

**AUTHORS**

Santás-Miguel V.<sup>1</sup>

Díaz-Raviña M.<sup>2</sup>

Martín A.<sup>2</sup>

García-Campos E.<sup>2</sup>

Barreiro A.<sup>2</sup>

Núñez-Delgado A.<sup>3</sup>

Fernández-Sanjurjo M.J.<sup>3</sup>

Álvarez-Rodríguez E.<sup>3</sup>

Arias-Estévez M.<sup>1</sup>

Fernández-Calviño D.<sup>1, @</sup>  
davidc@uvigo.es

@ Corresponding Author

<sup>1</sup>Área de Edafología y Química Agrícola, Departamento de Biología Vegetal e Ciencia do Solo, Facultade de Ciencias, Universidade de Vigo. Campus As Lagoas, s/n. 3204 Ourense, Spain.

<sup>2</sup>Departamento de Bioquímica del Suelo, Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC). Apartado 122, Avda Vigo s/n. 15780 Santiago de Compostela, Spain.

<sup>3</sup>Departamento de Edafología e Química Agrícola, Escola Politécnica Superior de Enxeñaría, Universidade de Santiago de Compostela. Campus Universitario, s/n. 27002 Lugo, Spain

# Medium-term influence of tetracyclines on total and specific microbial biomass in cultivated soils of Galicia (NW Spain)

*Influencia a medio plazo de las tetraciclinas en la biomasa microbiana total y específica en suelos de cultivo de Galicia (NO España)*  
*Influência a médio prazo das tetraciclinas na biomassa microbiana total e específica de em solos cultivados da Galiza (NO Espanha)*

Received: 07.05.2020 | Revised: 29.09.2020 | Accepted: 30.09.2020

## ABSTRACT

This work examines the results of a soil incubation experiment in the laboratory, under controlled conditions of humidity and temperature. The purpose was to determine the medium-term influence of the presence of antibiotics on the total and specific microbial biomass, determined by means of the phospholipid fatty acids (PLFAs) analysis (total microbial biomass, and specific fungal, bacterial, actinobacterial, Gram-negative bacterial and Gram-positive bacterial biomass), as well as the relationship between some of these groups (fungal biomass/bacterial biomass, Gram-negative-bacterial/Gram-positive bacterial). The experiment was performed with four different cultivated soils with a similar pH but different organic matter (OM) content, to which eight doses of three antibiotics of the tetracycline group (tetracycline, oxytetracycline and chlorotetracycline) were added. Microbial biomass measurements (total and specific groups) were performed after 42 days of incubation. As expected, the total and specific microbial biomass values were different in the four soils studied. Both the total and the specific microbial biomass showed a similar response to the presence of antibiotics, although in several cases the data were inconsistent and difficult to interpret. In general, in all soils the addition of chlorotetracycline and tetracycline slightly modified or increased, to a greater or lesser extent, the values of both total and specific microbial biomass, particularly at higher doses. However, in certain cases, biomass values decreased due to the addition of the highest dose of oxytetracycline. With regard to fungal/bacterial and Gram<sup>-</sup>bacteria/Gram<sup>+</sup> bacterial biomass ratios, values slightly changed after the addition of the antibiotics.

## RESUMEN

*En este trabajo se examinan los resultados de una experiencia de incubación de suelos en el laboratorio, bajo condiciones controladas de humedad y temperatura, cuya finalidad fue determinar la influencia a medio plazo de la presencia de antibióticos en la biomasa microbiana total y específica, establecida a partir de los ácidos grasos de los fosfolípidos (biomasa microbiana total, biomasa fúngica, bacteriana, de actinobacterias, de bacterias Gram-negativas y de bacterias Gram-positivas) y de la relación entre algunos de estos grupos (hongos/bacterias, bacterias Gram-negativas/bacterias Gram-positivas). La experiencia se realizó con cuatro suelos de cultivo diferentes con un pH similar y con un diferente contenido de materia orgánica, a los que se aplicaron 8 dosis de tres antibióticos del grupo de las tetraciclinas (tetraciclina, oxitetraciclina y clorotetraciclina). Las medidas de biomasa microbiana (total y grupos específicos) se realizaron después de 42 días de incubación. Tal como era de esperar, los valores de la biomasa*

*microbiana total y específica fueron diferentes en los cuatro suelos estudiados. Tanto la biomasa microbiana total como la específica mostraron una respuesta similar frente a la presencia de los antibióticos, aunque en varios casos los datos fueron inconsistentes y difíciles de interpretar. En general, en todos los suelos la adición de la clorotetraciclina y la tetraciclina modificó o incrementó en mayor o menor medida los valores tanto de la biomasa microbiana total e específica, particularmente a las dosis más altas. Sin embargo, en determinados casos, como consecuencia de la adición de la dosis más alta de oxitetraciclina, los valores de biomasa disminuyeron. En lo que respecta a las relaciones biomasa fúngica/bacteriana y biomasa bacterias Gram-positivas/bacterias Gram-negativas, los valores apenas variaron tras la adición de los antibióticos.*

## RESUMO

*Este artigo examina os resultados de uma experiência de incubação do solo em laboratório, sob condições controladas de humidade e temperatura. O objetivo foi determinar a influência, a médio prazo, da presença de antibióticos na biomassa microbiana total e em grupos específicos de microrganismos, a partir da análise de ácidos gordos fosfolipídicos (biomassa microbiana total, biomassa fúngica, biomassa bacteriana e de actinobactérias, e de biomassa de bactérias Gram-negativas e de bactérias Gram-positivas), assim como a relação entre alguns desses grupos (biomassa fúngica/biomassa bacteriana, bactérias Gram-negativas/bactérias Gram-positivas). A experiência foi realizada com quatro solos cultivados diferentes, com pH semelhante e com um conteúdo diferente de matéria orgânica, aos quais foram aplicadas oito doses de três antibióticos do grupo das tetraciclina (tetraciclina, oxitetraciclina e clorotetraciclina). As determinações de biomassa microbiana (total e grupos específicos) foram realizadas após 42 dias de incubação. Como esperado, os valores da biomassa microbiana total e específica foram diferentes nos quatro solos estudados. Tanto a biomassa total como a específica mostraram uma resposta semelhante à presença de antibióticos, embora em vários casos os dados sejam inconsistentes e difíceis de interpretar. Em geral, em todos os solos, a adição de clorotetraciclina e tetraciclina modificou ou aumentou, em maior ou menor grau, os valores da biomassa microbiana total e específica, particularmente nas doses mais altas. Contudo, em certos casos, como resultado da adição de uma dose mais alta de oxitetraciclina, os valores de biomassa diminuíram. Em relação às razões biomassa fúngica/biomassa bacteriana e biomassa de bactérias Gram-negativas/biomassa de bactérias Gram-positivas, os valores variaram ligeiramente após a adição de antibiótico.*

## 1. Introduction

Organic manures are often used as fertilizers and/or amendments to improve soil quality and hence crop production in both agricultural and forest soils, as well for recovery of degraded soils due to natural or anthropic degradation processes (Gregorich et al. 1994; Villar et al. 2004; Briceño et al. 2007; Basanta et al. 2017). The reasons for their application in cultivated soils (mainly as fertilizer function) or degraded soils (mainly as amendments) are the benefits to most soil characteristics, such as an increase of organic matter (short-term liberation of carbon and nutrients directly from residue, available fractions, and medium- and long term liberation due to the mineralization process throughout the microorganisms), and other properties related to it, including improvement of soil structure, water retention, density, microbial activity, etc. (Lehman and Kleber 2015). Therefore, application of organic manures to soils play a key role in the quality and quantity of organic matter, which is the basis of the quality and the sustainability of soils by improving many physical, chemical and biological properties (Gregorich et al. 1994; Liu et al. 2006; Lichtfouse 2009; Fageria 2012). However, in determined soil types, when organic manures are used they can also have negative effects on soil quality due mainly to an imbalance of nutrients and the presence of toxic compounds at high concentrations (heavy metals, pathogenic microorganisms, excess or deficit of nutrients, high salinity, herbicides, fungicides, etc.), which depends mainly on the

**KEYS WORDS**  
Polluted soils,  
oxytetracycline,  
chlorotetracycline,  
microorganisms,  
phospholipid fatty  
acids.

**PALABRAS  
CLAVE**  
Suelos  
contaminados,  
oxitetraciclina,  
clorotetraciclina,  
microorganismos,  
ácidos grasos de los  
fosfolípidos.

**PALAVRAS-  
CHAVE**  
Solos contaminados,  
oxitetraciclina,  
clorotetraciclina,  
microorganismos,  
ácidos gordos  
fosfolípidos.

type of residue, the soil where it is applied, and inadequate agricultural or forestry soil practices (dose, manner and time of application) (Bååth et al. 1998; Lakhdar et al. 2010). The timing of the doses and time passed after application are also determinant factors. As expected, the more accentuated effects are detected at the highest doses in the short-term and decrease over time; however, in some cases, medium- and long-term effects (residual effects) occur, mainly due to the presence of inorganic compounds such as heavy metals worldwide (Petruzzelli 1989; Burgos et al. 2002). Likewise, studies of several authors showed a residual effect of the application of sludge contaminated with different doses of heavy metals on soil microorganisms even 150 years after its application (Bååth et al. 1998). In the last few decades, a residual effect of organic compounds such as herbicides or their metabolites has been observed in some acid soils of NW Spain, even one year after application despite the fact that these compounds are considered to have a short half-life of several days (Mahía and Díaz-Raviña 2007; Mahía et al. 2008). In this sense, organic residues can also be used as immobilizing agents for both inorganic and organic compounds in soil (Briceño et al. 2007; Alvarenga et al. 2009) improving the soil quality.

