Tolerance of soil bacterial community to tetracycline antibiotics induced by As, Cd, Zn, Cu, Ni, Cr and Pb pollution

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Abstract. The widespread use of both heavy metals and antibiotics in livestock farming, followed by their subsequent arrival on agricultural soils through manure/slurry spreading, has become a problem of vital importance for human health and the environment. In the current research, a laboratory experiment was carried out for 42 days to study tolerance and co-tolerance of three tetracycline antibiotics (tetracycline, TC; oxytetracycline, OTC; chlortetracycline, CTC) in soils polluted with heavy metals (As, Cd, Zn, Cu, Ni, Cr and Pb) at high concentrations (1000 mg kg^{-1} of each one, separately). Pollution Induced Community Tolerance (PICT) of the bacterial community was estimated using the leucine incorporation technique. The Log IC_{50} (logarithm of the concentration causing 50% inhibition in bacterial community growth) values obtained in uncontaminated soil samples for all the heavy metals tested showed the following toxicity sequence: Cu>As>Cr≥Pb≥Cd>Zn>Ni. However, in polluted soil samples the toxicity sequence was Cu>Pb≥As≥Cd≥Cr≥Ni≥Zn. Moreover, at high heavy metal concentrations the bacterial communities showed tolerance to the metal itself, this taking place in the long term for all the metals tested. The bacterial communities of the soil polluted with heavy metals showed also long-term co-tolerance to TC, OTC, and CTC. This kind of studies, focusing on the eventual increases of tolerance and co-tolerance of bacterial communities in agricultural soil, favored by the presence of different kinds of pollutants, is of crucial importance, mostly bearing in mind that the appearance of antibiotic resistance genes in soil bacteria could be transmitted to human pathogens.

Keywords: Chlortetracycline; heavy metals; trace elements; oxytetracycline; PICT; tetracycline

1. Introduction

The accumulation of heavy metals in soils is a widespread problem all over the world. Some anthropogenic activities such as mining, industrial production, agriculture, or livestock farming, are among the main sources of release of heavy metals to the terrestrial environment (Abdu et al., 2017). The use of heavy metals in livestock production is a common practice, since commercial feeds are enriched with essential elements (such as Cu and Zn) to prevent diseases and obtain an optimal growth rate (Sager, 2007; Wu et al., 2013). In fact, the of presence heavy metals in livestock manure can be seen as generalized, with really high concentrations in swine manure reported by Hölzel et al. (2012) for Zn (up to 8239 mg kg^{-1}), and for Cu (up to 3387 mg kg^{-1}), while Wang et al. (2013) found up to 1601.7 mg kg^{-1} for Cr in layer manure, Zhang et al. (2012) detected up to 300 mg kg^{-1} for Pb, and up to 100 mg kg^{-1} for Ni, in poultry manure, and finally Liu et al. (2020) reported up to 59.7 mg kg^{-1} for Cd, and up to 89.3 mg kg^{-1} for As, in pig manure. In view of that, these and other heavy metals are considered a serious problem for public health, especially because these toxic elements tend to accumulate in soils, where they may reach very high and progressively increasing concentrations (Kabata-Pendias, 2000; Huang et al., 2007). Moreover, veterinary antibiotics are also widely used for the treatment of bacterial infections (Boxal et al., 2003), and their consumption has been increasing in recent decades, with the estimate of world use for 2030 being 105,596 tons (Van Boeckel et al., 2015). The antibiotics most widely used in veterinary medicine in the European Union are tetracyclines (TCs), and specifically tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) (European Medicines Agency, 2016). Both heavy metals and antibiotics for veterinary use are poorly absorbed by the intestines of animals, causing that a high percentage of them is excreted in feces and urine (Kornegay et al., 1976; Sarmah et al., 2006). In this sense, it has been reported that tetracycline antibiotics may reach in manures concentrations as high as 746 mg kg^{-1} for CTC (Pan et al., 2011), 211 mg kg^{-1} for OTC, and 300 mg kg^{-1} for TC (Widyasari-Mehta et al., 2016). The presence of high levels of heavy metals and veterinary antibiotics in farmland soils is due to the repeated applications of manure and slurries (as well as sewage sludge) as organic fertilizers (Hamscher et al., 2002; Nicholson et al., 2003). Once in the soil, these compounds can interact with soil microbial communities and modify their structure and function (Hattori, 1992; Thiele-Bruhn and Beck, 2005; Chien et al., 2008; Giller et al., 2009; Caban et al., 2018).

The increase of the concentration of any pollutant in soils may suppose a selection pressure for soil bacterial communities, causing tolerance to that pollutant (Blanck, 2002). This effect may be useful to quantify the harmful repercussions produced by pollutants on soil bacteria and is called pollution-induced community tolerance (PICT). Agricultural soils, highly influenced by anthropogenic activities, deserve special attention since they are recognized as the largest reservoirs of antibiotic-resistant genes, receiving antibiotics from veterinary use (through repeated applications of manure and slurries, as indicated above), as well as heavy metals (Ji et al., 2012). Therefore, the resistance of soil bacterial communities to antibiotics has become a crucial threat at a world scale, and the study of whether bacterial communities generate co-tolerance to antibiotics in the presence of heavy metals (as well as on how and in which degree is developed) is of vital importance.

