1 **Increase of bacterial community induced-tolerance to Cr in response to soil**

2 **properties and Cr level in the soil**

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10 **ABSTRACT**

11 Chromium (Cr) pollution in soils is a global concern that should be assessed. Pollution

12 Induced Community Tolerance (PICT) methodology is a highly sensitive tool that can

13 directly indicate metal toxicity in the microbial community. Ten soils with a wide range

14 of properties were spiked with 31.25, 62.5, 125, 250, 500, 1000 and 2000 mg Cr·kg^{-1}, in

15 addition to the control. Bacterial growth (using [^{3}H]-leucine incorporation technique) was

16 used to determine PICT, that is, whether bacterial communities developed tolerance in

17 response to Cr additions to different soil types. Some bacterial communities did not grow

18 normally at 1000 or 2000 mg Cr·kg^{-1}, probably due to high Cr toxicity, while others did.

19 Regarding below 500 mg Cr·kg^{-1}, bacterial communities showed two responses

20 depending on soil type: 7 of the 10 studied soils showed increased tolerance to Cr, while

21 for the remaining 3 soils did not develop tolerance to Cr. Furthermore, the Cr level from

22 which bacterial communities developed tolerance was dependent on the soil, i.e. Cr was

23 more toxic in some of studied soils. The Cr effect on microbial communities was mainly

24 determined by Dissolved Organic Carbon (DOC) and the fraction of Cr extracted with

25 distilled water (H$_2$O-Cr) (R^2 = 95.6 %). Their effect on Cr in the soil might lead to an

26 increase in toxicity (selection phase of PICT).

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30 Keywords:

31 PICT (Pollution-Induced Community Tolerance), bacterial growth, Cr (chromium),

32 dissolved organic carbon, metal bioavailability, risk assessment

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1. Introduction

Chromium (Cr) is a highly toxic non-essential metal for microorganisms and plants, that may naturally occur at high concentrations from parent materials, e.g. serpentine rocks (Adriano, 2001; Cervantes et al., 2001). The world average content of Cr in soils is 60 mg·kg^{-1}, but in soils developed from mafic and volcanic rocks can reach up to 10000 mg·kg^{-1} (Gonnelli and Renella, 2013). Cr contents up to 2879 and 3865 mg·kg^{-1} were reported for serpentine soils in Galicia (NW Spain) and Albania, respectively, (Covelo et al., 2007; Shallari et al., 1998). Anthropogenic activities, e.g. metallurgical industry, also lead to Cr accumulation in soils (Kabata-Pendias, 2011). Up to 195, 88 and 6228 mg·kg^{-1} Cr were found in urban, agricultural and industrial soils, respectively (Srinivasa Gowd et al., 2010; Wei and Yang, 2010). Speciation and adsorption on soil solid surfaces are the main processes controlling Cr toxicity in soils (Adriano, 2001; Shahid et al., 2017). Despite the various Cr oxidation states, Cr(III) and Cr(VI) are the most stable and common forms in soils. Cr(VI) is considered the most toxic form of Cr, while Cr (III) is less mobile, less toxic and presents mostly precipitated (Kabata-Pendias, 2011). The adsorption of Cr on soil solid surfaces depends on several factors, e.g. soil pH, clay content, organic matter or Fe hydroxides (Bolan and Thiagarajan, 2001; Bradl, 2004; Dias-Ferreira et al., 2015; Gonnelli and Renella, 2013; Kabata-Pendias, 2011).

In the assessment of metal pollution, the toxic metal effect on soil microorganisms should be considered, because of their key role in maintaining soil ecosystem functions (Nannipieri et al., 2003). Lower microbial diversity, enzymatic activity, C mineralization and microbial biomass were found in Cr-polluted soil in comparison to unpolluted soil (Dotaniya et al., 2017; He et al., 2016; Pradhan et al., 2019). The potential nitrification and microbial abundance were inhibited with the increase of Cr level in the soil (Zhang

60   et al., 2022). Bacterial diversity was negatively correlated with total and available Cr,

61   while microbial community structure was altered (Zhang et al., 2021). However,

62   sometimes differentiating if the microbial response is due to Cr toxicity or to soil

