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Effects of ciprofloxacin, trimethoprim, and amoxicillin on microbial structure and growth as emerging pollutants reaching crop soils

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ABSTRACT

The presence of emerging pollutants, and specifically antibiotics, in agricultural soils has increased notably in recent decades, causing growing concern as regards potential environmental and health issues. With this in mind, the current study focuses on evaluating the toxicity exerted by three antibiotics (amoxicillin, trimethoprim, and ciprofloxacin) on the growth of soil bacterial communities, when these pollutants are present at different doses, and considered in the short, medium, and long terms (1, 8 and 42 days of incubation). Specifically, the research was carried out in 12 agricultural soils having different physicochemical characteristics and was performed by means of the leucine (^{3}H) incorporation method. In addition, changes in the structure of soil microbial communities at 8 and 42 days were studied in four of these soils, using the phospholipids of fatty acids method for this. The main results indicate that the most toxic antibiotic was amoxicillin, followed by trimethoprim and ciprofloxacin. The results also show that the toxicity of amoxicillin decreases with time, with values of Log IC_{50} ranging from 0.07 ± 0.05 to 3.43 ± 0.08 for day 1, from 0.95 ± 0.07 to 3.97 ± 0.15 for day 8, and from 2.05 ± 0.03 to 3.18 ± 0.04 for day 42, during the incubation period. Regarding trimethoprim, 3 different behaviors were observed: for some soils the growth of soil bacterial communities was not affected, for a second group of soils trimethoprim toxicity showed dose-response effects that remained persistent over time, and, finally, for a third group of soils the toxicity of trimethoprim increased over time, being greater for longer incubation times (42 days). As regards ciprofloxacin, this antibiotic did not show a toxicity effect on the growth of soil bacterial communities for any of the soils or incubation times studied. Furthermore, the principal component analysis performed with the phospholipids of fatty acids results demonstrated that the microbial community structure of these agricultural soils, which persisted after 42 days of incubation, depended mainly on soil characteristics and, to a lesser extent, on the dose and type of antibiotic (amoxicillin, trimethoprim or ciprofloxacin). In addition, it was found that, in this research, the application of the three antibiotics to soils usually favored the presence of fungi and Gram-positive bacteria.

1. Introduction

The high consumption of antibiotics used in the treatment and prevention of infectious diseases in humans and animals favors their subsequent presence in different environmental compartments after being excreted (Gothwal and Shashidhar, 2015). This makes that these compounds can be considered as environmental contaminants, and specifically included within the so called emerging pollutants (Khan et al., 2020; Richardson et al., 2005). The most important reason causing this is that antibiotics are poorly metabolized, and a significant portion of them are excreted as an original compound through feces and urine (Magesh et al., 2020; Sarmah et al., 2006), entering wastewater treatment plants (WWTPs) through the sewerage system (Salgado et al., 2010).

Antibiotics are not easily eliminated in WWTPs (Lindberg et al., 2006; Barber et al., 2009; Sabri et al., 2020) and therefore high amounts

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of these substances are accumulated in sewage sludge (Kolpin et al., 2002; Sorinolu et al., 2021) especially in those without treatments such as anaerobic digestion (Aziz et al., 2022), composting (Ezzariai et al., 2018) or hydrothermal carbonization (Vom Eyser et al., 2015). Taking into account that sewage sludge with high amounts of antibiotics is used as organic amendment in agricultural soils, its repeated applications constitute one of the main entry routes for antibiotics to terrestrial ecosystems (Buta et al., 2021; Du and Liu, 2012; Ternes et al., 2004; Yudong et al., 2010).

Once antibiotics reach the soil, these pollutants can affect non-target organisms such as soil microbial communities and modify their structure and function (Zielezny et al., 2006; Hammesfahr et al., 2008; Caban et al., 2018; Urra et al., 2019). Soil microorganisms play a fundamental role in many ecosystem processes, such as the biogeochemical cycle of nutrients and the recycling of organic matter, making that their correct maintenance is of main importance (Singh and Gupta, 2018; Swift, 1994).

Regarding specific antibiotics, AMX (amoxicillin (a penicillin)), TMP (trimethoprim (a diaminopyrimidine)), and CIP (ciprofloxacin (a fluoroquinolone)), are widely used and appear in high concentrations in both soils and waters (Kemper, 2008; Kinigopoulou et al., 2022). The maximum values for CIP, TMP and AMX concentrations found in water were up to 1168, 820 and 2.7 ng/L, respectively (Azanu et al., 2018), and the maximum values for concentrations in natural soils were up to 2.15 ng/g for TMP and 6.70 ng/g for CIP (Chen et al., 2011), reaching up to 76,620 ng/g for AMX (Borquaye et al., 2019). These concentrations cause soil microorganisms to be highly exposed to the antibiotics mentioned above. However, the effect of these three antibiotics on the growth of soil bacterial communities and on structural changes in soil microbial communities has been poorly studied. The originality of this study lies in the fact that it is not frequent (but clearly interesting) performing research where these aspects are addressed using simultaneously the Leucine incorporation technique to study the effect on bacterial growth, and the Phospholipid Fatty Acids (PLFA) technique to study the effect on the structure of soil microbial communities. This is one of the few works where both techniques are used together to evaluate the temporary toxicity exerted by antibiotics for human use on soil microbial communities.

In view of the above, the main objectives of this study were: i) to determine the eventual toxicity of AMX, TMP, and CIP on the growth of bacterial communities in 12 agricultural soils; ii) to shed light on the effect of these antibiotics on the structure of soil microbial communities using the PLFA technique. The results of this research would increase the knowledge on the potential harmful effects that these antibiotics may have on soil microbial communities, which could be highly relevant as regards environmental and health aspects.

2. Materials and methods

2.1. Chemicals

The antibiotics used were amoxicillin (AMX), CAS number: 61,336-70-7, 95% purity; trimethoprim (TMP), CAS: 738-70-5, 95% purity; and ciprofloxacin (CIP), CAS number: 85,721-33-1, 95% purity, which were supplied by Sigma-Aldrich (Steinheim, Germany), as well as talc (CAS number 14807-96-6).

2.2. Soil samples

Twelve different soils sampled in Galicia (NW Spain), were used in the current study, which included marked differences as regards various properties, such as organic carbon content and pH. The selected soils had not been previously treated with antibiotics. At the time of sampling, in each sampling site 10–20 sub-samples were taken in the soil surface horizon (0–20 cm) using an Edelman probe, and were subsequently mixed to constitute a composite sample. The soils used in the current study were previously analyzed by Rodríguez-González et al. (2021). The general characteristics for these 12 soils samples are listed in Table S1 (Supplementary Material). Briefly, these soils present different textures, with sand content ranging from 34 ± 2% to 81 ± 7%, silt content from 10 ± 0% to 38 ± 3%, and clay content from 9 ± 0% to 28 ± 2%. The pH values measured in water (pH_W) and in 1 M KCl (pH_{KCl}) ranged from 4.1 ± 0.1 to 6.1 ± 0.2, and from 3.7 ± 0.2 to 5.3 ± 0.2, respectively. The organic carbon and nitrogen contents vary between 0.6 ± 0.2% and 6.80 ± 0.3%, and between 0.1 ± 0.0 and 0.6 ± 0.3%, respectively, whereas OC (organic carbon) ranged between 121 ± 10 and 634 ± 47 mg kg^{-1}. The values corresponding to the effective cation exchange capacity (eCEC) ranged from 3.2 ± 0.1 to 37.2 ± 1.3 $cmol_c$ kg^{-1}.

2.3. Experimental design

The 12 soil samples were moistened up to 70% of their water holding capacity and incubated in the dark at 22 °C for 15 days, which is time enough to recover the microbial activity and achieve the stabilization of soil bacterial community growth (Meisner et al., 2013). After the incubation period, the 12 soils were spiked with AMX TMP, and CIP (each antibiotic separately, and by triplicate in all cases). The soils were spiked in dry, using talc as a carrier for equalizing the amount of dry material added to each microcosm and facilitating the mixture with the soil (Rousk et al., 2008). Different doses of the antibiotics (talc + antibiotic) were used to achieve the following eight concentrations: 0, 0.49, 1.95, 7.81, 31.25, 125, 500, and 2000 mg kg^{-1} (values referred to mass of soil). These concentrations will allow obtaining appropriate short-term dose-response curves, and the correct estimation of toxicity indices (Fox and Landis, 2016). In fact, these same concentrations were previous used in researches performed to study the repercussions on soil bacterial communities caused by the application of a variety of tetracyclines (Rodríguez-González et al., 2021; Santás-Miguel et al., 2020a, 2020b, 2020c, 2020d, 2021, 2022).

