

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 IC₅₀ (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.

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

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34 1. Introduction

35 The accumulation of heavy metals in soils is a widespread problem all over the world. Some
36 anthropogenic activities such as mining, industrial production, agriculture, or livestock farming, are among
37 the main sources of release of heavy metals to the terrestrial environment (Abdu et al., 2017). The use of
38 heavy metals in livestock production is a common practice, since commercial feeds are enriched with
39 essential elements (such as Cu and Zn) to prevent diseases and obtain an optimal growth rate (Sager, 2007;
40 Wu et al., 2013). In fact, the presence of high heavy metal concentrations in swine manure has been reported
41 by Hölzel et al. (2012), specifically for Zn (up to 8239 mg kg⁻¹), and for Cu (up to 3387 mg kg⁻¹). In addition,
42 Wang et al. (2013) found up to 1602 mg kg⁻¹ for Cr in layer manure, while Zhang et al. (2012) detected up to
43 300 mg kg⁻¹ for Pb, and up to 100 mg kg⁻¹ for Ni, in poultry manure. Finally, Liu et al. (2020) reported up to
44 60 mg kg⁻¹ for Cd, and up to 89 mg kg⁻¹ for As, in pig manure. In view of that, these and other heavy metals
45 are considered a serious problem for public health, especially because these toxic elements tend to
46 accumulate in soils, where they may reach high and progressively increasing concentrations (Kabata-Pendias,
47 2000; Huang et al., 2007). Moreover, veterinary antibiotics are also widely used for the treatment of bacterial
48 infections (Boxal et al., 2003), and their consumption has been increasing in recent decades, with the estimate
49 of world use for 2030 being 105,596 tons (Van Boeckel et al., 2015). The antibiotics most widely used in
50 veterinary medicine in the European Union are tetracyclines (TCs), and specifically tetracycline (TC),
51 oxytetracycline (OTC) and chlortetracycline (CTC) (European Medicines Agency, 2016). Both heavy metals
52 and antibiotics for veterinary use are poorly absorbed by the intestines of animals, causing that a high
53 percentage of them is excreted in feces and urine (Kornegay et al., 1976; Sarmah et al., 2006). In this sense, it
54 has been reported that tetracycline antibiotics concentrations in manures may reach values as high as 746 mg
55 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.,
56 2016). The presence of high levels of heavy metals and veterinary antibiotics in farmland soils is due to the
57 repeated applications of manure and slurries (as well as sewage sludge) as organic fertilizers (Hamscher et
58 al., 2002; Nicholson et al., 2003). Once in the soil, these compounds can interact with soil microbial
59 communities and modify their structure and function (Hattori, 1992; Thiele-Bruhn and Beck, 2005; Chien et
60 al., 2008; Giller et al., 2009; Caban et al., 2018).

61 The increase of the concentration of any pollutant in soils may suppose a selection pressure for soil
62 bacterial communities, causing tolerance to that pollutant (Blanck, 2002). This effect may be useful to
63 quantify the harmful repercussions produced by pollutants on soil bacteria and is called pollution-induced

64 community tolerance (PICT). Agricultural soils, highly influenced by anthropogenic activities, deserve
65 special attention since they are recognized as the largest reservoirs of antibiotic-resistant genes, receiving
66 antibiotics from veterinary use (through repeated applications of manure and slurries, as indicated above), as
67 well as heavy metals (Ji et al., 2012). Therefore, the resistance of soil bacterial communities to antibiotics has
68 become a crucial threat at a world scale, and the study of whether bacterial communities generate co-
69 tolerance to antibiotics in the presence of heavy metals (as well as **on** how and in which degree is developed)
70 is of vital importance.

71 Several previous studies have focused **on co-tolerance** among different heavy metals and antibiotics (Berg et
72 al., 2010; Sarma et al., 2010; Fernández-Calviño and Bååth, 2013; Song et al., 2017; Santás-Miguel et al.,
73 2020a; Zhong et al., 2021). Specifically, Fernández-Calviño and Bååth (2013) evaluated the tolerance of soil
74 bacterial community to three antibiotics (vancomycin, tetracycline and tylosin), in one soil polluted with
75 different Cu concentrations (2-32 mmol Cu kg⁻¹) and observed an increase in the co-tolerance of soil bacterial
76 communities to tetracycline (at ≥ 8 mmol Cu kg⁻¹), tylosin (at ≥ 16 mmol Cu kg⁻¹) and vancomycine (at ≥ 16
77 mmol Cu kg⁻¹). Song et al. (2017) assessed the effects of adding different Cu and Zn concentrations (33-
78 1000 mg kg⁻¹, and 165-5000 mg kg⁻¹, respectively) on the tolerance of soil bacterial communities to
79 tetracycline, finding that the co-tolerance to the antibiotic significantly increased in soils where Cu
80 concentration were ≥ 365 mg kg⁻¹, and Zn concentrations were ≥ 264 mg kg⁻¹. Zhong et al. (2021) studied
81 the tolerance to tetracycline and vancomycin in 10 mine soil samples with the presence of Cu (361-4399 mg
82 kg⁻¹), Zn (33-3811 mg kg⁻¹) and Pb (195-20,239 mg k⁻¹). These authors found that, in general, there were
83 increases in tolerance of soil bacterial communities to antibiotics, although they did not detect a systematic
84 pattern in the co-tolerance to tetracycline associated to heavy metals concentrations, whereas the induced
85 levels of heavy metal tolerance coincided with elevated levels of tolerance to vancomycin. However, until
86 now, there were not studies evaluating the effect of a wide range of different heavy metals **present in**
87 **livestock manure** on the tolerance shown by soil bacterial communities against each of the three tetracycline
88 antibiotics most used in animal **husbandry**. Therefore, the objective of this study is to determine the eventual
89 development of tolerance **in** soil bacterial communities to heavy metals in agricultural soils contaminated
90 individually with 1000 mg kg⁻¹ of As, Cd, Zn, Cu, Ni, Cr and Pb, and also the eventual generation of co-
91 tolerance to the antibiotics tetracycline, oxytetracycline and chlortetracycline, using the leucine incorporation
92 technique as the endpoint. Previous works using Cu and/or Zn showed that the heavy metal concentrations
93 needed to induce bacterial community tolerance to antibiotics in soils are high, generally ≥ 1000 mg kg⁻¹