Several previous studies have focused on co-tolerance among different heavy metals and antibiotics (Berg et al., 2010; Sarma et al., 2010; Fernández-Calviño and Bååth, 2013; Song et al., 2017; Santás-Miguel et al., 2020a; Zhong et al., 2021). Specifically, Fernández-Calviño and Bååth (2013) evaluated the tolerance of soil bacterial community in one soil polluted with different Cu concentrations (2-32 mmol Cu kg^{-1}) to three antibiotics (vancomycin, tetracycline and tylosin) and observed an increase in the co-tolerance of soil bacterial communities to tetracycline (at ≥ 8 mmol Cu kg^{-1}), tylosin (at ≥ 16 mmol Cu kg^{-1}) and vancomycine (at ≥ 16 mmol Cu kg^{-1}). Song et al. (2017) assessed the effects of adding different concentrations of Cu (33.3- 1000 mg kg-^{1}) and Zn (165-5000 mg kg^{-1}) on the tolerance of soil bacterial communities to tetracycline, finding that the co-tolerance to the antibiotic significantly increased in soils where Cu concentration were ≥ 365 mg kg^{-1}, and Zn concentrations were ≥ 264 mg kg^{-1}. Zhong et al. (2021) studied the tolerance to tetracycline and vancomycin in 10 mine soil samples with the presence of Cu (361-4399 mg k^{-1}), Zn (33-3811 mg kg^{-1}) and Pb (195-20,239 mg k^{-1}). These authors found that, in general, there were increases in tolerance of soil bacterial communities to antibiotics, although they did not detect a systematic pattern in the co-tolerance to tetracycline associated to heavy metals concentrations, whereas the induced levels of heavy metal tolerance coincided with elevated levels of tolerance to vancomycin. However, until now, there were not studies evaluating the effect of a wide range of different heavy metals present in livestock manure on the tolerance shown by soil bacterial communities against each of the three tetracycline antibiotics most used in animal husbandry. Therefore, the objective of this study is to determine the eventual development of tolerance in soil bacterial communities to heavy metals in agricultural soils contaminated individually with 1000 mg kg^{-1} of As, Cd, Zn, Cu, Ni, Cr and Pb, and also the eventual generation of co-tolerance to the antibiotics tetracycline, oxytetracycline and chlortetracycline, using the leucine incorporation technique as the endpoint. Previous works using Cu and/or Zn showed that the heavy metal concentrations needed to induce bacterial community tolerance to antibiotics in soils is high, generally ≥ 1000 mg kg^{-1} (Fernández-Calviño and Bååth, 2013; Song et al., 2017). Although these values are nor very common, they may be reached after accumulation with time, since heavy metals are not degraded in soils. In this regard, Mirlean et al. (2007) found Cu concentrations higher than 1000 mg kg^{-1} in Brazilian vineyard soils. On the other hand, soils contaminated with Zn can reach values much higher than 1000 mg kg^{-1}, as is the case of soils dedicated to orchards in England, with concentrations of 1800 mg kg^{-1} (Brümmer and Herms, 1983), while amended agricultural soils may reach scores around 7000 mg kg^{-1} (Kabata-Pendias, 2000). Soils dedicated to gardens and orchards located in England also reached As concentrations of around 900 mg kg^{-1} (Xu and Thornton, 1985). The same occurs with soils contaminated with Pb, achieving up to 12000 mg kg^{-1} (Godzik et al., 1995) in soils of orchards and gardens in Poland. Therefore, the results of the current research could be relevant in order to define appropriate management practices for wastes and fertilizers in agricultural soils.

2. Material and methods

2.1. Chemicals

Tetracycline hydrochloride (CAS 64-75-5; ≥95% in purity), oxytetracycline hydrochloride (CAS 2058-46-0; ≥95% in purity) and chlortetracycline hydrochloride (CAS 64-72-2; ≥97% in purity), all three supplied by Sigma–Aldrich (Steinheim, Germany), were used for soil spiking.

The heavy metals added to the soil samples for determining bacterial community tolerance were arsenic (as Na_2HAsO_4 $7H_2O$, CAS; 10048-95-0), cadmium (as $Cd(NO_3)_2$ $4H_2O$, CAS 10022-68-1), zinc (as $Zn(NO_3)_2$ $6H_2O$, CAS; 10196-18-6), copper (as $Cu(NO_3)_2$ $2.5H_2O$, CAS 19004-19-4), nickel (as $Ni(NO_3)_2$ $6H_2O$, CAS 13478-00-7), chromium (as $K_2Cr_2O_7$, CAS 7778-60-9), and lead (as $Pb(NO_3)_2$, CAS 10099-74-8), all of them supplied by Panreac (Barcelona, Spain).

2.2. Soil samples

An agricultural soil from Sarria (NW of Spain) was selected form a set of soils previously analyzed by Conde-Cid et al. (2018). Total concentrations of Na, K, Ca, Mg, Al, Fe, Mn, as well as of As, Cd, Cr, Cu, Ni, Pb, and Zn, were determined using ICP-mass spectrometry (820-NS, Varian, Palo Alto, CA, USA), after nitric acid (65%) microwave assisted digestion. As, Cd, Cr, Cu, Ni, Pb, and Zn bioavailability at 0 and 42 days of incubation was assessed performing extractions with 0.01 M $CaCl_2$ (Novozamsky et al., 1993), and with EDTA (ethylenediamine tetra-acetic acid) (Lakanen and Erviö, 1971). The electrical conductivity (EC) was analyzed using distilled water as extraction solution ratio 1:5 (soil:water ratio 1:5) and measuring it in a conductivy-meter. Moreover, this soil was previously used for measurement of the bacterial growth after being polluted with antibiotics (tetracycline, oxytetracycline and chlortetracycline), with results shown in Santás-Miguel et al. (2020b) and Santás-Miguel et al. (2020c).

The main characteristic of the soil studied are shown in Table S1 (Supplementary Material). Briefly, its texture was silt loam, with pH in water of 6.0 and pH in KCl (0.1 M) of 5.2. The organic carbon (C) and total nitrogen (N) contents were 1.8% and 0.2%, respectively and the effective cation exchange capacity (eCEC) was 13.16 $cmol_c\ kg^{-1}$. The electrical conductivity measured was 298 $\mu S\ cm^{-1}$. The total contents (in $mg\ kg^{-1}$) for the measured heavy metals were the following: Cr_T 43.4, Ni_T 25.4, Cu_T 43.4, As_T 27.5, Cd_T <detection limit, Pb_T 17.1, and Zn_T 135.5. These values were similar to those found in non-polluted soils in the study area (Macías and Calvo, 2008).

2.3. Experimental design

The agricultural soil was polluted, separately, with 1000 $mg\ kg^{-1}$ of As, Cd, Zn, Cu, Ni, Cr, and Pb. The procedure was: an amount of 90 g of the air-dried soil was placed in a polypropylene tube (500 mL) and then moistened up to 60-80% of the water holding capacity and incubated for 1 week at 22 °C in the dark, this time being adequate for the recovery and stabilization of the growth of the bacterial communities after moisture adjustment (Meisner et al., 2013). After this time, the soil sample was distributed in 8 polypropylene tubes (100 mL) (putting 12 g of soil, dry weight, in each), with 7 of them being individually polluted with 1000 $mg\ kg^{-1}$ of one of the 7 heavy metals (As, Cd, Zn, Cu, Ni, Cr, or Pb), and the 8th tube acting as control (containing soil without metal). Then, the 8 microcosms were distributed in 24 polypropylene tubes (50 mL) (8 microcosms x 3 replicates), placing 3.75 g (dry weight) in each tube, and they were incubated for 42 days in the dark. After 1 and 42 days of incubation, 1 g of each tube was used to measure the growth of bacterial communities, and after 42 days, 1.75 g samples were used to estimate the bacterial community tolerance to heavy metals. A schematic description of the experimental design is shown in Figure S1 (Supplementary Material).