63   properties variation is a difficult task (Liu et al., 2019), in addition to the complex

64   biogeochemical behaviour of Cr in soils (Ao et al., 2022). Therefore, a microbial indicator

65   specifically related to Cr toxicity that reduces interference of other soil properties is

66   needed to assess the Cr toxicity, such as the Pollution Induced Community Tolerance

67   (PICT) methodology. PICT is a sensitive tool that can be used as a direct indicator of

68   metal toxicity in the microbial community (Blanck, 2002). PICT methodology is based

69   on the selective pressure that the metal exerts on a microbial community, which favoured

70   the proliferation of more tolerant species over the more sensitive ones. Thus, the microbial

71   community that was exposed to the pollutant should show higher tolerance than that of

72   the unexposed reference microbial community (Blanck, 2002; Tlili et al., 2016). PICT

73   methodology has been successfully applied to assess Cr pollution in soils or sediments

74   (Gong et al., 2002; Ipsilantis and Coyne, 2007; Ogilvie and Grant, 2008; Santás-Miguel

75   et al., 2021; Shi et al., 2002a, 2002b; Van Beelen et al., 2004). The microbial community

76   tolerance should be quantified in a short-term assay by a sensitive endpoint, such as

77   bacterial growth measured using [^{3}H]-leucine incorporation (Berg et al., 2012; Boivin et

78   al., 2006; Lekfeldt et al., 2014). Despite the high sensitivity and specificity, the PICT

79   methodology might present some difficulties, mainly due to the influence of soil

80   properties (Blanck, 2002; Lekfeldt et al., 2014). Shi et al. (2002b) found similar values of

81   PICT to Cr and Pb both at low and high Cr (263 g·kg^{-1}) and Pb (10000 mg·kg^{-1}) levels,

82   respectively, suggesting that different soils affected Cr and Pb bioavailability. Similarly,

83   Shi et al. (2002a) did not found bacterial community tolerance to Cr (or Pb), regardless

84   of exposure history to Cr (or Pb), suggesting that several factors (organic matter, pH,

85   redox potential) might influence metal availability. Boivin et al. (2006), Fernández-

86   Calviño et al. (2012) and Fernández-Calviño and Bååth (2016) also reported different

87   tolerance values to heavy metals in soils with similar values of metals but different soil

88   properties. Soil properties may affect PICT development due to effects on metals

89   speciation, adsorption and bioavailability (Bradl, 2004; Shahid et al., 2017).

90   We hypothesize that soil pollution with Cr induces the development of bacterial

91   community tolerance to Cr, but the magnitude of the increases depends on soil

92   physicochemical characteristics. Therefore, we aim to determine the induced bacterial

93   community tolerance to Cr in response to the addition of different Cr levels to 10 soils

94   with variable properties. We also aim to assess the importance of soil properties on the

95   increase of bacterial community tolerance to Cr.

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2. Materials and Methods

2.1 Soil samples

Soil samples were the same used previously in Campillo-Cora et al. (2021a, 2020) to study Cr adsorption and fractionation in soils with different properties, mainly in terms of organic matter and pH. In brief, ten remote forest locations in Galicia (NW Spain) were selected to avoid heavy metal pollution. Locations were also selected to obtain soil samples with a range of different physicochemical properties (Macías-Vázquez and Calvo de Anta, 2009). Superficial soil samples (0-20 cm) were taken using an Edelman probe and, once in the laboratory, were air-dried, homogenized, sieved (2 mm mesh) and stored until analysis.

2.2 Soil properties

A detailed description of the chemical analysis is given in Campillo-Cora et al. (2020) and in Supplementary Information. The properties of the 10 soils can be found in Tables S1 and S2. In brief, soil samples presented a wide range of textures (19-71 % Sand, 13-67 % Silt, 14-32 % Clay). A wide range of soil pH_W and pH_K was found: 4.0-7.5 and 3.0-6.9, respectively. Similarly, OM oscillated between 10-29 %. A range from 2 to 29 $cmol_c \cdot kg^{-1}$ was obtained for eCEC. A large range was obtained for DOC: 0.14 to 0.70 $g \cdot kg^{-1}$. Chromium total content varied from 7 up to 394 $mg \cdot kg^{-1}$.

