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Time-course evolution of bacterial community tolerance to tetracycline antibiotics in agricultural soils: A laboratory experiment

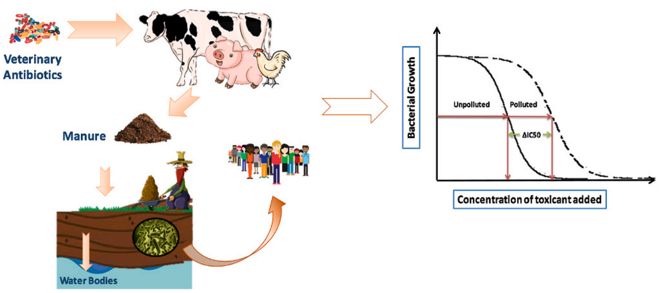
Vanesa Santás-Miguel [a,*], Laura Rodríguez-González [a], Avelino Núñez-Delgado [b], Esperanza Álvarez-Rodríguez [b], Montserrat Díaz-Raviña [c], Manuel Arias-Estévez [a], David Fernández-Calviño [a]

[a] *Área de Edafoloxía e Química Agrícola, Facultade de Ciencias, Universidade de Vigo, As Lagoas 1, 32004, Ourense, Galiza, Spain*
[b] *Departamento de Edafoloxía e Química Agrícola, Escola Politécnica Superior de Enxeñaría, Universidade de Santiago de Compostela, Campus de Lugo, Galicia, Spain*
[c] *Departamento de Bioquímica Del Suelo, Instituto de Investigaciones Agrobiológicas de Galicia (IIAG/CSIC), Santiago de Compostela, Galicia, Spain*

HIGHLIGHTS

- Soil pollution with tetracyclines may increase bacterial community tolerance.
- Antibiotic concentrations needed to cause those increases are higher than 500 mg/kg.
- The increases were higher in soils with low organic carbon content.
- The magnitude of the increases in bacterial community tolerance was time dependent.
- Bacterial community tolerance to tetracyclines was maximum after 45–100 days.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of antibiotics in soils may increase the selection pressure on soil bacterial communities and cause tolerance to these pollutants. The temporal evolution of bacterial community tolerance to different concentrations of tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) was evaluated in two soils. The results showed an increase of soil bacterial community tolerance to TC, CTC and OTC only in samples polluted with the highest antibiotic concentrations tested (2000 mg kg^{-1}). The magnitude of those increases was higher in the soil with the lower organic carbon content (1.6%) than in the soil with an organic carbon content reaching 3.4%. In the soil with low organic carbon content, the time-course evolution showed a maximum increase in the tolerance of bacterial communities to tetracycline antibiotics between 45 and 100 incubation days, while for longer incubation times (360 days) the tolerance decreased. In the soil with high organic carbon content, a similar behavior was found for OTC. However, for CTC and TC, slightly increases and decreases (respectively) were found in the bacterial community tolerance at intermediate incubation times, followed by values close to zero for TC after 360 days of incubation, while for CTC they remained higher than in the control. In conclusion, soil pollution due to tetracyclines may cause bacterial community tolerance to these antibiotics when present at high concentrations. In addition, the risk is higher in soils with low organic matter content, and it decreases with time.

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* Corresponding author.
E-mail address: vsantas@uvigo.es (V. Santás-Miguel).

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1. Introduction

Veterinary antibiotics (VAs) have been widely used as a treatment for infectious diseases and even as growth promoters in animals (Sapkota et al., 2008; Li et al., 2011). The consumption of veterinary antibiotics has been increasing in recent decades, with the overall world consumption of antibiotics estimated for 2030 being 105,596 tons (Van Boeckel et al., 2015). When these compounds are administered to cattle, they are poorly absorbed in the animal intestine and between 30 and 60% are excreted as the original molecule (Sarmah et al., 2006; Massé et al., 2014). Terrestrial ecosystems are exposed to veterinary antibiotics mainly through repeated applications of manure/slurry and sludge to agricultural soils (Montforts et al., 1999) and, once there, antibiotics can affect non-target organisms such as bacterial communities (Warman, 1980). The effect of antibiotics on soil microbial communities has been studied previously by various authors, who observed how the presence of these compounds in the soil can cause changes in the microbial community structure (Hammesfahr et al., 2008; Unger et al., 2013; Urra et al., 2019) and functions (Zielezny et al., 2006; Toth et al., 2011; Liu et al., 2015; Ma et al., 2016).

