Elsas *et al.*, 2007). The resulting DGGE fingerprinting of microbial soil community discriminates nucleotide fragments according to T_m, i.e. all single base pair substitutions in short 16S rRNA gene fragments could be distinguished (Nübel *et al.*, 1996).

In this work we used the 16S rRNA-PCR DGGE to compare:

- The effect of manure, unspiked and spiked with Tc, on the bacterial community structure in PU and SA soils. Treatments were performed three times and, 60 days after each amendment, bacterial fingerprints were performed by comparing the effects of manure and Tc at a specific time point (60, 120 and 180 days);
- The effect of repeated amendments in a soil (PU or SA) by comparing the results after the first and the third amendment.

All DGGE gels were analysed by the software GelCompar. Dendrograms, calculated by Pearson UPGMA correlation coefficient, were used to compare microcosm fingerprints using band-based indices to calculate a matrix of pairwise similarities. The report is a group of clusters including samples of similar microbial communities, on the basis of the 16S rRNA gene fragment fingerprint (van Elsas *et al.*, 2007). Similar matrixes were then used to calculate, statistically, significant effects of Tc and manure by random permutations within treatments and between treatments (van Elsas *et al.*, 2007) by Permutation test (P<0.05) as described by Kropf *et al.* (2004).

Effect of repeated application on the bacteria community analysed by DGGE

The DGGE fingerprinting of the bacterial community in PU soil showed a clear effect of treatments 60 days after each amendment (60, 120 and 180 days). The UPGMA analysis on PU soil showed, 60 days after the first amendment (60-days), samples grouped in three distinct clusters (Figure 11a). The untreated soil was clustered with a low similarity of 55% to other treatments. Moreover, PU+M+Tc500 showed about 70% similarity from the cluster including treatments PU+M and PU+M+Tc100 (Figure 11a). Sixty days after the second amendment (120-days) untreated PU samples still clustered, sharing 35% similarity to other treatments (Figure 11b). After the third amendment (180-days) the similarity between untreated and treated PU soil decreased again (20%) (Figure 11c). In SA soil the effect of treatments was investigated, as described for PU soil, through UPGMA analysis (Figure 12). Sixty days after the first amendment, untreated soil shared 50% similarity to other samples (Figure 12a) and, after the second amendment, similarity between control SA and soil treatments decreased (<40%) (Figure 10b). After the third amendment untreated SA samples still clustered, sharing high similarity among replicates (ca. 85%) and the SA+M+Tc500 shared 73% similarity to other treatments (Figure 12c).

The UPGMA analysis calculated by Pearson's correlation test showed samples clustered on the basis of their DGGE fingerprinting. To understand better the effects of the treatments, Pearson's correlation indexes were used to calculate distances between treatments by Permutation statistic test. In PU soil, at all time points, all soil treatments were statistically (P<0.05) different from untreated PU but no statistically difference was calculated between PU+M and PU+M+Tc100 and between PU+M+Tc100 and PU+M+Tc500 (Table 8). After the first amendment (60-days), a significant (P<0.05) effect between PU+M and PU+M+Tc500 was also observed.

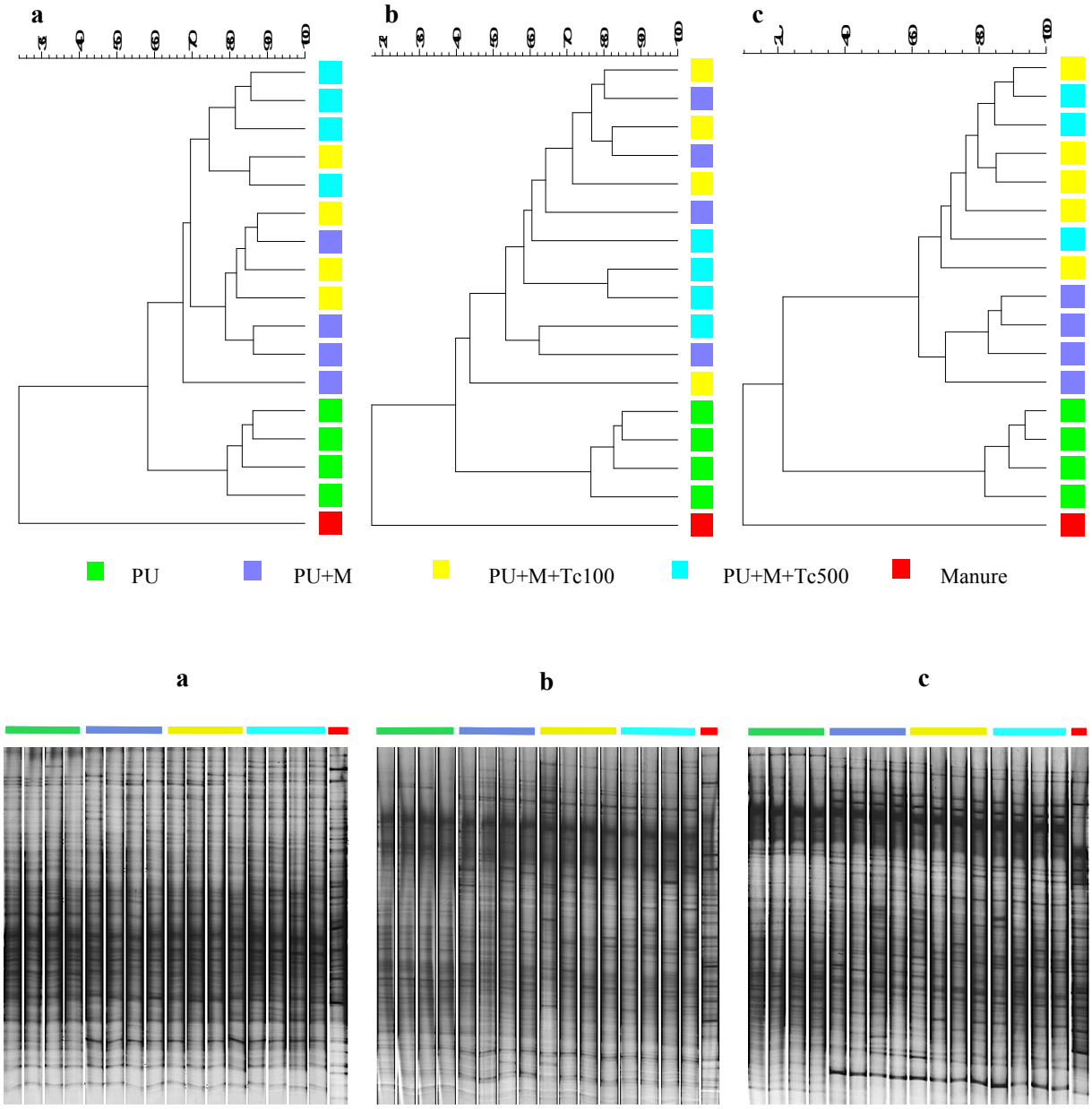

Figure 11. UPGMA analysis of 16S rRNA DGGE in PU soil after the first (60 days, a), second (120 days, b) and third (180 days, c) amendment. At the bottom are reported the respective DGGE gels. M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Table 8. Permutation statistic test (P<0.05) in PU soil after the first (60 days), second (120 days) and third (180 days) amendment expressed through d-value [*].