The impact of organic manure amendment on soil quality is very difficult to determine by measuring physical and chemical soil properties; this is due to the fact that the data vary depending on the single soil property considered (no effect, increases or decreases). Its impact on soil quality will be the result of the balance between these positive and negative effects on these properties. In contrast, microorganisms can be successfully used as bioindicators of the influence of different soil degradation processes and/or soil conservation practices on soil quality due to the fact that they reflect "in situ" balances of the interaction of all these factors with opposite trends on microorganisms and plant growth. In addition, they are more sensitive and, due to their fast turnover, they are early indicators of soil quality changes when compared with physical and chemical properties (Allen et al. 2011).

Recently, the presence of other compounds in animal feces and urine, such as veterinary

antibiotics used extensively on farms for disease control, is detected frequently from low to high doses; hence through its application to soil as amendment they can enter into the environment (soils, waters, etc.) and have negative consequences in the food chain and human health (Sarmah et al. 2006; Brandt et al. 2015; Caracciolo et al. 2015; Cycón et al. 2019). Research on these emerging contaminants is necessary due to potential risk assessment of the terrestrial ecosystems; however, unlike other priority pollutants (e.g. heavy metals or POPs), the behavior and the fate of veterinary compounds in the environment have not been extensively studied. In addition, information concerning their impact on soil microorganisms, which are the main responsible for soil functioning (Nannipieri et al. 2003), is scarce. Therefore, investigations concerning the presence, dynamics, behavior and impact of antibiotics added directly with the contaminated organic manures in relation to soil microorganisms is only limited in some agricultural soils.

The tetracyclines group, chlortetracycline (CTC), tetracycline (TC) and oxytetracycline (OTC), is one of the most extensively used as antibiotics both on humans and farm animals worldwide, including Spain. Nevertheless, only scarce studies concerning its presence in waters and recent papers concerning its impact on soil-plant ecosystems in Galicia (NW Spain) are available. These preliminary studies concerning the presence of tetracyclines in manures (cows, pigs and chicken), soils and potato crops (Conde-Cid et al. 2018a), adsorption-desorption processes (Fernández-Calviño et al. 2015), biotic and abiotic dissipation (Conde-Cid et al. 2018b) and bacterial toxicity (Santás-Miguel et al. 2018b; 2020a) are those published by our research group. The results showed the potential risk of these compounds on terrestrial and aquatic ecosystems, and hence the need of their investigation. In this line, there is a special interest in studying the influence of residual antibiotics of veterinary origin on several aspects of native soil agricultural microorganisms such as number, mass, activity, structure and diversity. The aim of this research is to increase our knowledge on the fate and behavior of tetracyclines (CTC, TC, OTC) by evaluating their effects in these agricultural soils of Galician (NW Spain).

## 2. Materials and Methods

### 2.1. Chemicals

Chlortetracycline (CTC), tetracycline hydrochloride (CAS.64-75-5; (TC), oxytetracycline (OTC), all three as hydrochloride, were supplied by Sigma-Aldrich (USA) and used in the present laboratory study.

### 2.2. Soil samples and general characterization

The study was performed with four agricultural soils collected in A Limia (Galicia, Spain), with a potato-wheat rotation culture. This area has an average altitude of 640 m above sea level. The mean annual temperature is 11 °C, with a total mean annual precipitation of 881 mm, irregularly distributed through the year. The four soils were classified as Mollic Umbrisols (Anthric) according to IUSS Working Group WRB (2015). Due to the abundance of poultry farms in the area, poultry manure (with average dry matter 50%) is routinely used as organic fertilizer and also for its liming effect, with usual yearly doses averaging around 40 m<sup>3</sup> ha<sup>-1</sup>. Inorganic fertilizers are also added, in average yearly 1000 kg ha<sup>-1</sup>

dose for NPK formulations of 8:24:16. Main soil characteristics are given in **Table 1**. During July-August, when the soil was without culture, after harvests of the potato crop and before sowing of wheat, a total of 10 soil subsamples was collected from the Ae horizon (0-20 cm depth) of each agricultural soil and subsequently mixed into a composite representative soil sample (approximately 3 kg) and refrigerated (4 °C) until processing in the laboratory. The soil samples were passed through a sieve with a 2 mm diameter mesh; fractions bigger than 2 mm were discarded and the soil fractions < 2 mm thoroughly homogenized used in all the subsequent analysis. They were divided into: a) fresh subsamples lyophilized and frozen to -15 °C for the assessment of phospholipid fatty acids (PLFAs) analysis; (b) air-dried subsamples used for analysis of the physical, chemical and biochemical properties. Soil pH in water ranged from 4.7 to 5.0, and pH in KCl from 4.25 to 4.23; total organic carbon (OC) ranged from 10.7 to 33.9 g kg<sup>-1</sup> dissolved organic carbon (DOC) from 211 to 306 mg kg<sup>-1</sup> and total nitrogen (N) from 0.9 to 3.1 g kg<sup>-1</sup> (**Table 1**). The effective cation exchange capacity ranged from 4.1 to 6.4 cmol<sub>c</sub> kg<sup>-1</sup> and the texture from sandy-loam to sandy clay loam. More details about the experiment and physical and chemical characterization are included in Santás-Miguel et al. (2020b).

**Table 1.** Some properties of soils studied in the laboratory experiment

	Soil		
	1	2	3
pH <sub>w</sub>	4.8	5.0	5.0
pH <sub>KCl</sub>	4.25	4.43	4.43
C (g kg <sup>-1</sup> )	10.7	21.4	25.3
N (g kg <sup>-1</sup> )	0.9	2.0	2.3
C/N	12	11	11
eCEC* (cmol <sub>c</sub> kg <sup>-1</sup> )	4.1	5.3	6.4
DOC** (mg kg <sup>-1</sup> )	211	279	306
Sand (%)	70	61	59
Silt (%)	12	16	19
Clay (%)	18	23	22
Texture	Sandy-Loam	Sandy Clay Loam	Sandy Clay Loam

\*Effective cation exchange capacity; \*\*Water dissolved organic carbon.

### 2.3. Experiment design

Dried soil samples were rewetted up to 60-80% of water holding capacity and incubated at 22 °C for one week, enough time for stabilization of biomass, activity and structure or composition of soil microbial communities (Meisner et al. 2013). Afterwards, three antibiotics of the tetracycline group (CTC, TC, OTC) were added in triplicate to the different soils (up to 8 different antibiotic concentrations to each soil), resulting in a total of 288 microcosms (4 soils x 3 antibiotics x 8 concentrations x 3 replications), 25 grams each. The final tetracycline concentration in each microcosms was 0.00, 0.49, 1.95, 7.81, 31.25, 125, 500 and 2000 mg kg<sup>-1</sup> of soil. Tetracyclines were added to the soils separately, using talc powder as a carrier, for equalizing the amount of dry material added to each microcosm, thus facilitating the mixture with soils (Rousk et al. 2008). Once the soil was spiked with the tetracyclines, each microcosm was incubated at 22 °C in the dark maintaining the moisture content (60-80% of water holding capacity, in other words, weighing the soil containers and adding the lost water as needed) for 42 days. These 96 composite soil samples (a mixture of three incubation replicates per treatment; four soils: 1, 2, 3 and 4) were used to see the medium term effect of tetracyclines samples on total and specific microbial biomass.

### 2.4. Total and specific microbial biomass by phospholipid fatty acids (PLFA) analysis

The total and specific microbial biomass values were estimated using the procedure described by Frostegård et al. (1993). Briefly, lipids were extracted from the soil with a chloroform:methanol:citrate buffer mixture (1:2:0.8 v/v/v) and separated into neutral lipids, glycolipids and phospholipids using a pre-packed silica column. The phospholipids were subjected to a mild alkaline methanolysis and the fatty acid methyl esters were identified by gas chromatography (flame ionization detector) by the relative retention times of the fatty acids, using methyl nondecanoate (19:0) as internal standard.

The total microbial biomass (TotPLFAs) was estimated as the sum of all the extracted

PLFAs. The sum of the PLFAs, considered to be predominantly of bacterial origin (i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7t, i17:1ω8, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0), was used as an index of the bacterial biomass (bactPLFAs), and the quantity of the 18:2ω6 as fungi origin (Frostegård and Bååth 1996). The sum of 16Me16:0, 17Me17:0 and 16Me18:0 was used as an index of actinobacteria (actPLFAs). The i14:0, i15:0, i16:0 and 10Me18:0 PLFAs are predominantly found in gram-positive (G<sup>+</sup>) bacteria, and the cy17:0, cy19:0, 16:1ω7c and 18:1ω7 PLFAs characterize gram-negative (G<sup>-</sup>) bacteria (Díaz-Raviña et al. 2006).

All results were obtained from triplicate determinations and were expressed on the basis of oven-dry (105 °C) weight of soil. For the non-contaminated control soil, the mean ± standard error of three replicates obtained from the experiments with the three antibiotics (CTC, TC, OTC) was calculated. Pearson's correlation coefficients were used to determine the relationships between the total microbial biomass and the biomass of specific groups.