Tetracycline antibiotics are one of the groups most widely used in the European Union, and ranking second place worldwide (Bansal, 2012), mainly due to their low cost, effectiveness, broad-spectrum, and high solubility in water. Besides, within the group of tetracyclines the most consumed are tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) (ESVAC, 2016). In addition, tetracyclines are characterized by long half-lives that can last beyond 100 days in soils (Cycon et al., 2019). The high persistence of these antibiotics in the soil is of particular concern, as it may lead to the increase of antibiotic tolerance by soil bacterial communities. In fact, the increase in the concentration of any pollutant (organic or inorganic) in soil may exert a selection pressure on the soil bacterial communities, and hence cause tolerance to that specific pollutant (Blanck, 2002). This effect is called pollution-induced community tolerance (PICT), and its measurement can be useful in the quantification of the harmful effects of toxic substances on soil microorganisms and quantifying the appearance of resistance of soil bacterial communities to toxic substances. It should be noted that the increase in antibiotic resistance of bacterial communities in soils has become a crucial threat to public health. Exposure of environmental organisms to antimicrobial agents can create reservoirs of resistance in soil organisms that could potentially confer resistance to pathogens through horizontal gene transfer and other mechanisms (Davies, 1994; Knapp et al., 2010; Serwecińska, 2020). The resulting increase in infections by pathogens and the problem of bacteria resistant to antibiotics have become a threat to global public health, which needs addressing this problem from two complementary concepts: One Health and Global Health (Manyi-Loh et al., 2018; Hernando -Amado et al., 2019; Serwecińska, 2020).

Regarding tolerance, studies related to this topic can be carried out using DNA techniques (presence of resistance genes) or from a functional point of view (focusing on PICT) (Milenkovski et al., 2010). The use of techniques such as PICT instead DNA fingerprinting has two clear advantages: i) the easy interpretation of the data, and ii) the detection of the presence of tolerance in the bacterial communities together with the confirmation of the toxicity of the contaminant on the bacterial communities (Milenkovski et al., 2010). Although there are some studies on functional tolerance (PICT) to antibiotics performed on bacterial communities (Schmitt et al., 2004, 2006; Demoling and Bååth, 2008; Brandt et al., 2009; Demoling et al., 2009; Liu et al., 2012), limited research has been carried out measuring this functional tolerance to tetracycline antibiotics (Schmitt, 2005; Schmitt et al., 2006; Fang et al., 2014; Song et al., 2017; Han et al., 2019). Moreover, none of these works focused on the time-course evolution of bacterial community tolerance to antibiotics. This kind of research could be improved taking into account that the duration of the exposure of bacterial communities to toxicants must be long compared to the time required for the community to go through its succession to a more tolerant state (Blanck, 2002). In general, in those works where the PICT method has been used for antibiotics, the times of exposition were lower than 9 weeks (Schmitt, 2005; Schmitt et al., 2006; Fang et al., 2014; Song et al., 2017; Han et al., 2019). Therefore, long-term studies dealing with eventual increases in bacterial community tolerance to tetracycline antibiotics are needed to clarify the potential persistence of the functional tolerance to these antibiotics with time. These data could delineate trends in antibiotic resistance potentially valuable for epidemiological studies (Knapp et al., 2010).

In view of this background, the aim of the present work is to obtain new information as regards the long-term time-course evolution of the bacterial community tolerance to three tetracycline antibiotics (TC, OTC, and CTC) in two soils with different organic carbon content that were polluted with different concentrations of these substances. Specifically, 8 different concentrations of each antibiotic were used (2000, 500, 125, 31.3, 7.8, 2, 0.5 and 0 mg kg^{-1}), and soil bacterial community tolerance to these antibiotics was assessed after 45, 100, 180 and 360 days of incubation, using the polluted induced community tolerance (PICT) methodology. The results of the study could be relevant to shed further light on the fate of these pollutants when spread on environmental compartments, and specifically regarding its effect on soil microbial communities over time.

2. Material and methods

2.1. Chemicals

Tetracycline hydrochloride (TC, CAS. 64-75-5; ≥95% in purity), Oxytetracycline hydrochloride (OTC, CAS, 2058-46-0; ≥95% in purity), and Chlortetracycline hydrochloride (CTC, CAS 64-72-2; ≥97% in purity) were supplied by Sigma–Aldrich (Steinheim, Germany). Talc (CAS 14807-96-6) was supplied by Sigma–Aldrich (Steinheim, Germany).

2.2. Soil samples

Two agricultural soils were selected from a soil pool previously characterized by Conde-Cid et al. (2018). Soils were sampled from superficial horizons (0–20 cm) with an Edelman probe. Once in the laboratory, these samples were air-dried, sieved through a 2 mm mesh and stored in polyethylene bottles until analysis. The main characteristics of the studied soils are shown in Table S1 (Supplementary Material). Briefly, these soils presented similar particle size distribution and texture, being sandy clay loam for soil 1 (sand: 54.6%; clay: 23.4%; silt: 22%) and sandy loam for soil 2 (sand: 58.5%; clay: 22.5%; silt: 19.1%). Organic carbon contents were 1.6% and 3.4% for soils 1 and 2, respectively. The pH values were similar in both soils, being 4.65 for soil 1 and 4.74 for soil 2. The effective cation exchange capacity was 4.7 cmol$_c$ kg^{-1} for soil 1, and 5.9 cmol$_c$ kg^{-1} for soil 2. The concentrations of heavy metals determined in the selected soils were low and similar to those found in non-polluted soils in the study area (Macías and Calvo, 2008). In addition, the levels of tetracycline, oxytetracycline and chlortetracycline were below the detection limit (50 ng g^{-1}) (Conde-Cid et al., 2018).