94 (Fernández-Calviño and Bååth, 2013; Song et al., 2017). Although these values are nor very frequent, could
95 be reached after enough time elapsed to accumulate, since heavy metals are not degraded in soils. In this
96 regard, Mirlean et al. (2007) found Cu concentrations higher than 1000 mg kg⁻¹ in Brazilian vineyard soils.
97 Furthermore, Zn polluted soils can reach values higher than 1000 mg kg⁻¹, as reported by (Brümmer and
98 Herms, 1983) for soils dedicated to orchards in England, with concentrations of 1800 mg kg⁻¹, while some
99 amended agricultural soils may reach scores around 7000 mg kg⁻¹ (Kabata-Pendias, 2000). Soils dedicated to
100 gardens and orchards located in England also reached arsenic concentrations of around 900 mg kg⁻¹ (Xu and
101 Thornton, 1985). The same occurs with soils contaminated with Pb, achieving up to 12,000 mg kg⁻¹ in soils
102 of orchards and gardens in Poland (Godzik et al., 1995). Therefore, the results of the current research could
103 be relevant in order to define appropriate management practices for wastes and fertilizers in agricultural soils.

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107 **2. Material and methods**

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109 **2.1. Chemicals**

110 Tetracycline hydrochloride (CAS 64-75-5; ≥95% in purity), oxytetracycline hydrochloride (CAS
111 2058-46-0; ≥95% in purity) and chlortetracycline hydrochloride (CAS 64-72-2; ≥97% in purity), all three
112 supplied by Sigma–Aldrich (Steinheim, Germany), were used for soil spiking.

113 The heavy metals added to the soil samples for determining bacterial community tolerance were
114 arsenic (as Na₂HAsO₄ 7H₂O, CAS; 10048-95-0), cadmium (as Cd(NO₃)₂ 4H₂O, CAS 10022-68-1), zinc (as
115 Zn(NO₃)₂ 6H₂O, CAS; 10196-18-6), copper (as Cu(NO₃)₂ 2.5H₂O, CAS 19004-19-4), nickel (as Ni(NO₃)₂
116 6H₂O, CAS 13478-00-7), chromium (as K₂Cr₂O₇, CAS 7778-60-9), and lead (as Pb(NO₃)₂, CAS 10099-74-
117 8), all of them supplied by Panreac (Barcelona, Spain).

118

119 **2.2. Soil samples**

120 An agricultural soil from Sarria (NW of Spain) was selected from a set of soils previously analyzed
121 by Conde-Cid et al. (2018). Total concentrations of Na, K, Ca, Mg, Al, Fe, Mn, as well as of As, Cd, Cr, Cu,
122 Ni, Pb, and Zn, were determined using ICP-mass spectrometry (820-NS, Varian, Palo Alto, CA, USA),
123 which was carried out after performing a microwave-assisted digestion using 65% nitric acid. As, Cd, Cr, Cu,

124 Ni, Pb, and Zn bioavailability at 0 and 42 days of incubation was assessed performing extractions with 0.01
125 M CaCl₂ (Novozamsky et al., 1993), and with EDTA (ethylenediamine tetra-acetic acid) (Lakanen and Erviö,
126 1971). The electrical conductivity (EC) was analyzed using distilled water for extraction, with 1.5 as
127 soil:water ratio, and measuring it in a conductivity-meter. Moreover, this soil was previously used for
128 measurement of the bacterial growth after being polluted with antibiotics (tetracycline, oxytetracycline and
129 chlortetracycline), with results shown in Santás-Miguel et al. (2020b) and Santás-Miguel et al. (2020c).
130 The main characteristic of the soil studied are shown in Table S1 (Supplementary Material). Briefly, its
131 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
132 nitrogen (N) contents were 1.8% and 0.2%, respectively and the effective cation exchange capacity (eCEC)
133 was 13.2 cmol_c kg⁻¹. The electrical conductivity value was 298.0 μS cm⁻¹. The total contents (in mg kg⁻¹) for
134 the determined heavy metals were the following: Cr_T 43.4, Ni_T 25.4, Cu_T 43.4, As_T 27.5, Cd_T <detection
135 limit, Pb_T 17.1, and Zn_T 135.5. These values were similar to those found in non-polluted soils in the study
136 area (Macías and Calvo, 2008).