Time-point	Treatment	PU	PU+M	PU+M+Tc100	PU+M+Tc500
60 days	**PU**	-			
	PU+M	11.8	-		
	PU+M+Tc100	15.1	ns	-	
	PU+M+Tc500	25.9	5.7	ns	-
120 days	**PU**	-			
	PU+M	28.7	-		
	PU+M+Tc100	29.0	ns	-	
	PU+M+Tc500	33.2	6.0	ns	-
180 days	**PU**	-			
	PU+M	62.2	-		
	PU+M+Tc100	61.7	ns	-	
	PU+M+Tc500	53.7	16.2	ns	-

[*] ns, not significant; M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

With repeated amendments, all distances increased over time. Moreover, the d-value was statistically significant (P<0.05) between PU+M and PU+M+Tc500 but not between PU+M and PU+M+Tc100. By the way, UPGMA at time point 180-days showed a clear distinction, in PU soil, between manure and manure-contaminated treatments that clustered separately (Figure 11) but Permutation test revealed no statistical significance between PU+M and PU+M+Tc100 (Table 8).

The d-values calculated for SA soil fingerprints (Table 9) showed, at the first time point, no significant (P<0.05) differences with exception between SA+M and SA+M+Tc100. At the second and third time points, all d-values became statistically significant (P<0.05) but, curiously, the d-value between SA+M and SA+M+Tc100, the only one significant at 60-day time point, was no longer significant. At the last time point d-values increased, except between SA+M+Tc100 and SA+M+Tc500 which, though, was significant in SA but not in PU soil (Table 8-9).

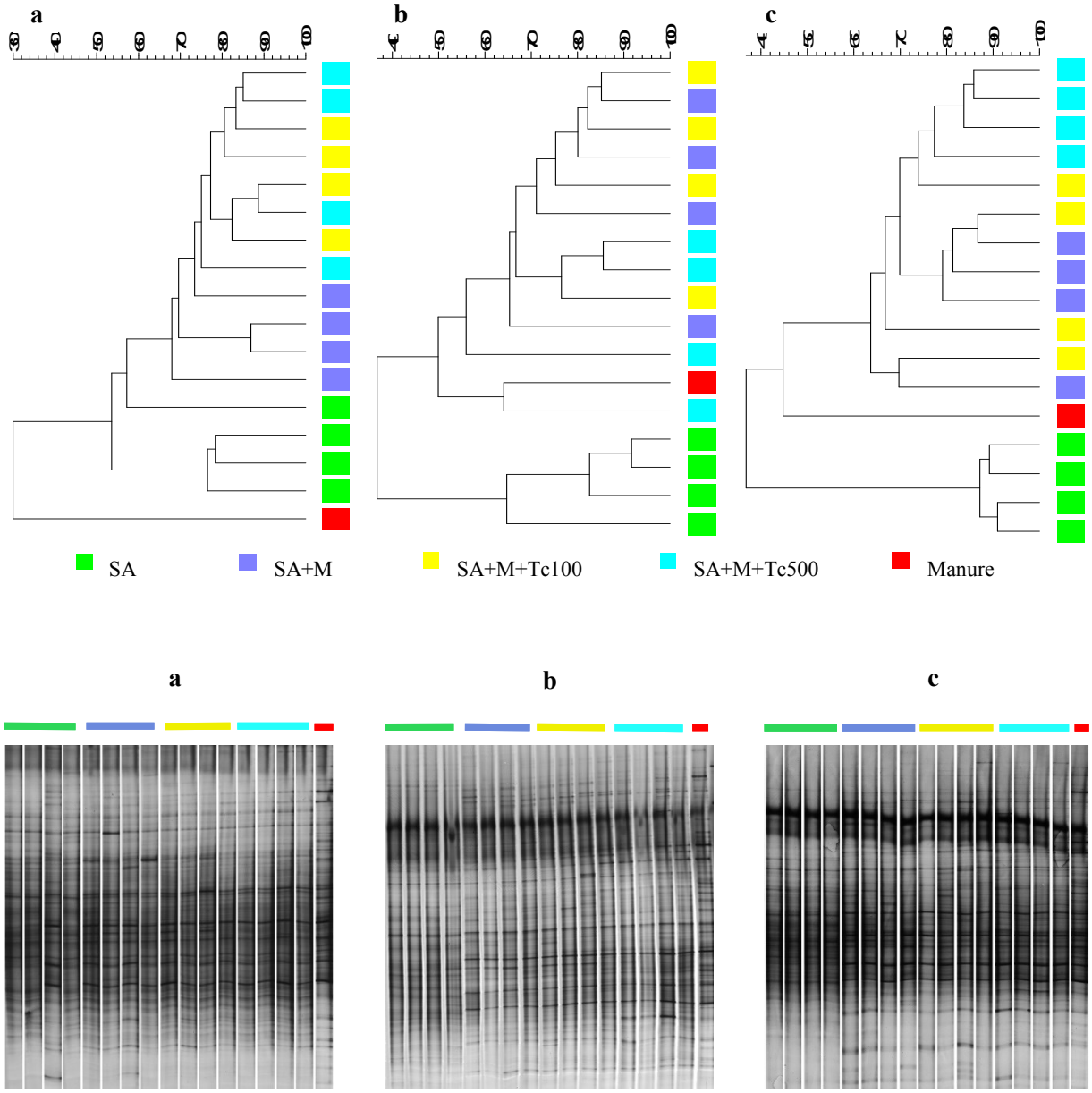

Figure 12. UPGMA analysis of 16S rRNA DGGE in SA soil after the first (60 days, a), second (120 days, b) and third (180 days, c) amendment. At the bottom are reported the respective DGGE gels. M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Table 9. Permutation statistic test (P<0.05) in SA soil after the first (60 days), second (120 days) and third (180 days) amendment expressed through d-value [*].

Time-point	Treatment	SA	SA+M	SA+M+Tc100	SA+M+Tc500
60 days	**SA**	-			
	SA+M	ns	-		
	SA+M+Tc100	ns	10.1	-	
	SA+M+Tc500	ns	ns	ns	-
120 days	**SA**	-			
	SA+M	30.9	-		
	SA+M+Tc100	35.4	ns	-	
	SA+M+Tc500	37.9	13.8	9.7	-
180 days	**SA**	-			
	SA+M	45.5	-		
	SA+M+Tc100	41.1	ns	-	
	SA+M+Tc500	44.2	12.9	4.1	-

[*] ns, not significant; M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Comparison of treatments effect between the first and the third amendment by bacterial DGGE fingerprint

UPGMA analysis and Permutation test were also used to analyse manure and Tc effects on soil microbial community after the first and the third amendment. For this purpose, bacterial DGGE fingerprinting of PU or SA soils, 60 days after the first and 60 days after the third amendment (60 and 180 days time points respectively), were compared on the same DGGE gel. This fingerprint allows to compare better how repeated addition of manure and Tc treatments affected the bacterial structure. Otherwise, it is not thorough to observe effects on different gels. The UPGMA dendrogram of PU soil showed treatments grouped in two big clusters sharing 25% similarity between them (Figure 13a). All treatments clustered separately. The first big cluster was characterized by soil treatments at 60 days after the first amendment and untreated PU at 60 days after the first and the third amendments (60 and 180 days respectively). Here, soil treated with contaminated manure at 60 days time point (PU+M+TC100 and PU+M+TC500, 55% similarity between them) shared <40% similarity from cluster with untreated PU at 60 days after the first and the third amendment (ca. 55% similarity between them) and PU+M at 60 days after the first amendment. The second big cluster grouped all manure treatments at 180 days. Here, contaminated manure (PU+M+Tc100 and PU+M+Tc500) shared ca. 45% similarity from PU+M

(Figure 13a). The UPGMA of SA soil was quite similar to PU soil. Two big clusters were observed, sharing 73% similarity. In the first big cluster, treatments SA+M+Tc100 and SA+M+Tc500 at 180 days shared 74% similarity to SA+M at 180 days (Figure 13b).