2.3. Experimental design

The selected soil samples were moistened up to 60–80% of water holding capacity and incubated at 22 °C in the dark during 1 week, an adequate time to recover and stabilize soil bacterial community growth after moisture adjustment (Meisner et al., 2013). After this time, each soil was distributed in 72 polypropylene tubes (100 mL) (3 antibiotics x 3 replicates x 8 antibiotic concentrations), adding 20 g of soil (dry weight) in each tube. The total number of microcosms was 144 (72 per each soil). Then, different concentrations of the three tetracycline antibiotics (tetracycline, oxytetracycline and chlortetracycline) were added to the soil microcosm individually (24 per antibiotic and soil) using talc powder as a carrier (Rousk et al., 2008), reaching a final concentration in the soil samples of 0, 0.5, 2, 7.8, 31.3, 125, 500 and 2000 mg antibiotic per kg^{-1} of soil. These concentrations were used previously in works testing direct toxicity of tetracycline antibiotics on the growth of bacterial communities (Santás-Miguel et al., 2020a, 2020b, 2020c, 2020d). The concentrations were previously selected in order to obtain dose-response curves that included: a) low concentrations that almost did not affect bacterial growth, and b) very high concentrations that caused almost complete inhibition of soil bacterial growth (>90%) and thus offer estimates of toxicity indices in a more reliable way (Fox and Landis, 2016). After performing soil spiking with tetracycline antibiotics, the soil microcosms were incubated at 22 °C in the dark during 360 days. During this period, bacterial community tolerance to the three tetracycline antibiotics was estimated in the microcosms polluted with each of them after 45, 100, 180 and 360 days. The soil microcosms were frequently aerated, while the soil moisture was maintained by adding water if necessary.

Tolerance of bacterial communities to tetracycline antibiotics was measured for all microcosms by means of a short-term toxicity test according to Bååth (1992) and Díaz-Raviña et al. (1994) with certain modifications indicated below, and using the leucine (Leu) incorporation method (Bååth, 1994; Bååth et al., 2001) to estimate bacterial community growth. Briefly, 3.5 g of soil (fresh weight) was mixed with 50 mL of distilled water using a multivortex shaker for 3 min at maximum intensity, followed by low-speed centrifugation at 1000×*g* for 10 min, thus creating a bacterial suspension in the supernatant. An aliquot (1.5 mL) of this suspension was transferred to 2 mL micro-centrifugation tubes. Then, volume aliquots of 0.15 mL containing different concentration of the tetracycline antibiotics were added before measuring the leucine incorporation. Seven different concentrations of the three antibiotics (TC, OTC or CTC) were used, going from 0.1 to 400 mg L^{-1}. A control with distilled water was also used for each microcosm. Then, the bacterial community growth was estimated after a pre-incubation step of 24 h for bacterial suspensions with the different antibiotics concentrations added before the leucine incorporation assay (Berg et al., 2010; Fernández-Calviño et al., 2013). After this pre-incubation time, 2 μL [^{3}H] Leu (3.7 MBq mL^{-1} and 0.574 TBq $mmol^{-1}$; PerkinElmer, USA) were added with non-labeled Leu to each tube, resulting in a final concentration of 275 nM Leu in the bacterial suspension. After incubation at 22 °C for 3 h, the bacterial growth was stopped with 75 μL of 100% trichloroacetic acid. Washing was performed as described by Bååth et al. (2001), and radioactivity was determined by scintillation liquid counting (Tri-Carb 2810 TR, PerkinElmer, USA).

2.4. Data analysis

The data corresponding to estimated bacterial community growth (leucine incorporation) as a function of the concentration of antibiotics (TC, OTC o CTC) added to the bacterial suspension was normalized dividing all values by the control (sample without antibiotic) for each microcosm, in order to allow the subsequent comparison among different dose-response curves.

The tolerance of the bacterial communities to TC, OTC or CTC was estimated as log IC_{50}, the logarithm of the concentration of antibiotic that resulted in 50% inhibition of bacterial community growth (leucine incorporation) in each dose-response curve. Log IC_{50} was estimated using a logistic model (Rousk et al., 2011; Sebaugh et al., 2011; Rath et al., 2016), $Y = c/[1+e^{b(a-x)}]$, where Y is Leu incorporation for each antibiotic concentration, x is the logarithm of the concentration of antibiotic added, a is the value of log IC_{50}, b is a parameter related with the slope of inhibition curves, and c is the bacterial growth without antibiotic added (control sample). A higher value of log IC_{50} indicates a higher community tolerance, while a lower value indicates that the antibiotics are more toxic to the bacterial community, i.e., it is less tolerant. The increases of bacterial community tolerance to antibiotics in soils spiked with them (PICT = Pollution Induced Community Tolerance) was calculated as $\Delta logIC_{50}$, specifically by subtracting $logIC_{50}$ values estimated in spiked soils minus the $logIC_{50}$ values found in the unpolluted controls for each soil.

