

29 from 5 to 36%, respectively). However, CTC toxicity did not change, or even increased in response
30 to the PB amendment. Regarding soil amendment with CMS, it was not effective to prevent the
31 toxicity of any of the three antibiotics studied.

32

33 Keywords: Crushed mussel shell; leucine incorporation; pine bark; oxytetracycline;
34 chlortetracycline

35

36

37 **1. Introduction**

38 In recent years, the spread of antibiotics in the environment has raised special concern. Antibiotics
39 are widely used for the medical treatment of microbial infectious diseases, and therefore tons of
40 antibiotics are administered annually to humans and animals (FEDESA, 2001; Boxal et al., 2003;
41 Kumar et al., 2005). Within the latter, which are called veterinary antibiotics (VA) (Cabello, 2006),
42 those used with livestock are of main relevance. In general, these antibiotics are rapidly excreted
43 from the treated body, since they are poorly absorbed by the animal intestine, and between 30-
44 90% are excreted in the form of the original compounds, some of which are bioactive (Sarmah et
45 al., 2006). Thus, manure and slurry used as fertilizers on agricultural land are often contaminated
46 with veterinary antibiotics, with repeated applications of them being the main route of entry of
47 antibiotics into soil ecosystems (Hamscher et al., 2002; Thiele-Bruhn, 2003). Once in the soil,
48 antibiotics are potentially dangerous for soil microorganisms (Baguer et al., 2000) since they can
49 cause changes in microbial structure, affecting their biodiversity (Hammesfahr et al., 2008), and
50 these antibiotics can also cause changes in the various environmental functions of the affected
51 microorganisms (Zielezny et al., 2006; Aminov and Mackie, 2007; Liu et al., 2009; Toth et al.,
52 2011; Ma et al., 2016; Santás-Miguel et al., 2020a; Santás-Miguel et al., 2020b). Tetracyclines are
53 the veterinary antibiotics most used in the European Union (33.4% of the total) (ESVAC, 2016),
54 with three of them (tetracycline, oxytetracycline and chlortetracycline) being the most consumed.
55 The maintenance of the correct growth of the bacterial communities in the soil is of vital
56 importance, since it contributes to maintaining the recycling of nutrients and the renewal of organic
57 matter (Thiele-Bruhn et al., 2012), which are crucial aspects for the maintenance and sustainability
58 of an optimal soil fertilization. However, there are few studies evaluating the effect of tetracycline
59 antibiotics on the growth of soil bacterial communities (Rousk et al., 2008; Rousk et al., 2009;
60 Santás-Miguel et al., 2020a; Santás-Miguel et al., 2020b), even if is clearly relevant, as these
61 studies showed negative effects of TC, OTC and CTC on the growth of these communities of

62 microorganisms. Therefore, finding appropriate means to prevent the toxicity of tetracycline
63 antibiotics on soil bacterial communities can be considered an important objective for soils
64 polluted with these substances.

65 A good alternative to minimize the negative effect of contaminants on soil is the use of bio-
66 adsorbents materials, due to their low cost and adsorption capacity for toxic substances
67 (Fernández-Calviño et al., 2016; Fernández-Pazos et al., 2013; Romar-Gasalla et al., 2018). Pine
68 bark (PB) and crushed mussel shell (CMS) are abundant waste/by-products with potential for
69 retaining pollutants (Nehrenheim and Gustafsson, 2008; Gundogdu et al., 2009; Cutillas-Barreiro
70 et al., 2014; Farouq and Yousef, 2015). In addition, PB is mainly characterized by having high
71 organic carbon content (Cutillas-Barreiro et al., 2014), while CMS is characterized by their
72 potential for increasing soil pH (Garrido-Rodríguez et al., 2013). For organic pollutants such as
73 antibiotics, and specifically for tetracyclines, PB was much more effective than mussel shell as
74 regard its adsorption potential (despite similar surface areas), which was attributed to the organic
75 matter content of the pine bark (Conde-Cid et al., 2020a), whose interactions occur mainly through
76 cation exchange, through the formation of ternary complexes and through hydrogen bonds.
77 However, the effect of the addition of PB and CMS on soil bacterial communities has been poorly
78 studied (Fernández-Calviño et al., 2015; Fernández-Calviño et al., 2018; Santás-Miguel et al.,
79 2018), but with promising results. Specifically, Fernández-Calviño et al. (2018) found that the
80 amendment of mining soils with PB, CMS and a mixture of both had positive effects on microbial
81 growth due to increases in organic matter and pH, respectively. However, the effect on soil
82 bacterial communities due to the amendment with PB and CMS in agricultural soils as a preventive
83 tool against antibiotics pollution was not studied yet.

84 In view of this background, the main hypothesis of the current work is that adding
85 appropriate doses of PB and CMS to soils polluted with tetracycline antibiotics, the effects due to
86 the toxicity of these compounds could be decreased. Therefore, the main objective of this work is

87 to evaluate the effect of PB and CMS amendments on the toxicity exerted by tetracycline
88 antibiotics (tetracycline, oxytetracycline and chlortetracycline) on the growth of bacterial
89 communities in soils.

90

91 **2. Material and methods.**

92 **2.1. Chemicals**

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

97

98 **2.2. Soil, pine bark and crushed mussel shell**

99 Four agricultural soils were selected for this study, using a pool of previously studied soils
100 characterized by Conde-Cid et al. (2018). The main characteristics of the selected soils are shown
101 in Table S1 (Supplementary Material). Briefly, the pH measured in water (pH_W) varied between
102 4.9 and 5.7, while the pH measured in KCl (pH_{KCl}) ranged from 4.1 up to 4.5. The total organic
103 carbon values varied between 1.1 and 3.8% and the effective cation exchange capacity (eCEC)
104 between 4.1 and 6.8 $\text{cmol}_c \text{kg}^{-1}$.