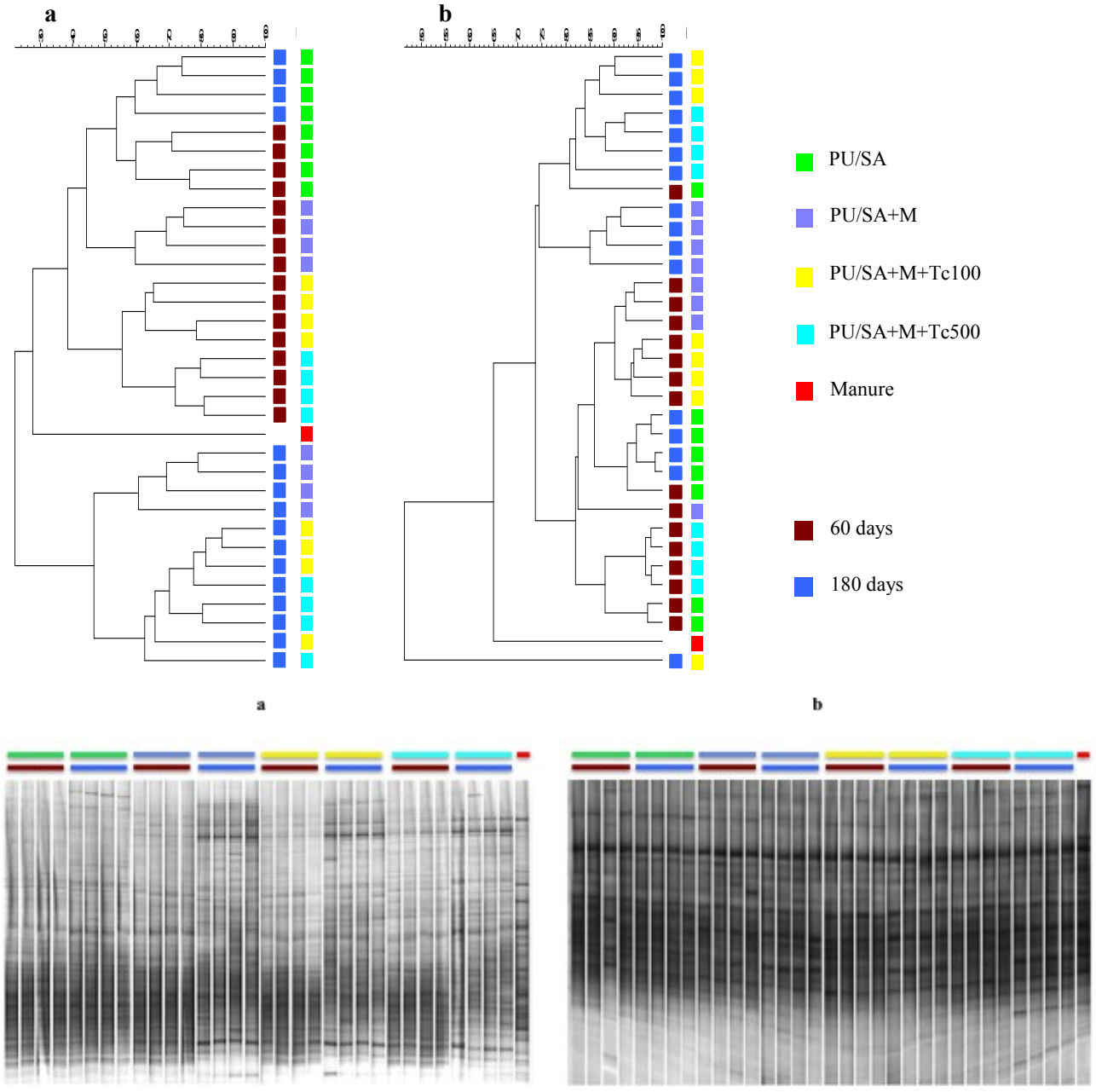

Figure 13. UPGMA analysis of 16S rRNA DGGE comparing the first and the third amendment (60 and 180 days time point, respectively) in PU soil (a) and in SA soil (b). Samples were loaded in block-row manner adjacent to each other. M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

In the second big cluster, untreated SA at 180 days shared <87% similarity to SA+M and SA+M+Tc100 ad 60 days, which shared 86% similarity to SA+M+Tc500 (Figure 13b).

Permutation test was used to compare effects of treatments 60 days after the first and the third amendments, and all d-values were mighty significant. In both soil, d-values of manure treatments, contaminated and not (PU/SA+M, PU/SA+M+Tc100 and PU/SA+M+Tc500) increased by increasing Tc concentration, compared to their respective controls (untreated PU or SA soils), with the only exception SA+M+Tc500 (Table 10).

Table 10. Permutation statistic test (P<0.001) in PU and SA soils to compare effect of treatment 60 days after the first and the second amendment [*].

	PU	**PU+M**	**PU+M+Tc100**	**PU+M+Tc500**
PU	-			
PU+M	34.4	-		
PU+M+Tc100	39.7	22.1	-	
PU+M+Tc500	42.0	32.7	11.2	-

	SA	**SA+M**	**SA+M+Tc100**	**SA+M+Tc500**
SA	-			
SA+M	14.4	-		
SA+M+Tc100	19.7	6.5	-	
SA+M+Tc500	11.1	13.9	7.3	-

[*] M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Also d-values between manure treatment (PU/SA+M) and contaminated manure treatments (PU/SA+M+Tc100 and PU/SA+M+Tc500) increased with increasing Tc concentration. Furthermore a Tc concentration effect was observed (Table 10). The d-values were also calculated, by Permutation statistic test, to express the overall distance between the first and the third amendment in soils. These values were highly significant (P<0.001) with 32.3 for PU and 11.6 for SA soils, respectively. This finding suggests that repeated amendments of manure, unspiked and spiked with Tc, significantly modified the bacterial structure in soils.

In order to compare the first and the third amendments, DGGE fingerprints, were also repeated by loading samples randomly on the gel to easily compare treatments effect, on the same gel, at different time points (Figure 14). The UPGMA dendrogram was similar to that described previously for PU (Figure 13a) and SA soils (Figure 13b). Manure treatments at 60 days after the

third amendment were clustered together (PU/SA+M, PU/SA+M+Tc100 and PU/SA+M+Tc500). All d-values calculated by Permutation test were significant (P<0.001) in SA soil whereas in PU only manure treatments (PU+M, PU+M+Tc100 and PU+M+Tc500) were significant, compared to control (Table 11).