The total number of mixtures of antibiotic and soil resulted in 864 microcosms (12 soils x 8 concentrations x 3 replications x 3 antibiotics). Once the soils were spiked with AMX TMP, and CIP, the soil microcosms were incubated at 22 °C in the dark and the bacterial community growth was determined after 1, 8, and 42 days. These incubation periods have been successfully tested in previous works (Santás-Miguel et al., 2020a, 2020b) and they have been selected to estimate toxicities in the short term (immediate toxicity, day 1), medium term (day 8) and long term (day 42). The high number of samples to be processed and the highly time-consuming analyses did not allow performing PLFA estimates on all 864 soil microcosms. Thus, PLFA analyses were carried out on four selected soils with similar pH values and different organic matter contents (soils 3, 5, 10, and 12) (see Table S1, Supplementary Material), both after 8 and 42 days. For each soil, the three incubation replicates of the same antibiotic concentration were mixed and carefully homogenized to get a representative composite soil sample. A total of 192 composite soil samples (4 soils x 3 antibiotics x 8 concentrations x 2 incubation times) were analyzed as regards PLFA characteristics.

2.4. Estimation of bacterial community growth

The bacterial community growth was estimated using the leucine incorporation technique (Bååth, 1994; Bååth et al., 2001).

Briefly, 1 g of soil (fresh weight) was mixed with 10 mL of distilled water using a multivortex shaker at maximum intensity for 3 min, followed by low-speed centrifugation at 1000×*g* for 10 min to create a bacterial suspension in the supernatant. An aliquot (1 mL) of this suspension was transferred to 2 mL microcentrifugation tubes. Then, volumes of 2 μL [^{3}H] Leu (3.7 MBq ml^{-1} and 0.574 TBq $mmol^{-1}$; PerkinElmer, Waltham, MA, USA) were added with non-labeled Leu to each tube, resulting in 300 nM Leu in the bacterial suspensions. After incubation for 2 h at 22 °C, the growth was stopped with 75 μL of 100%

trichloroacetic acid. Finally, the bacteria in the microcentrifugation tubes were washed as described by Bååth et al. (2001) and radioactivity determined using a scintillation liquid counting device (Tri-Carb 2810 TR, PerkinElmer, USA).

The resulting bacterial community growth data were normalized dividing each value by mean control values (without antibiotic) for each soil sample. The logarithm of the added AMX TMP, and CIP concentrations that inhibited 50% of bacterial community growth (Log IC_{50}) was estimated for each soil using the following logistic model (1):

$$Y = c/[1+e^{b(a-x)}]$$

Where Y is the Leu incorporation (bacterial community growth) determined for each concentration of AMX TMP, and CIP added; x is the logarithmic value of the concentration of antibiotic added; a is the value of Log IC_{50}; b is a parameter related to the slope of the inhibition curve; and c is the bacterial growth rate observed in the control sample (without antibiotic). High Log IC_{50} values indicate low antibiotic toxicity on bacterial growth, whereas low Log IC_{50} values indicate high antibiotic toxicity.

The differences between the Log IC_{50} values obtained at days 1, 8 and 42 of incubation were checked using a paired t-test. Statistical analysis was performed with SPSS Statistics 21.0 software (IBM, Armonk, NY, USA). KaleidaGraph software (Synergy Software, Reading, PA, USA) was used to drawn figures corresponding to dose-response curves.

2.5. Phospholipid fatty acids (PLFA) analysis

Phospholipids fatty acids (PLFA) were estimated using the procedure described by Frostegård et al. (1993). All glass material used in the procedure were heated at 400 °C overnight to remove lipid contaminants. Briefly, lipids present in the soil (1.5 g weight) were extracted 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 (Bond Elut-C solid phase extraction cartridge) and solvents of increasing polarity (chloroform, acetone and methanol). The phospholipids were subjected to a mild alkaline methanolysis and the fatty acid methyl esters were separated, identified by the relative retention times and quantified in a gas chromatograph with flame ionization detector (TraceGCultra of Finnigan-Thermo, USA) using methyl nonadecanoate (19:0) as internal standard. A 50 m HP5 capillary column was used and the carrier gas was helium. Fatty acids are designated in terms of the total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule. Cis and trans configurations are indicated by "c" and "t", respectively. The prefixes "a" and "i" indicate anteiso- or iso-branching, "br" indicates an unknown methyl branching position, "10Me" indicates a methyl group on the 10th carbon atom from the carboxyl end of the molecule, and "cy" refers to cyclopropane fatty acids.

The total microbial biomass (TotPLFA) 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 (BactPLFA), and the quantity of the 18:2ω6 PLFA was used as an indicator of the fungal biomass (FungPLFA). The sum of 16Me16:0, 17Me17:0 and 16Me18:0 was used as an index of actinobacteria (ActPLFA). The i14:0, i15:0, i16:0 and 10Me18:0 PLFAs are predominantly found in Gram-positive (G^+) bacteria, and the cy17:0, cy19:0, 16:1ω7c and 18:1ω7 PLFAs characterize Gram-negative (G^-) bacteria (Santás-Miguel et al., 2020c).

The use of PLFA in combination with Principal Component Analyses (PCA) has a great potential in soil microbial studies in order to detect environmental effects (Frostegård et al., 2011). Thus, to determine the main differences in the PLFA patterns of the microbial communities of the soils under study, the concentrations of all the individual PLFAs (expressed in mole percent and logarithmically transformed), were used for performing principal component analyses (PCA). Statistical analyses were carried out by means of the SPSS 21.0 statistical package.

3. Results and discussion

3.1. Toxicity of AMX TMP, and CIP on the growth of soil bacterial communities

The addition of AMX to soils caused toxicity on soil bacterial communities, resulting in sigmoid dose-response curves for most soils and incubation times studied. Specifically, low doses of AMX did not inhibit soil bacterial growth, whereas the degree of inhibition increased as a function of rising doses of AMX added. As a general trend, the magnitude of toxicity exerted by AMX on the growth of bacterial communities decreased with incubation time, causing that the dose-response curves shift to the right (Fig. 1, Fig. S1). In addition, in the long term (day 42) there was a total recovery and even an exponential increase in bacterial growth in many of the soils studied. Although this is the first work to study the toxicity of AMX on the growth of soil bacterial communities, a similar behavior was previously observed for clarithromycin (Rodríguez-González et al., 2021). The reduction in AMX toxicity over time may be due to the low persistence of AMX in soils, where this antibiotic is generally readily degraded. Braschi et al. (2013) found that AMX has a half-life of 0.43–0.57 days in non-autoclave-treated soils, and 0.59–1.74 days in autoclaved soils. However, its persistence in the soil depends on the initial concentration of the antibiotic (Bansal, 2012), as well as on its degree of sorption to soil colloids. Although AMX sorption onto soils has not been extensively studied, in general it has been to be high (Kim et al., 2012; Cela-Dablanca et al., 2022). Subsequently, the recovery and/or stimulation of soil bacterial communities could be due to the fact that, after the incubation time, the antimicrobial power of AMX decreases and the molecules of the antibiotc can be used as a source of carbon and nitrogen by the bacterial communities of the soil, producing its stimulation (Abd-El-Malek et al., 1961; Schofield, 2018). In addition, there are certain bacterial species in soils and in activated sludge that are able to biodegrade AMX (Längin et al., 2009; Qin et al., 2021; Yang et al., 2020).

The dose-response curves obtained for AMX were well described by the logistic model, with R^2 values ranging from 0.947 to 0.998 (mean = 0.980) for day 1, R^2 between 0.865 and 0.998 (mean = 0.974) for day 8, and R^2 between 0.995 and 0.999 (mean = 0.997) for day 42, in the few cases that could be estimated by total recovery of bacterial growth (n = 2) (Table S2, Supplementary Material). The Log IC_{50} values obtained for day 1 ranged between 0.07 ± 0.05 and 3.43 ± 0.08 (mean = 1.90), for day 8 ranged between 0.95 ± 0.07 and 3.97 ± 0.15 (mean = 2.70), and, for day 42, the soils where it was possible to estimate (n = 2) ranged between 2.05 ± 0.03 and 3.18 ± 0.04 (mean = 2.62) (Table S2, Supplementary Material).