3. Results and discussion

3.1. Increases in bacterial community tolerance to tetracycline antibiotics

The inhibition curves of bacterial growth obtained for each tetracycline antibiotic, for each tetracycline concentration, each soil sample, and each time tested are shown in Figures S1-S3 (Supplementary Material). All microcosms showed sigmoid dose-response curves, i.e., low tetracycline antibiotics did not inhibit bacterial growth, but at high doses, the extent of inhibition increased with the dose. As a general trend, there is no clear shift to the right in the dose-response curves obtained for tetracycline antibiotic concentrations ≤500 mg of antibiotic per kg^{-1} of soil. However, at higher concentrations of added antibiotic (2000 mg kg^{-1}), a shift to the right is observed in the dose-response curves. However, this shifting is more clear in the soil with lower organic carbon content (Soil 1; C = 1.6%) than in the soil with a higher organic carbon content (Soil 2; C = 3.4%). The shifts in the dose-response curves to the right suggest increases in the tolerance of bacterial communities to the antibiotics tested. These increases in response to soil pollution with this type of substances may be due to physiological or genetic adaptations, as was suggested previously for heavy metals pollution (Díaz-Raviña and Bååth, 1996). These adaptations may include: 1) killing of sensitive species due to immediate antibiotic toxicity; 2) selection of tolerant species due to different competitive abilities to growth in presence of antibiotics; 3) physiological or behavioral responses in individual cell populations; and 4) adaptive evolution via mutation or acquisition of antibiotic resistance via horizontal gene transfer (Brandt et al., 2015). In the case of tetracycline antibiotics, the first mechanisms may be discarded because molecules of the tetracycline group, being bacteriostatic antibiotics (Smilack, 1999), may inhibit the growth of bacteria but do not kill them.

The dose-response curves were generally well described by the logistic model (Tables S2 and S3, Supplementary Material), with R^2 values ranging from 0.899 to 0.998 for tetracycline (mean $R^2 = 0.967$), from 0.815 to 0.999 for oxytetracycline (mean $R^2 = 0.953$), and from 0.877 to 0.988 for chlortetracycline (mean $R^2 = 0.980$).

Fig. 1 shows the Log IC_{50} values corresponding to soil 1 for all TC, OTC and CTC concentrations and incubation times. In general, the log IC_{50} values show similar behaviors for the different tetracycline antibiotics. Overall, tolerance of soil bacterial communities to tetracycline antibiotics was not observed in soil 1 at concentrations ≤500 mg kg^{-1} since no increases of Log IC_{50} values were observed for any of the tetracycline antibiotics tested. However, the Log IC_{50} values obtained for the maximum concentration tested (2000 mg kg^{-1}) show, in general, an increase in tolerance to antibiotics with respect to the values obtained for the control (0 mg of antibiotic kg^{-1} of soil), for any of the incubation-times tested. These increases observed in Log IC_{50} values ranged between 0.6 and 1.9 units for TC, between 0 and 1.6 units for OTC, and ranged between 0.9 and 3.0 units for CTC. Therefore, soil bacterial communities showed increases in tolerance to tetracycline antibiotics in soils for the antibiotics concentration of 2000 mg kg^{-1}. The results obtained in this study agree with those reported by other authors using the PICT method (Hund-Rinke et al., 2004; Schmitt et al., 2006). These authors did not observe increases in soil bacterial community tolerance to tetracycline antibiotics at low antibiotics concentrations (Hund-Rinke et al., 2004; Song et al., 2017), however, for higher concentrations (1000 mg of antibiotic kg^{-1} of soil) increases in bacterial community tolerance have been found after one week of incubation (Schmitt et al., 2006). Schmitt et al. (2006) also observed that increases in tolerance to tetracycline antibiotics can occur at lower concentrations (100 mg kg^{-1}), but this was not observed in our study.