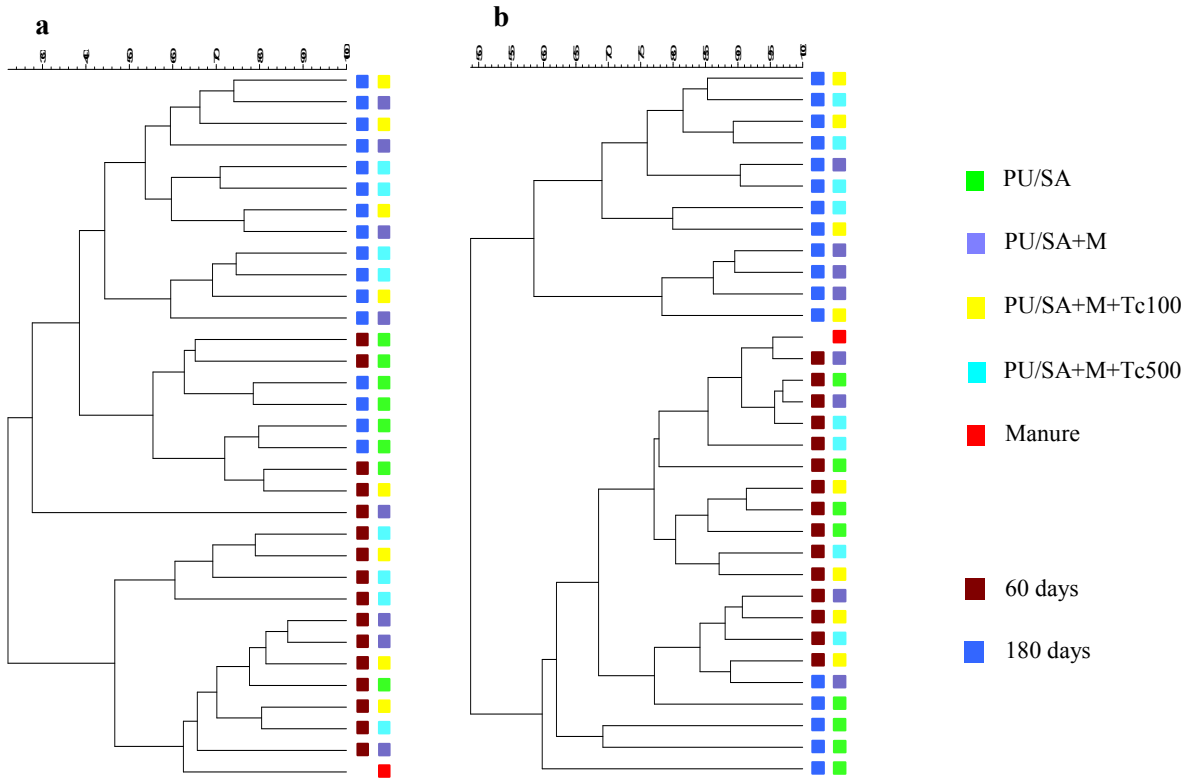

Figure 14. UPGMA analysis of 16S rRNA DGGE comparing the first and the third amendment (60 and 180 days time point, respectively) in PU soil (a) and in SA soil (b). Samples were loaded randomly on the gel. M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Table 11. Permutation statistic test (P<0.001) in PU and SA soils to compare effect of treatment 60 days after the first and the second amendment on randomly loaded samples [*].

	PU	PU+M	PU+M+Tc100	PU+M+Tc500
PU	-			
PU+M	1.0	-		
PU+M+Tc100	16.8	ns	-	
PU+M+Tc500	23.1	ns	ns	-

	SA	SA+M	SA+M+Tc100	SA+M+Tc500
SA	-			
SA+M	24.8	-		
SA+M+Tc100	18.3	2.2	-	
SA+M+Tc500	22.3	12.1	1.3	-

[*] ns, not significant; M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Several papers reported studies about the effect manure, spiked and not with antibiotic, on soil microbial community, as observed by 16S rRNA gene fragment DGGE (Ellis *et al.*, 2003; Binh *et al.*, 2007; Hammesfahr *et al.*, 2008; Heuer *et al.*, 2008; Gielen *et al.*, 2011).

Our fingerprints confirm the effect of manure amendment on the bacterial soil structure by nutrient addition, already after the first amendment in PU soil and from the second amendment in SA soil. These results were also observed for the bacterial community investigated by the Biolog® CLPP method (Cap. 4.6). In PU soil 60 days after the single amendment, the bacterial community in manure treatments, spiked and not with Tc, clustered separately from untreated PU (Figure 10c), as observed in DGGE fingerprint. Furthermore in SA soil were not observed statistical differences, 60 days after single amendment, both by DGGE and Biolog® (Figure 10f) analyses.

Amplicons of each treatment were first loaded on the gel adjacent to each other (block-wise) since it was easier to compare treatments effect. However, it is recommended to load amplicons randomly to avoid biases caused by gel position of lanes (Smalla *et al.*, 2007). The two fingerprints (random and not) confirmed the important effect of both manure treatment and repeated amendments on soils bacterial communities. Several studies regarded antibiotic and manure effects on soil microbial structure (Alexander *et al.*, 2011; Nelson *et al.*, 2011; Reichel *et al.*, 2013) and authors found only a short-term effect (1-7 days). Other authors found strong manure effect on microbial fingerprints and transient antibiotic effect after single amendment (Binh *et al.*, 2007; Heuer and Smalla, 2007; Hammesfahr *et al.*, 2008; Heuer *et al.*, 2008).

Parallel in SA soil we did not find any effect after the first amendment, by DGGE fingerprinting, but after three repeated amendments we observed manure and also Tc concentration effects on the soil bacterial community structure. Probably, effects of manure and Tc were transient and restored within 60 days in SA soil, but after three repeated amendments we could observed meaningful effect of manure and also a Tc concentration effect. Conversely, in PU soil 60 days after the first amendment we found a strong manure effect and also Tc effect, only at highest concentration (500 mg kg^{-1}).

Normally, as the common agricultural practices, manure, contaminated and not, is spread on soil several times and this could intensify the effects on the structure of the soil microbial community (Hammesfahr *et al.*, 2008). After three amendments we observed that differences in bacterial community structure between soil treated and the untreated soil were amplified.

Both in PU and SA soils, the repeated application of manure and Tc-spiked manure increased differences in bacterial populations, compared to the untreated soils (Figure 11-12). Other authors observed that repeated amendments of manure in long-term experiments influenced the microbial community (Hopkins and Shiel, 1996; Sun *et al.*, 2004). Parham *et al.* (2002) reported a clear effect of manure amendments in soil on increasing microbial biomass, and Marschner *et al.* (2003) found a manure effect on bacterial populations over a long-time period of about 30 years. We compared, in the same gel, the fingerprints of bacterial populations 60 days after the first and the third amendments to better understand the effect of repeated manure and Tc additions. The statistical analysis revealed that repeated applications significantly affected the bacterial structure in all treatments, probably by adaptive mechanisms. Moreover, Tc affected the bacterial structure after three repeated amendments, in PU soil already after the first amendment.

4.3.2 Investigation of resistance development by quantification of genes related to antibiotic resistance and mobile genetic elements

Effects of repeated manure and tetracycline amendments on Tc resistance genes

***tet*(Q).** The rt-qPCR revealed that *tet*(Q) was not detectable in the untreated PU soil due to its low abundance, below the instrumental detection limit. The addition of manure to PU soil highly increased the abundance of *tet*(Q), already 60 days after the first amendment (Figure 15a). However Tc addition, with manure, did not significantly (Tukey-Kramer statistic test, $P<0.05$) increase its relative abundance, with respect to manure treatment, at all time points (Figure 15a). Conversely, *tet*(Q) was detected also in untreated SA soil and no statistical effects ($P<0.05$) of manure and/or Tc were observed 60 days after the first and the second amendments (Figure 15b). After the third amendment, the relative abundance of *tet*(Q) in manure, unspiked and spiked with TC, was statistically higher ($P<0.05$) than the untreated SA, due to decreasing in *tet*(Q) relative abundance in the control soil. No Tc effect was observed in SA soil (Figure 15b).