In general, the addition of TMP to soils caused inhibition of bacterial growth, being greater as a function of increased TMP concentrations added, obtaining dose-response curves in most cases. However, the effects caused by TMP are not homogeneous, showing different behaviors depending on the various soils and incubation periods (Fig. 2, Fig. S2). In some soils it was a persistent toxicity (observed after 42 days of incubation) on soil bacterial communities due to TMP, with magnitude maintained over time (soils 6 and 7 are examples in this regard). This type of behavior was previously observed by Santás-Miguel et al. (2020a) for the antibiotic oxytetracycline. Toxicity exerted by TMP on soil bacterial communities may be due to the high persistence of this antibiotic in soils, with half-lives estimated to be between 36 and 139 days (Kodešová et al., 2016; Cycoń et al., 2019). In addition, TMP shows high sorption onto soils (Zhang et al., 2014; Kodešová et al., 2015; Kočárek et al., 2016). This high sorption to soil colloids could prevent TMP from being biodegraded by soil bacterial communities, as this antibiotic is susceptible to dissipation in soils and sludge through this

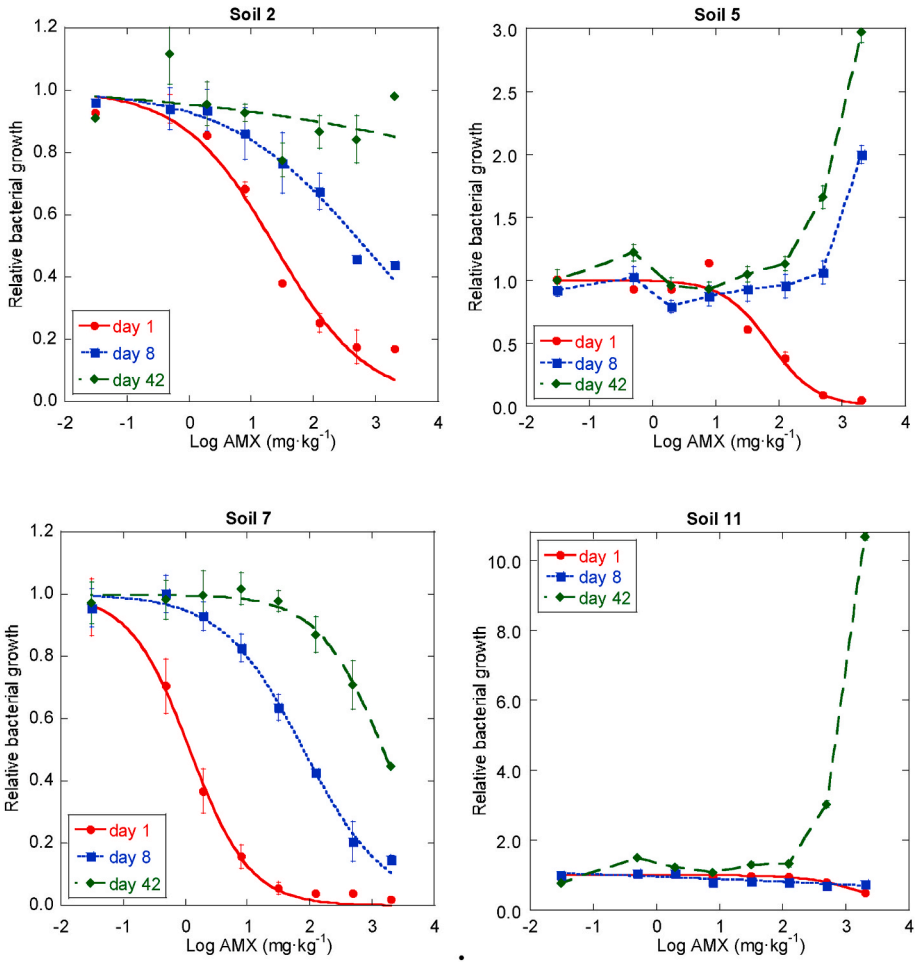

Fig. 1. Relative bacterial community growth as a function of amoxicillin (AMX) concentration in the soil, after 1, 8 and 42 days of incubation, as observed for 4 soil samples (shown as example).

mechanism (Gulkowska et al., 2008; Dalkmann et al., 2014a; Reis et al., 2020).

In addition, TMP does not show dose-response curves for some soils, indicating that in these cases bacterial growth is independent of the dose of antibiotic added (Fig. 2, Fig. S2). This non-inhibition of bacterial growth is observed in the short, medium and long term (1, 8 and 42 days) (soils 10 and 12 are examples of this). The non-toxic effect of TMP on the growth of soil bacterial communities may be due, as discussed above, to the increased sorption of this antibiotic in some soils (Zhang et al., 2014; Kodešová et al., 2015; Kočárek et al., 2016), causing that TMP is less bioavailable for the bacterial communities of these soils and, therefore, its toxicity is diminished.

Finally, it can be observed that TMP toxicity increases with incubation time, that is, the toxicity exerted by TMP on soil bacterial communities at day 1 (direct toxicity) is lower than that observed at day 42 of incubation (soils 3 and 4 are examples of this). The increase in toxicity over time is a phenomenon scarcely observed for antibiotics and other environmental pollutants; however, there are studies focused on antibiotics, such as sulfadiazine (Santás-Miguel et al., 2020d), as well as on pesticides, such as terbutryn (Fernández-Calviño et al., 2021), where it was shown that their toxicities on soil bacterial communities increased with incubation time. This may be because antibiotics that show high persistence in soils, such as TMP, may be more susceptible to mineralization over time (Kim et al., 2004; Bouju et al., 2012), becoming more bioavailable to soil microorganisms, and this higher bioavailability would make them more toxic. In addition, increased toxicity over time may be due to the appearance of secondary metabolites of these antibiotics, which may be more toxic than the original compound (Majewsky et al., 2014; Perry et al., 2021).

The dose-response curves obtained show a good fit to the logistic model, obtaining values of $R^2 = 0.750$ and $R^2 = 0.996$ (average = 0.924) for day 1, $R^2 = 0.878$ and $R^2 = 0.997$ (average = 0.972) for day 8, and $R^2 = 0.868$ and $R^2 = 0.995$ (average = 0.956) for day 42. From the dose-response curves obtained above, the $LogIC_{50}$ values were estimated for each soil and incubation time tested (Table S3, Supplementary Material). Log IC_{50} values obtained for day 1 ranged between 2.92 ± 0.09 and 4.00 ± 0.4 (mean = 3.45), while for day 8 ranged between 2.75 ± 0.11 and 4.10 ± 0.35 (mean = 3.46), and for day 42 ranged between 1.85 ± 0.25 and 4.20 ± 0.70 (mean = 3.26).

When examining the data as a whole, TMP toxicity on bacterial communities only shows variation between day 1 and day 8 of incubation, although an increase in the toxicity of TMP can be observed at 42 days of incubation.

Regarding the antibiotic CIP, in general it did not cause inhibition of soil bacteria communities at any of the concentrations tested (0–2000 mg kg^{-1}) or incubation times (1, 8, and 42 days) (Fig. 3, Fig. S3). Due to the lack of toxicity shown by this antibiotic and, therefore, to the absence of dose-response curves, no Log IC_{50} values could be calculated for any of the soils or incubation times studied. The results of this research are similar to those obtained by Lin et al. (2016), which studied the effect on the bacterial community of adding 100 mg kg^{-1} of CIP, for incubations lasting 7 and 20 days, and found that the addition of CIP to the soil did not cause changes in the number of bacteria with respect to the 7-day incubation control, while at 20 days of incubation there was