The concentration of bioavailable heavy metals at days 0 and 42 is shown in Table S2. The concentrations of bioavailable heavy metals at day 0, determined in polluted soil samples extracted with $CaCl_2$, were 617.4, 333.4, 834.8, 71.1, 429.0, 220.3, and 302.2 $mg\ kg^{-1}$ (for As, Cd, Cr, Cu, Ni, Pb, and Zn respectively), whereas, for those extracted with EDTA, they were 917.4, 1016.9, 904.4, 982.1, 802.6, 1080.7, and 953.2 mg kg^{-1} for As, Cd, Cr, Cu, Ni, Pb, and Zn respectively (Table S2, Supplementary Material). The bioavailable concentrations determined in polluted soil samples at day 42 of incubation were 95.7, 140.9, 191.3, 17.4, 252.2, 3.5 and 146.1 mg kg^{-1}, when extracted with $CaCl_2$, while they were 426.1, 852.2, 252.2, 687.0, 800.0, 765.2 and 713.0 mg kg^{-1} when extracted with EDTA, corresponding to As, Cd, Cr, Cu, Ni, Pb, and Zn, respectively, in both extractants (Table S2, Supplementary Material).

The toxicity exerted individually by the heavy metals on the bacterial communities was measured by estimating the bacterial growth on days 1 and 42 of incubation, following the protocol established by Bååth (1994) and Bååth et al. (2001). The bacterial community tolerance was determined according to Bååth (1992), and to Díaz-Raviña et al. (1994), with certain modifications indicated below, and using the leucine incorporation method (Bååth, 1994; Bååth et al., 2001). Briefly, soil samples obtained from each microcosm were mixed with distilled water (rate 1:20 (w/v)), using a multi-vortex shaker for 3 min at maximum intensity. Then, the soil/water mixture was centrifuged at 1000 x *g* for 10 min to obtain the soil bacterial suspension (supernatant). Aliquots of 1.50 mL of the supernatant were transferred to 2 mL centrifugation tubes, in which 150 µL of pollutant solutions (containing heavy metals or antibiotics) were subsequently added. As a result, a total of 7 heavy metals (As, Cd, Zn, Cu, Ni, Cr and Pb) and 3 antibiotics (TC, OTC and CTC) were added to the bacterial suspensions (thus, a total of 10 pollutants were studied), and these plus a control (only water added), were used for each sample. The final concentrations of heavy metals added ranged between 10^{-2} and 10^{-6} mol L^{-1}, while the range for antibiotics went from 400 to $6x10^{-3}$ mg L^{-1}. The bacterial community growth was estimated after a pre-incubation step of 24 h, where bacterial suspensions were added with the different concentrations of antibiotics before the leucine incorporation assay (Berg et al., 2010; Fernández-Calviño and Bååth, 2013). The [^{3}H] leucine incorporation was then measured on each micro-centrifugation tube, as follows: a volume of 0.2 µL [^{3}H] Leu (3.7 MBq mL^{-1} and 0.574 TBq mmol^{-1}; Amersham) was added with non-labeled Leu to each tube, resulting in 275 nM Leu in the bacterial suspensions. After 3 h of incubation, the bacterial growth was terminated by adding 75 mL of 100% trichloroacetic acid. Washing was performed as described by Bååth et al. (2001), and a subsequent measurement of radioactivity was carried out using a liquid scintillation counter (Tri-Carb 2810 TR, PerkinElmer, USA).

2.4. Data analyses

The tolerance of the bacterial community to the seven heavy metals (As, Cd, Zn, Cu, Ni, Cr and Pb) and the three tetracycline antibiotics (TC, OTC and CTC) was estimated as Log IC_{50}, the logarithm of the concentration that resulted in 50% inhibition of bacterial community growth. Log IC_{50} was calculated using a logistic model, as follows:

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

where Y is the measured level of Leu incorporation, X is the logarithm of the contaminant substance (heavy metals (As, Cd, Zn, Cu, Ni, Cr, Pb) or antibiotics (TC, OTC or CTC)) added to the bacterial suspension, a is the log IC_{50}, c the bacterial growth rate in absence of the toxic substance, and b is a slope parameter indicating the inhibition rate. A higher value of log IC_{50} indicates a higher community tolerance, while a lower value indicates that the pollutants (heavy metals or antibiotics) are more toxic to the bacterial community.

3. Results and Discussion

3.1. Toxicity of heavy metals on soil bacteria

The toxicity of the heavy metals on soil bacterial communities was tested in the agricultural soil in the short and long terms (1 and 42 days of incubation). The bacterial growth results obtained for the soil samples polluted with 1000 mg kg^{-1} of As, Cd, Zn, Cu, Ni, Cr, or Pb (separately), and for the unpolluted soil (0 mg kg^{-1}), allowed to stablish a toxicity index for these heavy metals (Table 1). On day 1, the toxicity sequence was the following: Cr≥Cu≥Ni>Cd≥Zn>As≥Pb. After 42 days of incubation the sequence was similar, with changes mainly affecting to Ni and Cd: Cr>Cu>Cd>Ni≥Zn≥As>Pb.

The data obtained in this study differ from those reported by other authors, which was expected, since the toxicity of heavy metals on soil microorganisms depends on the physicochemical characteristics of the soils (such us pH and carbon content), the concentrations of heavy metals added, and the incubation period (Hattori, 1992; Diaz-Raviña and Bååth, 1996; Nannipieri et al., 1997; Ahmad et al., 2005). Hattori (1992) evaluated the influence of Cd, Cr, Cu, Ni, Pb, and Zn on the microorganisms present in two soils after four weeks, and obtained the toxicity sequence Cd> Cu> Ni> Cr> Zn for one of them (a Gleysol, with pH = 5.8 and C = 0.5%), and the sequence Cu> Cd> Ni> Zn> Cr for the other soil (an Andosol, with pH = 6.4 and C = 3.2%). Ahmad et al. (2005) measured the response of the microbial populations of one soil (pH = 7.6 and C = 0.39%) to Cu, Cd, Cr, Hg, Mn, Ni, Pb and Zn at five incubation times, finding that the sensitivity of the bacterial populations depended on the functional group to which they belonged, being the aerobic-heterotrophic bacteria more sensitive to Ni, Pb and Cu, while nitrogen-fixing bacteria were more sensitive to Cd and Pb. Bååth (1989) observed that the relative toxicity of metals decreased in the order Cd> Cu> Zn> Pb, although some differences were found among different investigations. Analyzing data obtained in different studies, it can be observed that, in general, the heavy metals that exert a higher effect on soil microorganisms are Cd and Cu. These results agree with those obtained using as endpoints other organisms, such as *Daphnia magna*, *Vibrio fischeri*, *Lemma minor* and *Eisenia fetida*, which show that there is great variability in the toxicities caused by heavy metals, but also that Cd and Cu are the most toxic (Neuhauser et al., 1985; Arambašić et al., 1995; Huynh and Bulich, 1995; Dirilgen, 2001; Hsieh et al., 2004; Teodorovic et al., 2009).