## 3. Results

The values of total biomass [total PLFAs (totPLFAs)] and biomass of specific groups of microorganisms (FungPLFAs, BactPLFAs, ActPLFAs), G-BactPLFA, G<sup>+</sup>BactPLFA varied depending on soil studied (**Figures 1-3**). Total biomass in control soils showed values (mean ± standard error of three incubation replicates) of 50 ± 0.7, 120 ± 36, 119 ± 14 and 97 ± 12 nmol g<sup>-1</sup>, for soils 1, 2, 3 and 4, respectively. The data of the composite soil samples with the three added antibiotics (mean value of three measurement replicates) were also shown in **Figures 1-3**. For each soil, the high variability found in mean values of three incubation replicates in the control soil (see data in the above paragraph) was similar or even higher than the differences observed among soil values added with increasing antibiotic dose. Thus, based on this fact, it can be deduced that the antibiotic effect was not significant in most soil treatments.



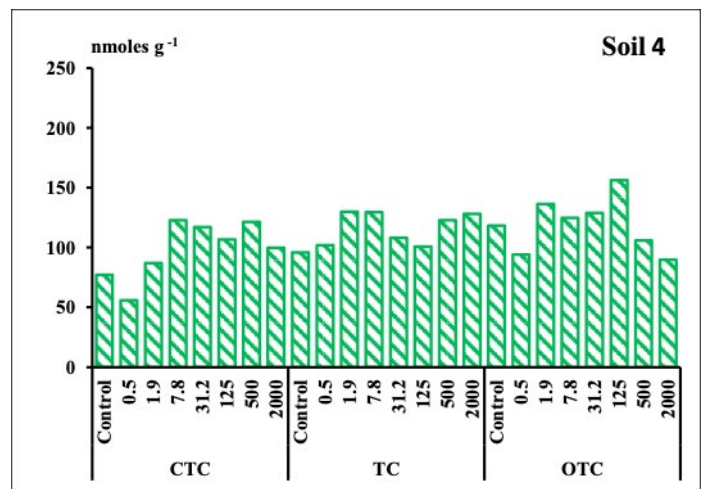
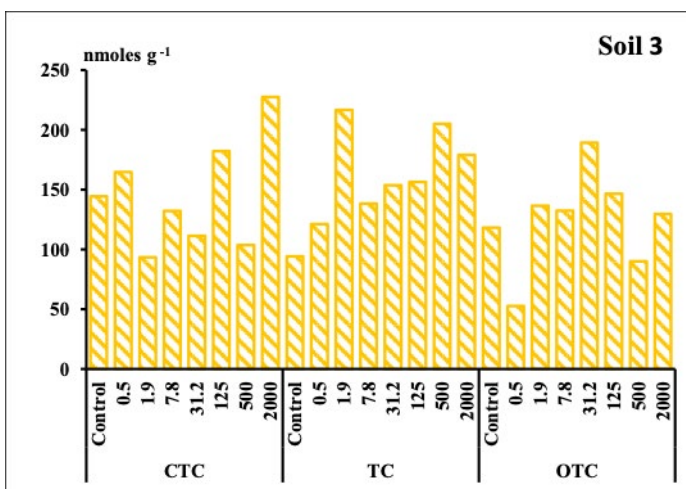
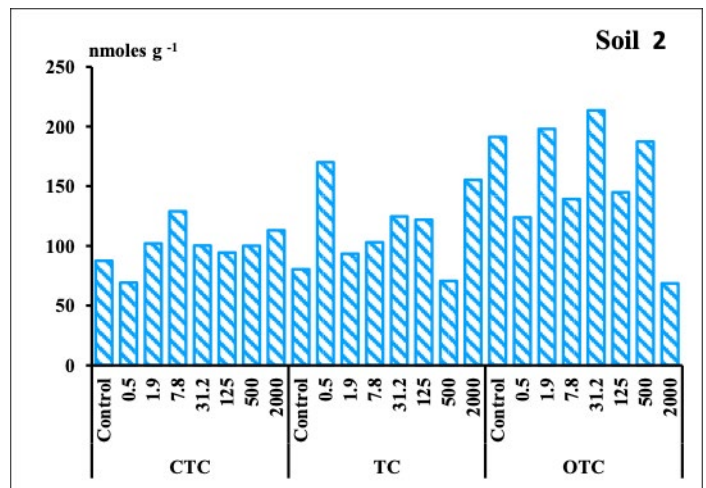
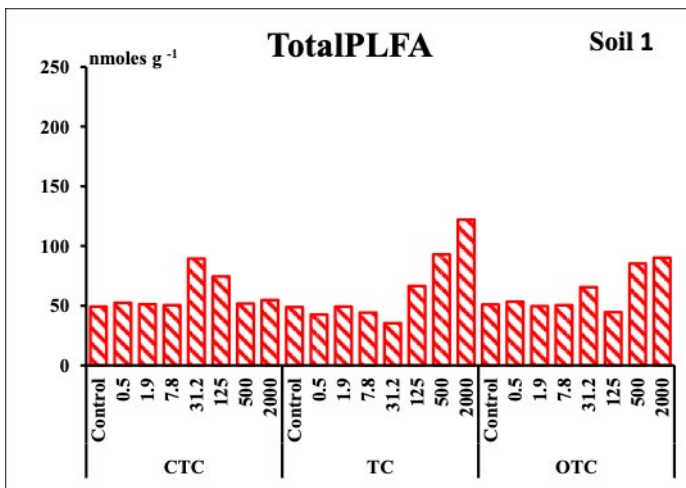
The results concerning the antibiotic effects on total and specific microbial biomass varied depending on microbial group, soil type, dose and antibiotic considered (CTC, TC, OTC). Therefore, due to inconsistent data, no general trends can be observed (the effect was not dose dependent, different behavior can be detected at low, medium and high doses), although some considerations can be done.

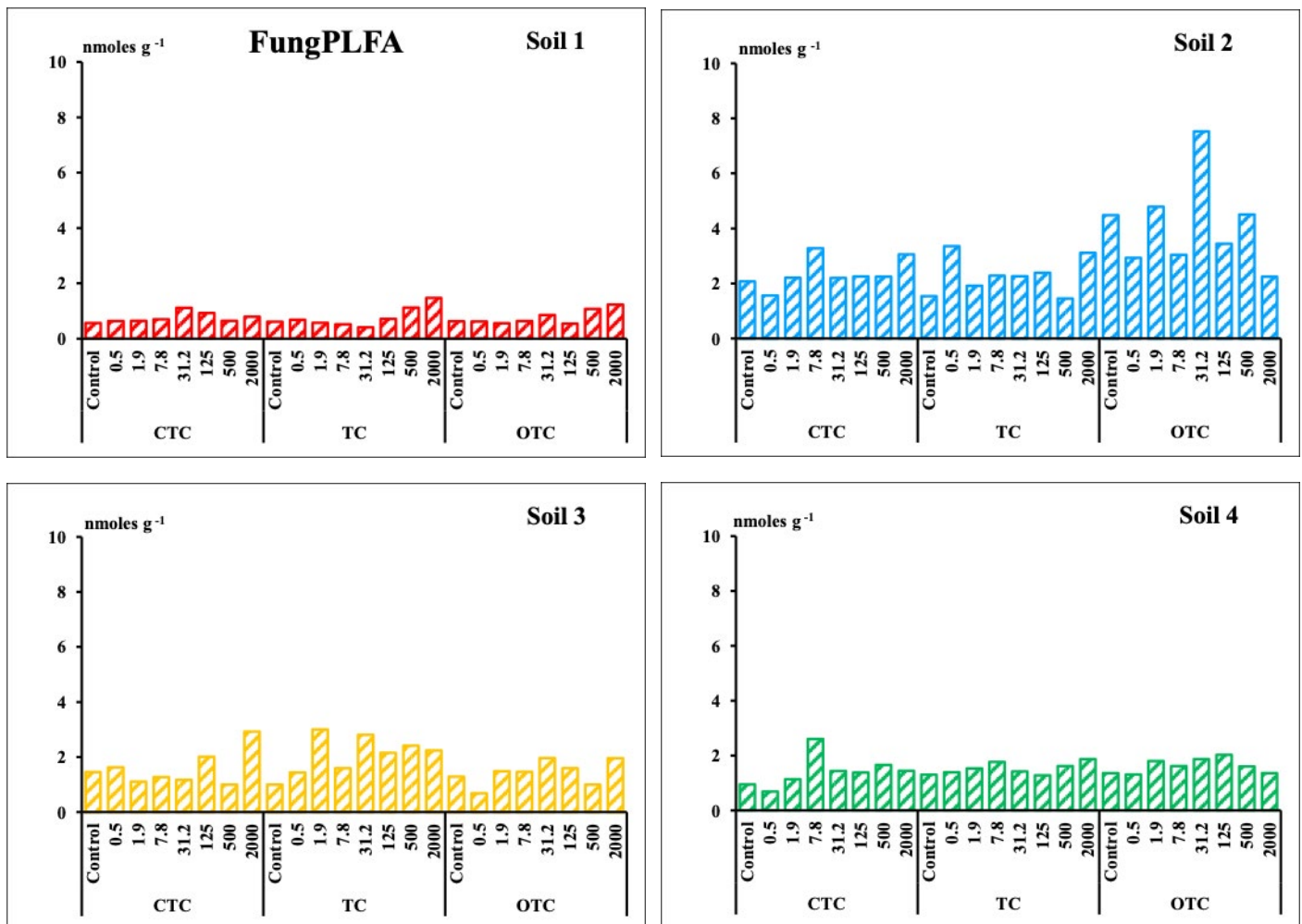
The addition of CTC had a slight or even no effect on total microbial biomass in soil 1 and 2, but increased their value at higher doses in soils 4 and 3, particularly the latter.