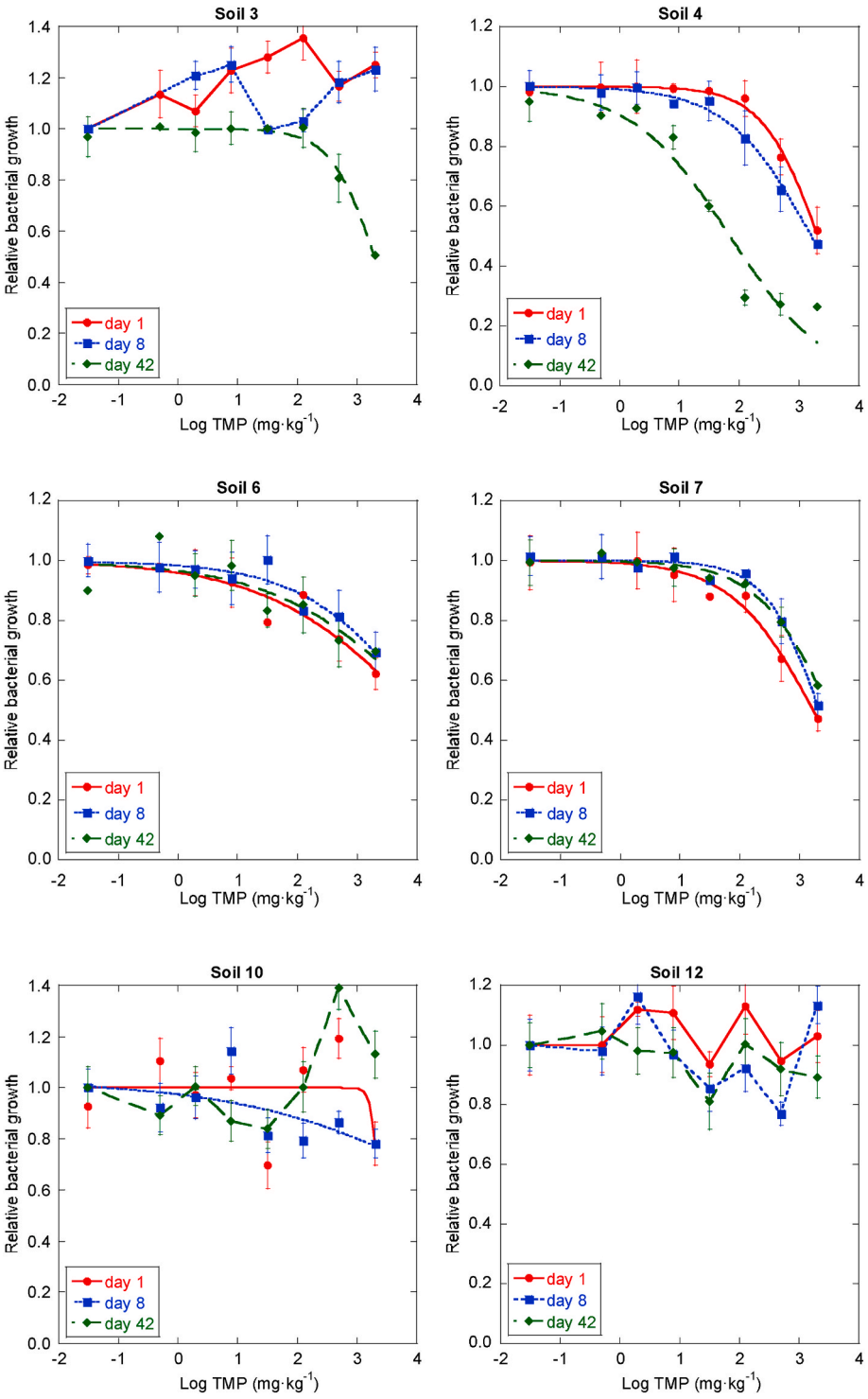

Fig. 2. Relative bacterial community growth as a function of trimethoprim (TMP) concentration in the soil, after 1, 8 and 42 days of incubation, as observed for 6 soil samples (shown as example).

an increase with respect to the control. This tendency of non-inhibition has also been observed for other antibiotics, as in the work by Santás-Miguel et al. (2020d), who studied the long-term (100 days) effect of different concentrations of sulfadiazine (0.002–2000 mg kg^{-1}) on 2 agricultural soils and did not observe any negative effect of SDZ on the bacterial growth for incubation times ≤64 days in one of the soils studied. In addition, Córdova-Kreylos and Scow (2007) noted that the addition of CIP had a positive effect on the number of bacteria in marsh sediments. However, there is also a study by Girardi et al. (2011) showing that the addition of CIP to soils could significantly inhibit bacteria from agricultural soils. These different results can be attributed to several mechanisms. First, CIP shows high sorption in soils (Picó and Andreu, 2007; Uslu et al., 2008; Leal et al., 2012). The high adsorption of CIP on soil colloids causes a low bioavailability of this antibiotic in soils and, therefore, its effective toxicity on soil bacterial communities is reduced (Girardi et al., 2011). This may be due to the association of the bioactive functional groups of antibiotics to soil exchange sites, this binding being particularly efficient to decrease CIP toxicity on soil

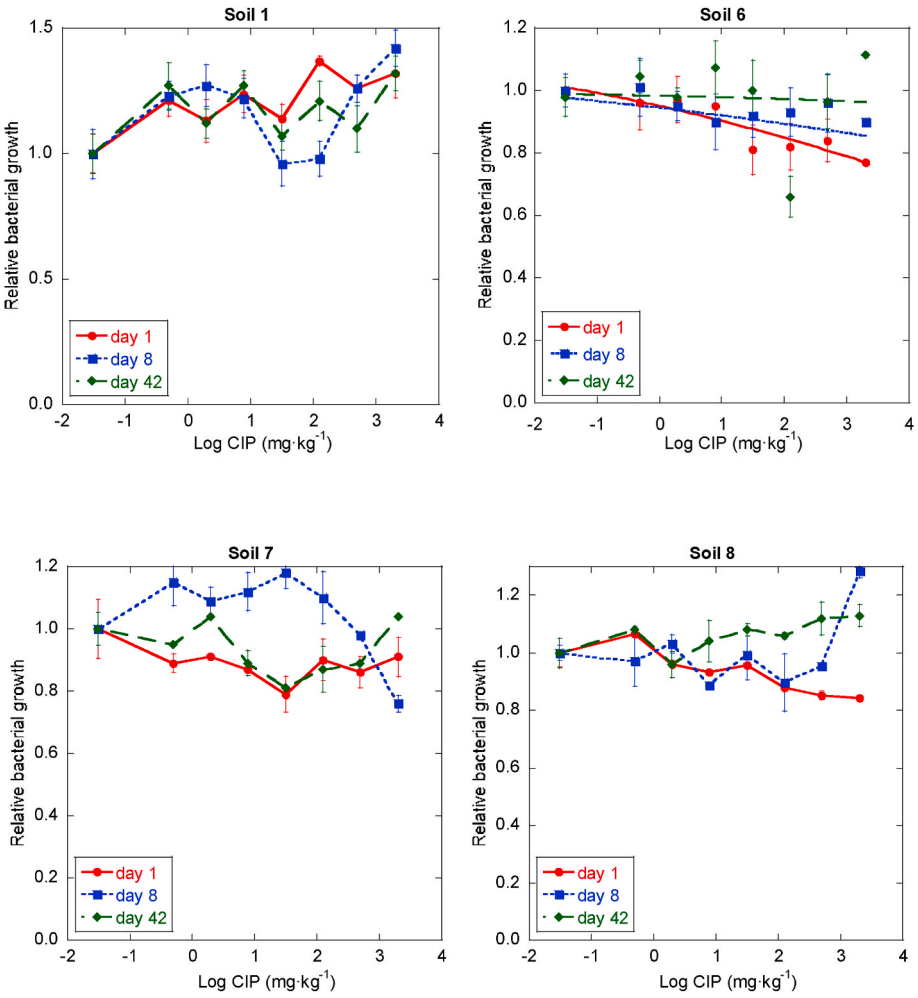

Fig. 3. Relative bacterial community growth as a function of ciprofloxacin (CIP) concentration in the soil, after 1, 8 and 42 days of incubation, as observed for 4 soil samples (shown as example).

microorganisms (Thiele, 2000). Second, the high adsorption of CIP in soils causes a very high persistence in soils, reaching half-lives of up to 3466 days (Dalkmann et al., 2014b; Cycoń et al., 2019). Therefore, studying toxicity after 42 days of incubation may result in a short time scale in order to observe the effects of CIP on soil bacterial communities. Third, the high adsorption of CIP in soils and its subsequent inactivation could allow its biodegradation over time. Although the results obtained in this regard are contradictory, several studies show that CIP is a biodegradable antibiotic (Girardi et al., 2011; Zhang et al., 2012; Liu et al., 2013; Liao et al., 2016). The biodegradation of antibiotics can be carried out by different microorganisms such as fungi (Wetzstein et al., 1999; Zhang et al., 2012) and bacteria (Amorim et al., 2014).