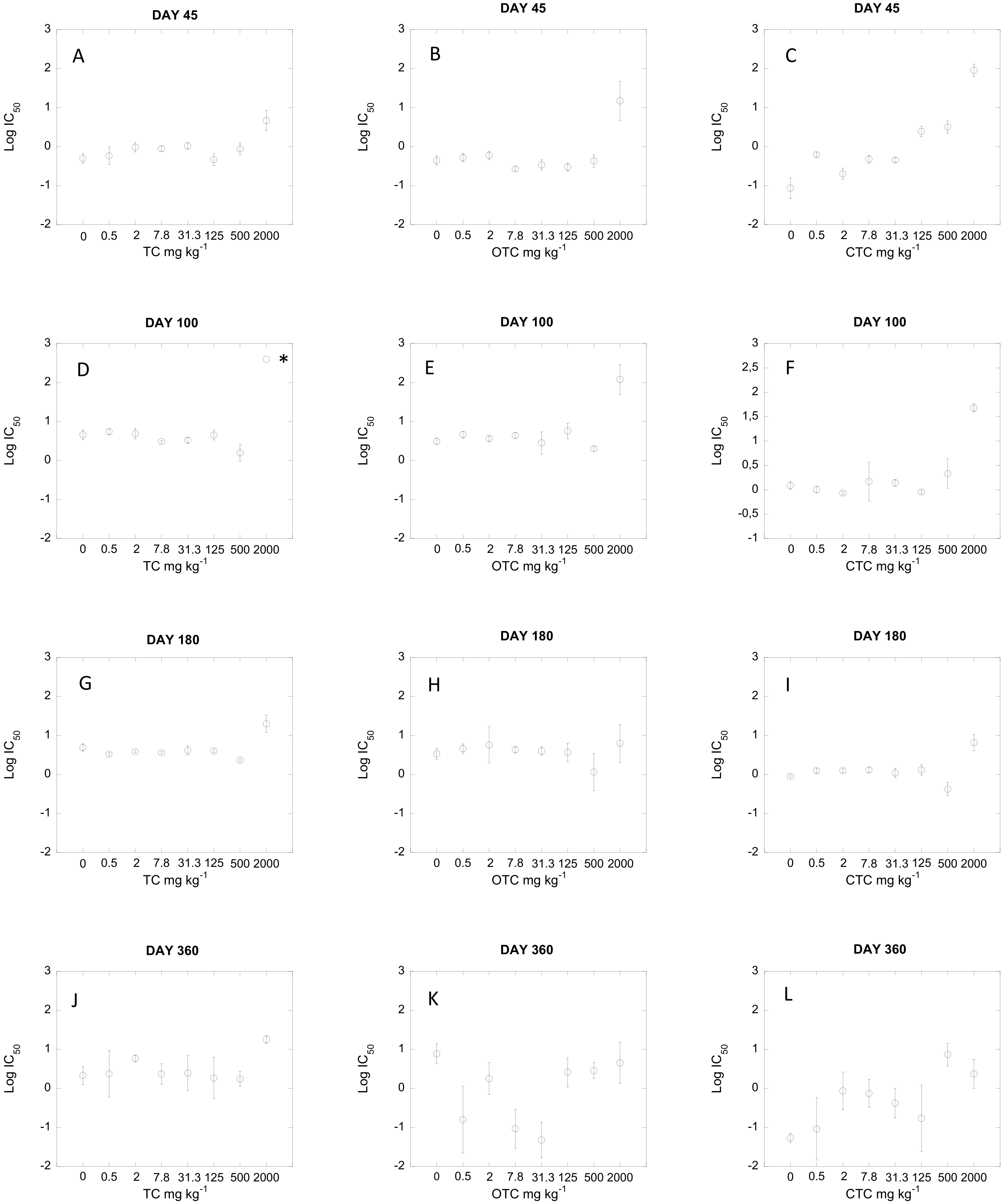

Fig. 1. Time-course variation of bacterial community tolerance to tetracycline (TC; A, D, G and J), oxytetracycline (OTC; B, E, H and K) and chlortetracycline (CTC; C, F, I and L) (expresed as Log IC_{50}), as a function of antibiotic concentration added to soil 1, after 45, 100, 180 and 360 days of incubation. *The concentration inhibiting the 50% of population was not estimated because the bacterial communities showed total recovery, therefore the Log IC_{50} asigned was 2.6, corresponding to the maximum tetracycline concentration tested in the PICT tests.

The log IC_{50} values obtained for soil 2, regarding TC, OTC and CTC, and all incubation times are shown in Fig. 2. The increases observed in Log IC_{50} values were ≤ 0.1 units for TC, and ≤ 0.5 units for CTC, for all antibiotic concentrations and incubation times. However, in the case of OTC there was a small increase in bacterial community tolerance to the antibiotic after soil spiking with 2000 mg kg^{-1} and at lower incubation times (45 and 100 days), but being ≤ 0.9 units in both cases. Therefore, most of the increases in the tolerance of bacterial communities to