105 The pine bark (PB) used was supplied by Geolia (Madrid, Spain) and the crushed mussel shell
106 (CMS) was supplied by Abonomar S.L. (Illa de Arousa, Galicia, Spain). Both materials were
107 ground and grinded by a mesh with 2 mm light. The PB and the CMS samples were previously
108 analyzed by Romar-Gasalla et al. (2018), and their main characteristics and analytical
109 methodologies used are shown in Table S2 (Supplementary Material). Briefly, PB presented an
110 acid pH (4.0), organic carbon content of 486 g kg^{-1} and nitrogen content of 0.8 g kg^{-1} . The CMS

111 batch used presented a strongly alkaline pH of 9.4 and a total carbon concentration of 124 g kg⁻¹
112 (mainly as inorganic carbon \geq 99%), both due to the high presence of carbonates.

113

114 **2.3. Experimental design**

115 Each soil was divided in 7 portions (72 g dry weight each) using 500 mL polypropylene bottles
116 with lids. Three portions were amended with PB (6, 24 and 48 g kg⁻¹), other three portions with
117 CMS separately (6, 24 and 48 g kg⁻¹) and the last one was not amended (acting as control, without
118 any waste/by-product). The doses of the different by-products added to soils were calculated in
119 dry weight. The doses of 6, 24 and 48 g kg⁻¹ corresponded to 12, 48, and 96 Mg ha⁻¹ (considering
120 an effective soil depth of 20 cm and a bulk density of 1 kg dm⁻³). These concentrations were
121 satisfactorily used in previous works testing the effects of different by-products on soils (Ramírez-
122 Pérez et al., 2013; Fernández-Calviño et al., 2015; Conde-Cid et al., 2020b, 2020c; Santás-Miguel
123 et al., 2020c). Soils and waste/by-products were mixed using a spatula and shaking the bottles by
124 hand, resulting in a total of 28 soil/amendment mixtures (4 soils x 7 portions). The procedure has
125 been carried out in the same way as in the previous studies, due to comparative purposes
126 (Fernández-Calviño et al., 2015; 2018). Once these soil mixtures were homogenized, the samples
127 were moistened at 60-80% of water holding capacity, and incubated for 30 days at 22 °C in the
128 dark. After this time, soil pH was measured in water (1:10 soil:water ratio), and the
129 soil/amendment mixtures were spiked with antibiotics. Briefly: different amounts of tetracycline,
130 oxytetracycline and chlortetracycline were added separately to the 28 soil mixtures using talc as a
131 carrier. For this, each soil mixture was divided into 24 polypropylene tubes (15 mL), and then 12
132 mg of the talc and antibiotic mixtures were added, reaching a final concentration of 0, 0.5, 2, 7.8,
133 31.3, 125, 500 and 2000 mg antibiotic per kg⁻¹ of soil in the mixtures. These concentrations of the
134 antibiotics tested have been successfully used in previously studies (Santás-Miguel et al., 2020a,
135 2020b, 2020c). The use of high concentrations of antibiotics to cause almost complete inhibition

136 of bacterial communities is necessary for the correct estimation of toxicity indices in a more
137 reliable way (Fox and Landis, 2006). This process has been carried out in triplicate, resulting in a
138 total of 672 microcosms (3 grams each).
139 The mixtures spiked with the different concentrations of each antibiotic were incubated for 1 day
140 at 22 °C in the dark, and subsequently bacterial community growth was estimated using the ³H
141 Leucine incorporation method (Bååth, 1994; Bååth et al., 2001). Briefly, 1 g of soil (fresh weight)
142 was mixed with 10 mL of distilled water using a multivortex shaker for 3 min at maximum
143 intensity, followed by low-speed centrifugation at 1000 *x g* for 10 min, thus creating a bacterial
144 suspension in the supernatant. An aliquot (1 mL) of this suspension was transferred to 2 mL micro-
145 centrifugation tubes. Then, 2 µL of [³H]Leu (3.7 MBq mL⁻¹ and 0.574 TBq mmol⁻¹; Perkin Elmer,
146 USA) were added with non-labeled Leu to each tube, resulting in 410 nM Leu in the bacterial
147 suspensions. After incubation for 2 h at 22 °C, bacterial growth was stopped using 75 µL of 100%
148 trichloroacetic acid. The amount of Leu incorporated into bacteria cells was determined using
149 scintillation liquid counting (Tri-Carb 2810 TR).

150

151 **2.4. Data analysis**

152 The bacterial growth data estimated as a function of antibiotics concentration were
153 normalized dividing all values by the control (sample without antibiotic) for each soil, waste dose
154 and antibiotic, in order to allow comparison among different dose-response curves.

155 Dose-response curves were plotted (relative bacterial growth vs log antibiotic
156 concentration) using data obtained for each soil mixture and antibiotic. Using these curves, a
157 toxicity index (log IC₅₀) was calculated. Log IC₅₀ (logarithm of concentration that inhibits 50% of
158 bacterial growth) was calculated using the following logistic model (1):

$$159 \quad Y = c/[1+eb(a-x)] \quad (1)$$

160 where Y is Leu incorporation for each antibiotic concentration, x is the logarithm of the
161 concentration of antibiotic added, a is the value of $\log IC_{50}$, b is a parameter related with the slope
162 of inhibition curves, and c is the bacterial growth without antibiotic added (control sample). High
163 $\log IC_{50}$ values indicate that the toxicity of the antibiotics added is low as regards the growth of
164 the bacterial communities, while low $\log IC_{50}$ values implicate high toxicity of the antibiotics
165 added. The differences between the pH values obtained after adding the different doses of each
166 by-products were estimated by analysis of variance (ANOVA) followed by Dunnett's posthoc test.
167 This test allowed the comparison of the statistical differences between the control soils (without
168 by-product) and the soils amended with different doses of by-products.