Adsorption constants determined from Freundlich and Langmuir models (batch experiments) are presented in Table S3, obtained from Campillo-Cora et al. (2020). The different Cr fractions from extractions using distilled water, $CaCl_2$ and DTPA are shown in Table S4, obtained from Campillo-Cora et al. (2021a)

123   *2.3 Experimental design and bacterial community tolerance to Cr determination*

124   Sieved soil samples were rewetted until reaching 60 – 80% of water holding capacity

125   (Meisner et al., 2013). To rewet, soil samples were spiked with seven Cr solutions (made

126   from $K_2Cr_2O_7$) and one of distilled water, to obtain the following final Cr levels in soils:

127   2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 mg Cr$\cdot$kg^{-1} soil. Each Cr solution was added

128   separately and in triplicate, finally obtaining 240 microcosms (10 soils x 8 [Cr] x 3

129   replicates). Once soil samples were spiked with Cr, microcosms were incubated in the

130   dark at 22 ºC for two months, to ensure the reactivation of bacterial communities (Meisner

131   et al., 2013).

132   After the incubation period, bacterial community tolerance to Cr was estimated

133   through the PICT methodology (Blanck, 2002). The homogenization-centrifugation

134   technique was performed to extract soil bacterial communities (Bååth, 1992). The

135   bacterial community tolerance to Cr was determined as previously for Cu (Fernández-

136   Calviño et al., 2011), with modifications based on suggestions by Lekfeldt et al. (2014).

137   For this purpose, each microcosm was distributed in three 50 mL centrifuge tubes and

138   MES buffer was added in a ratio 1:10 soil/buffer (20 Mm pH 6; 4-

139   Morpholineethanesulfonic acid, CAS no: 4432-31-9) (Lekfeldt et al., 2014). The

140   suspensions soil/MES were mixed using a multi-vortex at maximum intensity for 3 min.

141   This step was followed by low-speed centrifugation to remove most of the fungal biomass

142   (1000 x g, 10 min) (Bååth, 1994; Bååth et al., 2001; Rousk and Bååth, 2011). Soil

143   supernatants, i.e. bacterial suspensions, were filtered through glass wool and 1.5 mL

144   aliquots were transferred into 2 mL micro-centrifugation tubes. A volume of 0.15 mL of

145   different Cr concentrations (made from $K_2Cr_2O_7$) was added to micro-centrifugation

146   tubes, obtaining nine Cr concentrations (3.3×10^{-4} to 10^{-8} M) plus a blank (0.15 mL of

147   distilled water). Then, the ^{3}H-leucine incorporation method was used to estimate bacterial

148   growth (Bååth et al., 2001). A volume of 0.2 μL [^{3}H]Leu (37 MBq mL^{-1} and 5.74 TBq

149   mmol^{-1}. Amersham) with non-labelled Leu (19.8 μL) was added to each tube, resulting

150   in 300 nM Leu in the bacterial suspensions. Bacterial suspensions were incubated for 8 h

151   at 22ºC. Bacterial growth was stopped with 75 μL of 100% trichloroacetic acid. The

152   washing procedure and subsequent radioactivity measurement were carried out according

153   to Bååth et al. (2001). Radioactivity was measured by liquid scintillation counting using

154   a Tri-Carb 2810 TR (PerkinElmer, USA)

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156   *2.4 Data analysis*

157   *2.4.1 Estimation of bacterial community tolerance to Cr (log IC$_{50}$)*

158   A dose-response curve was obtained for each soil microcosm. To compare the dose-

159   response curves, i.e. inhibition curves, with each other, bacterial growth was expressed

160   as relative bacterial growth. For each inhibition curve, generally, the four lowest added

161   metal concentrations to bacterial suspensions not showed bacterial growth inhibition

162   (Figure 1). Thus, relative bacterial growth was calculated by dividing all bacterial growth

163   data by the average of results from the four lowest added metal concentrations (including

164   blank), obtaining comparable dose-response curves. From each dose-response curve, log

165   IC$_{50}$ was determined as a tolerance index, i.e. Cr concentration resulting in 50% inhibition

166   of bacterial community growth. Higher log IC$_{50}$ values mean higher bacterial community

167   tolerance to Cr, and lower log IC$_{50}$ values mean lower bacterial community tolerance to

168   Cr. Log IC$_{50}$ was calculated using the following logistic model (Fernández-Calviño et al.,

169   2011):

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

171   where Y is the measured level of Leu incorporation, c is the bacterial growth rate without

172   added Cr, b is a slope parameter indicating the inhibition rate, X is the logarithm of Cr

173   added, and a is log IC_{50}.