137

138 **2.3. Experimental design**

139 The agricultural soil was polluted, separately, with 1000 mg kg⁻¹ of As, Cd, Zn, Cu, Ni, Cr, and Pb.
140 The procedure was: an amount of 90 g of the air-dried soil was placed in a polypropylene tube (500 mL) and
141 then moistened up to 60-80% of the water holding capacity and incubated for 1 week at 22 °C in the dark,
142 this time being adequate for the recovery and stabilization of the growth of the bacterial communities after
143 moisture adjustment (Meisner et al., 2013). After this time, the soil sample was distributed in 8
144 polypropylene tubes (100 mL) (putting 12 g of soil, dry weight, in each), with 7 of them being individually
145 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
146 acting as control (containing soil without metal). Then, the 8 microcosms were distributed in 24
147 polypropylene tubes (50 mL) (8 microcosms x 3 replicates), placing 3.8 g (dry weight) in each tube, and they
148 were incubated for 42 days in the dark. After 1 and 42 days of incubation, 1 g of each tube was used to
149 measure the growth of bacterial communities, and after 42 days, 1.8 g samples were used to estimate the
150 bacterial community tolerance to heavy metals. A schematic description of the experimental design is shown
151 in Figure S1 (Supplementary Material).

152 The concentration of bioavailable heavy metals at days 0 and 42 is shown in Table S2
153 (Supplementary Material). The concentrations of bioavailable heavy metals at day 0, determined in polluted

154 soil samples extracted with CaCl₂, were: 617.4, 333.4, 834.8, 71.1, 429.0, 220.3, and 302.2 mg kg⁻¹ (for As,
155 Cd, Cr, Cu, Ni, Pb, and Zn respectively), whereas, for those extracted with EDTA, the concentrations were
156 917.4, 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
157 (Table S2, Supplementary Material). The bioavailable concentrations determined in polluted soil samples at
158 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 CaCl₂,
159 while the values were 426.1, 852.2, 252.2, 687.0, 800.0, 765.2 and 713.0 mg kg⁻¹ when extracted with EDTA,
160 corresponding to As, Cd, Cr, Cu, Ni, Pb, and Zn, respectively, in both extractants (Table S2, Supplementary
161 Material).

162 The toxicity exerted individually by the heavy metals on the bacterial communities was measured by
163 estimating the bacterial growth on days 1 and 42 of incubation, following the protocol established by Bååth
164 (1994) and Bååth et al. (2001). The bacterial community tolerance was determined according to Bååth
165 (1992), and to Díaz-Raviña et al. (1994), with certain modifications indicated below, and using the leucine
166 incorporation method (Bååth, 1994; Bååth et al., 2001). Briefly, soil samples obtained from each microcosm
167 were mixed with distilled water (rate 1:20 (w/v)), using a multi-vortex shaker for 3 min at maximum
168 intensity. Then, the soil/water mixture was centrifuged at 1000 x g for 10 min to obtain the soil bacterial
169 suspension (supernatant). Aliquots of 1.5 mL of the supernatant were transferred to 2 mL centrifugation
170 tubes, in which 150 µL of pollutant solutions (containing heavy metals or antibiotics) were subsequently
171 added. As a result, a total of 7 heavy metals (As, Cd, Zn, Cu, Ni, Cr and Pb) and 3 antibiotics (TC, OTC and
172 CTC) were added to the bacterial suspensions (thus, a total of 10 pollutants were studied), and these plus a
173 control (only water added), were used for each sample. The final concentrations of heavy metals added
174 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
175 bacterial community growth was estimated after a pre-incubation step of 24 h, where bacterial suspensions
176 were added with the different concentrations of antibiotics before the leucine incorporation assay (Berg et al.,
177 2010; Fernández-Calviño and Bååth, 2013). The [³H] leucine incorporation was then measured on each
178 micro-centrifugation tube, as follows: a volume of 0.2 µL [³H] Leu (3.7 MBq mL⁻¹ and 0.574 TBq mmol⁻¹;
179 Amersham) was added with non-labeled Leu to each tube, resulting in 275 nM Leu in the bacterial
180 suspensions. After 3 h of incubation, the bacterial growth was terminated by adding 75 mL of 100%
181 trichloroacetic acid. Washing was performed as described by Bååth et al. (2001), and a subsequent
182 measurement of radioactivity was carried out using a liquid scintillation counter (Tri-Carb 2810 TR,
183 PerkinElmer, USA).

184

185 **2.4. Data analyses**

186 The tolerance of the bacterial community to the seven heavy metals (As, Cd, Zn, Cu, Ni, Cr and Pb)
187 and the three tetracycline antibiotics (TC, OTC and CTC) was estimated as Log IC₅₀, the logarithm of the
188 concentration that resulted in 50% inhibition of bacterial community growth. Log IC₅₀ was calculated using a
189 logistic model, as follows:

$$190 Y = c/[1 + e^{b(X-a)}]$$

191 where Y is the measured level of Leu incorporation, X is the logarithm of the **contaminant** substance (heavy
192 metals (As, Cd, Zn, Cu, Ni, Cr, Pb) or antibiotics (TC, OTC or CTC)) added to the bacterial suspension, a is
193 the log IC₅₀, c the bacterial growth rate in absence of the toxic substance, and b is a slope parameter
194 indicating the inhibition rate. A higher value of log IC₅₀ indicates a higher community tolerance, while a
195 lower value indicates that the pollutants (heavy metals or antibiotics) are more toxic to the bacterial
196 community.