3.2. Differences among the toxicities of AMX, TMP and CIP

The dose-response curves for AMX, TMP and CIP obtained at day 1 of incubation (direct toxicity) in 4 representative soils are shown in Fig. 4. In general, there is a displacement to the right of the curves corresponding to TMP and CIP with respect to AMX, as well as displacement of the curve corresponding to CIP with respect to TMP. This indicates that AMX is the antibiotic presenting the highest toxicity on the growth of the soil bacterial communities, followed by TMP and CIP (the latter hardly exhibiting any toxicity). It is evident that there are differences regarding the toxicity exerted by CIP compared with AMX and TMP, since CIP does not inhibit the growth of bacterial communities. However, to check if there are further significant differences between AMX and TMP, a paired T test was performed with the values of Log IC_{50} at day 1 of incubation, and it was obtained that indeed there are differences that are statistically significant between the toxicity of AMX and TMP ($t = -3.712$; $P < 0.01$). Therefore, the sequence of toxicities obtained for the 3 antibiotics studied is: AMX > TMP > CIP.

3.3. Influence of AMX, TMP and CIP on total and specific microbial biomass

The values of total biomass (TotalPLFA) and biomass of specific microbial groups (FungPLFA, BactPLFA, ActPLFA, G^-BactPLFA, and G^+BactPLFA) in the four soils studied by means of the PLFA technique (soils 3, 5, 10, and 12), which were added with different doses of AMX, CIP and TMP, are shown in Figs. 5 and 6 and Tables S5–S7 (Supplementary Material). The results showed that total and specific microbial biomass values varied depending on soil characteristics, with scores being about 2 times lower in soil 3 than in the rest of soils studied. Overall, the effects caused by the antibiotics were similar for the four soils under study. Specifically, the addition of the antibiotics in general increased TotPLFA, FungPLFA, BactPLFA, ActPLFA, G^-BactPLFA, and G^+BactPLFA, after 8 and 42 days of incubation. However, the results showed a more consistent and marked trend for the soil 10 (Figs. 5 and 6). For each soil, a different effect was observed depending on the microbial group, dose and antibiotic considered. In the short term (8 days), the total and specific microbial biomass increased notably due to the addition of AMX, CIP and TMP. However, the magnitude of the increase

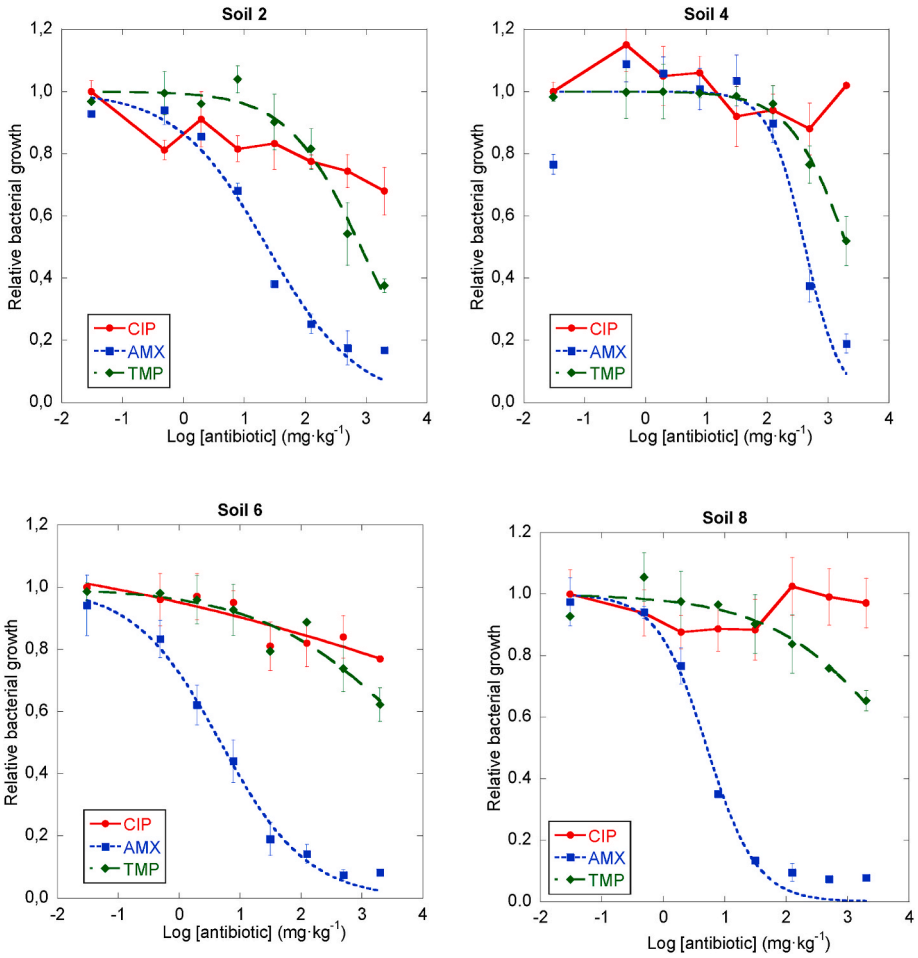

Fig. 4. Comparison of amoxicillin (AMX), trimethoprim (TMP) and ciprofloxacin (CIP) toxicity for four soils (shown as example) after 1 day of incubation period.

varied depending on the microbial group considered, as clearly indicated by the values of the Fung/BactPLFA and G^{-}Bact/G^{+}BactPLFA ratios. The data demonstrated that, in general, the fungi and Gram-positive bacteria are favored by the addition of the three antibiotics. In contrast, in some cases the G^{-}Bact/G^{+}BactPLFA ratio decreased (Table S7, Supplementary Material). A dose effect of the antibiotic was only observed for AMX. In the long term (42 days) the antibiotics effects were diminished, particularly in the case of TMP.

The results of the current study are in line with those of Córdova-Kreylos and Scow (2007), showing that the Total-PLFA was substantially increased after adding CIP, while the opposite behavior (Total-PLFA decrease) was found by Cui et al. (2014). However, other studies have showed that Total-PLFA levels were unaffected by the presence of antibiotics (Thiele-Bruhn and Beck, 2005; Kotzerke et al., 2011; Santás-Miguel et al., 2020c). These different effects on non-target soil microbial communities are due to the balance of different processes affecting positively or negatively the antibiotic dynamics in soil (adsorption-desorption and degradation processes, extractable fraction, half-life, bactericidal or bacteriostatic mechanism of action, toxicity of the antibiotics and derived metabolites, growth of resistant microorganisms that use C and nutrients derived from dead bacterial and/or the degradation of the antibiotics) (Caracciolo et al., 2015; Cycoń et al., 2019).

The data showed that the dynamics of AMX in soils differed notably from that observed for CIP and TMP. In this regard, initially the higher toxicity of AMX with respect to CIP and TMP provoked a significant reduction in bacterial growth, which was dose dependent, as previously indicated (Fig. 1). Later on, the antibiotic resistant microorganisms grew using dead bacteria as substrate, leading to a dose dependent increase in the total and specific microbial biomass (Figs. 1, 5 and 6). In contrast, the low toxicity of CIP and TMP did not decrease substantially the bacterial growth and therefore the bacterial biomass (Figs. 2, 3, 5 and 6). However, the presence of these antibiotics favored notably both the FungPLFA and TotalPLFA contents. For CIP and TMP, the soils treated with these antibiotics exhibited similar microbial biomass estimates independently of the application dose (Figs. 5 and 6).

3.4. Microbial community structure in soils added with amoxicillin, trimethoprim and ciprofloxacin

The results of the principal component analyses (samples and variables) performed with the whole PLFA data set obtained for different concentrations of AMX, TMP and CIP added to four soils after 8 and 42 days of incubation are shown in Fig. 7. After 8 days of incubation, the plane defined by factor 1 and 2, explaining the 44% of the variance, separated the samples of different soils. The samples of soils 3 and 10, with a low content of organic matter, are situated in the positive part of factor 1, while samples of soils 5 and 12 were located in the negative part one. Similar results were observed after 42 days of incubation, although in this case the soils were differentiated along factor 2, which explained 20% of total variance. Soil 12 was characterized by having higher concentrations of PLFAs br18:0, i15:0, i17:0 and 16:1ω7t, whereas in soil 5 the predominance of PLFAs 18:1ω9, 18:1ω7, 16:1ω9, 16:ω7c and 16:0 was observed, and soil 10 exhibited higher concentrations of PLFAs 10Me17:0, 10Me18:0, i16:0 and a17:0. This is consistent with the results of other authors showing that microbial community structure varied greatly with soil type and hence with soil properties (Díaz-Raviña et al., 2006; Mahía et al., 2011; Santás-Miguel et al., 2021). Thus, the general