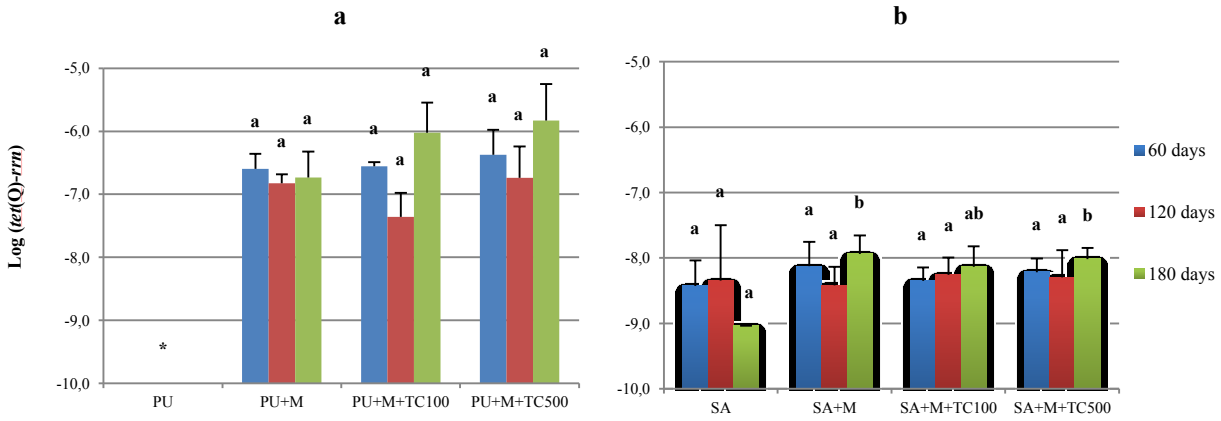

Figure 15. *tet*(Q) gene relative abundance in PU (a) and SA (b) soils measured by rt-qPCR after the first (60 days), second (120 days) and third (180 days) amendment. Tukey-Kramer statistic test ($P<0.05$). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level ($P<0.05$) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

***tet*(W).** The trend of *tet*(W) in PU soil was quite similar to that described for *tet*(Q). The relative abundance of *tet*(W) was always under the detection limit in the untreated soil (Figure 16.b). Significant (P<0.05) manure effect was observed in PU soil but Tc did not further increase *tet*(W) after repeated amendments (Figure 16.a). In SA soil the relative abundance of *tet*(W), at the first time point, was equal in all samples but, in the untreated soil, it decreased over time (Figure 16.b) and, 60 days after the first and the second amendment, its relative abundance was significantly lower than in soil treatments. No Tc effect was observed (Figure 16.b).

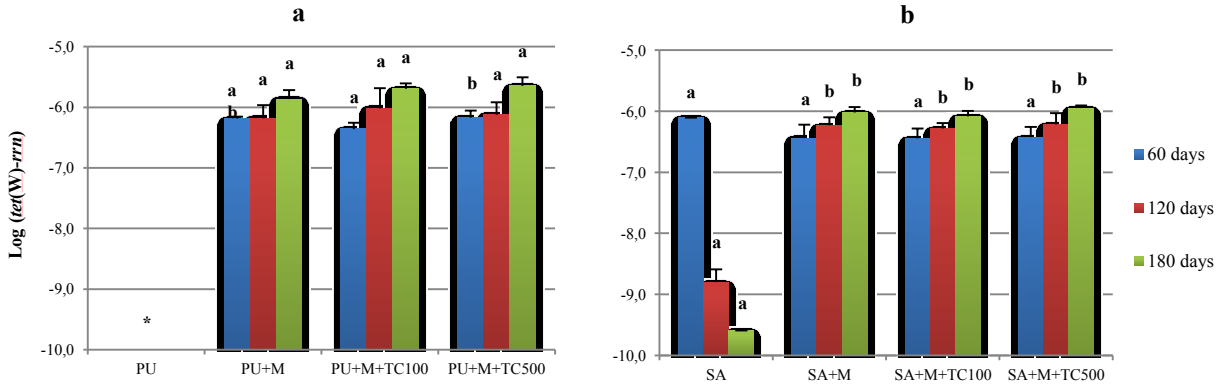

Figure 16. *tet*(W) gene relative abundance in PU (a) and SA (b) soils measured by rt-qPCR after the first (60 days), second (120 days) and third (180 days) amendment. Tukey-Kramer statistic test (P<0.05). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

***tet*(A).** tet(A) and tet(M) were detected by Souther blot hybridization by comparing their prevalence at 60 days after the first and the third amendment. After the first amendment in PU soil, *tet*(A) gene was found only in soil amended with manure (spiked and not with Tc) but in SA soil *tet*(A) was found also in the untreated soil (Figure 17a). 60 days after the third amendment in PU soil, the treatment PU+M+Tc500 was positive and PU+M+Tc100 was only slightly positive. *tet*(A) was not detected in untreated PU and PU+M 60 days after the third amendment (Figure 17b). Conversely, in SA soil, SA+M was slight positive and in SA+M+Tc100 a strong signal was detected and its intensity increased more in SA+M+Tc500 (Figure 17b). Moreover the *tet*(A) gene was also detected in the manure sample (Figure 17).

***tet*(M).** The *tet*(M) gene was neither detected in soil samples nor in manure sample (data not shown).

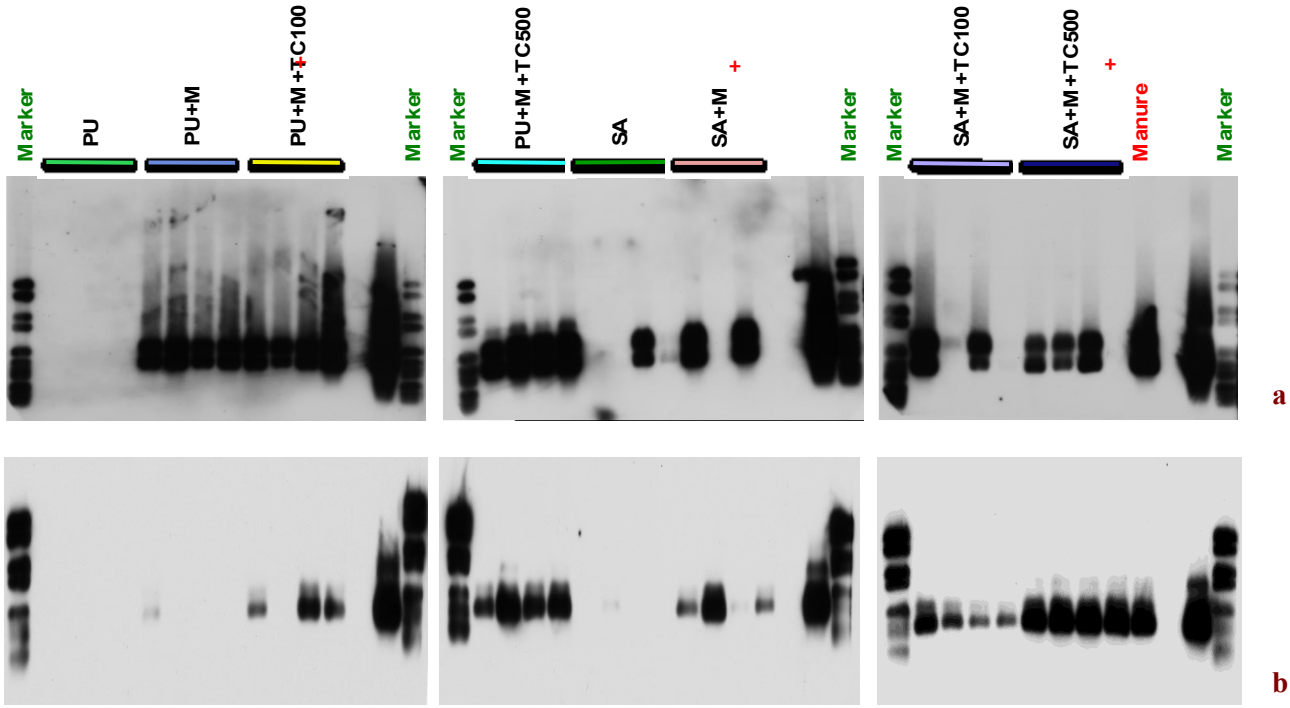

Figure 17. PCR Southern blot hybridization of *tet*(A) gene in PU and SA soils after the first amendment (a), and third amendment (b). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight.