197

198 3. Results and Discussion

199 3.1. Toxicity of heavy metals on soil bacteria

200 The toxicity of the heavy metals on soil bacterial communities was tested in the agricultural soil in
201 the short and long terms (1 and 42 days of incubation). The bacterial growth results obtained for the soil
202 samples polluted with 1000 mg kg⁻¹ of As, Cd, Zn, Cu, Ni, Cr, or Pb (separately), and for the unpolluted soil
203 (0 mg kg⁻¹), allowed to establish a toxicity index for these heavy metals (Table 1). On day 1, the toxicity
204 sequence was the following: Cr≥Cu≥Ni>Cd>Zn>As>Pb. After 42 days of incubation the sequence was
205 similar, with changes mainly affecting to Ni and Cd: Cr>Cu>Cd>Ni≥Zn≥As>Pb.

206 The data obtained in this study differ from those reported by other authors, which was expected,
207 since the toxicity of heavy metals on soil microorganisms depends on the physicochemical characteristics of
208 the soils (such as pH and carbon content), the concentrations of heavy metals added, and the incubation
209 period (Hattori, 1992; Diaz-Raviña and Bååth, 1996; Nannipieri et al., 1997; Ahmad et al., 2005). Hattori
210 (1992) evaluated the influence of Cd, Cr, Cu, Ni, Pb, and Zn on the microorganisms present in two soils after
211 four weeks, and obtained the toxicity sequence Cd> Cu> Ni> Cr> Zn for one of them (a Gleysol, with pH =
212 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
213 C = 3.2%). Ahmad et al. (2005) measured the response of the microbial populations of one soil (pH = 7.6 and
214 C = 0.4%) to Cu, Cd, Cr, Hg, Mn, Ni, Pb and Zn at five incubation times, finding that the sensitivity of the
215 bacterial populations depended on the functional group to which they belonged, being the aerobic-
216 heterotrophic bacteria more sensitive to Ni, Pb and Cu, while nitrogen-fixing bacteria were more sensitive to
217 Cd and Pb. Bååth (1989) observed that the relative toxicity of metals decreased in the order Cd> Cu> Zn>
218 Pb, although some differences were found among different investigations. Analyzing data obtained in
219 different studies, it can be observed that, in general, the heavy metals that exert a higher effect on soil
220 microorganisms are Cd and Cu. These results agree with those obtained using as endpoints other organisms,
221 such as *Daphnia magna*, *Vibrio fischeri*, *Lemma minor* and *Eisenia fetida*, which show that there is great
222 variability in the toxicities caused by heavy metals, but also that Cd and Cu are the most toxic (Neuhauser et
223 al., 1985; Arambašić et al., 1995; Huynh and Bulich, 1995; Dirilgen, 2001; Hsieh et al., 2004; Teodorovic et
224 al., 2009).

225

226 3.2. Soil bacterial communities' tolerance to heavy metals in the long-term

227 Figure 1 shows the dose-response curves obtained for each heavy metal after 42 days of incubation,
228 including data for each microcosm. These dose-response curves are sigmoidal, such as those reported by
229 other authors (Díaz-Raviña et al., 1994; Cruz-Paredes et al., 2017; Song et al., 2017; Santás-Miguel et al.,
230 2020a). The inhibition curves show the absence of bacterial growth inhibition at low heavy metals'
231 concentrations, but inhibition takes place at high doses, and it increased with dose. As general trend, it is
232 observed that the inhibition curves obtained for all heavy metals tested shift to the right with respect to the
233 control (unpolluted soil). This shift to the right in the dose-response curves suggests the existence of soil
234 bacterial community tolerance to the high heavy metals' concentrations added on the soil used in this
235 research (1000 mg kg⁻¹).

236 In this study, the dose-response curves fitted well to the logistic model, with R² ranging between
237 0.966 and 0.991 (mean R²=0.983) for the control sample (0 mg kg⁻¹), and R² between 0.861 and 0.987 (mean
238 R²= 0.950) for samples polluted with heavy metals (1000 mg kg⁻¹ of each one, individually).

239 Table 2 shows the Log IC₅₀ values obtained from each dose-response curve. The unpolluted soil
240 showed the lowest Log IC₅₀ value for Cu (-4.17±0.05), and the highest value for Ni (2.56±0.08), i.e., the
241 bacteria found in the bacterial suspension of unpolluted soils show high toxicity effects due to Cu, and lower
242 toxicity due to Ni. As regards the Log IC₅₀ values obtained for the whole set of heavy metals tested, the
243 following toxicity sequence was observed for the control (unpolluted soil): Cu>As>Cr≥Pb≥Cd>Zn>Ni. This
244 toxicity sequence is similar to that reported by Díaz-Raviña et al. (1994), who studied the bacterial
245 community tolerance to Cu, Cd, Zn, Ni and Pb using the thymidine incorporation technique, and found that
246 Cu was the most toxic, while Ni showed the lowest toxicity on bacterial communities. However, the order of
247 the rest of heavy metals differed from that observed in the current study, with the sequence being: Cu> Cd>
248 Zn> Pb> Ni.

249 Regarding the samples polluted with 1000 mg kg⁻¹ of each of the heavy metals separately, the lowest
250 Log IC₅₀ score corresponded to Cu (-2.61±0.12), and the highest to Zn (-1.63±0.09) (Table 2), i.e., the
251 bacterial communities present in these microcosms are more sensitive to Cu than to Zn. The toxicity
252 sequence, in function of the Log IC₅₀ values, was: Cu>Pb≥As≥Cd≥Cr≥Ni≥Zn.