The addition of TC to the soil seems to slightly increase the total biomass values in soil 4,

moderately in soil 1 and be more accentuated in soil 3, while the opposite behavior, a negative effect, was detected in soil 2.

For the addition of OTC to soil, inconsistent total microbial biomass values were observed; at the highest doses no effect or even a slight effect was detected in soils 4 and 3, and a negative marked effect was detected in soil 2. Total biomass values were positively and significantly correlated with the values of all specific microbial groups, fungi ( $r = 0.757$ ,  $p < 0.001$ ); bacteria ( $r = 0.996$ ,  $p < 0.001$ ); actinobacteria ( $r = 0.979$ ,  $p < 0.001$ ); Gram-negative bacteria ( $r = 0.995$ ,  $p < 0.001$ ); and Gram-positive bacteria ( $r = 0.998$ ,  $p < 0.001$ ).



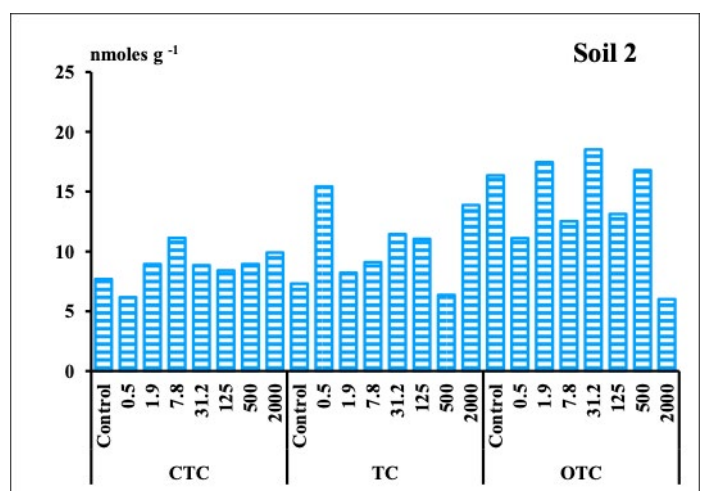
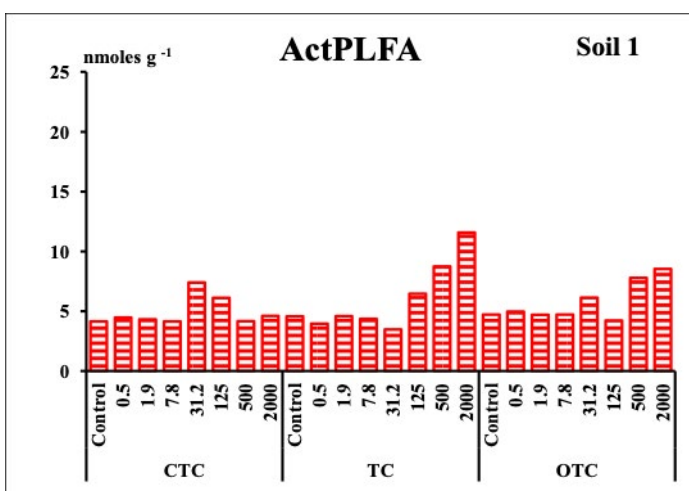
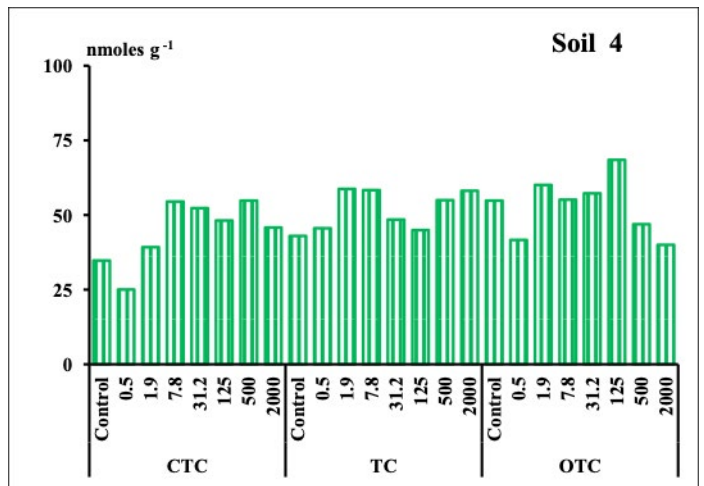
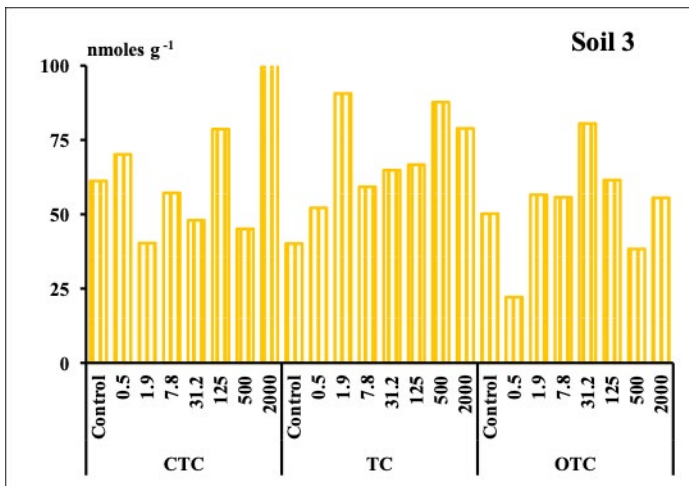
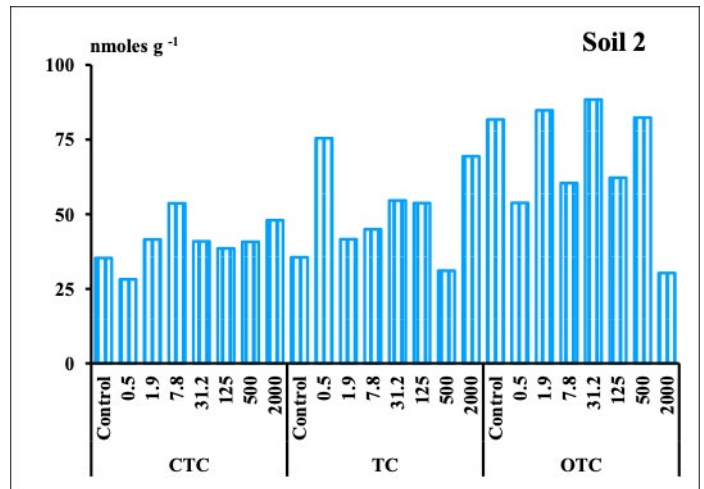
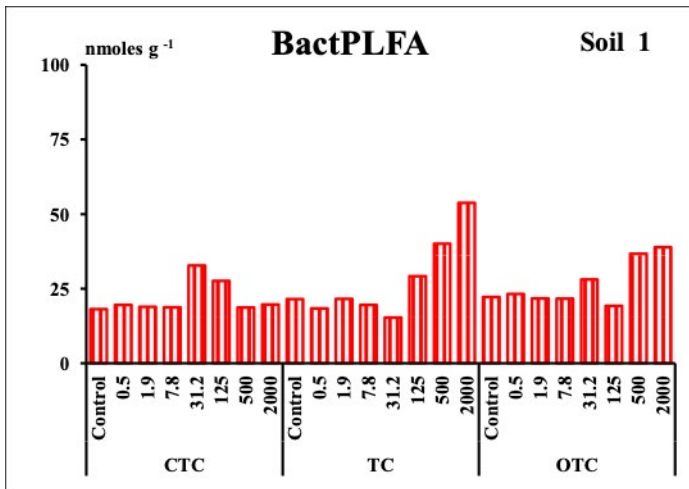


**Figure 1.** Total biomass (TotPLFA) and fungal biomass (FungPLFA) in the four studied soils with different doses of the tetracycline antibiotics after 42 days of application. CTC, chlorotetracycline; TC, tetracycline; OTC, oxytetracycline.