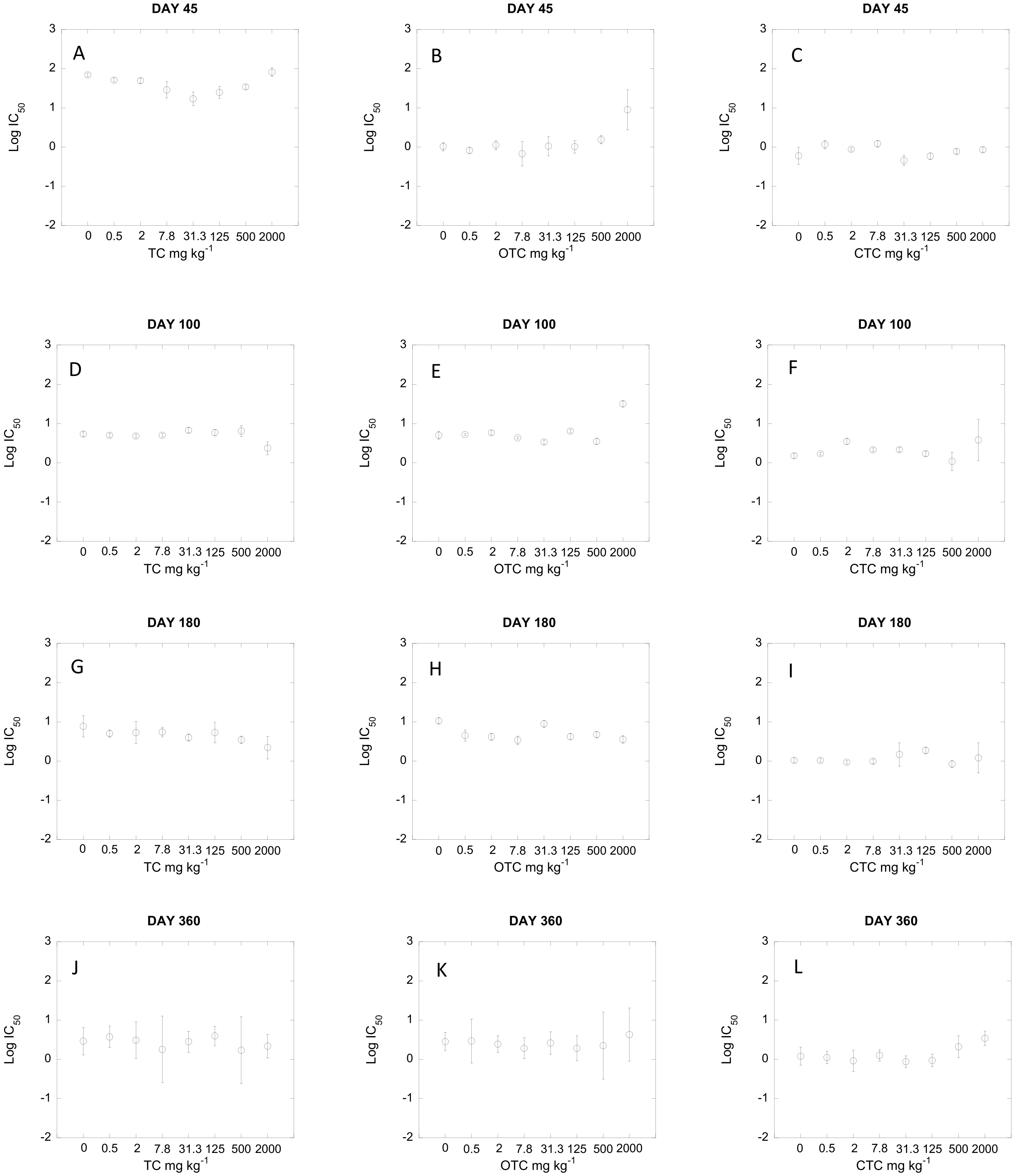

Fig. 2. Time-course variation of bacterial community tolerance to tetracycline (TC; A, D, G and J), oxytetracycline (OTC; B, E, H and K) and chlortetracycline (CTC; C, F, I and L) (expresed as Log IC_{50}), as a function of antibiotic concentration added to soil 2, after 45, 100, 180 and 360 days of incubation.

tetracycline antibiotics occur at 2000 mg kg^{-1}. These results agree with the values observed for soil 1 and with data reported by other authors (Hund-Rinke et al., 2004; Schmitt et al., 2006; Song et al., 2017). However, in the current work the magnitude of these increases is different for each of the soils studied. Specifically, the increase in the magnitude of the tolerance to tetracycline antibiotics is lower for soil 2 (with high carbon content) than for soil 1 (with low carbon content), suggesting that these increases in the tolerance of the bacterial communities to antibiotics may be influenced by the organic matter content of the soil. This effect may be due to the key role played by organic matter in the adsorption of tetracycline antibiotics in soils (Tolls, 2001; Gu and Karthikeyan, 2008; Ling-Ling et al., 2010; Conde-Cid et al., 2019). The sorption of tetracycline antibiotics onto soils reduces the bioavailability of these molecules to bacterial communities and, therefore, it could make lower the selective pressure on bacteria and reduce the promotion of antibiotic resistance of the microorganisms.

3.2. *Time-course evolution of bacterial community tolerance to tetracycline antibiotics*

The bacterial communities showed increases in tolerance to antibiotic concentrations of 2000 mg kg^{-1}. Therefore, the time-course evolution (45, 100, 180 and 360 days) of Δlog IC_{50} values was evaluated for the maximum concentration tested (2000 mg kg^{-1}) (Fig. 3). In soil 1 (soil with low carbon content, C = 1.6%), the tolerance of bacterial communities to antibiotics increased with time up to a maximum Δlog IC_{50} value reached at 45 days for CTC (Fig. 3C), while it was achieved at incubation times between 45 and 100 days for OTC (Fig. 3B), and after 100 days of incubation for TC (Fig. 3A). After these incubation times, the tolerance of the bacterial communities to tetracycline antibiotics gradually decreased, reaching after 360 days Δlog IC_{50} values close to zero (−0.2) for OTC, but remaining close to 0.9 and 1.6 for TC and CTC, respectively. However, in soil 2 (with high carbon content, C = 3.4%) soil bacterial communities tolerance to CTC (Δlog IC_{50}) slightly increased with time, reaching the maximum value (0.5 units) after 360 days (Fig. 3F). The bacterial community tolerance to TC slightly decreases for intermediate incubation times (up to −0.5), reaching Δlog IC_{50} values close to zero (−0.1) after 360 days (Fig. 3D). However, the bacterial community tolerance to OTC showed a time-course evolution similar to the trend found in soil 1 for all three antibiotics, with a maximum increase of tolerance to OTC between 45 and 100 days (Fig. 3E), but showing values lower than those found for soil 1 (0.9, 0.8, instead of 1.5, 1.6). For longer incubation times bacterial community tolerance to OTC gradually decreased, reaching a Δlog IC_{50} value close to zero (0.2) after 360 days of incubation.