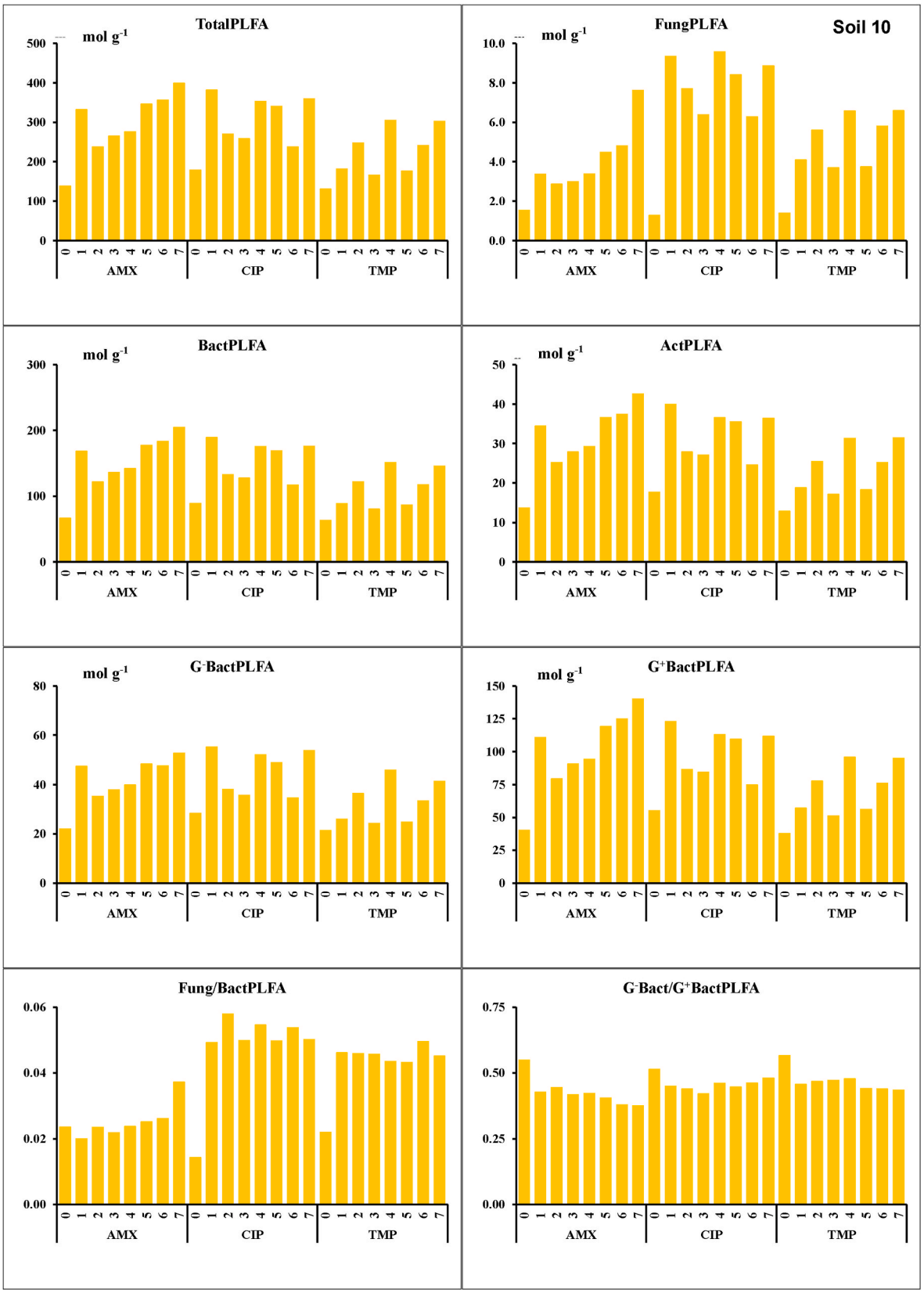

Fig. 5. Total and specific microbial biomass values (fungal, bacterial, actinobacteria, Gram-negative bacteria, and Gram-positive bacteria) in soil S10 after being added with different doses of amoxicillin (AMX), ciprofloxacin (CIP) and trimethoprim (TMP), after 8 days of incubation. Doses: 0 (C), 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), 125 (5), 500 (6) and 2000 (7) mg kg^{-1} of soil.

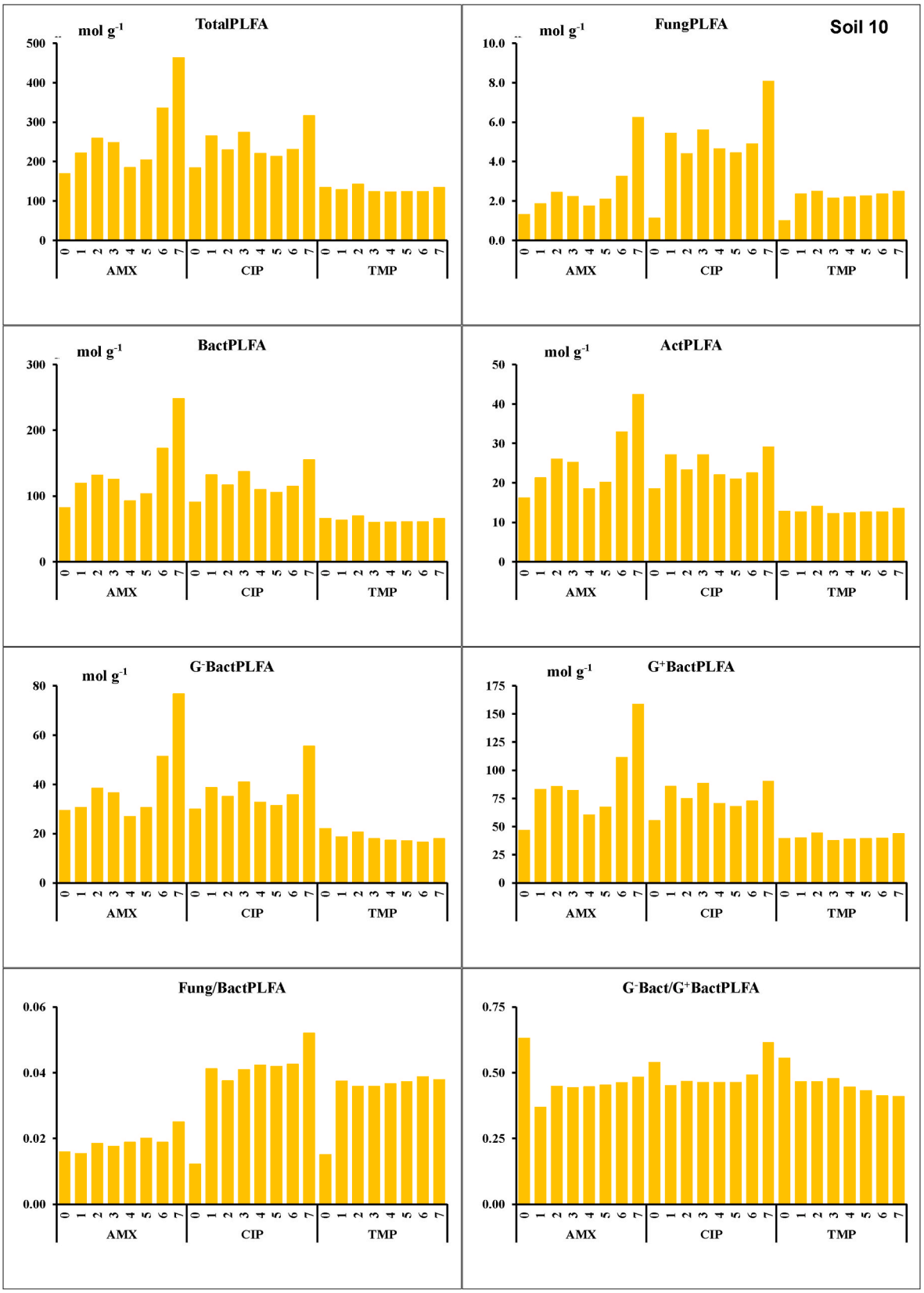

Fig. 6. Total and specific microbial biomass values (fungal, bacterial, actinobacteria, Gram-negative bacteria, and Gram-positive bacteria) in soil S10 after being added with different doses of amoxicillin (AMX), ciprofloxacin (CIP) and trimethoprim (TMP), after 42 days of incubation. Doses: 0 (C), 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), 125 (5), 500 (6) and 2000 (7) mg kg^{-1} of soil.