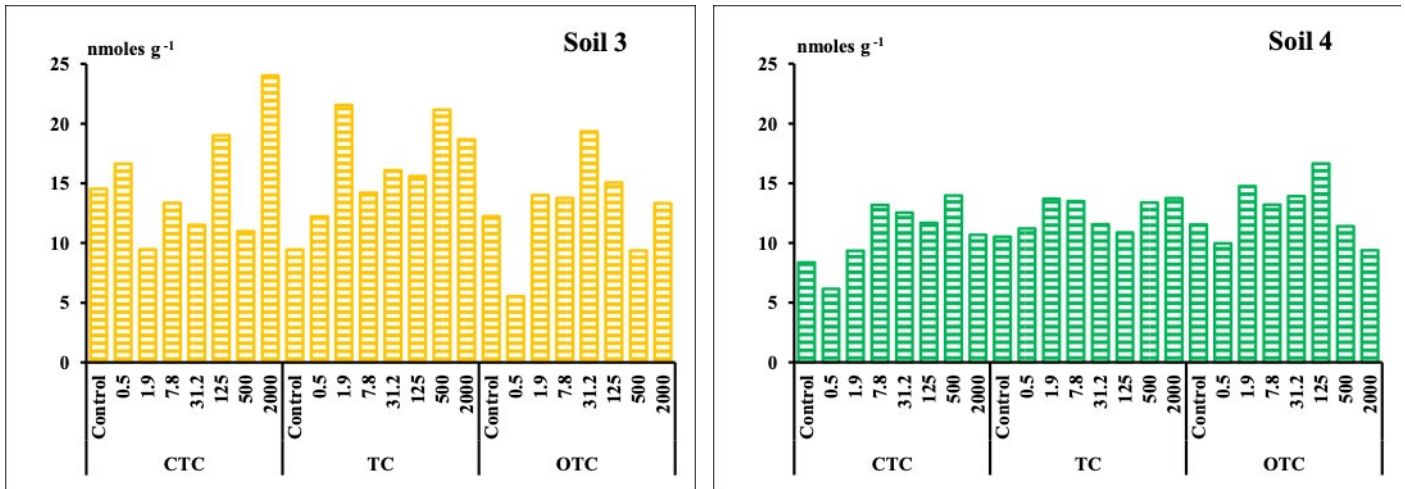
Fungal biomass in control soils showed values of  $0.60 \pm 0.02$ ,  $2.70 \pm 0.91$ ,  $1.24 \pm 0.13$  and  $1.20 \pm 0.13$   $\text{nmol g}^{-1}$ , for soils 1, 2, 3 and 4, respectively (Figure 1). As consequence of the application of the different doses of CTC to soils, the low values of fungal biomass hardly change in soil 1, or increase slightly in the rest of the soils. The addition of TC showed a similar trend with slight positive changes at high values of antibiotic in all soils (1, 2 3 and 4). In contrast, fungal biomass after OTC addition did not change in soil 4, slightly increased in soils 1 and 3 and generally decreased at several concentrations of the

antibiotic (except for 31.2 doses when a marked positive effect was detected).

Bacterial biomass in control soils showed values of  $21 \pm 1.51$ ,  $16.50 \pm 6$  and  $44 \pm 6$   $\text{nmol g}^{-1}$ , for soils 1, 2, 3 and 4, respectively (Figure 2). The results of bacterial biomass showed a similar trend with respect of the antibiotic addition (CTC, TC, OTC) on the four studied soils than that observed for the total microbial biomass (see above).





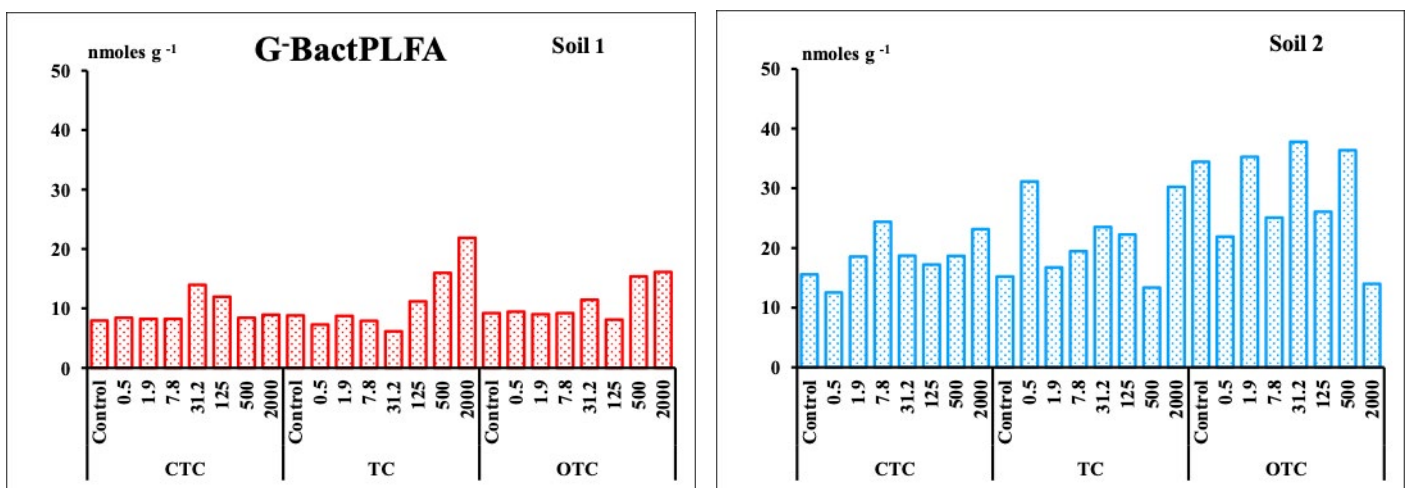


**Figure 2.** Bacterial biomass (BactPLFA) and actinobacteria biomass (ActPLFA) in the four studied soils with different doses of the tetracycline antibiotics after 42 days of application. CTC, chlorotetracycline; TC, tetracycline; OTC, oxytetracycline.

Biomass of Gram-negative bacteria in control soils showed values of  $8.7 \pm 0.4$ ,  $21.8 \pm 6.4$ ,  $21.3 \pm 6.7$  and  $17.9 \pm 2.1$  nmol g<sup>-1</sup>, for soils 1, 2, 3 and 4, respectively (Figure 3). The results of bacterial biomass showed a similar trend with respect of the antibiotic addition (CTC, TC, OTC) on the four soils than that observed for the total microbial biomass.

Biomass of Gram-positive bacteria in control soils showed values of  $5.6 \pm 0.3$ ,  $13.7 \pm 4.4$ ,

$14.4 \pm 1.6$  and  $12.5 \pm 1.3$  nmol g<sup>-1</sup>, for soils 1, 2, 3 and 4, respectively (Figure 3). Actinobacterial biomass in control soils showed values of  $4.5 \pm 0.2$ ,  $10.5 \pm 3.0$ ,  $12.0 \pm 1.5$  and  $10.1 \pm 0.9$  nmol g<sup>-1</sup>, for soils 1, 2, 3 and 4, respectively (Figure 2). In the four studied soils the effect of the application of three antibiotic studied (OTC, TC, CTC) on biomass of Gram-positive bacteria, Gram-negative bacteria and actinobacteria was similar than that observed for total and bacterial biomass (Figure 3).