There is a lack of research dealing with the study of time-course evolution of soil bacterial community tolerance to antibiotics in soils polluted with this type of emerging pollutants. The present work is one of those focusing on this field. Some other interesting and recent studies are those by Santás-Miguel et al. (2020e), or the paper by Zhong et al. (2021), where the authors investigated the potential effect of Cu and other heavy metals on the bacterial tolerance or resistance to antibiotics. Previously, a similar research was performed, but studying the time-course evolution of bacterial community tolerance to different heavy metals (Diaz-Raviña and Bååth, 1996). Results from that previous study showed that the bacterial community tolerance to Cu, Zn and Cd in soils polluted with these heavy metals increased with time, reaching maximum values at the end of the experiment (14 months of incubation). In the case of soils polluted with Zn, Diaz-Raviña and Bååth (1996) also studied the bacterial community tolerance to Zn up to 1024 days, finding the higher values at this prolonged incubation time. In the current work, the results partially fit with those found for heavy metals, i.e. initial bacterial community tolerance increases with time. However, for longer incubation times the bacterial community tolerance to antibiotics decreased, reaching in some cases Δlog IC_{50} values close to zero. In addition, our results also fit, at least partially, with studies measuring the abundance of antibiotics resistance genes (ARGs) in agricultural soils treated with antibiotic-polluted manures, which in some cases showed a similar time-course evolution as that we found for bacterial community tolerance to antibiotics (Hu et al., 2016; Zhang et al., 2017). Hu et al. (2016) reported increases of β-lactam ARGs up to 63 incubation days followed by decreases at 140 days, while, for tetracycline and other antibiotics, ARGs increases were detected at 63 days and maintained until the end of the incubation (140 days). Zhang et al. (2017) assessed the abundance of ARGs in agricultural soils after the addition of different types of manures spiked and no-spiked with tylosin, showing that the general abundance of ARGs decreased with time.

The increase of tolerance with time may be due to the slow rate of

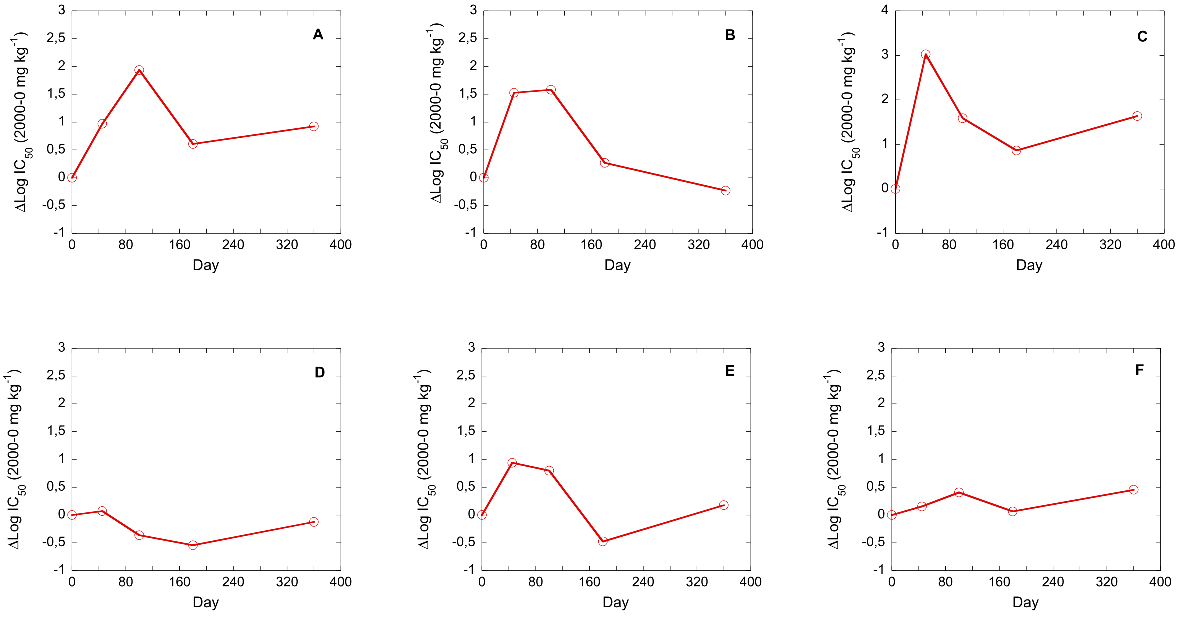

Fig. 3. Time-course evolution of increases in bacterial community tolerance (ΔLog IC_{50}) to tetracycline antibiotics: Tetracycline (TC), Oxytetracycline (OTC) and Chlortetracycline (CTC). Soil 1: TC (A), OTC (B) and CTC (C). Soil 2 TC (D), OTC (E) and CTC (F). ΔLog IC_{50} = Log IC_{50} for samples polluted with 2000 mg kg^{-1} of tetracycline antibiotic (TC, OTC or CTC) minus Log IC_{50} for 0 mg of antibiotic kg^{-1} (control soil).