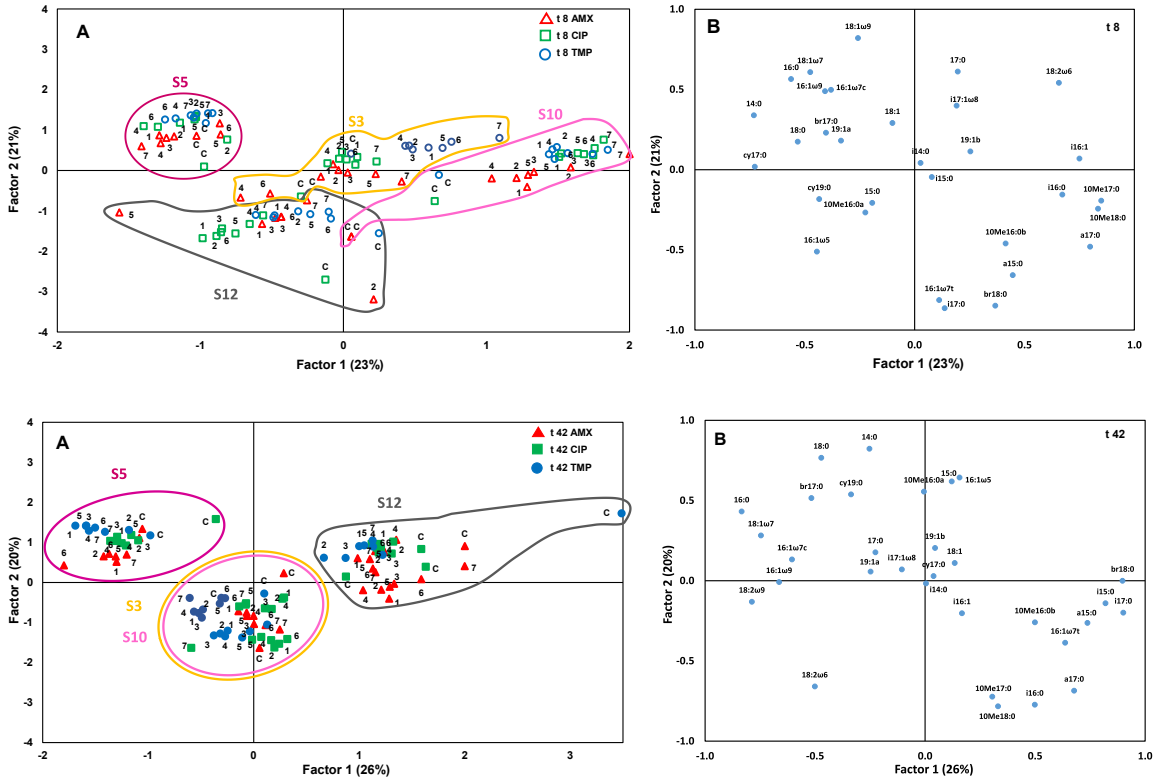

Fig. 7. Principal component analysis (A, variables; B, samples) performed with the PLFA dataset for the 4 soils studied (S3, S5, S10 and S12) added with different doses of amoxicillin (AMX), ciprofloxacin (CIP) and trimethoprim (TMP), after 8 (t8) and 42 (t42) days of incubation. Doses: 0 (C), 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), 125 (5), 500 (6) and 2000 (7) mg kg^{-1} of soil.

trend was that soil samples of the same soil were grouped together, independently of the antibiotic (AMX, CIP, or TMP) and dose added, and separated from the rest of samples of other soils.

In order to discard the influence of soil properties on microbial community structure, the whole data set of samples added with the three antibiotics for two incubation times was analyzed separately for each soil (Figs. 8 and 9). The effects of the factors considered were more clearly observed in soils with low organic matter contents. Thus, for soil 3 the influence of incubation time was observed along PC1, which explained a 24% of variance, while the influence of dose was exhibited along PC2, which explained 18% of the variance. It should be also noticed that PCA of PLFAs discriminates as well among type and dose of antibiotics (AMX, CIP, TMP), the dose effect being more evident for AMX. The same trend was observed for the rest of soils, but the magnitude of the changes increased with the soil organic matter content, following the order soil 3 > soil 10 > soil 5 > soil 12. Thus, for soil 12 the PCA separated the control samples from those added to the different doses of antibiotics at both incubation times. The interpretation of the results of the variable's distribution was complex and did not allow to identify the PLFAs affected directly by the addition of antibiotic, probably due to the interactions among different factors considered in the analysis. In this sense, the results of the impact of the addition of AMX, CIP and TMP on the Fung/Bact PLFA and G-/G+ ratios were more adequate (Figs. 5 and 6).

The results obtained in the present study are consistent with previous investigations performed with the same soils concerning the effects of antibiotics of veterinary use on microbial communities after 42 days of incubation (Santás-Miguel et al., 2021). Thus, PLFA pattern changes were induced by chlorotetracycline, tetracycline and oxytetracycline, and their magnitude varied depending on the soil studied and, in less extent, on the type and dose of antibiotic. Likewise, our results were coincident with those obtained previously in other studies showing a larger influence of incubation or sampling time than that observed for the impact of abiotic stress agents such as high temperatures (Barreiro et al., 2015; Fontúrbel et al., 2012) and antibiotics of both veterinary or human use (Böhme et al., 2005; Hund-Rinke et al., 2004). In contrast, Hammesfahr et al. (2008) detected that for pig liquid manure and sulfadiazine amendments the influence of treatments on PLFA pattern was more important than incubation time.

3.5. *Comparison of the results of the leucine incorporation technique and phospholipid fatty acid analyses*

The determination of bacterial community growth by means of the leucine incorporation technique, used to evaluate the risk of potential antibiotics toxicity on soil microbial communities, has been gaining considerable support in recent decades (Fernández-Calviño and Bååth, 2013; Rousk et al., 2008; Santás-Miguel et al., 2020a, 2020b, 2020d). Another way of assessing the impact of antibiotics on soil microorganisms is to analyze the microbial community structure by means of the phospholipid fatty acids (PLFA pattern) (Santás-Miguel et al., 2021). Most investigations concerning this topic were performed using these two approaches separately in different soils, which makes not possible to compare properly the data obtained. Both kinds of measurements have shown that the response of microbial communities to the stress provoked by the presence of antibiotics is variable and depended on soil type, dose and type of antibiotic, and the time passed after the antibiotic application. However, the results as regards the relative importance of these factors determining the antibiotic effects was different depending on the methodology used to detect this impact.

In the current study, based on the bacterial growth estimates (Figs. 1–3), AMX and TMP had a toxic effect, but showing differences for