169

170 **3. Results and Discussion**

171 **3.1. Changes in soils characteristics after the addition of pine bark and crushed mussel**

172 **shell**

173 The amendments with PB and CMS caused changes in soil properties. The pH_w values
174 measured in non-amended soils (control soils) varied between 4.9 and 5.7. After the addition of
175 PB to the soils, the pH_w values decreased in 0.1 units for all PB doses, except in soil 1 (the one
176 with lower organic carbon content), for which pH values decreased in a range going from 0.2 units
177 (for PB dose of 6 g kg^{-1}) up to 0.5 units (for PB dose of 48 g kg^{-1}) (Table 1). In addition, the CMS
178 amendment caused marked and significant ($P < 0.05$) changes in soil pH_w (Table 2). Specifically,
179 the amendment with 6 g kg^{-1} of CMS caused that soil pH_w increased between 0.1 and 0.5 units.
180 The amendments with doses of 24 g kg^{-1} and 48 g kg^{-1} of CMS caused increases in pH values
181 ranging between 0.3 and 1.5 units, and between 0.7 and 1.8 units, respectively. As in the case of
182 PB, the highest variations in pH values were found in the soil which shows the lowest carbon
183 content (soil 1).

184 Carrying out a theoretical calculation, the addition of PB to the soils contributes to increase
185 total carbon and nitrogen contents in 2.9 g kg⁻¹ and 0.02 g kg⁻¹ respectively (for PB dose of 6 g kg⁻¹
186 ¹), 11.7 g kg⁻¹ and 0.08 g kg⁻¹ respectively (for PB dose of 24 g kg⁻¹), and 23.5 g kg⁻¹ and 0.15 g
187 kg⁻¹ respectively (for PB dose of 48 g kg⁻¹), being mainly organic carbon (Cutillas-Barreiro et al.,
188 2014). Due to the high C/N value of PB, its addition to the soil contributed to increase the overall
189 C/N ratio. The addition of CMS contributes to increase total soil carbon and nitrogen in 0.7 g kg⁻¹
190 and 0.01 g kg⁻¹ respectively (for CMS dose of 6 g kg⁻¹), 3.0 g kg⁻¹ and 0.05 g kg⁻¹ respectively (for
191 CMS dose of 24 g kg⁻¹), and 6.0 g kg⁻¹ and 0.1 g kg⁻¹ respectively (for CMS dose of 48 g kg⁻¹), in
192 this case mainly as inorganic carbon (Ramírez-Pérez et al., 2013).

193 The changes in the chemical properties of the soils obtained in this study after the
194 amendment with PB and CMS are similar to those observed by other authors (Munksgaard and
195 Lottermoser, 2010; Álvarez et al., 2012; Paz-Ferreiro et al., 2012; Abd El-Azeem et al., 2013;
196 Garrido-Rodríguez et al., 2013; Pérez-Esteban et al., 2012). In addition to the modification of
197 chemical properties, the amendment with these materials may cause changes in the biological
198 properties of the soils. In this regard, increases in medium- and long-term microbial activity were
199 found in previous studies (Kokalisburelle et al., 1994; Paz-Ferreiro et al., 2012; Fernández-Calviño
200 et al., 2015; Fernández-Calviño et al., 2018), together with increases in soil fertility (Schulte and
201 Whitcomb, 1975; Álvarez et al., 2012; Paz-Ferreiro et al., 2012).

202

203 **3.2. Effect of pine bark and crushed mussel shell amendments on the toxicity exerted by** 204 **tetracycline antibiotics on bacterial community growth**

205 *3.2.1. Pine bark effects*

206 Fig. 1 show the inhibition curves corresponding to the different antibiotics (tetracycline,
207 oxytetracycline and chlortetracycline) obtained for 4 soils and 4 PB doses (0, 6, 24, and 48 g kg⁻¹
208 ¹). In general, two different behaviors were observed. On the one hand, TC and OTC behave

209 similarly, with dose-response curves shifting to the right respect to the control in response to PB
210 additions, i.e., the toxicity exerted by TC and OTC decreases with the addition of higher PB doses.
211 On the other hand, CTC had a different behavior to that observed for the other 2 antibiotics (TC
212 and OTC) since, in general, the dose-response curves almost did not change, or even shift to the
213 left, i.e., the toxicity of CTC increases with the addition of PB.

214 The dose-response curves obtained for the 3 antibiotics, 4 soils and 4 PB doses (0, 6, 24, and 48 g
215 kg⁻¹) were generally well described by the logistic model, with R² values ranging from 0.899 to
216 0.997 (mean R²=0.975). The results from these equations (Table 3) provided the estimated log
217 IC₅₀. For control soils (un-amended), the logIC₅₀ values after the addition of different
218 concentrations of antibiotics (Table 3) ranged from 2.26 to 2.71 for TC, from 1.91 to 2.70 for OTC,
219 and from 1.83 to 2.42 for CTC. These results showed that CTC was the most toxic antibiotic for
220 soil bacterial communities in the studied soils, with TC and OTC showing quite similar effect on
221 bacterial community growth. These results are consistent with those found in previous works
222 (Santás-Miguel et al., 2020a; 2020b), which showed the toxicity sequence: CTC >> OTC ≥ TC.
223 Furthermore, this sequence is similar to those found by other authors using other microbial activity
224 endpoints, such as population dynamics (Bansal and Srivastava, 2014), enzymatic activity (Liu et
225 al., 2009; Bansal 2015), or pour plate method (Halling-Sørensen et al., 2002). The toxicity
226 sequence found for bacterial community growth in the present work is also in accordance with the
227 results found by other authors using other types of organisms such as various algal species
228 (Lützhøft et al., 1999; Halling-Sorensen, 2000), *Carassius auratus* (Wang et al., 2008), *Daphnia*
229 *magna* (Park and Choi, 2008; Wang et al., 2008; Ji et al., 2012), *Danio rerio* (Wang et al., 2008),
230 *Oryzias latipes* (Park and Choi, 2008; Ji et al., 2012), *Vibrio fischeri* (Park and Choi, 2008).