Determination of relative abundance of class 1 integron integrase genes

***intI1*.** In PU soil, the relative abundance of the *intI1* gene increased significantly (P<0.05) after manure and Tc amendments, with respect to control, at all time points (Figure 18a). Already 60 days after the first amendment, a Tukey-Kramer statistic test showed a significant effect (P<0.05) of manure treatments on increasing *intI1* relative abundance. Besides, in PU+M+Tc500 the relative abundance of *intI1* further increased, with respect to PU+M (Figure 18a). Sixty days after the second amendment, differences among treatments increased and after the third amendment in all treatments, both manure and Tc at two concentrations, the relative abundance of *intI1* synergistically increased showing a Tc-concentration effect (P<0.05) (Figure

18a). In SA soil, 60 days after the first amendment, no statistical difference (P<0.05) was observed among treatments and untreated soil. The relative abundance of *intI1* in untreated SA decreased over time and 60 days after the second amendment the differences between manure treatments (SA+M, SA+M+Tc100 and SA+M+Tc500) and untreated SA became significant (P<0.05) (Figure 18b). After the third amendment, was also observed a Tc effect, only at the concentration 500 mg kg^{-1}, which further increased *intI1* abundance with respect to SA+M and SA+M+Tc100 (Figure 18b).

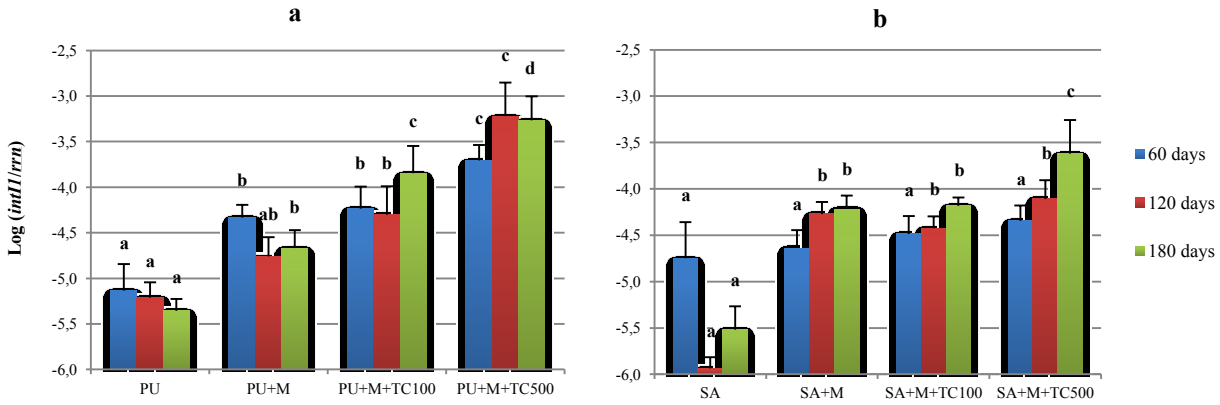

Figure 18. *intI1* gene relative abundance in PU (a) and SA (b) soils measured by rt-qPCR after the first (60 days), second (120 days) and third (180 days) amendment. Tukey-Kramer statistic test (P<0.05). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

Evaluation of abundance of IncP-1ε plasmid by quantification of *trfA* related gene

trfA. In PU soil, treatments and amendments did not produce a significant (P<0.05) effect on the relative abundance of *trfA* gene (Figure 19a). In SA soil, both manure and TC addition synergistically increased the relative abundance of *trfA* already 60 days after the first amendment. Sixty days after the second and the third amendments, the *trfA* abundance increased further and all treatments were statistically different (P<0.05).

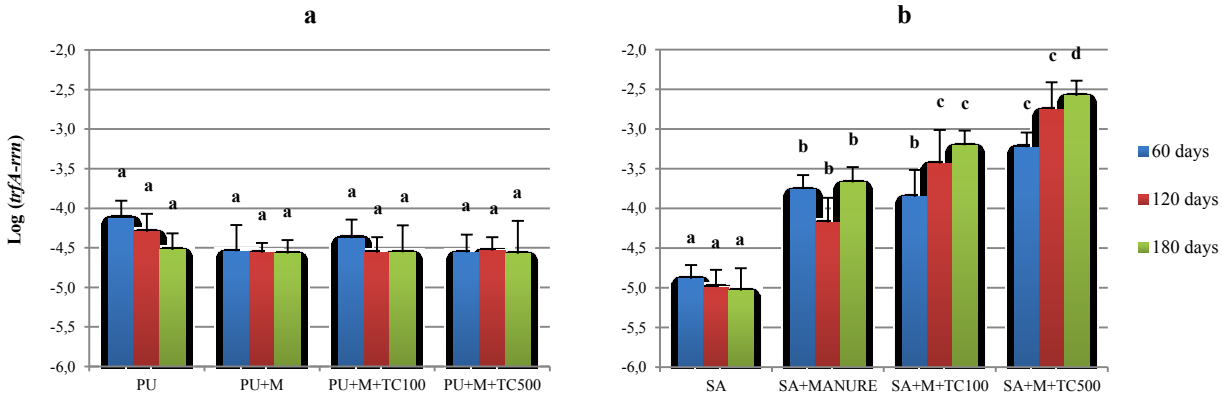

Figure 19. *trfA* gene relative abundance in PU (a) and SA (b) soils measured by rt-qPCR after the first (60 days), second (120 days) and third (180 days) amendment. Tukey-Kramer statistic test (P<0.05). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

Quantification of relative abundance of sulfonamides resistance genes *sul1* and *sul2*.

In both soils the relative abundance of *sul*1 and *sul*2 genes increased significantly (P<0.05) after manure and Tc application, with the exception of *sul2* genes in SA soil. In PU soil, the effect of manure and Tc was synergic and resulted in an increased (P<0.05) relative abundance of *sul1* gene already 60 days after the first amendment. Further amendments amplified this effect (Figure 20a). Conversely, in SA soil manure and Tc addition did not affect *sul1* relative abundance (Figure 20b). The abundance of *sul2* gene, in PU soil, strongly increased after manure addition. Sixty days after the first amendment we observed only a manure effect (P<0.05) but after the second and third amendments also the effect of Tc concentration was detected (Figure 21a). After the third amendment, the abundance of *sul2* gene in untreated PU decreased below the instrumental detection limit. In SA soil, manure statistically increased (P<0.05) the abundance of *sul2* gene and in SA+M+Tc500 further increased (Figure 21b). After the second and third amendments, in all manured treatments, both unspiked and spiked with Tc, the relative abundance of sul2 gene was significantly (P<0.05) greater than in untreated SA soil (Figure 21b).