3.2. Soil bacterial communities' tolerance to heavy metals in the long-term

Figure 1 shows the dose-response curves obtained for each heavy metal after 42 days of incubation, including data for each microcosm. These dose-response curves are sigmoidal, such as those reported by other authors (Díaz-Raviña et al., 1994; Cruz-Paredes et al., 2017; Song et al., 2017; Santás-Miguel et al., 2020a). The inhibition curves show the absence of bacterial growth inhibition at low heavy metals' concentrations, but inhibition takes place at high doses, and it increased with dose. As general trend, it is observed that the inhibition curves obtained for all heavy metals tested shift to the right with respect to the control (unpolluted soil). This shift to the right in the dose-response curves suggests the existence of soil bacterial community tolerance to the high heavy metals' concentrations added on the soil used in this research (1000 mg kg^{-1}).

In this study, the dose-response curves fitted well to the logistic model, with R^2 ranging between 0.966 and 0.991 (mean R^2=0.983) for the control sample (0 mg kg^{-1}), and R^2 between 0.861 and 0.987 (mean R^2= 0.950) for samples polluted with heavy metals (1000 mg kg^{-1} of each one, individually).

Table 2 shows the Log IC_{50} values obtained from each dose-response curve. The unpolluted soil showed the lowest Log IC_{50} value for Cu (-4.17±0.05), and the highest value for Ni (2.56±0.08), i.e, the bacteria found in the bacterial suspension of unpolluted soils show high toxicity effects due to Cu, and lower toxicity due to Ni. As regards the Log IC_{50} values obtained for the whole set of heavy metals tested, the following toxicity sequence was observed for the control (unpolluted soil): Cu>As>Cr≥Pb≥Cd>Zn>Ni. This toxicity sequence is similar to that reported by Díaz-Raviña et al. (1994), who studied the bacterial community tolerance to Cu, Cd, Zn, Ni and Pb using the thymidine incorporation technique, and found that Cu was the most toxic, while Ni showed the lowest toxicity on bacterial communities. However, the order of the rest of heavy metals differed from that observed in the current study, with the sequence being: Cu> Cd> Zn> Pb> Ni.

Regarding the samples polluted with 1000 mg kg^{-1} of each of the heavy metals separately, the lowest Log IC_{50} score corresponded to Cu (-2.61±0.12), and the highest to Zn (-1.63±0.09) (Table 2), i.e., the bacterial communities present in these microcosms are more sensitive to Cu than to Zn. The toxicity sequence, in function of the Log IC_{50} values, was: Cu>Pb≥As≥Cd≥Cr≥Ni≥Zn.

The LogIC$_{50}$ values obtained for each soil sample polluted with the highest heavy metal concentrations increased with respect to the control (unpolluted soil), indicating that the bacteria exposed to high levels of heavy metals developed tolerance to these pollutants. The highest increases in Log IC_{50} (ΔLog IC_{50}, Table 2) corresponded to Cr and Cu (1.6 units), followed by As, Zn, Cd and Pb (1.4, 1.3, 1.2 and 1.1 units, respectively), whereas the increase was of 0.7 units for Ni. To note that the presence of high amounts of heavy metals in the soil causes that bacterial communities increase their tolerance to these pollutants (Díaz-Raviña et al. 1994; Díaz-Raviña and Bååth, 1996; Díaz-Raviña and Bååth, 2001; Fernández-Calviño and Bååth, 2013; Santás-

Miguel et al., 2020a; Zhong et al., 2021), although the magnitude of these increases seems to depend on the toxicity or initial pressure exerted by the metal on the soil bacterial communities.

Figure S2 (Supplementary Material) shows the relation between the increase in tolerance to heavy metals and the toxicity exerted by each metal on the bacterial growth in the short term (1 day) and in the long term (42 days). It can be observed that the higher is the bacterial growth inhibition on days 1 and 42, the greater is the increase in tolerance generated by bacterial communities to heavy metals. This behavior was observed for all the heavy metals studied except Ni. In fact, eliminating Ni the relation becomes significant at day 1 ($r=0.789$, $P <0.05$) and at day 42 ($r=0.730$; $P <0.05$). The toxicity of heavy metals on soil bacterial communities is related to the bioavailability of these heavy metals in the soil. The time-course evolution of heavy metals' bioavailability is shown in Table S2 (Supplementary Material), indicating that, in general, they have a high bioavailability in the short term (Ni being the least bioavailable), while, in the long term (concentrations in EDTA), the bioavailability of heavy metals decreases in a percentage of between 20 and 70%, being less in the case of Ni, which remains constant over time.

The results suggest that the increase in soil bacterial community tolerance after the addition of heavy metals can be attributed to the immediate effect caused by the decay of sensitive species, and to a posterior effect caused by different competitive capacities and ulterior adaptation in the bacteria remaining. This effect of heavy metals in soil has been observed previously by Diaz-Raviña and Bååth (1996) for Zn, Cd, Ni, Cu and Pb, and by Fernandez-Calviño and Bååth (2016) for Cu.

3.3. Soil bacterial communities' tolerance to tetracycline antibiotics in the long-term

Figures 2, 3, and 4 show dose-response curves obtained at 42 days of incubation for each heavy metal added to the soil at 1000 mg kg^{-1}, after the exposure of bacterial suspension from each microcosm to concentrations of tetracyclines antibiotics (TC, OTC and CTC) ranging from 400 to 6x10^{-3} mg L^{-1}. The inhibition curves obtained for these tetracycline antibiotics are sigmoidal. As general trend, the dose-response curves are shifted to the right respect to the control, and in some cases the tetracycline antibiotics did not show an inhibitory effect on bacterial communities.

These dose-response curves fitted well to the logistic model, with R^2 ranging between 0.925 and 0.982 (mean $R^2=0.956$) for TC samples, between 0.771 and 0.997 (mean $R^2= 0.952$) for OTC samples, and from 0.959 to 0.992 (mean $R^2=0.976$) for CTC samples.