253 The LogIC₅₀ values obtained for each soil sample polluted with the highest heavy metal
254 concentrations increased with respect to the control (unpolluted soil), indicating that the bacteria exposed to
255 high levels of heavy metals developed tolerance to these pollutants. The highest increases in Log IC₅₀ (ΔLog
256 IC₅₀, Table 2) corresponded to Cr and Cu (1.58 and 1.56 units, respectively), followed by As, Zn, Cd and Pb

257 (1.35, 1.26, 1.16 and 1.08 units, respectively), whereas the increase was of 0.74 units for Ni. To note that the
258 presence of high amounts of heavy metals in the soil causes that bacterial communities increase their
259 tolerance to these pollutants (Díaz-Raviña et al. 1994; Díaz-Raviña and Bååth, 1996; Díaz-Raviña and Bååth,
260 2001; Fernández-Calviño and Bååth, 2013; Santás-Miguel et al., 2020a; Zhong et al., 2021), although the
261 magnitude of these increases seems to depend on the toxicity or initial pressure exerted by the metal on the
262 soil bacterial communities.

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

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

279

280 3.3. Soil bacterial communities' tolerance to tetracycline antibiotics in the long-term

281 Figures 2, 3, and 4 show dose-response curves obtained at 42 days of incubation for each heavy
282 metal added to the soil at 1000 mg kg^{-1} , after the exposure of bacterial suspension from each microcosm to
283 concentrations of tetracyclines antibiotics (TC, OTC and CTC) ranging from 400 to $6 \times 10^{-3} \text{ mg L}^{-1}$. The
284 inhibition curves obtained for these tetracycline antibiotics are sigmoidal. As general trend, the dose-response
285 curves are shifted to the right respect to the control, and in some cases the tetracycline antibiotics did not
286 show an inhibitory effect on bacterial communities.

287 These dose-response curves fitted well to the logistic model, with R^2 ranging between 0.925 and
288 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
289 from 0.959 to 0.992 (mean $R^2=0.976$) for CTC samples.

290 Table 3 shows the Log IC_{50} values obtained from each dose-response curve, being 1.83 ± 0.06 for the
291 control sample after exposure of the bacterial suspension to different concentrations of TC. In general, the
292 Log IC_{50} values obtained for each microcosm polluted with 1000 mg kg^{-1} of each heavy metal, and
293 subsequently exposed to TC, increased with respect to the control. The magnitude of these **increases** varies in
294 function of the heavy metal, being 0.40, 0.45 and 0.43 units for Pb, Zn and As, and 0.50 units to Ni.
295 However, the bacterial communities of soils contaminated with Cd, Cu and Cr do not show inhibition in their
296 growth when exposed to TC, **and**, therefore, their Log IC_{50} cannot be determined due to the high community
297 tolerance to TC. Consequently, heavy metals that do not show inhibition have been assigned a Log IC_{50} value
298 of >2.60 , since it is the maximum concentration tested in the current study.

299 The Log IC_{50} value obtained for the control soil in presence of OTC was 1.11 ± 0.08 . Regarding the
300 Log IC_{50} values obtained for the samples polluted with heavy metals, it can be observed that, after the
301 exposure of the bacterial suspension to OTC, there was an increase in these values with respect to the control,
302 going from 0.23 to 0.57 for Pb, Cd, Zn, As and Ni, and being 1.56 for Cu. Regarding Cr, the exposure of the
303 soil bacterial suspension to OTC did not cause inhibition of its growth, **and**, therefore, as in the case of Cd,
304 Cu and Cu for TC, the value of >2.60 (maximum concentration tested) was assigned.

305 Finally, the Log IC_{50} value obtained for the control soil added with CTC was 1.52 ± 0.10 . After
306 exposure of the bacterial suspensions to CTC, the Log IC_{50} values obtained for the samples polluted with
307 heavy metals showed an increase with respect to the control, going from 1.75 to 2.19 for all **the** heavy metals
308 except for Cr, which did not show inhibition of bacterial growth when exposed to different concentrations of
309 CTC. Therefore, it can be indicated that bacterial communities exposed to high concentrations of heavy
310 metals show great co-tolerance to CTC. In general, the increase in tolerance obtained for TC, OTC and CTC
311 cannot be related to the toxicity of heavy metals in the soil, at 1 and 42 days of incubation (data not **shown**).

312 Co-tolerance to TC generated by soil bacterial communities exposed to heavy metals has been
313 previously demonstrated (Berg et al., 2010; Fernández-Calviño and Bååth, 2013; Song et al. 2017). Song et
314 al. (2017) observed the existence of co-tolerance to TC in a microcosm exposed to concentrations of $\text{Cu} \geq 333$
315 mg kg^{-1} and $\text{Zn} \geq 500 \text{ mg kg}^{-1}$. Fernández-Calviño and Bååth (2013) found co-tolerance **to TC** of soil bacterial
316 communities in contaminated soils with concentrations of $\text{Cu} \geq 500 \text{ mg kg}^{-1}$. The results shown in these

317 studies agree with what was observed in the present work, indicating that soil bacterial communities exposed
318 to high concentrations of heavy metals (1000 mg kg⁻¹) generate co-tolerance to tetracycline. However, Zhong
319 et al. (2021) did not observe co-tolerance to TC in soils contaminated with Cu, Zn and Pb, while they found
320 co-tolerance of soil bacterial communities to vancomycin.