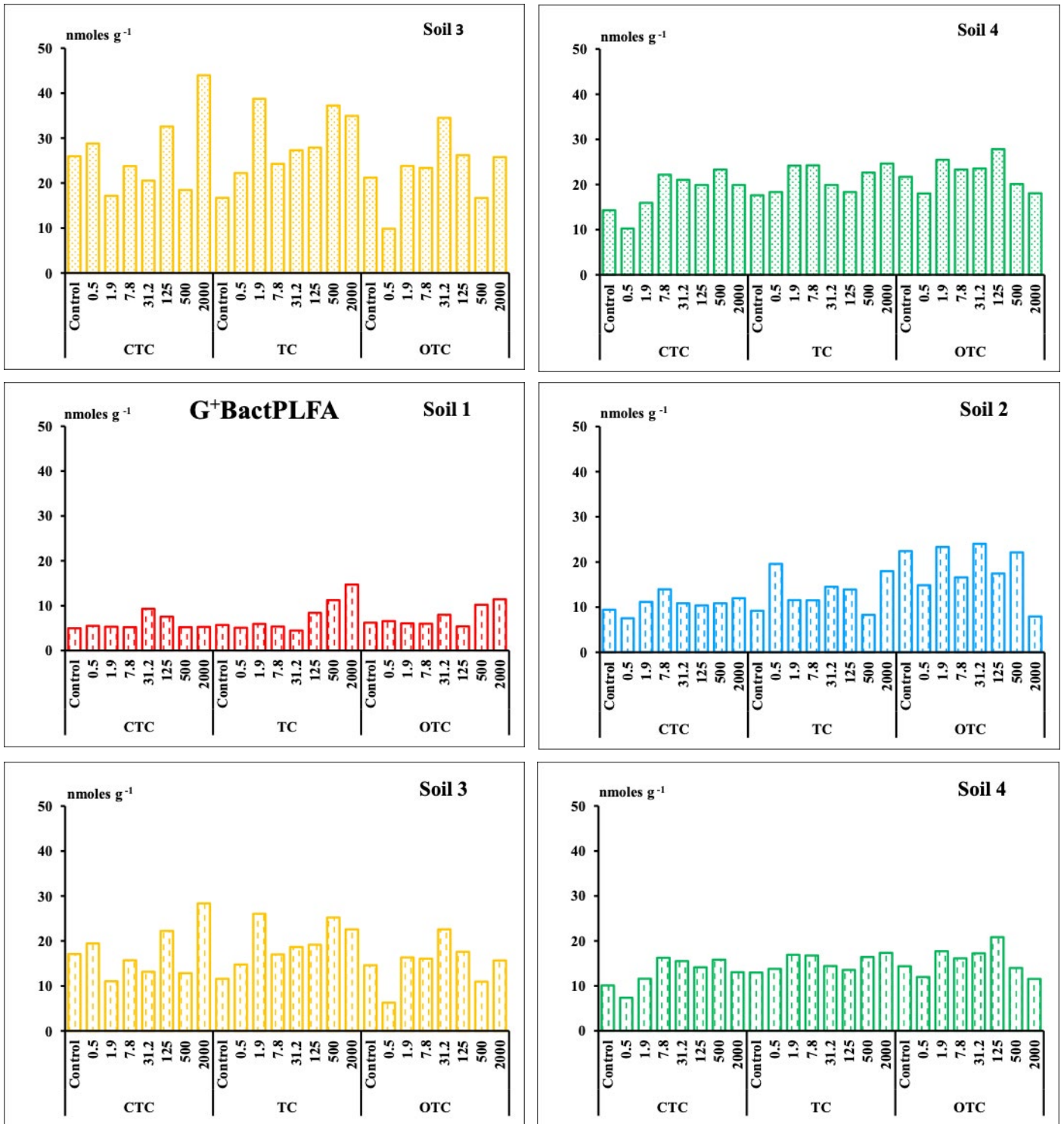
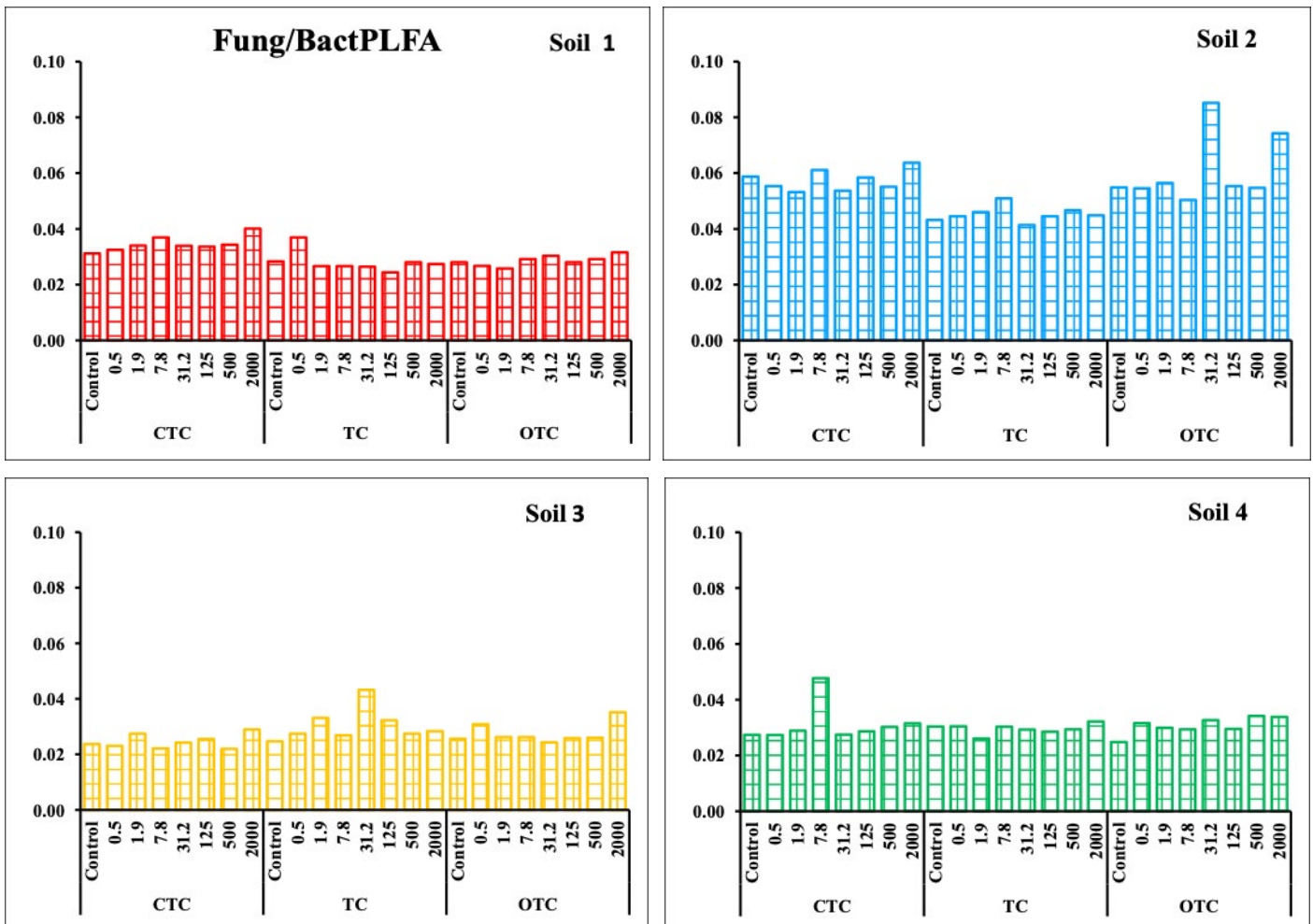


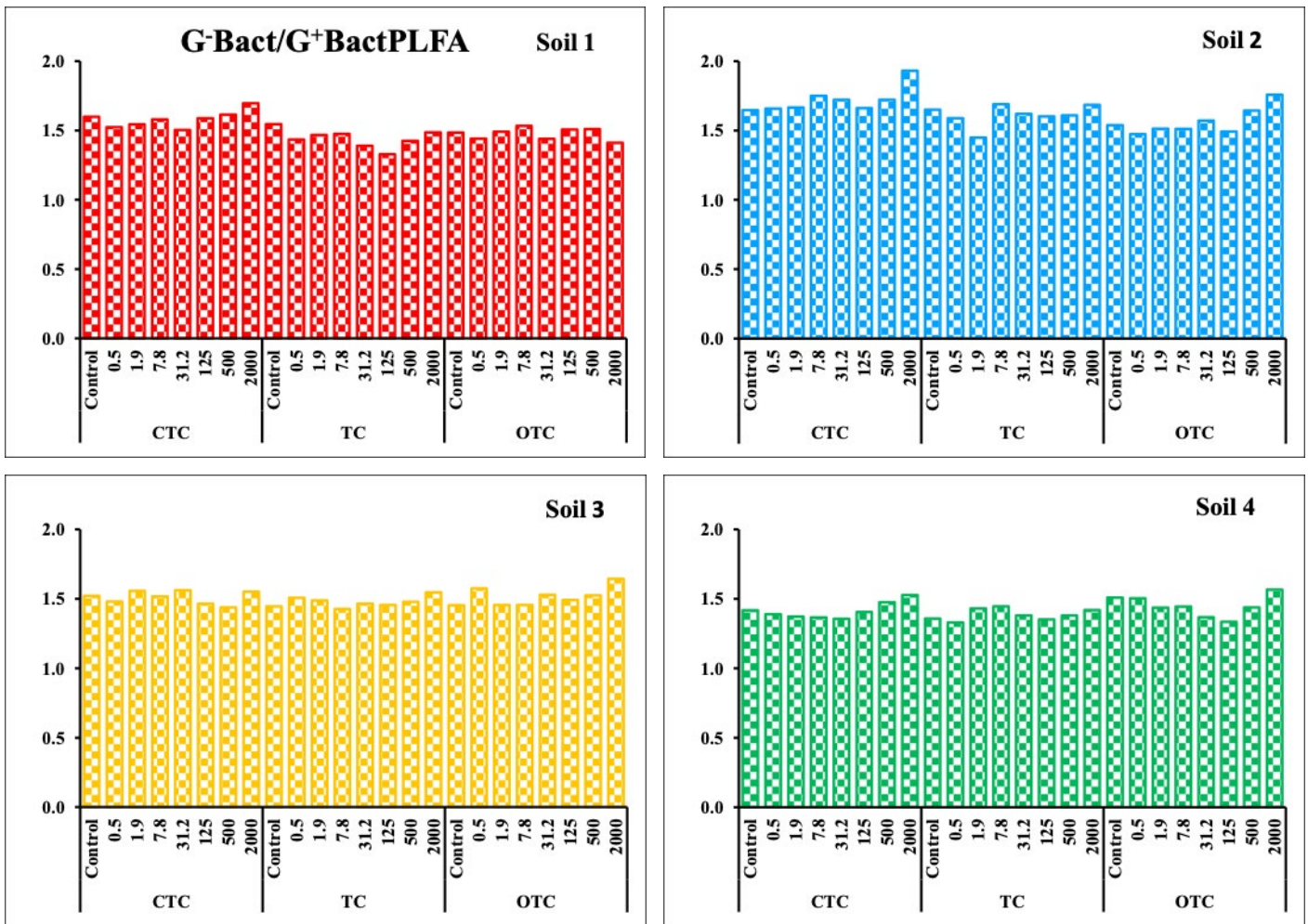
Figure 3. Gram-negative bacterial biomass (G-BactPLFA) and Gram-positive bacterial biomass (G+BactPLFA) in the four studied soils with different doses of the tetracycline antibiotics after 42 days of application. CTC, chlorotetracycline; TC, tetracycline; OTC, oxytetracycline.