Table 3 shows the Log IC_{50} values obtained from each dose-response curve, being 1.83±0.06 for the control sample after exposure of the bacterial suspension to different concentrations of TC. In general, the Log IC_{50} values obtained for each microcosm polluted with 1000 mg kg^{-1} of each heavy metal, and subsequently exposed to TC, increased with respect to the control. The magnitude of these increases varies in function of the heavy metal, being around 0.4 units for Pb, Zn and As, and 0.5 units to Ni. However, the bacterial communities of soils contaminated with Cd, Cu and Cr do not show inhibition in their growth when exposed to TC, and, therefore, their $LogIC_{50}$ cannot be determined due to the high community tolerance to TC. Consequently, heavy metals that do not show inhibition have been assigned a Log IC_{50} value of >2.6, since it is the maximum concentration tested in the current study.

The Log IC_{50} value obtained for the control soil in presence of OTC was 1.11±0.08. Regarding the Log IC_{50} values obtained for the samples polluted with heavy metals, it can be observed that, after the exposure of the bacterial suspension to OTC, there was an increase in these values with respect to the control, going from 0.2 to 0.6 for Pb, Cd, Zn, As and Ni, and being 1.6 for Cu. Regarding Cr, the exposure of the soil bacterial suspension to OTC did not cause inhibition of its growth, and, therefore, as in the case of Cd, Cu and Cu for TC, the value of >2.6 (maximum concentration tested) was assigned.

Finally, the Log IC_{50} value obtained for the control soil added with CTC was 1.52±0.10. After exposure of the bacterial suspensions to CTC, the Log IC_{50} values obtained for the samples polluted with heavy metals showed an increase with respect to the control, going from 1.7 to 2.0 for all the heavy metals except for Cr, which did not show inhibition of bacterial growth when exposed to different concentrations of CTC. Therefore, it can be indicated that bacterial communities exposed to high concentrations of heavy metals show great co-tolerance to CTC. In general, the increase in tolerance obtained for TC, OTC and CTC cannot be related to the toxicity of heavy metals in the soil, at 1 and 42 days of incubation (data not shown).

Co-tolerance to TC generated by soil bacterial communities exposed to heavy metals has been previously demonstrated (Berg et al., 2010; Fernández-Calviño and Bååth, 2013; Song et al. 2017). Song et al. (2017) observed the existence of co-tolerance to TC in a microcosm exposed to concentrations of Cu ≥333 mg kg^{-1} and Zn ≥500 mg kg^{-1}. Fernández-Calviño and Bååth (2013) found co-tolerance to TC of soil bacterial communities in contaminated soils with concentrations of Cu ≥500 mg kg^{-1}. The results shown in these studies agree with what was observed in the present work, indicating that soil bacterial communities exposed to high concentrations of heavy metals (1000 mg kg^{-1}) generate co-tolerance to tetracycline. However, Zhong et al.

(2021) did not observe co-tolerance to TC in soils contaminated with Cu, Zn and Pb, while they found co-tolerance of soil bacterial communities to vancomycin.

This co-tolerance of soil bacterial communities to TC at high concentrations of heavy metals is also observed for OTC and CTC. The results obtained in this study agree with the data reported in studies previously carried out by Santás-Miguel et al. (2020a), where co-tolerance of soil bacterial communities to tetracycline antibiotics (TC, OTC and CTC) was observed in soils with concentrations of Cu ≥1000 mg kg^{-1} after 42 days of incubation.

It is relevant that co-tolerance studies, in general, focus on a reduced number of heavy metals (generally Cu) and tetracycline antibiotics, while the present work shows for the first time the effect of 7 different heavy metals as regards the co-tolerance to the 3 most used tetracycline antibiotics (TC, OTC and CTC).

It is also remarkable that the role of heavy metals as a co-selection factor to generate antibiotic resistance genes has been studied for a wide variety of heavy metals and antibiotics (Berg et al., 2010; Dickinson et al., 2019; Cao et al., 2020; Mazhar et al., 2021), and, although in the present study antibiotic resistance genes (ARGs) have not been studied directly, the resistance of soil bacterial communities to heavy metals and tetracycline antibiotics (TC, OTC, CTC) could be detected using the Leucine incorporation method. The heavy metal levels of the tested microcosms (1000 mg kg^{-1}) are really very high, but they should not be considered unrealistic, since heavy metals are not easily biodegradable, and therefore their concentrations can increase over time (Li et al., 2015), reaching very high levels in the soil (Smolders et al., 2004; Spiteri et al., 2005; Zhuang et al., 2009; Komárek et al., 2010). Therefore, soils contaminated with high concentrations of heavy metals could become hotspots of ARGs, which would pose a potential danger to public health, since they could be transmitted to humans (Forsberg et al., 2012).

4. Conclusions

In the current study it was evidenced that heavy metals present in soils show wide variability as regards the toxicity they exert on bacterial communities, with Cd and Cu being the most toxic. Specifically, soil samples contaminated with a high concentration (1000 mg kg^{-1}) of each of 7 different heavy metals (As, Cd, Zn, Cu, Ni, Cr and Pb), added separately, showed tolerance of the soil bacterial communities to the heavy metals themselves, compared to the control soil. Besides, it was shown that the higher the effect of heavy metals in the short and long terms, the greater the increase in tolerance to metals generated in the soil bacterial communities (except for Ni). In addition, soil samples contaminated with high concentrations of heavy metals also showed co-tolerance of soil bacterial communities to tetracycline, oxytetracycline and chlortetracycline for all the metals tested, compared to the control soil. However, in general, there was no correlation between the initial damage exerted by heavy metals in the short and long terms and the co-tolerance shown by soil bacterial communities to tetracycline antibiotics. Future studies could give further details about the potential interactions of different pollutants present in agricultural soils, which can be considered relevant as regards environmental preservation and public health.

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Tables and figures

Table 1. Toxicity index values ± error of soil samples containing 0 mg kg^{-1} (control soil) and 1000 mg kg^{-1} of As, Cd, Zn, Cu, Ni, Cr or Pb (individually), after 1 and 42 days of incubation.