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

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

330 It is also remarkable that the role of heavy metals as a co-selection factor to generate antibiotic
331 resistance genes has been studied for a wide variety of heavy metals and antibiotics (Berg et al., 2010;
332 Dickinson et al., 2019; Cao et al., 2020; Mazhar et al., 2021), and, although in the present study antibiotic
333 resistance genes (ARGs) have not been studied directly, the resistance of soil bacterial communities to heavy
334 metals and tetracycline antibiotics (TC, OTC, CTC) could be detected using the Leucine incorporation
335 method. The heavy metal levels of the tested microcosms (1000 mg kg⁻¹) are really very high, but they should
336 not be considered unrealistic, since heavy metals are not easily biodegradable, and therefore their
337 concentrations can increase over time (Li et al., 2015), reaching very high levels in the soil (Smolders et al.,
338 2004; Spiteri et al., 2005; Zhuang et al., 2009; Komárek et al., 2010). Therefore, soils contaminated with high
339 concentrations of heavy metals could become hotspots of ARGs, which would pose a potential danger to
340 public health, since they could be transmitted to humans (Forsberg et al., 2012).

341

342 **4. Conclusions**

343 In the current study it was evidenced that heavy metals present in soils show wide variability as
344 regards the toxicity they exert on bacterial communities, with Cd and Cu being the most toxic. Specifically,
345 soil samples contaminated with a high concentration (1000 mg kg⁻¹) of each of 7 different heavy metals (As,
346 Cd, Zn, Cu, Ni, Cr and Pb), added separately, showed tolerance of the soil bacterial communities to the heavy

347 metals themselves, compared to the control soil. Besides, it was shown that the higher the effect of heavy
348 metals in the short and long terms, the greater the increase in tolerance to metals generated in the soil
349 bacterial communities (except for Ni). In addition, soil samples contaminated with high concentrations of
350 heavy metals also showed co-tolerance of soil bacterial communities to tetracycline, oxytetracycline and
351 chlortetracycline for all the metals tested, compared to the control soil. However, in general, there was no
352 correlation between the initial damage exerted by heavy metals in the short and long terms and the co-
353 tolerance shown by soil bacterial communities to tetracycline antibiotics. Future studies could give further
354 details about the potential interactions of different pollutants present in agricultural soils, which can be
355 considered relevant as regards environmental preservation and public health.

356

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362

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562 **Tables and figures**

563

564 **Table 1.** Toxicity index values \pm error of soil samples containing 0 mg kg⁻¹ (control soil) and 1000 mg kg⁻¹ of

565

As, Cd, Zn, Cu, Ni, Cr or Pb (individually), after 1 and 42 days of incubation.

566

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

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568

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

Heavy metal	Control soil		Heavy metal-polluted soil (1000 mg kg^{-1})		$\Delta \text{log IC}_{50}$
	$\text{Log IC}_{50 \pm \text{error}}$	R^2	$\text{Log IC}_{50 \pm \text{error}}$	R^2	
As	-3.63±0.11	0.970	-2.28±0.04	0.987	1.35
Cd	-3.37±0.07	0.988	-2.21±0.06	0.966	1.16
Zn	-2.89±0.06	0.988	-1.63±0.09	0.966	1.26
Cu	-4.17±0.05	0.991	-2.61±0.12	0.973	1.56
Ni	-2.56±0.08	0.966	-1.82±0.11	0.861	0.74
Cr	-3.44±0.07	0.987	-1.86±0.19	0.938	1.58
Pb	-3.42±0.07	0.989	-2.34±0.06	0.977	1.08

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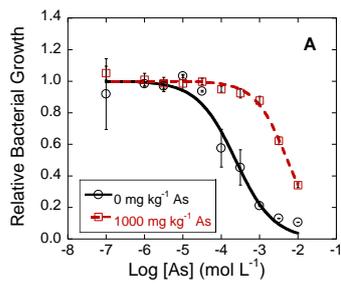
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574 **Table 3.** Estimated values of $\text{LogIC}_{50} \pm \text{error}$ and R^2 from fits to a logistic model of soil samples polluted with
575 1000 mg kg^{-1} of As, Cd, Zn, Cu, Ni, Cr and Pb. The increases in tolerance ($\Delta \text{log IC}_{50} = \text{LogIC}_{50} \text{ polluted} - \text{LogIC}_{50}$
576 control) were calculated for tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC)

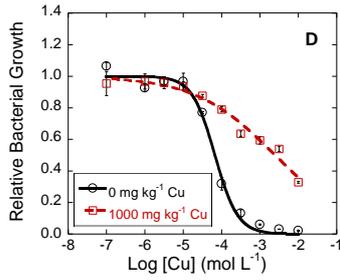
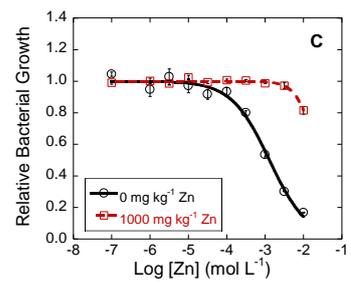
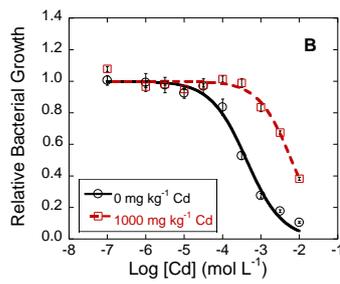
	TC			OTC			CTC		
	$\text{Log IC}_{50} \pm \text{error}$	R^2	$\Delta \text{log IC}_{50}$	$\text{Log IC}_{50} \pm \text{error}$	R^2	$\Delta \text{log IC}_{50}$	$\text{Log IC}_{50} \pm \text{error}$	R^2	$\Delta \text{log IC}_{50}$
Control	1.83±0.06	0.975	--	1.11±0.08	0.982	--	1.52±0.10	0.959	--
As	2.26±0.09	0.925	0.43	1.65±0.02	0.997	0.54	1.91±0.04	0.985	0.39
Cd	≥2.6*	--	0.77	1.47±0.09	0.973	0.36	1.91±0.01	0.982	0.39
Zn	2.28±0.05	0.971	0.45	1.64±0.04	0.991	0.53	2.19±0.11	0.977	0.67
Cu	≥2.6*	--	0.77	2.67±0.37	0.771	1.56	1.82±0.08	0.959	0.30
Ni	2.33±0.04	0.982	0.50	1.68±0.07	0.968	0.57	1.75±0.04	0.992	0.23
Cr	≥2.6*	--	0.77	≥2.6*	--	1.49	≥2.6*	--	1.08
Pb	2.23±0.08	0.926	0.40	1.34±0.08	0.981	0.23	1.99±0.03	0.976	0.47