231 Log IC₅₀ values support that there are 2 different types of behavior for the antibiotics studied: TC
232 and OTC toxicity decreases with the addition of PB respect to the control soil (log IC₅₀ increased),
233 while CTC toxicity did not change, or even increased, with PB additions (log IC₅₀ decreased).

234 However, the magnitude of those increases/decreases was dependent on the soil type (Table 3). In
235 soil 1 (lowest organic carbon: 1.1%), TC toxicity decreased in a 25% for the lowest dose of PB
236 added to the soil ($\geq 6 \text{ g kg}^{-1}$) in relation to the control. In soils 3 and 4 (with organic carbon between
237 3.1-3.8%), a decrease (14 and 6%, respectively) in the effect of antibiotic toxicity on bacterial
238 growth was observed for doses $\geq 24 \text{ g kg}^{-1}$, while for soil 2 (with organic carbon 3.8%), the decrease
239 in the effect of TC on bacterial community growth was only observed for the highest PB dose (48
240 g kg^{-1}), with a magnitude of 9%. Regarding OTC, the behavior observed in soil 1 (lowest organic
241 carbon) is the same as for TC, i.e. the decrease in OTC toxicity occurs for the lowest dose of PB
242 ($\geq 6 \text{ g kg}^{-1}$), being the toxicity reduction of 15% respect to the control. However, the behavior of
243 soils with a higher percentage of carbon (soils 2, 3 and 4), despite significant differences ($P < 0.05$)
244 in carbon contents, differs together from that observed for TC. The decrease in OTC toxicity on
245 bacterial community growth in soils 2 and 4 (with reduction of toxicity in 28 and 11%,
246 respectively) occurs at PB doses of $\geq 24 \text{ g kg}^{-1}$, while, soil 3 did not show significant differences
247 for the various doses of PB added to the soil compared to the control soil (un-amended). Therefore,
248 the PB amendment with the aim of preventing TC and OTC toxicities is more effective in soils
249 with low organic carbon contents than in soils with high organic carbon values. Regarding CTC
250 toxicity on soil bacterial communities, it was negligible for some soils (soils 1 and 4) and even
251 increased with the PB amendment for other soils (Soils 2 and 3), with increases in CTC toxicity
252 reaching up to 27 and 17%, respectively. Therefore, the addition of PB to prevent CTC toxicity in
253 soils polluted with this antibiotic cannot be considered an effective management practice.

254 In general, the reduction in the antibiotics toxicity on soil bacterial communities may be attributed
255 to increases in organic matter caused by PB additions. Previous works showed that the log IC_{50}
256 values obtained for TC, OTC and CTC after 1 day of incubation were significantly and positively
257 correlated with the carbon content of the soil, i.e, as higher the carbon content of the soil was, the
258 lower was the toxicity exerted by these antibiotics on the growth of soil bacterial communities

259 (Santás-Miguel et al., 2020a; 2020b). This effect may be due to the key role played by organic
260 matter in the adsorption of tetracycline antibiotics in soils (Gu et al., 2007; Pils and Laird, 2007;
261 Ling-Ling et al., 2010; Zhao et al., 2011; Fernández-Calviño et al., 2015). In this way, Conde-Cid
262 et al. (2019) studied the adsorption/desorption of TC, OTC and CTC in 3 waste materials,
263 observing that PB showed a high adsorption capacity for the same three antibiotics here
264 considered. This high adsorption, in acid conditions, was attributed to an exchange of protons
265 between tetracyclines charged positively and H^+ from components of PB. Therefore, this high
266 affinity of tetracyclines for organic compounds present in PB may decrease the negative effects of
267 TC and OTC on bacterial community growth. Other soil characteristic with a potential influence
268 on the effect caused by PB on the toxicity exerted by tetracycline antibiotics on bacterial
269 community growth in soils is soil pH. The sorption of tetracyclines antibiotics on soils compounds
270 is strongly governed by pH (Gu et al., 2007; Parolo et al., 2008; Chang et al., 2009), also affecting
271 the chemical speciation of antibiotics and of sorbent surfaces (Figueroa-Diva et al., 2010). In
272 addition, changes in antibiotics speciation may directly affect the antibiotics toxicity (Yamaguchi
273 et al., 1991). However, in the current study, the addition of PB to the soils produced negligible
274 changes in soil pH, and hence in speciation (Table S3; Supplementary Material). Therefore, effects
275 due to pH resulting in decreases of TC and OTC toxicity may be discarded. According to the
276 literature previously described, the no-effect and negative effect of PB amendments on the
277 reduction of the toxicity exerted by CTC on the growth of bacterial communities is an unexpected
278 result. In previous works where PB was used, some toxic effects on soil bacterial communities due
279 to PB were observed for high PB doses (Fernández-Calviño et al., 2018), which were attributed to
280 the presence of toxic substances in the bark, such as polyphenols (Bailey et al., 1999; Kofujita et
281 al., 1999). One possibility is that the interaction of CTC with PB may release these toxic substances
282 to the bacterial suspensions, and another possibility is the presence of synergistic toxic effects of
283 CTC and those substances. However, results from soil 1 were different, i.e. slight decreases in

284 OTC toxicity were found when PB was added. Therefore, the effect of PB to the soil was different
285 in soils with low organic carbon (soil 1) than in soils with high organic carbon contents (soils 2-
286 4). These discrepancies make that further research is needed to clarify these hypotheses.