The FungPLFA/BactPLFA biomass ratio in control soils showed values of  $0.029 \pm 0.001$ ,  $0.052 \pm 0.005$ ,  $0.025 \pm 0.001$  and  $0.028 \pm 0.002$  for soils 1, 2, 3 and 4, respectively (Figure 4). The Gram<sup>-</sup>BactPLFA/Gram<sup>+</sup>BactPLFA biomass ratios in control soils showed values of  $1.543 \pm 0.033$ ,  $1.612 \pm 0.037$ ,  $1.473 \pm 0.024$  and  $1.428 \pm 0.044$ , for soils 1, 2, 3 and 4, respectively. In these relationships (FungPLFA/BactPLFA and Gram<sup>-</sup>BactPLFA/Gram<sup>+</sup>BactPLFA biomass), the

addition of three different antibiotics (CTC, TC and OTC) to the four soils (1, 2, 3 and 4) hardly changed the initial values of unamended soils and generally a slight increase was observed at some concentrations for both ratios, especially in the highest dose (2000 mg kg<sup>-1</sup>). However, especially for fungi/bacteria ratio, a positive effect of 31.2 mg kg<sup>-1</sup> of OTC and TC was observed in soils 2 and 3, respectively, and of 7.8 mg CTC kg<sup>-1</sup> for soil 4 (Figure 4).







**Figure 4.** Ratios Fungal biomass/Bacterial biomass (FungPLFA/BactPLFA) and Gram-negative/Gram-positive biomass (G-BactPLFA/G+BacPLFA) in the four studied soils with different doses of the tetracycline antibiotics after 42 days of application. CTC, chlorotetracycline; TC, tetracycline; OTC, oxytetracycline.

## 4. Discussion

The PLFAs analysis indicated that the microbial biomass estimated as TotPLFA of selected cultivated soils was lower (Barreiro et al. 2010) and higher (Arias-Estévez et al. 2018) than that exhibited by forest and vineyard soils located in the same area, respectively. However, as expected, the values lay in the reported range given for agricultural soils (Mahía et al. 2011). Likewise, the same trend was observed for the biomass of specific groups of microorganisms (bacteria, fungi, actinobacteria, Gram-negative and Gram-positive bacteria) as well as for the percentage of each specific group with respect

to TotPLFA and the FungPLFA/bactPLFA and G-BactPLFA/G+BacPLFA biomass ratios. It is well-known that the total organic matter and, hence, available carbon, is closely and positively related with the microbial biomass carbon determined by the fumigation-incubation method (Díaz-Raviña et al. 1988) as well as other soil properties such as pH, available nutrients, moisture, temperature, presence of inorganic or organic toxic compounds, etc. In the present study the microbial biomass varied depending on soil considered ( $50 \pm 0.7$  to  $120 \pm 36$  nmol g<sup>-1</sup>), the values being about 2 times lower in soil 1 than in the rest of the soils. Since pH is similar in all soils, these data can mainly be explained on the basis of OC content, DOC,



CICe and texture, which are more favorable for microorganism growth in the later ones.

The response of the microbial communities of the four soils studied to the addition of the antibiotics of the tetracycline group after 42 days of incubation were quite similar, although small differences could be observed depending on soil, antibiotic and dose of application. This fact can partly explain on the basis of close relationships observed between TotPLFA and FungPLFA; BactPLFA; G-BactPLF; and G<sup>+</sup>BacPLFA biomass. However, in some cases the trends of effects (comparison between soil with and without antibiotics addition) differed. These results were variable and showed no consistent trend, therefore the results interpretation was very difficult. The three possible impacts were observed, no effect, positive and negative effects. Only in some cases the trend seems to be dose dependent, increasing the effect with the dose increment, but in most cases this was not observed and inconsistent effects depending of doses were observed. In order to analyze the effects, the general trend with the dose increment (independent of anomalous results of some doses) and the comparison of control soil with respect to the highest doses (2000 mg kg<sup>-1</sup>) were used. Thus, the results seem to show a small residual effect of the three antibiotics (CTC, TC and OTC) on the soils studied. A slight positive effect of three antibiotics seems to be observed in soils 1 and 3, no effect or a small effect in soil 4 while soil 2 seems to show a different behavior with an increase of values after CTC addition and a decrease after TC or OTC addition. With respect to the ratios FungPLFA/BactPLFA and G-BactPLFA/G<sup>+</sup>BactPLFA, the results showed that values did not vary appreciably after antibiotic addition independently of the soil, antibiotic or dose considered (only there is a slight increase in soil 2). Our results (no effect, positive effect and negative effect) under laboratory conditions (temperature and moisture are kept constant) can be explained only on the basis of antibiotic effect and the availability of C and nutrients. It shows that in these acid soils located in Galicia (NW Spain), the availability of carbon is the limiting factor of microbial growth and that diminished during the incubation (Díaz-Raviña et al. 1988). Since only compounds present in soil solution can affect microbial communities, these results can be partially explained as

follows: a) no effect, the absence of available antibiotic and/or the lack of available carbon, b) negative effect, the presence of available antibiotic that inhibited microbial growth and/or the lack of available carbon, and c) positive effect, the presence of microorganisms that use the antibiotic as substrate and hence a source of carbon and/or the increase of available carbon. De Nobili et al (2001) and Mahía et al. (2011) found that the presence of trace concentrations of appropriate “trigger solutions” derived or degradable substrates or organic residues can accelerate the mineralization of native organic matter, resulting a “priming effect”, and providing, hence, available carbon and nutrients. This is observed for some exogenous organic compound added to soils such as herbicides (atrazine) (Mahía et al. 2011) therefore, this behavior cannot be discarded for other organic compounds such as antibiotics.

Investigations of several authors analyzing the impact of antibiotic on microbial biomass determined by means as PLFA analysis, showed an increase of fungi with respect to bacteria and of Gram-negative bacteria with respect with Gram-positive bacteria, therefore the ratios FungPLFA/BactPLFA and G-BactPLFA/G<sup>+</sup>BactPLFA (Hund-Rinke et al. 2004; Hammersfahr et al. 2008; Rousk et al. 2009; Chen et al. 2013; Cycón et al. 2019). It should be noted, however, that these results were observed at short-term, around 1 week following antibiotic addition and that this effect was diminished with time. Therefore, our results are not in contrast with values obtained in these studies since we examined the antibiotic effects at long-term (42 days after the incubation).

Previous laboratory studies of Santás-Miguel et al. (2020b) concerning the evolution of the toxicity of tetracycline in 22 agricultural soils of the same area during 42 days of incubation (measurements of microbial growth determined by means of the leucine incorporation technique following 1, 8 and 42 days of antibiotic addition), showed that tetracycline toxicity was highly dependent on soil characteristics, decreasing as a function of increasing organic matter, clay and effective exchange cation capacity (eCEC) contents and of decreasing soil pH values. In our case, obviously the influence of pH is discarded (pH similar in all soils). They also found that

toxicity decreased notably depending on antibiotic dose and time passed after addition. However, a marked residual antibiotic effect was still observed 42 days after the addition. This is in line with our results since a small residual effect was often found after this incubation time and the different level of magnitude was explained by the different sensitivity of the microbial parameters used in the experiments, microbial growth determined by the leucine incorporation technique and biomass determined by the biomass of the PLFA analysis. It is well known that microbial parameters had a different sensitivity to the impact of different agricultural and forestry practices and that the order of sensitivity of microbial growth is higher than that observed by biomass parameters (Brandt et al. 2015; Cycón et al. 2019).

Further studies are now being conducted in our laboratory with the same soils samples used in the present work (four soils added with different doses of tetracycline antibiotics after 42 days) measuring other microbial parameter of the, microbial community, such as microbial structure by means of PLFA pattern, which is known to be a good tool to detect the impact of different soil disturbance at short-, medium- and long-term due to its higher sensitivity in order examine properly the residual antibiotic changes at long-term.

## 5. Conclusions

As consequence of the addition of eight increasing doses of three antibiotics of tetracyclines group (chlortetracycline, tetracycline and oxytetracycline) in four agricultural soils of Galicia (NW of) no effects or slight inconsistent positive or negative effects were observed in total biomass or biomass of specific microbial groups (fungi, bacteria, actinobacteria, Gram-positive negative and Gram-positive bacteria) after 42 days of incubation under controlled conditions of moisture and temperature. This clearly showed that biomass PLFA measurements were not a good indicator for determining the residual effect of antibiotics addition on soil at medium- or long-term.