577 * The value of 2.6 is the maximum value tested

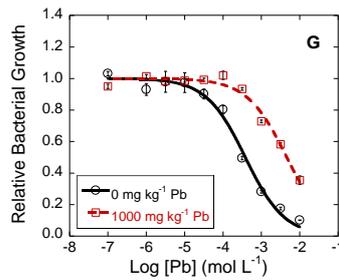
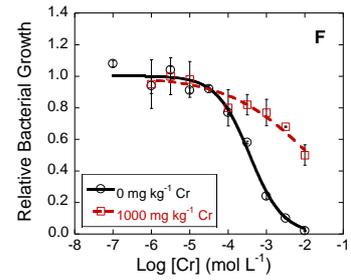
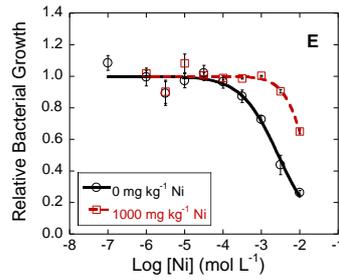
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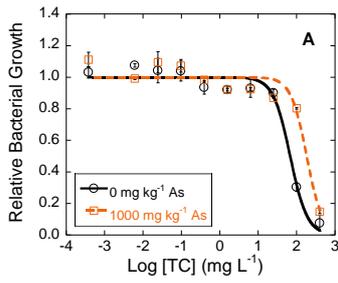


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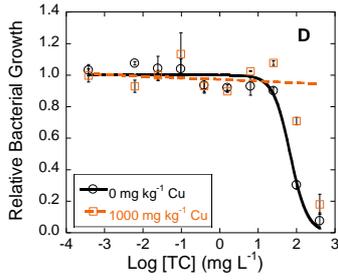
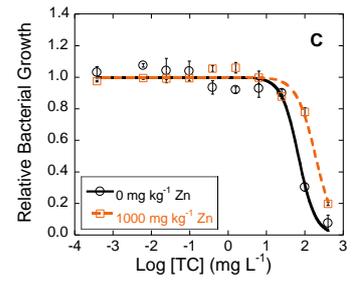
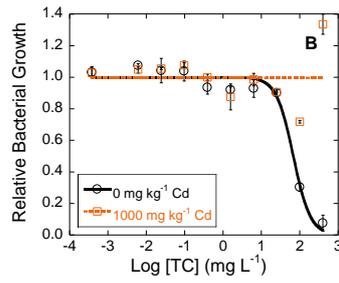
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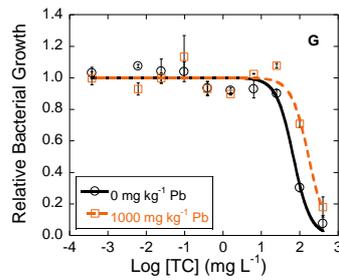
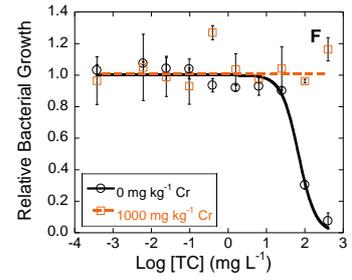
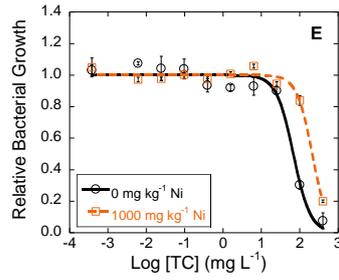
584 **Figure 1.** Dose-response curves obtained after the addition of 10 different concentrations of the heavy metals As (A),
 585 Cd (B), Zn (C), Cu (D), Ni (E), Cr (F) and Pb (G) to bacterial suspensions extracted from soils polluted with 1000 mg
 586 kg^{-1} of each of the same heavy metals, separately,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