287

288 3.2.2. Crushed mussel shell effects

289 The dose-response curves obtained for the 3 tetracycline antibiotics, 4 CMS doses (control-0, 6,
290 24, and 48 g kg⁻¹) and 4 studied soils, are shown in Fig. 2. In general, dose-response curves do not
291 move clearly to the right or to the left, meaning that these curves do not show a determinate pattern,
292 as in the case of PB. Therefore, a clear increase or decrease of tetracycline antibiotics toxicity on
293 the growth of bacterial communities, as a function of the CMS doses added, was not found. The
294 dose-response curves were generally well described by the logistic model, with R² values ranging
295 from 0.880 to 0.996 (mean R²=0.968), showing log IC₅₀ values ranging from 1.61 up to 2.84 (Table
296 4). The log IC₅₀ values showed that the addition of CMS to the soil caused negligible effects on
297 the toxicity exerted by TC, OTC and CTC on the growth of bacterial communities. Although
298 adsorption of tetracycline antibiotics on CMS is relatively low (Conde-Cid et al., 2020a), this
299 finding is surprising, because the addition of CMS to the soil, in different doses, highly and
300 significantly (P<0.05) modified the soil pH (Table 2). The pH modification may affect the
301 chemical speciation of tetracycline antibiotics in the soil (Figueroa-Diva et al., 2010), and therefore
302 their adsorption, availability and toxicity (Gu et al., 2007; Parolo et al., 2008; Chang et al., 2009).
303 Tetracycline antibiotics (TC, OTC and CTC) presented amphoteric character, and depending on
304 soil pH they may be in cationic, zwitterionic or anionic form. The chemical speciation of the
305 antibiotics will determine their adsorption to the different components of the soil due to changes
306 in the charge of the antibiotic. Moreover, antibiotics speciation (charge) determines the
307 incorporation/passage of the antibiotics into the bacteria, and therefore, the toxic effect that it could
308 produce (Yamaguchi et al., 1991; Stock et al., 1977). Table 5 shows the different species in which

309 TC, OTC and CTC may be found in the 4 soils studied for the different doses of CMS added. In
310 general, the change in pH due to the addition of CMS causes antibiotics to be mainly in zwitterionic
311 and anionic form. In the soil with the lowest organic C content (soil 1), increases in the dose of
312 CMS added favor the presence of negatively charged species of the tetracycline antibiotics (Table
313 5). The pH changes caused by the addition of CMS resulted in changes of the TC, OTC and CTC
314 speciation in soil 1 (the one with the lowest organic carbon content, and hence less buffer capacity).
315 In the other soils, the tetracycline speciation remains similar to the values found for the control
316 soil. However, no effects were found for soil 1 (neither for the other soils) due to CMS as regards
317 eventual decreases in antibiotics toxicity. These results suggest that the pH variation observed in
318 soils after the addition of CMS (reaching up to 7.5) was not high enough to generate changes in
319 the toxicity of the tetracycline antibiotics. Previously, Santas-Miguel et al. (2020c) tested the effect
320 of biomass ash on the toxicity exerted by the same three tetracycline antibiotics (TC, OTC and
321 CTC) on the growth of bacterial communities. Biomass ash (BA) has a high pH (11.3) and its
322 addition to the soil in similar doses as those used for CMS increased soil pH values from 4.6-5.1
323 up to 8.6-9.7. In general, after the addition of biomass ash, decreases in tetracycline toxicity on
324 bacterial communities were observed, going from 5% to 100% (a full recovery of the bacterial
325 communities in the soil). These differences may be also due to different organic carbon contents
326 between CMS and BA, or even to differences in surface area. However, PB provided much more
327 organic carbon than BA, but the reduction in toxicity is much higher using BA than that due to
328 PB. Moreover, biomass ash presents a surface area of 1.3 g m^{-2} (Conde-Cid et al., 2020a), very
329 similar to the value found for CMS (1.1 g m^{-2} ; Table S2; Supplementary Material). Therefore, it
330 seems that pH increases caused that tetracycline toxicity decreased only for pH values higher than
331 7.5. Since CMS present a limited capacity for pH increases, and it presents a low tetracycline
332 antibiotics adsorption capacity (Conde-Cid et al., 2019), this material cannot be considered useful
333 to prevent the toxicity exerted by tetracycline antibiotics on the growth of bacterial communities.

334 Previous works showed significant correlations between TC, OTC and CTC toxicities and soil pH,
335 in a pH range of 4.1-7.4 (Santás-Miguel et al., 2020a; 2020b). However, in view of the results
336 observed in the current work, the influence of pH is not clear. In those previous studies, a
337 correlation was also found between soil texture and antibiotics toxicity, but it was attributed to a
338 high correlation between pH and particle-size parameters (sand, silt and clay). However, the results
339 found in the present work support that changes in soil pH do not cause changes in tetracycline
340 antibiotics toxicity, and therefore, the toxicity of antibiotics may be influenced by soil texture,
341 together with other characteristics such as organic carbon content (Santás-Miguel et al., 2020a;
342 2020b).