	Day 1	**Day 42**
Heavy metal	**Toxicity Index ± error**	**Toxicity Index ± error**
As	0.69±0.04	2.39±0.18
Cd	0.12±0.01	0.15±0.00
Zn	0.15±0.04	2.36±0.04
Cu	0.03±0.01	0.06±0.01
Ni	0.05±0.02	2.18±0.39
Cr	0.01±0.01	0.01±0.00
Pb	0.72±0.04	2.76±0.06

Table 2. Estimated values of LogIC_{50}±error and R^2 from fits to a logistic model of soil samples containing 0 mg kg^{-1} (control soil) and 1000 mg kg^{-1} of As, Cd, Zn, Cu, Ni, Cr or Pb (individually). The increases in tolerance ($\Delta\log \text{IC}_{50}= \text{LogIC}_{50}$ polluted-LogIC_{50} control) were calculated for each heavy metal

	Control soil		**Heavy metal-polluted soil (1000 mg kg^{-1})**		
Heavy metal	**Log IC_{50}±error**	**R^2**	**Log IC_{50}±error**	**R^2**	**Δlog IC_{50}**
As	-3.63±0.11	0.970	-2.28±0.04	0.987	1.4
Cd	-3.37±0.07	0.988	-2.21±0.06	0.966	1.2
Zn	-2.89±0.06	0.988	-1.63±0.09	0.966	1.3
Cu	-4.17±0.05	0.991	-2.61±0.12	0.973	1.6
Ni	-2.56±0.08	0.966	-1.82±0.11	0.861	0.7
Cr	-3.44±0.07	0.987	-1.86±0.19	0.938	1.6
Pb	-3.42±0.07	0.989	-2.34±0.06	0.977	1.1

Table 3. Estimated values of LogIC$_{50}$±error and R^2 from fits to a logistic model of soil samples polluted with 1000 mg kg^{-1} of As, Cd, Zn, Cu, Ni, Cr and Pb. The increases in tolerance (Δlog IC$_{50}$= LogIC$_{50}$ polluted-LogIC$_{50}$ control) were calculated for tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC)

	TC			**OTC**			**CTC**		
	Log IC$_{50}$±error	**R^2**	**Δlog IC$_{50}$**	**Log IC$_{50}$±error**	**R^2**	**Δlog IC$_{50}$**	**Log IC$_{50}$±error**	**R^2**	**Δlog IC$_{50}$**
Control	1.83±0.06	0.975	--	1.11±0.08	0.982	--	1.52±0.10	0.959	--
As	2.26±0.09	0.925	0.4	1.65±0.02	0.997	0.5	1.91±0.04	0.985	0.4
Cd	≥2.6*	--	0.8	1.47±0.09	0.973	0.4	1.91±0.01	0.982	0.4
Zn	2.28±0.05	0.971	0.4	1.64±0.04	0.991	0.5	2.19±0.11	0.977	0.7
Cu	≥2.6*	--	0.8	2.67±0.37	0.771	1.6	1.82±0.08	0.959	0.3
Ni	2.33±0.04	0.982	0.5	1.68±0.07	0.968	0.6	1.75±0.04	0.992	0.2
Cr	≥2.6*	--	0.8	≥2.6*	--	1.5	≥2.6*	--	1.1
Pb	2.23±0.08	0.926	0.4	1.34±0.08	0.981	0.2	1.99±0.03	0.976	0.5

* The value of 2.6 is the maximum value tested

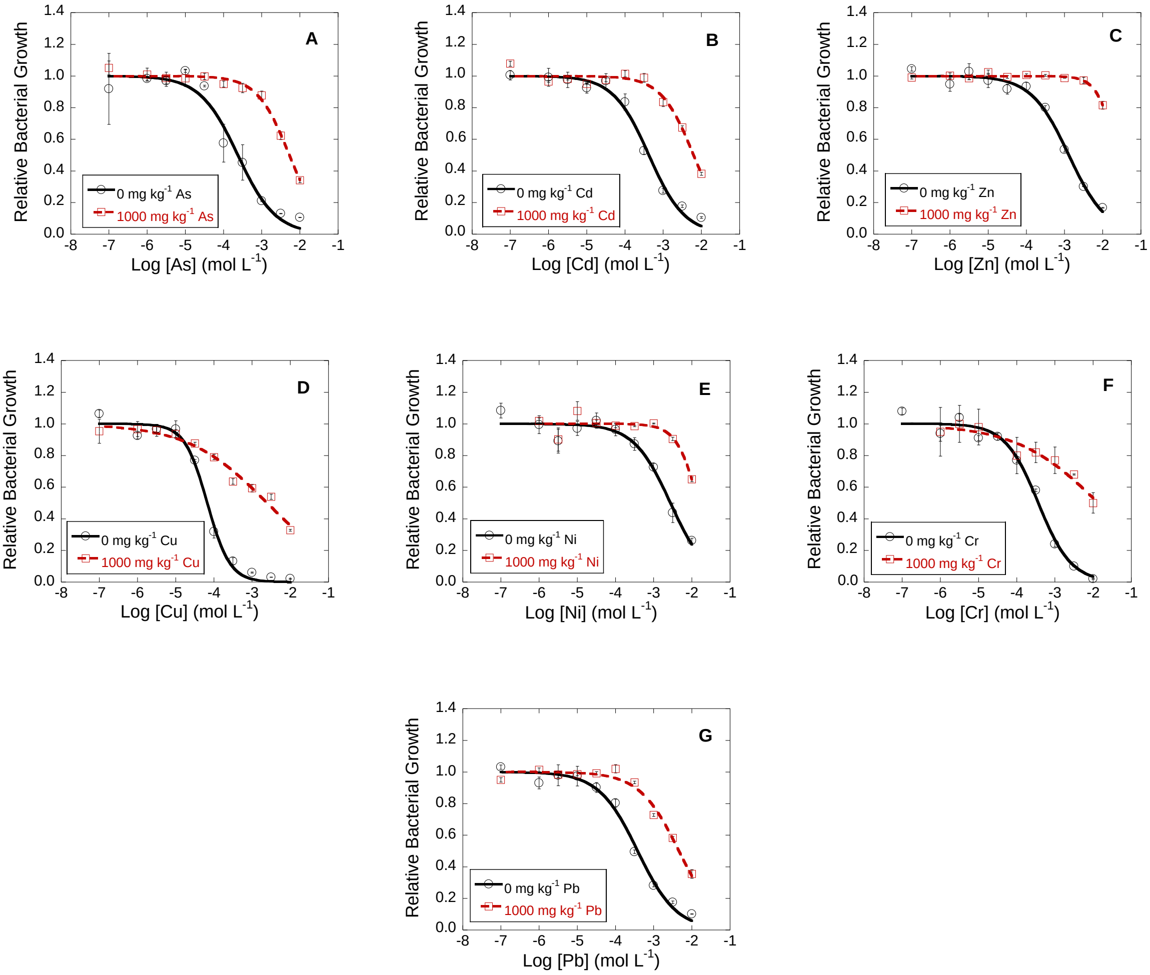

Figure 1. Dose-response curves obtained after the addition of 10 different concentrations of the heavy metals As (A), Cd (B), Zn (C), Cu (D), Ni (E), Cr (F) and Pb (G) to bacterial suspensions extracted from soils polluted with 1000 mg kg^{-1} of each of the same heavy metals, separately, after 42 days of incubation. Black lines represent the dose-response curves corresponding to the unpolluted soil (control) and discontinued red lines are dose-response curves corresponding to the soil polluted with 1000 mg kg^{-1} of heavy metal. Average values (n=3) with coefficients of variation always <5%