## 6. Acknowledgements

This study has been funded by the Spanish Ministry of Economy and Competitiveness through the projects CGL2015-67333-C2-1-R and -2-R (FEDER Funds). David Fernández Calviño holds a Ramón y Cajal contract (RYC-2016-20411), financed by the Spanish Ministry of Economy, Industry and Competitiveness. Vanesa Santás Miguel holds a predoctoral fellowship founded by the University of Vigo

---

## REFERENCES

- Allen DE, Singh BP, Dalal RC. 2011. Soil health indicators under climate change: A review of current knowledge. In: Singh BP, Cowie AL, Chan KY, editors. *Soil Health and Climate Change*. Heidelberg: Springer-Verlag, Berlin. p. 25-35.
- Alvarenga P, Gonçalves AP, Fernández RM, de Varennes A, Vallini G, Duarte E, Cunha-Quecha AC. 2009. Organic residues as immobilizing agents in aided phytostabilisation: I. Effects on soil chemical characteristics. *Chemosphere* 74:1292-1300.
- Arias-Estévez M, Díaz-Raviña M, Fernández-Sanjurjo MJ, Álvarez-Rodríguez A, Núñez-Delgado A, Fernández-Calviño D. 2018. By-products as an amendment of a mine soil: effects on microbial biomass determined using phospholipid fatty acids. *Spanish J Soil Sci.* 8:1-11.
- Bååth E, Díaz-Raviña M, Frostegård Å, Campbell D, Colin CP. 1998. Effect of metal-rich sludge amendments on the soil microbial community. *Appl Environ Microbiol.* 64:238-245.
- Barreiro A, Martín A, Carballas T, Díaz-Raviña M. 2010. Response of soil microbial communities to fire and firefighting chemicals. *Sci Total Environ.* 408:6172-6178.
- Basanta R, de Varennes A, Díaz-Raviña M. 2017. Microbial community structure and biomass of a mine soil with different organic and inorganic treatments and native plants. *J Soil Sci Plant Nutr.* 17:839-852.

- Brandt KK, Amézquita A, Backhaus T, Boxall A, Coors A, Heberer T, Lawrence JR, Lazorchak J, Schönfield J, Snape JR, Zhu YG, Toop E. 2015. Ecotoxicological assessment of antibiotics: a call for improved consideration of microorganisms. *Environ Int.* 85:189-205.
- Briceño G, Palma G, Durán N. 2007. Influence of organic amendment on the biodegradation and movement of pesticides. *Crit Rev Sci Tec.* 37:233-271.
- Burgos P, Madejón E, Cabrera F. 2002. Changes in soil organic matter, enzymatic activities and heavy metal availability induced by application of organic residues. *Develop Soil Sci.* 28:353-362.
- Caracciolo AB, Topp E, Grenni P. 2015. Pharmaceuticals in the environment: Biodegradation and effects on microbial communities. A review. *J Pharm.* 106:25-36.
- Chen W, Liu W, Pan N, Jiao W, Wang M. 2013. Oxytetracycline on functions and structure of soil microbial community. *J Soil Sci Plant Nutr.* 13:967-975.
- Conde-Cid M, Álvarez-Esmoris C, Paradelo-Núñez R, Nóvoa-Muñoz JC, Arias-Estévez M, Álvarez-Rodríguez E, Fernández-Sanjurjo MJ, Núñez-Delgado A. 2018a. Occurrence of tetracyclines and sulfonamides in manures, agricultural soils and crops from different areas in Galicia (NW Spain). *J Clean Prod.* 197:491-500.
- Conde-Cid M, Fernández-Calviño F, Nóvoa-Muñoz JC, Arias-Estévez M, Díaz-Raviña M, Fernández-Sanjurjo MJ, Núñez-Delgado A, Álvarez-Rodríguez E. 2018b. Biotic and abiotic dissipation of tetracyclines using simulated sunlight and in the dark. *Sci Total Environ.* 635:1520-1529.
- Cyczón M, Mroziak A, Piotowska-Seget Z. 2019. Antibiotic in the soil environment-Degradation and their impact on microbial activity and diversity: a review. *Front Microbiol* 10. <https://doi.org/10.3389/fmicb.2019.00338>.
- De Nobili M, Contin M, Mondini C, Brookes PC. 2001. Soil microbial biomass in triggered into activity by trace amounts of substrate. *Soil Biol Biochem.* 32:1163-1170.
- Díaz-Raviña M, Bååth E, Martín A, Carballas T. 2006. Microbial community structure in forest soils treated with a fire retardant. *Biol Fertil Soils* 42:465-471.
- Díaz-Raviña M, Carballas T, Acea MJ. 1988. Microbial biomass and metabolic activity in four acid soils. *Soil Biol Biochem.* 30:817-823.
- Fageria NK. 2012. Role of organic matter in maintaining sustainability of cropping systems. 2012. *Commun Soil Sci Plant* 43:2063-2013.
- Fernández-Calviño D, Bermúdez-Couso A, Arias-Estévez M, Nóvoa-Muñoz JC, Fernández Sanjurjo MJ, Álvarez-Rodríguez E, Núñez-Delgado A. 2015. Kinetics of tetracycline, oxitetracycline and chlortetracycline adsorption and desorption on two acid soils. *Environ Sci Pollut Res.* 22:425-433.
- Frostegård A, Bååth E. 1996. The use of the phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fertil Soils* 22:59-65.
- Frostegård A, Tunlid A, Bååth E. 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipids fatty acid analysis. *Soil Biol Biochem.* 25:723-730.
- Gregorich EG, Carter MR, Angers DA, Monreal B, Ellert H. 1994. Towards a minimum data set to assess soil organic matter in agricultural soil. *C J Soil Sci.* 74:367-385.
- Hammesfahr U, Heuer H, Manzke B, Smalla K, Thiele-Brunhn S. 2008. Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. *Soil Biol Biochem.* 40:1583-1591.
- Hund-Rinke K, Simon M, Lukow T. 2004. Effects of tetracyclines on the soil microflora: function, diversity and resistance. *J Soil Sediment* 4:11-16.
- IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update 2015 International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. World Soil Resources Reports No. 106. Rome: FAO.
- Lakhdar A, Scalza R, Sxotti R, Rao MA, Jedi N, Gianfreda L, Abdelly C. 2010. The effect of composts and sewage sludge on soil biological activities in salt affected soil. *Soil Nutri Ve.* 10:40-47.
- Lehman J, Kleber M. 2015. The contentious nature of soil organic matter. *Nature* 528:60-65.
- Lichtfouse E, editor. 2009. Sustainable Agriculture Reviews 1. Organic Farming Pest control and remediation of soil pollutants. London: Springer. 418 p.
- Liu X, Horbet SJ, Hashemi AM, Zhing X, Ding G. 2006. Effects of agricultural management on soil organic matter and carbon transformation- a review. *Plant Soil Environ.* 52:531-543.
- Mahía J, Cabaneiro A, Carballas T, Díaz-Raviña M. 2008. Microbial biomass and C mineralization in agricultural soils as affected by atrazine addition *Biol Fertil Soils* 45:99-105.
- Mahía J, Díaz-Raviña M. 2007. Atrazine degradation and residues distribution in two acid soils from temperate humid zone. *J Environ Qual.* 36:826-831.
- Mahía J, Martín A, Bååth E, González-Prieto SJ, Díaz-Raviña, M. 2011. Biochemical properties and community structure of five different incubated soils untreated and treated with atrazine. *Biol Fertil Soils* 47:577-584.
- Meisner A, Bååth E, Rousk J. 2013. Microbial growth responses upon rewetting soil dried for four days or one year. *Soil Biol Biochem.* 66:188-192.

- Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. 2003. Microbial diversity and soil functions. *Eur J Soil Sci.* 54:655-670.
- Petruzzelli G. 1989. Recycling wastes in agriculture: heavy metal bioavailability. *Agr Ecosystems Environ.* 27:493-503.
- Rousk J, Demoling LA, Bååth E. 2009. Contrasting short-term antibiotic effects on respiration and bacterial growth compromises the validity of the selective respiratory inhibition technique to distinguish fungi and bacteria. *Microb Ecol.* 58:75-85.
- Rousk J, Demoling LD, Bahr A, Bååth E. 2008. Examining the fungal and bacterial niche overlap using selective inhibitors in soil. *FEMS Microbiol Ecol.* 63:350-358.
- Santás-Miguel V, Cutillas-Barreiro L, Nóvoa-Muñoz JC, Arias-Estévez M, Díaz-Raviña M, Fernández-Sanjurjo MJ, Álvarez-Rodríguez E, Núñez-Delgado A, Fernández-Calviño D. 2018a. By-products as an amendment of a mine soil: effects on microbial biomass determined using phospholipid fatty acids. *Spanish J Soil Sci.* 8:1-11.
- Santás-Miguel V, Fernández-Sanjurjo MJ, Núñez-Delgado A, Álvarez-Rodríguez E, Carballas T, Díaz-Raviña M, Arias-Estévez M, Fernández-Calviño D. 2018b. Effect of tetracycline on soil bacterial communities. In: Abstracts fo the VIII Congreso Ibérico de las Ciencias del Suelo; 2018 Jun 20-22; Donostia, San Sebastián, Spain; p. 417-420.
- Santás-Miguel V, Fernández-Sanjurjo MJ, Núñez-Delgado A, Álvarez-Rodríguez E, Díaz-Raviña M, Arias-Estévez M, Fernández-Calviño D. 2020a. Use of biomass ash to reduce toxicity affecting soil bacterial community growth due to tetracycline antibiotics. *J Environ Manage.* 269:110838.
- Santás-Miguel V, Fernández-Sanjurjo MJ, Núñez-Delgado A, Álvarez-Rodríguez E, Díaz-Raviña M, Arias-Estévez M, Fernández-Calviño D. 2020b. Interactions between soil properties and tetracycline toxicity affecting to bacterial community growth in agriculture soils. *Appl Soil Ecol.* 147:103437.
- Sarmah AK, Meyer MT, Boxall ABA. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65:725-759.
- Villar MC, Petrikova V, Díaz-Raviña M, Carballas T. 2004. Changes in soil microbial biomass and aggregate stability following burning and soil rehabilitation. *Geoderma* 122:73-82.