343

344 **4. Conclusions**

345 The main objective of this study was to assess the effect of soil amendment with pine bark (PB)
346 and crushed mussel shell (CMS) in order to decrease toxicity of three tetracycline antibiotics
347 (tetracycline, oxytetracycline and chlortetracycline) on the growth of soil bacterial communities.
348 Adding PB to the soil prevents the toxicity of tetracycline (TC) and oxytetracycline (OTC)
349 antibiotics on soil bacterial communities, especially in soils with low organic carbon content, with
350 decrease in antibiotics toxicity reaching up to 25 and 15% for TC and OTC, respectively.
351 However, in soils contaminated with chlortetracycline (CTC), the addition of PB as a bio-
352 adsorbent is not suitable, as it does not reduce (or even increase up to 27%) the toxicity exerted by
353 this antibiotic on soil bacterial communities. Regarding soil amendment with CMS, it has not an
354 effect on the reduction of any tetracycline antibiotics toxicity on the growth of bacterial
355 communities, i.e. mussel shell cannot be considered an effective bio-adsorbent to prevent
356 tetracycline antibiotics toxicity in soils. This study also opened some questions which need further
357 research; the higher toxicity of OTC in soils with high organic matter content amended with PB,
358 but not in the soil with low organic content should be clarified by means of future investigations.

359

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367

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Tables and Figures.

561 **Table 1.**

562 Soil pH values after amendment with different doses of PB. Average values (n=3), with
563 coefficients of variation always <5%

	0 g kg ⁻¹ PB	6 g kg ⁻¹ PB	24 g kg ⁻¹ PB	48 g kg ⁻¹ PB
Soil 1	5.7	5.5	5.4	5.2
Soil 2	5.2	5.1	5.1	5.1
Soil 3	5.1	5.1	5.1	5.1
Soil 4	4.9	4.9	4.8	4.8

564

565 **Table 2.**

566 Soil pH values after amendment with different doses of CMS. Average values (n=3), with
567 coefficients of variation always <5%

	0 g kg ⁻¹ CMS	6 g kg ⁻¹ CMS	24 g kg ⁻¹ CMS	48 g kg ⁻¹ CMS
Soil 1	5.7	6.2	7.1	7.5
Soil 2	5.2	5.5	5.7	6.1
Soil 3	5.1	5.2	5.4	5.8
Soil 4	4.9	5.3	5.7	6.5

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569

570 **Table 3**

571 Estimated values of $\log IC_{50} \pm$ standard error and R^2 , derived from fits to the logistic model
 572 obtained from the soils studied after amendment with different doses of PB (control-0, 6, 24, and
 573 48 g kg⁻¹).

Antibiotic	PB dose	Soil 1		Soil 2		Soil 3		Soil 4	
		$\log IC_{50}$	R^2	$\log IC_{50}$	R^2	$\log IC_{50}$	R^2	$\log IC_{50}$	R^2
TC	0 g kg ⁻¹	2.26±0.08	0.976	2.71±0.06	0.984	2.51±0.05	0.989	2.70±0.08	0.973
	6 g kg ⁻¹	2.83±0.10	0.974	2.61±0.07	0.977	2.42±0.08	0.977	2.64±0.06	0.984
	24 g kg ⁻¹	2.59±0.12	0.955	2.62±0.04	0.991	2.86±0.07	0.965	2.86±0.05	0.985
	48 g kg ⁻¹	2.78±0.06	0.978	2.96±0.04	0.987	2.83±0.05	0.981	3.23±0.03	0.989
OTC	0 g kg ⁻¹	2.11±0.13	0.973	1.91±0.06	0.995	2.70±0.10	0.952	2.38±0.13	0.970
	6 g kg ⁻¹	2.41±0.08	0.982	1.92±0.13	0.978	2.44±0.05	0.988	2.52±0.03	0.997
	24 g kg ⁻¹	2.44±0.11	0.957	2.45±0.07	0.982	2.70±0.05	0.980	2.63±0.09	0.922
	48 g kg ⁻¹	2.50±0.06	0.987	2.59±0.12	0.930	2.83±0.11	0.938	3.03±0.05	0.981
CTC	0 g kg ⁻¹	1.83±0.12	0.986	1.99±0.09	0.981	2.15±0.08	0.983	2.42±0.03	0.996
	6 g kg ⁻¹	2.10±0.10	0.983	1.45±0.12	0.984	2.30±0.10	0.979	2.28±0.12	0.967
	24 g kg ⁻¹	1.86±0.16	0.965	1.74±0.08	0.992	1.88±0.12	0.986	2.05±0.13	0.976
	48 g kg ⁻¹	2.25±0.23	0.899	1.71±0.06	0.993	1.79±0.11	0.983	2.34±0.06	0.986

574 TC: tetracycline; OTC: oxytetracycline; CTC: chlortetracycline

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576

577

578 **Table 4**

579 Estimated values of $\log IC_{50} \pm$ standard error and R^2 , derived from fits to the logistic model obtained
 580 from the soils studied after amendment with different doses of CMS (control-0, 6, 24 and 48 g kg⁻¹
 581 ¹).