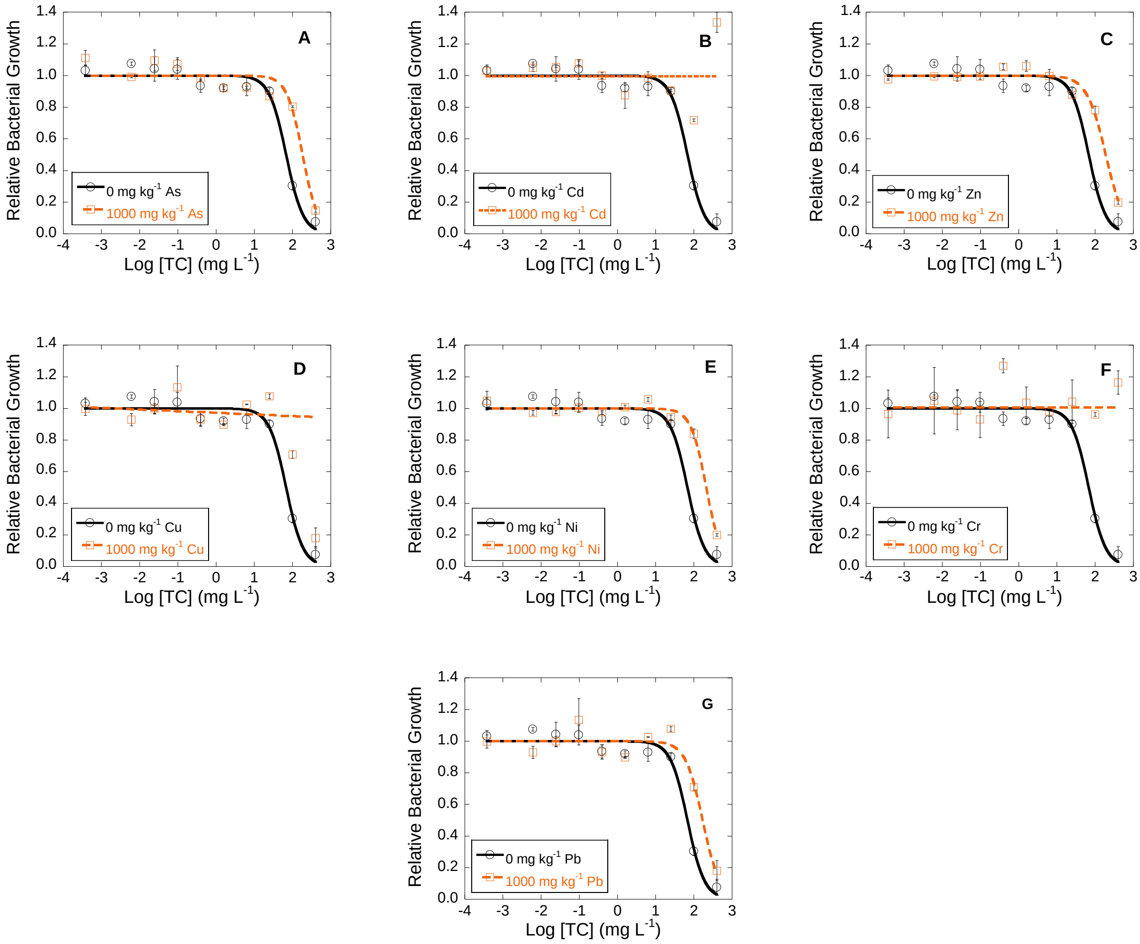

Figure 2. Dose-response curves obtained after the addition of 10 different concentrations of tetracycline (TC) to bacterial suspension extracted from soil samples polluted with 1000 mg kg^{-1} of As (A), Cd (B), Zn (C), Cu (D), Ni (E), Cr (F) or Pb (G), each of them added separately, after 42 days of incubation. Black lines represent the dose-response curves obtained for the unpolluted soil (control) and discontinued orange lines are dose-response curves for soils polluted with 1000 mg kg^{-1} of one of the heavy metals. Average values (n=3) with coefficients of variation always <5%.