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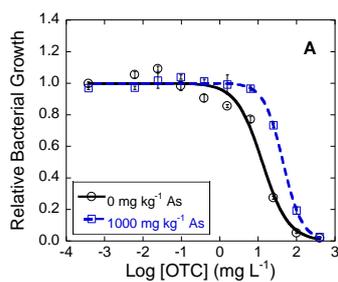
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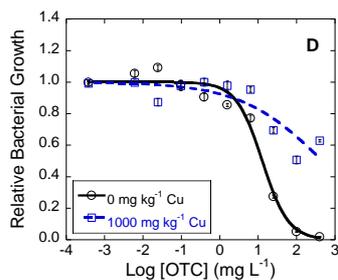
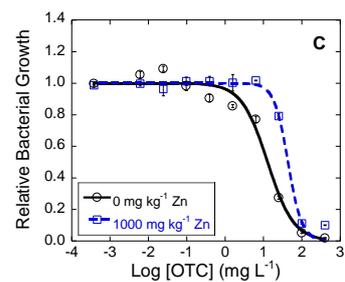
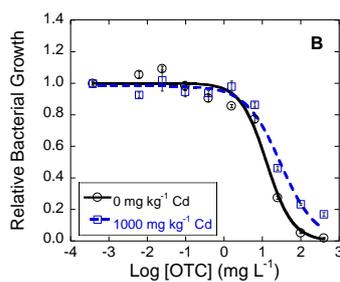
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594 **Figure 2.** Dose-response curves obtained after the addition of 10 different concentrations of tetracycline (TC) to
595 bacterial suspension extracted from soil samples polluted with 1000 mg kg⁻¹ of As (A), Cd (B), Zn (C), Cu (D),
596 Ni (E),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

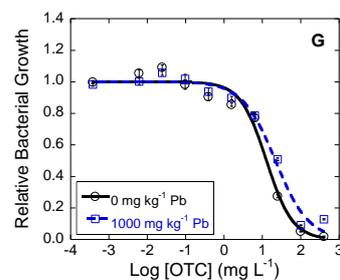
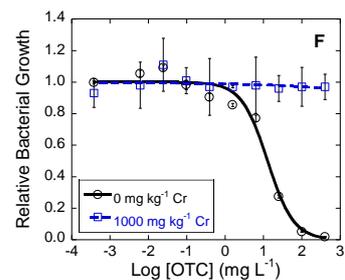
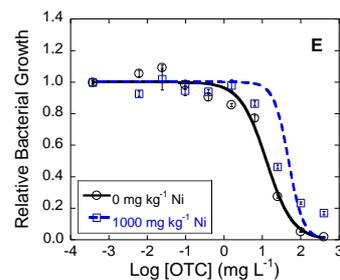
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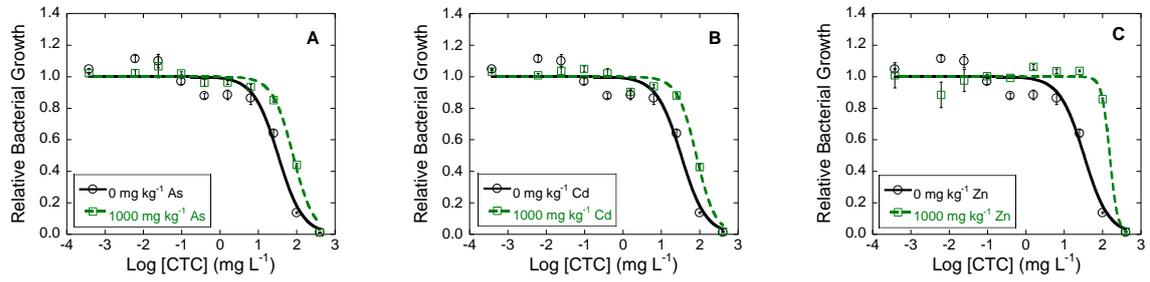
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606 **Figure 3.** Dose-response curves obtained after the addition of 10 different concentration of oxytetracycline
 607 (OTC) to bacterial suspension extracted from soil samples polluted with 1000 mg kg⁻¹ of As (A), Cd (B), Zn (C),
 608 Cu (D), Ni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

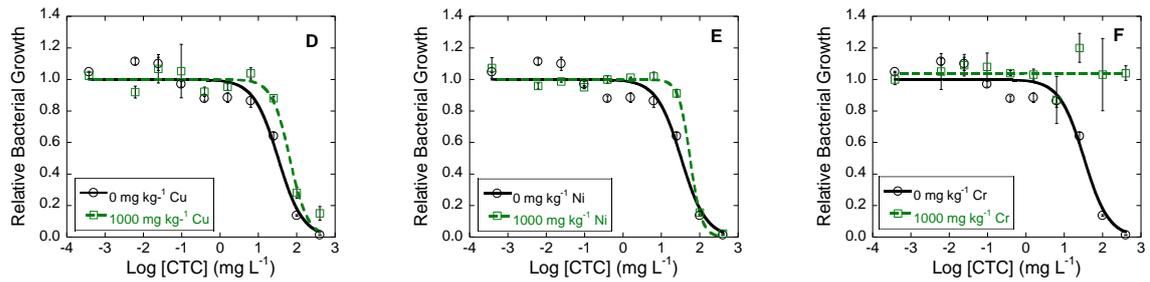
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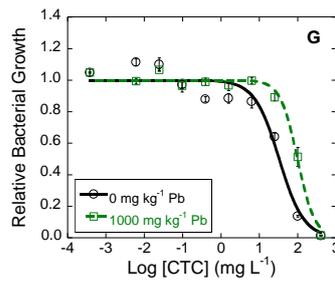
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621 **Figure 4.** Dose-response curves obtained after the addition of 10 different concentration of chlortetracycline
622 (CTC) to bacterial suspension extracted from soil samples polluted with 1000 mg kg⁻¹ of As (A), Cd (B), Zn (C),
623 Cu (D), Ni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

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