Antibiotic	CMS doses	Soil 1		Soil 2		Soil 3		Soil 4	
		Log IC ₅₀	R ²	Log IC ₅₀	R ²	Log IC ₅₀	R ²	Log IC ₅₀	R ²
TC	0 g kg ⁻¹	2.26±0.08	0.976	2.71±0.06	0.984	2.51±0.05	0.989	2.70±0.08	0.973
	6 g kg ⁻¹	2.31±0.08	0.979	2.57±0.09	0.961	2.68±0.07	0.969	2.25±0.14	0.959
	24 g kg ⁻¹	2.27±0.04	0.994	2.77±0.14	0.928	2.54±0.04	0.994	2.84±0.16	0.900
	48 g kg ⁻¹	2.17±0.11	0.968	2.36±0.11	0.985	2.63±0.10	0.954	2.68±0.14	0.953
OTC	0 g kg ⁻¹	2.11±0.13	0.973	1.91±0.06	0.995	2.70±0.10	0.952	2.38±0.13	0.970
	6 g kg ⁻¹	2.01±0.08	0.977	2.45±0.09	0.982	2.58±0.09	0.971	2.58±0.10	0.969
	24 g kg ⁻¹	2.19±0.14	0.970	2.00±0.10	0.990	2.31±0.05	0.995	2.42±0.15	0.945
	48 g kg ⁻¹	2.26±0.20	0.916	2.41±0.13	0.956	2.80±0.15	0.910	2.77±0.06	0.986
CTC	0 g kg ⁻¹	1.83±0.12	0.986	1.99±0.09	0.981	2.15±0.08	0.983	2.42±0.03	0.996
	6 g kg ⁻¹	1.78±0.31	0.938	1.89±0.11	0.975	1.92±0.15	0.971	2.14±0.13	0.964
	24 g kg ⁻¹	1.61±0.14	0.978	1.86±0.10	0.983	1.74±0.27	0.954	2.15±0.10	0.982
	48 g kg ⁻¹	1.84±0.06	0.992	1.82±0.16	0.970	2.77±0.04	0.992	2.36±0.30	0.880

582 TC: tetracycline; OTC: oxytetracycline; CTC: chlortetracycline

583

584

585 **Table 5**

586 Percentages corresponding to each species of the three antibiotics as a function of pH measured in
587 soils with different doses of CMS. Average values (n=3) with coefficients of variation always <5%

Soil	CMS dose (g kg ⁻¹)	pH	TC ⁺¹	TC ⁰	TC ⁻¹	TC ⁻²	OTC ⁺¹	OTC ⁰	OTC ⁻¹	OTC ⁻²	CTC ⁺¹	CTC ⁰	CTC ⁻¹	CTC ⁻²
1	0	5.7	0.4	98.8	0.8	0.0	0.3	98.0	1.7	0.0	0.4	98.8	0.8	0.0
	6	6.2	0.1	97.3	2.6	0.0	0.1	94.7	5.2	0.0	0.1	97.4	2.4	0.0
	24	7.1	0.0	82.7	17.3	0.1	0.0	69.3	30.3	0.4	0.0	83.3	16.6	0.1
	48	7.5	0.0	65.4	34.3	0.3	0.0	46.8	51.3	1.9	0.0	66.4	33.3	0.3
2	0	5.2	1.3	98.4	0.3	0.0	1.0	98.4	0.5	0.0	1.2	98.5	0.2	0.0
	6	5.5	0.7	98.8	0.5	0.0	0.5	98.4	1.1	0.0	0.6	98.9	0.5	0.0
	24	5.7	0.4	98.8	0.8	0.0	0.3	98.0	1.7	0.0	0.4	98.8	0.8	0.0
	48	6.1	0.2	97.8	2.0	0.0	0.1	95.7	4.2	0.0	0.2	97.9	2.0	0.0
3	0	5.1	1.6	98.2	0.2	0.0	1.3	98.3	0.4	0.0	1.6	98.2	0.2	0.0
	6	5.2	1.3	98.4	0.3	0.0	1.0	98.4	0.5	0.0	1.2	98.5	0.2	0.0
	24	5.4	0.8	98.8	0.4	0.0	0.7	98.5	0.9	0.0	0.8	98.8	0.4	0.0
	48	5.8	0.3	98.6	1.0	0.0	0.3	97.6	2.1	0.0	0.3	98.7	1.0	0.0
4	0	4.9	2.6	97.3	0.1	0.0	2.0	97.7	0.3	0.0	2.4	97.4	0.1	0.0
	6	5.3	1.0	98.6	0.3	0.0	0.8	98.5	0.7	0.0	1.0	98.7	0.3	0.0
	24	5.7	0.4	98.8	0.8	0.0	0.3	98.0	1.7	0.0	0.4	98.8	0.8	0.0
	48	6.5	0.1	95.0	5.0	0.0	0.0	90.0	9.9	0.0	0.1	95.2	4.8	0.0

588 TC: tetracycline; OTC: oxytetracycline; CTC: chlortetracycline. The superscript (TC^X.OTC^X and CTC^X is the
589 valence/charge of the antibiotic