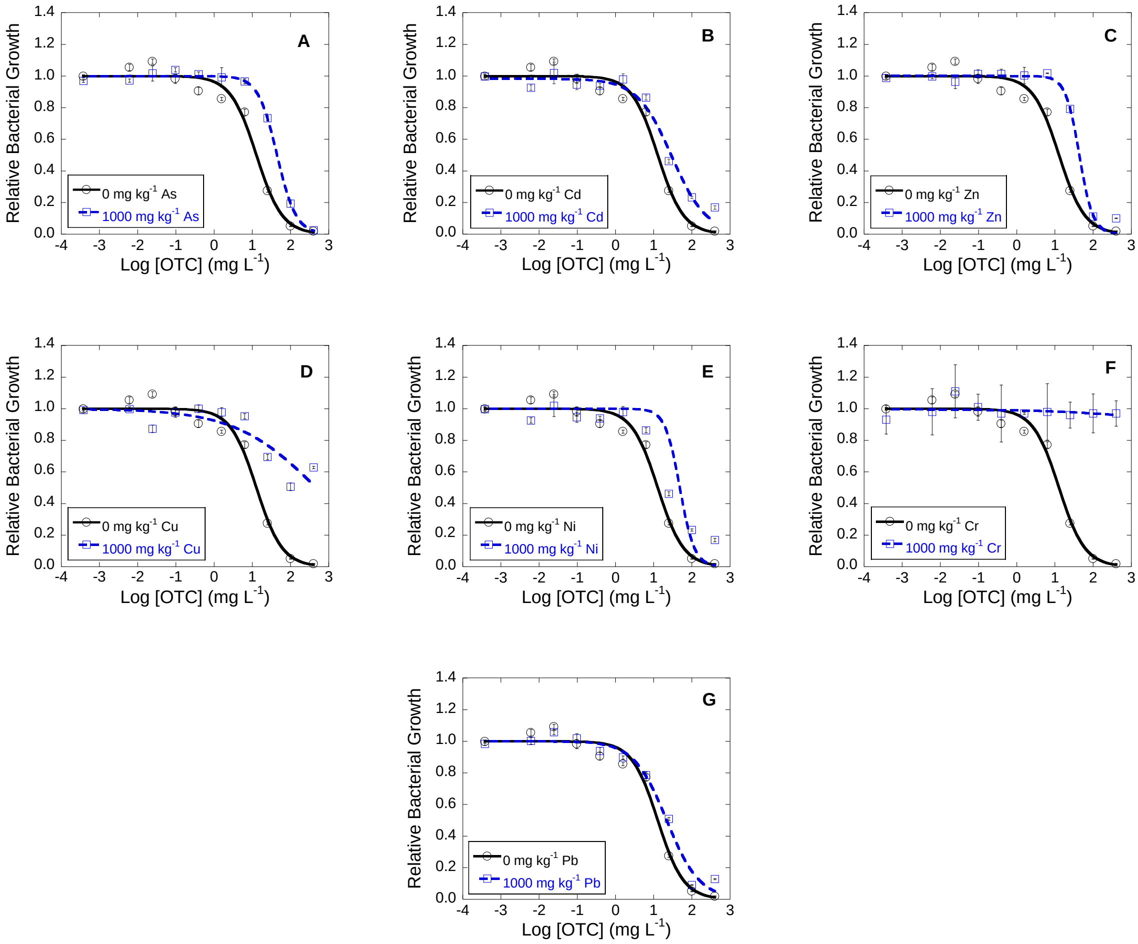

Figure 3. Dose-response curves obtained after the addition of 10 different concentration of oxytetracycline (OTC) to bacterial suspension extracted from soil samples polluted with 1000 mg kg^{-1} of As (A), Cd (B), Zn (C), Cu (D), Ni (E), Cr (F) or Pb (G), each of them added separately, after 42 days of incubation. Black lines represent the dose-response curves obtained for the unpolluted soil (control) and discontinued blue lines are dose-response curves for soils polluted with 1000 mg kg^{-1} of one of the heavy metals. Average values (n=3) with coefficients of variation always <5%.

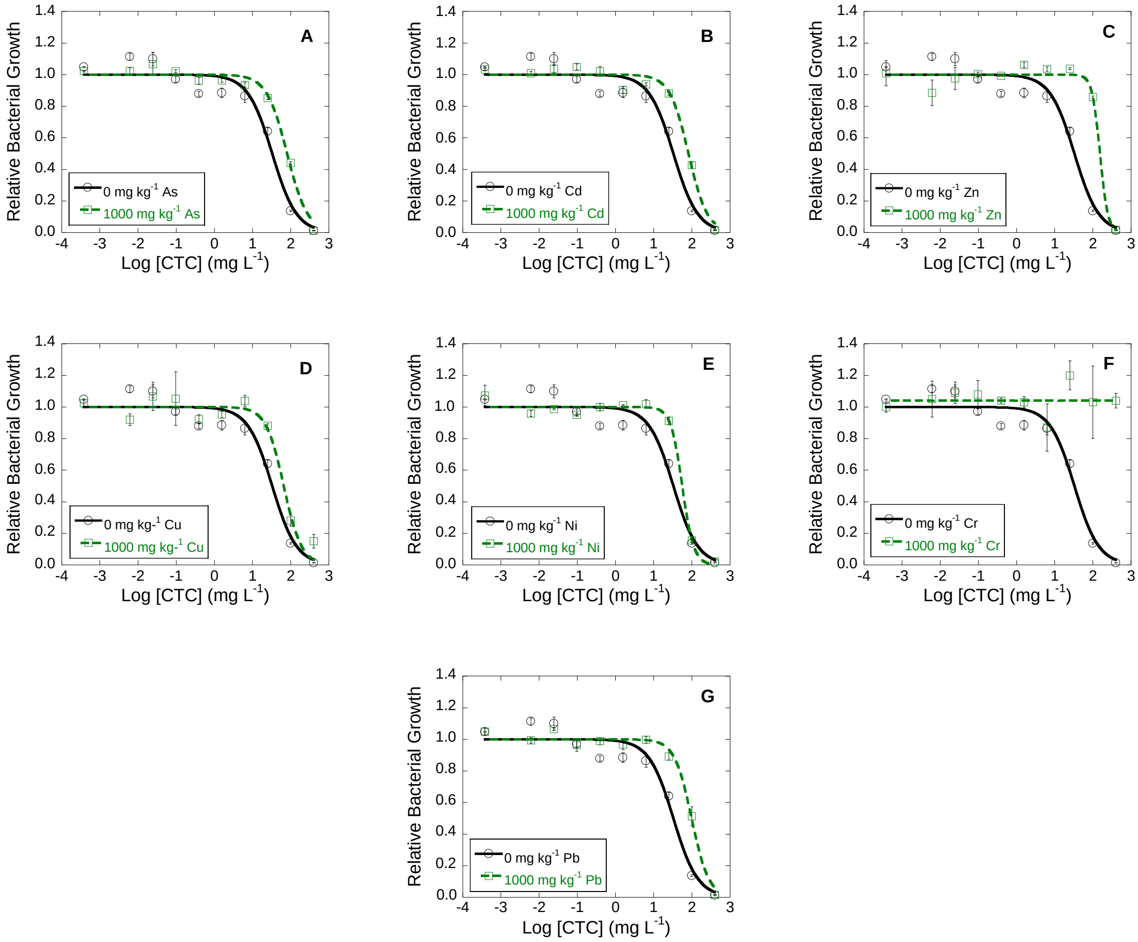

Figure 4. Dose-response curves obtained after the addition of 10 different concentration of chlortetracycline (CTC) to bacterial suspension extracted from soil samples polluted with 1000 mg kg^{-1} of As (A), Cd (B), Zn (C), Cu (D), Ni (E), Cr (F) or Pb (G), each of them added separately, after 42 days of incubation. Black lines represent the dose-response curves obtained for the unpolluted soil (control) and discontinued green lines are dose-response curves for soils polluted with 1000 mg kg^{-1} of one of the heavy metals. Average values (n=3) with coefficients of variation always <5%.