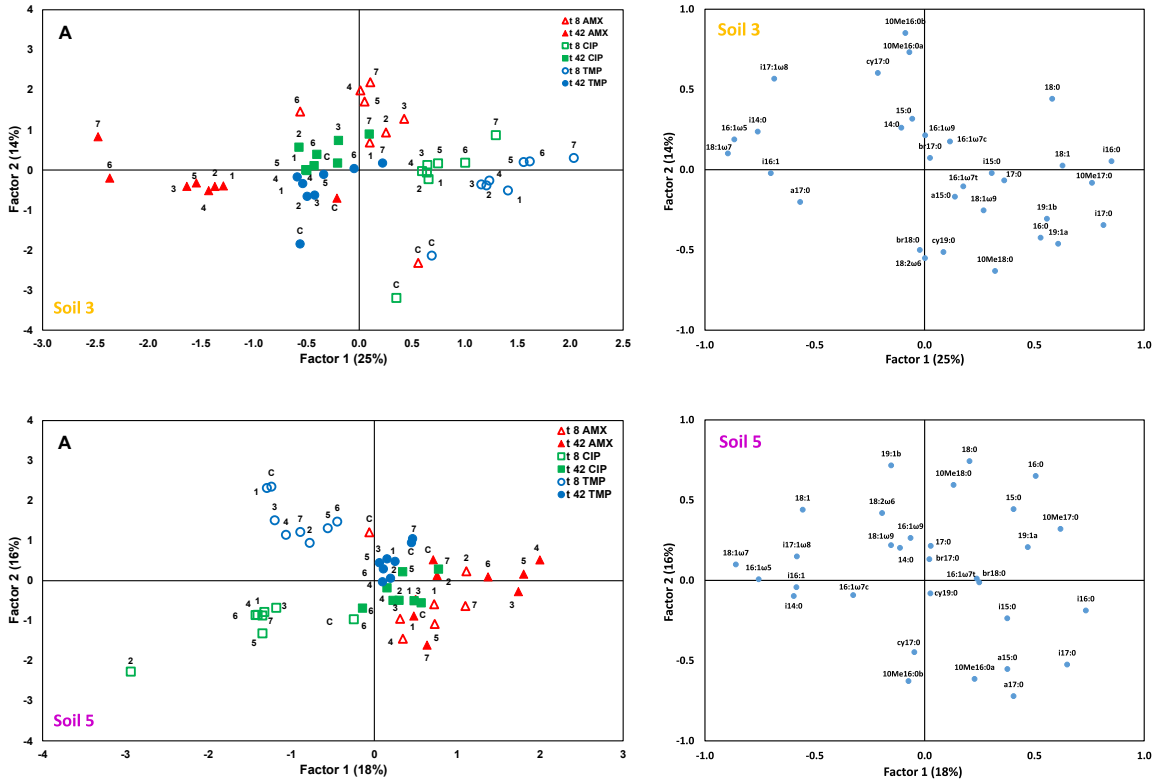

Fig. 8. Principal component analysis (A, variables; B, samples) performed with the PLFA dataset for the soils S3 and S5 added with different doses of amoxicillin (AMX), ciprofloxacin (CIP) and trimethoprim (TMP), after 8 (t8) and 42 (t42) days of incubation. Doses: 0 (C), 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), 125 (5), 500 (6) and 2000 (7) mg kg^{-1} of soil.

each of them. Specifically, for AMX, sigmoidal dose-response curves were observed for most soils and the toxicity was reduced with time; however, for TMP a different trend was observed depending on the soil, and its effect could persist or even increase 42 days after the application of this antibiotic. In contrast with these two biocides, CIP addition did not affect soil bacterial growth even 42 days after its application. The relative importance of the factor considered on the PLFA profiles followed the order: soil > incubation time > antibiotic (Figs. 7–9). The PLFA pattern showed shifts in microbial structure following the addition of AMX, TMP and CIP, both 8 and 42 days after their application. These effects, which were dose dependent, especially for AMX, were more accentuated in those soils with lower organic matter contents. The data suggest that the factors regulating bacterial activity were different that those regulating microbial composition or structure. This is coincident with reports from other authors showing that parameters based on mass, activity and diversity of soil microbial communities did not follow the same trend (Díaz-Raviña and Bååth, 1996; Bååth et al., 1998; Barreiro et al., 2015; Mahía et al., 2011; Santás-Miguel et al., 2021). In addition, another explanation is that these two different methodologies are providing information related to different soil microorganisms. Thus, the leucine incorporation technique reflects the activity of bacteria, while PLFA results integrate measurements of all living microorganisms present in soils, reflecting both species composition and relative species abundance (Bååth et al., 1998). In summary, the data obtained in the current study are explained by the fact that these two techniques analyzed different aspects and parts of soil microbial communities. However, the results and information extracted from these approaches are complementary, showing that the response of microbial communities to AMX, TMP and CIP was different and varied depending on the soil considered. This fact clearly demonstrates that the mechanisms of action and the dynamics of these antibiotics in soils are not coincident, and also that the results of the investigations made in one specific agricultural area cannot be extrapolated to another one. For this reason, it will be necessary to carry out future research with different antibiotics and different types of soils, as well as different physicochemical conditions that allow advancing in the dynamics of emerging contaminants and achieving a more sustainable agriculture.

4. Conclusions

The effect of amoxicillin (AMX), trimethoprim (TMP) and ciprofloxacin (CIP) on bacterial growth and on the structure or composition of soil microbial communities could be important in agricultural soils contaminated with high concentrations of these antibiotics. The results of this study indicate that the effects detected on soil microbial communities due to the presence of AMX, TMP and CIP depend on the methodology used, the type of antibiotic added and the characteristics of the soils under study. For the future, further studies should be performed including a wide range of soils and using both leucine incorporation and PLFA methodologies, which would allow gaining a deeper insight as regards risk assessment in relation to the presence of antibiotics on soil microbial communities and hence on soil quality.

Credit author statement

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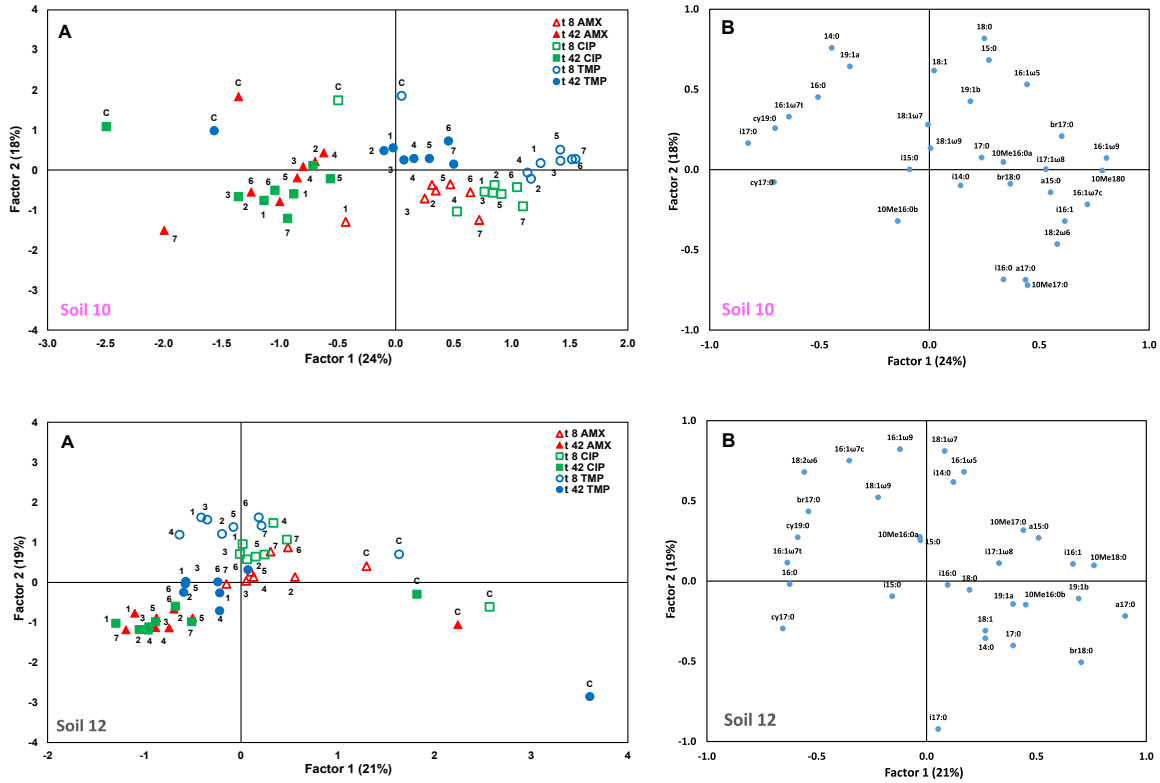

Fig. 9. Principal component analysis (A, variables; B, samples) performed with the PLFA dataset for the soils S10 and S12 added with different doses of amoxicillin (AMX), ciprofloxacin (CIP) and trimethoprim (TMP), after 8 (t8) and 42 (t42) days of incubation. Doses: 0 (C), 1 (0.49), 2 (1.95), 3 (7.81), 4 (31.25), 125 (5), 500 (6) and 2000 (7) mg kg^{-1} of soil.

Calviño, Ángela Martín, Montserrat Díaz-Raviña: Data curation, Vanesa Santás-Miguel, Manuel Arias-Estévez, David Fernández-Calviño, Ángela Martín, Montserrat Díaz-Raviña: Writing- Original draft preparation; Manuel Arias-Estévez, Esperanza Álvarez-Rodríguez, David Fernández-Calviño, Avelino Núñez-Delgado, Ángela Martín, Montserrat Díaz-Raviña, Laura Rodríguez-González, Elena García-Campos, Vanesa Santás-Miguel: Visualization; Laura Rodríguez-González, Elena García-Campos, Vanesa Santás-Miguel: Investigation; Manuel Arias-Estévez, Esperanza Álvarez-Rodríguez, David Fernández-Calviño, Avelino Núñez-Delgado, Ángela Martín, Montserrat Díaz-Raviña: Supervision; Manuel Arias-Estévez, Esperanza Álvarez-Rodríguez, David Fernández-Calviño, Avelino Núñez-Delgado, Ángela Martín, Montserrat Díaz-Raviña: Validation; Montserrat Díaz-Raviña, Avelino Núñez-Delgado: Writing-Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113916.

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