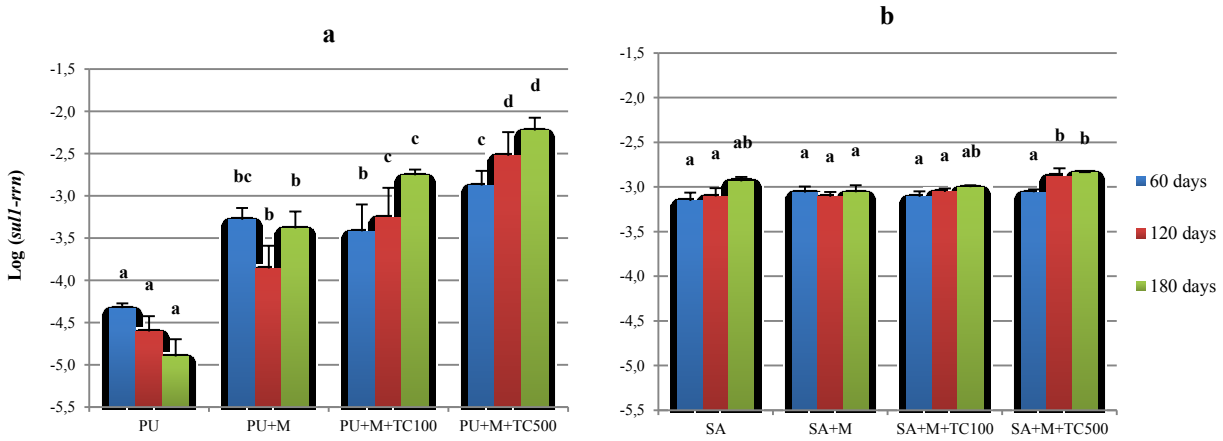

Figure 20. *sul1* gene relative abundance in PU (a) and SA (b) soils measured by rt-qPCR after the first (60 days), second (120 days) and third (180 days) amendment. Tukey-Kramer statistic test (P<0.05). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

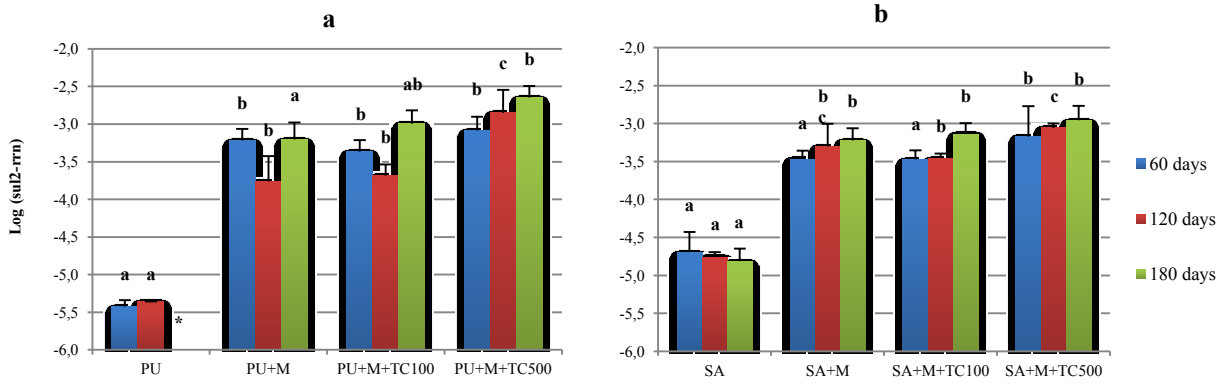

Figure 21. *sul2* gene relative abundance in PU (a) and SA (b) soils measured by rt-qPCR after the first (60 days), second (120 days) and third (180 days) amendment. Tukey-Kramer statistic test (P<0.05). M, manure; Tc100, Tc 100 mg kg^{-1} soil dry weight; Tc500, Tc 500 mg kg^{-1} soil dry weight. For each time-point, average values which share the same letter do not differ significantly at the 5% level (P<0.05) according to the Tukey-Kramer multiple comparison test. Error bars indicate the average of n=4 replicates.

Our data suggest a clear manure effect which increases antibiotic resistance carrying Tc resistance genes and mobile genetic elements in soil by amendments, although the cows from which the manure was obtained had never been treated with antibiotics. Also Heuer *et al.* (Heuer

et al., 2009) found that manure addition, from untreated animals, increases resistance in soil by carrying resistance genes in soil. Furthermore, during manure storage, not necessarily the abundance of resistance genes decreases in the bacterial manure population (Heuer *et al.*, 2009). We supposed that stronger effects of manure and Tc on the structure and the function of microbial community observed in PU soil, were attributable to higher susceptibility of its resident microbial community than in SA soil. For this reason, we checked and quantified some Tc resistance genes and some genes associated to mobile genetic elements, carrier of antibiotic resistance in the soil environment. We tested the genes *tet*(A), *tet*(M), *tet*(Q) and *tet*(W) since their high diffusion among all *tet* and *otr* resistance genes in the environmental bacteria (Roberts, 2005). *tet*(A) is involved in efflux resistance mechanism and encodes for a transmembrane efflux pump protein involved in reduction of intracellular Tc concentration (Roberts, 2011a). *tet*(M), *tet*(Q) and *tet*(W) are related to a ribosomal protection mechanism which prevents the Tc binding on 30S ribosomal subunit (Roberts, 2011a). Our results indicate that the *tet* genes under study (*tet*(A), *tet*(Q), *tet*(W)) were ubiquitous in SA soil and cow manure but were not found in PU soil before amendment. Conversely to other authors (Agersø *et al.*, 2002; Hund-Rinke *et al.*, 2004; Kyselková *et al.*, 2013), we did not find *tet*(M), involved in ribosomal protection and normally associated to enterobacteria (Roberts, 2011a), in soil neither in cow manure, according to Schmitt *et al.* (2006b). *tet*(W), *tet*(Q) and *tet*(A) are found in facultative anaerobic and aerobic bacteria, fastidious anaerobic bacteria and enterobacteria, respectively (Roberts, 2005; Popowska *et al.*, 2012). These data confirmed, as previously described by Heuer and Smalla, that antibiotic resistance genes were brought into soil by manure application (Binh *et al.*, 2007; Heuer and Smalla, 2007; Heuer *et al.*, 2008; Heuer *et al.*, 2009; Heuer *et al.*, 2011; Jechalke *et al.*, 2013b; Jechalke *et al.*, 2013c). Moreover repeated manure amendments continuously lead *tet* genes in soil (Schmitt *et al.*, 2006c). Continuous manure amendments added to soil, repeatedly, resistance genes which otherwise tend to decrease over time (Sengeløv *et al.*, 2003) as observed in SA soil (Figure 15a-16a). We observed meaningful manure effect on increasing *tet*(Q) and *tet*(W) genes abundance but Tc addition, even at concentration 500 mg kg^{-1}, did not further increase their abundance, in agreement to other authors (Schmitt *et al.*, 2006c; Kyselková *et al.*, 2013). Also *tet*(A) abundance increased after manure addition, already after the first amendment. In addition, also Tc further increased *tet*(A) abundance and, after three repeated amendments, Tc concentration effect was observed. The *tet*(A) gene is normally associated to intestinal microflora (Lanz *et al.*, 2003), reached the soil by manure amendment, in fact was also detected

in the manure sample. However, as reported by Sengeløv *et al.* (2003), the level of Tc resistance genes in untreated soil tends to decrease over time but repeated manure and Tc amendments synergistically increased *tet*(A) abundance.