tolerance development mechanisms, i.e. the process of increase of bacterial community tolerance to any toxicant needs enough time (Díaz-Raviña and Bååth, 1996). Furthermore, the decrease in bacterial community tolerance to different tetracycline antibiotics found for long incubation periods may be due to different causes:

1) tetracycline antibiotics show a strong adsorption by soil components such organic matter and clays (Tolls, 2001; Figueroa et al., 2004; Pils and Laird, 2007; Gu and Karthikeyan, 2008; Teixidó et al., 2012; Conde-Cid et al., 2019). This high sorption may increase over time via ageing processes (Loibner et al., 2006; He et al., 2019), causing that tetracycline antibiotics became less bioavailable for microorganisms, and therefore less toxic. To note that less toxicity also means less capacity to increase the bacterial community tolerance to antibiotics.
2) degradation of tetracycline antibiotics in soils with time (Maki et al., 2006; Wang and Yates, 2008; Pan and Chu, 2017). Although the degradation of tetracycline antibiotics in the soil is a slow process depending on the initial concentration of the antibiotic (Bansal, 2012; Cycoń et al., 2019), in 360 days they may be highly degraded because the half-life values are around 100 days when the concentrations of tetracycline antibiotics are high (Cycoń et al., 2019). Less concentration of tetracycline antibiotics with time lead to less toxicity pressure, and therefore to reductions in bacterial community tolerance to these substances.

3.3. *Environmental implications*

The concentrations of tetracycline antibiotics detected in agricultural soils are very variable among soil type and different regions (Karcı and Balcıoğlu, 2009; Hu et al., 2010; Conde-Cid et al., 2018), with maximum values ranging between 2.9×10^{-3} and 1 mg kg^{-1} for TC, between 7×10^{-4} and 2.7 mg kg^{-1} for OTC, and between 9×10^{-4} and 11 mg kg^{-1} for CTC (Conde-Cid et al., 2020). These values are much lower than values which caused increases in bacterial community tolerance to TC, OTC or CTC in the present work (>500 mg kg^{-1}). Therefore, for circumstances similar to those tested in the current research, tetracycline antibiotics concentrations usually found in agricultural soils would present low risk of increasing the bacterial community tolerance to these substances. This finding could have an important implication, in the sense that although many studies showed that the presence of low concentrations of antibiotics in the soil may increase the presence of antibiotics resistance genes, including tetracycline ARGs (Tang et al., 2015; Xie et al., 2018; Liu et al., 2020), our study shows that from a functional point of view the risk would be lower. In addition, if some type of antibiotic solutions spills happen in the farms, high concentrations of tetracycline antibiotics may be reached in limited soil surfaces as antibiotics hotspots (Kaeseberg et al., 2018), and therefore increases in bacterial community tolerance to this type of antibiotics could be possible. Identifying these hotspots is currently a challenge, deserving special attention because the presence of functional tolerance of soil bacterial communities to antibiotics may present important risk implications for human health, especially due to tolerance transmission to human pathogens (Forsberg et al., 2012). Therefore, the identification of environments where there are high densities of antibiotic-tolerant bacteria is important for founding the basis regarding the design of new or complementary mitigation strategies that will allow achieving progress in the control of antibiotic resistance (Berendonk et al., 2015).

4. Conclusions

The results from the present study showed that soil pollution due to the tetracycline antibiotics tetracycline, oxytetracycline and chlortetracycline may induce increases in bacterial community tolerance to these substances. However, the antibiotic concentrations needed are >500 mg kg^{-1}, values not usually found in agricultural soils. There was a difference in the magnitude of those increases as a function of soil type, being higher in the soil with lower organic carbon content (1.6%), compared with the soil with higher organic matter content (3.4%). Regarding the time-course evolution of bacterial community tolerance to tetracycline antibiotics, as general trend, it increased with time up to 45–100 incubation days, then decreasing for longer incubation periods. This effect was more evident in the soil with lower organic carbon content. These results could be of relevance to shed light on the environmental fate of this kind of emerging pollutants, and on the evolution of their effects on soil microbial communities over time.

Author statement

Vanesa Santás-Miguel: Methodology, Writing – original draft. Laura **Rodríguez-González**: Methodology **Avelino Núñez-Delgado**: Conceptualization, Writing- Reviewing and Editing. **Esperanza Álvarez-Rodríguez**: Methodology, Investigation. **Montserrat Díaz-Raviña** : Conceptualization, Writing – original draft. **Manuel Arias-Estévez**: Data curation, Investigation, Supervision. **David Fernández-Calviño**: Conceptualization, Investigation, 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.chemosphere.2021.132758.

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