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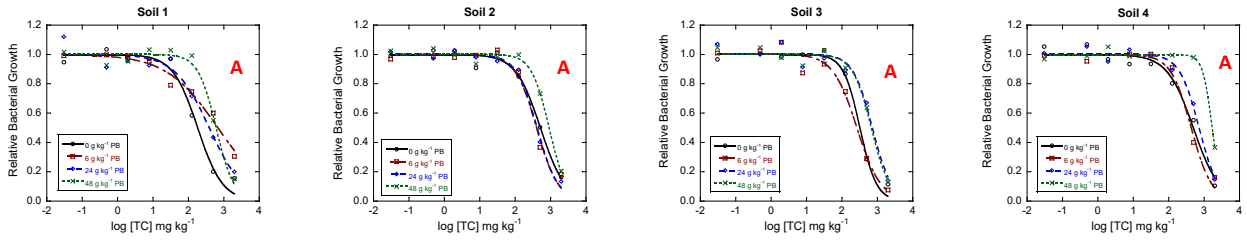
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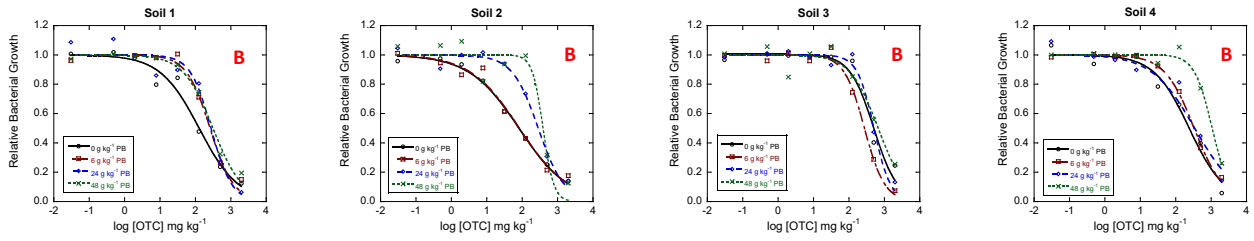
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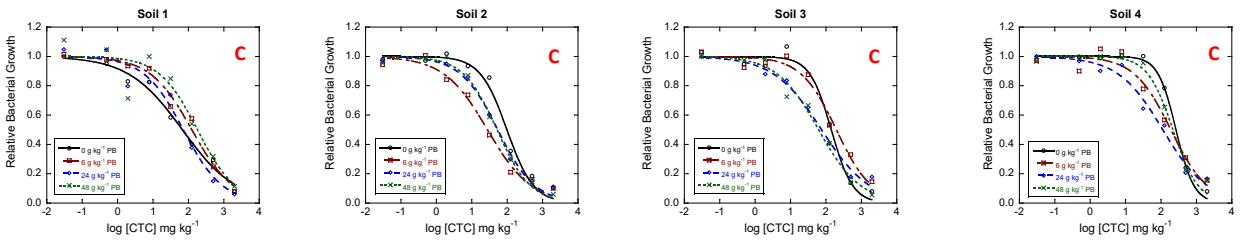
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603 **Fig. 1.** Dose-response curves obtained after the addition of 8 different concentrations of
 604 tetracycline (TC; A), oxytetracycline (OTC; B) and chlortetracycline (CTC; C) to 4 soils amended
 605 with 4 doses of PB (control-0, 6, 24 y 48 g kg⁻¹). Average values (n=3) with coefficients of
 606 variation always <5%

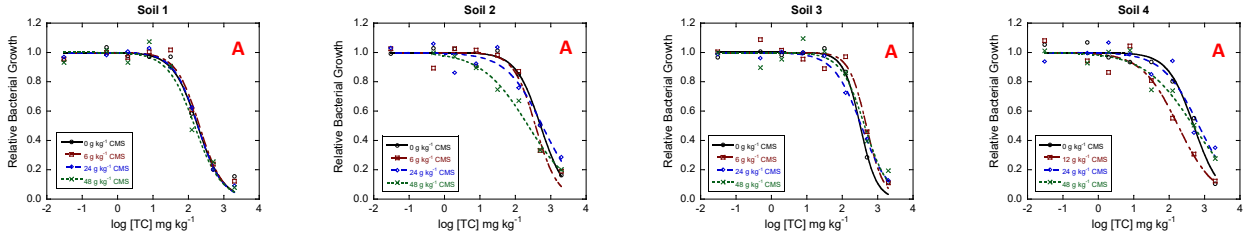
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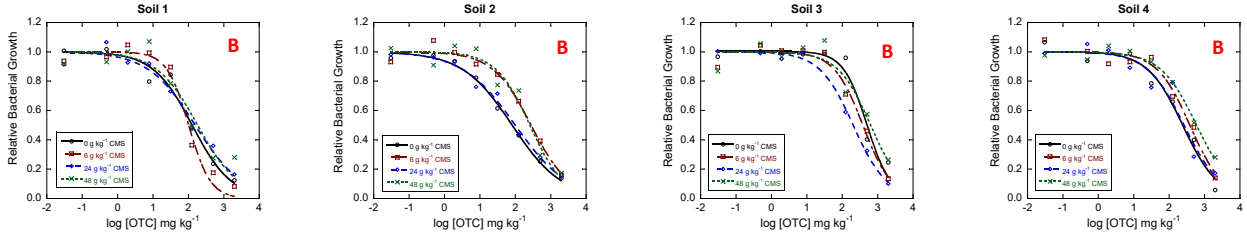
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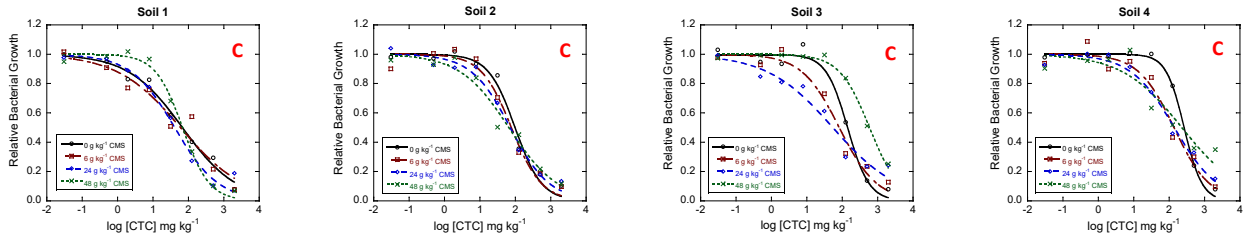
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615 **Fig. 2.** Dose-response curves obtained after the addition of 8 different concentrations of
 616 tetracycline (TC; A), oxytetracycline (OTC; B) and chlortetracycline (CTC; C) to 4 soils amended
 617 with 4 doses of CMS (control-0, 6, 24 y 48 g kg⁻¹). Average values (n=3) with coefficients of
 618 variation always <5%

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