Typically, *tet* genes are located on mobile genetic elements (Roberts, 2005; Roberts, 2011a; Popowska *et al.*, 2012). The relative abundance of genes related to this elements, like integrons (*intI1*) and conjugative plasmids (*trfA*), implicated on horizontal spreading of resistance genes in soil microbial community, was measured.

Integrons are mobile genetic elements able to capture genes related to gene-cassettes by integration or excision movements on bacterial genome and spread antibiotic resistance genes through the microbial community. Integration/excision reaction is catalysed by integron-encoded integrase IntI protein. The IntI classes 1, 2 and 3 are the most studied (Barraud *et al.*, 2010) and we observed the effects of manure and Tc amendments on the relative abundance of class 1 integron-integrase gene (*intI1*). This gene is normally related to resistant bacteria in manure (Lanz *et al.*, 2003). In our case, the addition of manure resulted in a significant effect in PU and SA soil which increased, by repeated amendments, the *intI1* gene abundance. Antibiotic resistance genes like *tet* and *sul1* are typically associated to class 1 integrons (Agerso and Sandvang, 2005; Graves *et al.*, 2011; Jechalke *et al.*, 2013c) which spread resistance among the bacterial community and to humans by the food chain or contact (Agerso and Sandvang, 2005; Heuer *et al.*, 2011). Also conjugative plasmids are involved in spreading of resistance genes in microbial soil communities (Roberts, 2011a). IncP-1 is one of the most studied plasmids in the environmental molecular biology due to its broad host range in Gram-negative bacteria (Bahl *et al.*, 2009; Heuer *et al.*, 2012) and was found not only in the soil environment but also in sewage, manure and clinical isolates (Heuer *et al.*, 2012). Plasmids belonging to IncP-1 incompatibility group are broad host range plasmids and can be divided into six groups: α, β, γ, δ, ε and ζ (Bahl *et al.*, 2007; Bahl *et al.*, 2009; Norberg *et al.*, 2011). *trfA*, associated to incP-1 conjugative plasmid (Jechalke *et al.*, 2013a), codes for a replication initiation protein (Bahl *et al.*, 2009) and a component of partition and replication system of incP-1 plasmid (Herman *et al.*, 2011) respectively. Quantification of *trfA* and gene by rt-qPCR was used to measure the spreading of the conjugative IncP-1 plasmids of the ε subgroup in soil after repeated amendments of manure unspiked and spiked with Tc. Manure and Tc addition to soil played an important role on increasing the relative abundance of incP-1ε plasmid by a synergistic action, in accordance with other authors (Heuer and Smalla, 2007; Heuer *et al.*, 2012). In this work was also investigated

the effect of repeated amendments, not investigated by Heuer *et al.* (2012), and we observed increasing of *rtfA* abundance in treatments with manure spiked by Tc, probably due to accumulation of these plasmids in microcosms, in accordance with Jechalke *et al.* (2013a).

Mobile genetic elements can be responsible for multidrug resistance in the soil environment and *tet* genes can be associated to resistance genes of other antibiotic classes, for example, sulfonamides (Agerso and Sandvang, 2005; Schmitt *et al.*, 2006a; Roberts, 2011b, a). Sulfonamides are, together with tetracyclines, the most used antibiotics in livestock (Dibner and Richards, 2005). They inhibit, in bacteria, the dihydropteroate synthase enzyme involved in acid folic synthesis (Heuer *et al.*, 2008). For this matter, we decided to observe the effects of manure and Tc amendments also on relative abundance of *sul1* and *sul2* genes. The addition of manure to soil increases the abundance of *sul* genes and Tc amplifies the effects on soil by further increments on their abundance, in accordance with other authors (Heuer and Smalla, 2007; Jechalke *et al.*, 2013c; Kopmann *et al.*, 2013), by synergistic action, with the exception of *sul1* gene in SA soil already present in untreated SA soil (Figure 20b). Furthermore, repeated manure amendments contribute to environmental contamination and amplify effects of antibiotic in soil (Heuer *et al.*, 2008) by continuous supply of resistance genes in the environment (Alexander *et al.*, 2011). The increasing on antibiotic resistance genes is correlated to increase on the abundance of mobile genetic elements which reach soil through manure addition and promote resistance development among microorganisms. The trend of the *intI1* gene was similar to that observed for *sul1* and *sul2* genes and in accordance with other authors, that is, class 1 integrons lead sulfonamide resistance in soil (Selvam *et al.*, 2012) by manure amendments (Heuer and Smalla, 2007) and horizontal gene transfer (Zhu *et al.*, 2013).

On the whole, the results on antibiotic resistance in soil are in accordance with our data about Tc and manure effects on microbial structure and activity. Effects of treatments were stronger in PU soil than in SA soil. This is reasonable because microbial community of SA soil already contained Tc resistance genes and was resistant to Tc action, whereas PU soil was totally susceptible to Tc action. Moreover, we affirmed the importance of manure on changing bacterial structure in soil and how repeated amendments of uncontaminated manure and manure contaminated by Tc can enhance this effect. Manure and Tc amendments can also increase the resistance development confirming that manure is an important carrier for resistance genes in soil and Tc acts synergistically further increasing their abundance in the soil microbial community. Then continuous spreading of manure and Tc on soil has a strong impact upon the

soil structure over time. It should be emphasized that Tc amendments increase not only *tet* genes but also related genes (*intI1*, *trfA* and *sul*) involved in gene spreading and multidrug resistance development.

Chapter 5
Conclusions

The results of this study indicate that Tc has strong affinity for clay soil and its bioavailability is mostly depending on pH and soil clay content and in minor extent on organic matter. The analyses of culturable and unculturable fractions of the microbial community, and soil microbial activity highlighted significant effects of Tc on the structure and function of the microbial communities after a single amendment. Short-term effects were particularly evident for bacteria, whose population significantly decreased after Tc addition. Long-lasting effects of manure and Tc were also evident on the structure of the bacterial community in PU soil but not in SA soil. Moreover, the microbial activity was influenced by short-time effect in both soils. After 60 days, the activity was restored in PU but not in SA soil. The inhibition of the microbial activity, i.e. FDA activity, was dependent in the short-term on the Tc affinity for soil.

According to 16S rRNA gene PCR-DGGE analysis, after 60 days the amendment with Tc contaminated manure had a stronger effect on the structure of the bacterial population of PU compared to SA soil. Most likely, this finding is due to the intrinsic resistance of the microbial community in SA soil which already contained Tc resistance genes and was resistant to its action, whereas bacterial community of PU soil was totally susceptible. A repeated soil amendment with contaminated manure was able to increase the development of the antibiotic resistance in the microbial communities confirming the crucial role of manure in the spread of antibiotic resistance genes. It should be emphasized that Tc-contaminated manure amendments increase, not only tetracycline resistance genes, but also related genes involved in spreading of multidrug resistance. Although manure is important for sustainable agriculture practices, the diffusion of antibiotic resistance in soil environment can become a serious global problem. The transfer of antibiotic to pathogenic or commensal bacteria could represent a major problem for treatment of infectious diseases and can be a risk for human and animal health. Further studies are therefore needed to estimate the actual extent of this phenomenon and possibly to state safe regulatory guidelines for the use and distribution of manure in agriculture.

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