174   To detect whether bacterial community tolerance increase from different studied

175   soils occurs, Δlog IC_{50} was determined as the difference between log IC_{50} value from each

176   Cr level in soil (2000, 1000, 500, 250, 125, 62.5 or 31.25 mg Cr·kg^{-1}) and the control soil

177   (0 mg Cr·kg^{-1}). A difference of 0.3 was taken as a reference value to determine if bacterial

178   community tolerance increased since it represents twice the Cr concentration in terms of

179   added Cr to bacterial suspensions. If Δlog IC_{50} is higher than 0.3, we will consider an

180   increase in bacterial community tolerance to Cr (Fernández-Calviño and Bååth, 2016,

181   2013).

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183   *2.4.2 Estimation of bacterial community tolerance increase to Cr (multiple linear*

184   *regression analyses)*

185   A multiple regression analysis, using the backward elimination method, was performed

186   to obtain an equation that allows estimating the increase in bacterial community tolerance

187   to Cr (Δlog IC_{50}) from soil properties. As the inhibition curves for some soils did not fit

188   the logistic model (equation 1) for the highest Cr concentrations (1000 and 2000 mg·kg^{-1}

189   ^{1}), Δlog IC_{50} from 500 mg·kg^{-1} was used for estimations. Once the equation was

190   estimated, determining factors were verified: linearity, error independency, residues

191   homoscedasticity, residuals normality, autocorrelation, collinearity and presence of

192   outliers. All statistics were performed using IBM SPSS Statistics 25 software (IBM,

193   USA).

194

3. Results and discussion

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196 *3.1 Bacterial community tolerance to Cr in Cr-polluted soils with different properties*

197 Figure 1 shows bacterial growth inhibition curves obtained for each microcosm.

198 Generally, a sigmoid dose-response behaviour is observed in the inhibition curves,

199 indicating that when the added Cr concentration to bacterial suspension was low, relative

200 bacterial growth was close to 1, while decreased when the Cr concentration increased.

201 Most of the bacterial growth data fitted the logistic model, obtaining $R^2 \geq 0.87$, (Table

202 S5). However, some data from 1000 and 2000 mg Cr·kg^{-1} did not fit the logistic model,

203 i.e. bacterial populations were not able to normally grow probably due to high Cr toxicity.

204 In the case of 2000 mg·kg^{-1}, bacterial populations only grew normally in 4 of the 10

205 studied soils, while at 1000 mg·kg^{-1} they grew normally in 7 soils. These differences in

206 bacterial growth for the same Cr levels may indicate the influence of soil properties on

207 Cr availability, as was previously suggested by Van Beelen et al. (2004). They found

208 tolerant communities to Cr(III) in polluted soils with high Cr levels (2894 mg·kg^{-1}) but

209 also reported that microbial communities from soils polluted with 3935 mg Cr·kg^{-1} did

210 not show tolerance to Cr(III), suggesting the influence of soil properties on metal toxicity.

211 Therefore, in order to determine which properties influence Cr toxicity, the data of 1000

212 and 2000 mg Cr·kg^{-1} were not considered in the following analysis.

213 The log IC$_{50}$ values determined from inhibition curves using the logistic model

214 (equation 1) are presented in Table 1. Bacterial community tolerance to Cr (log IC$_{50}$)

215 greatly varied between soils, even in the reference soils with no added Cr, log IC$_{50}$

216 oscillated from -6.40 (S8) up to -3.88 (S6) (log units). The variation of bacterial

217 community tolerance to Cr in the reference soils may be an indicator that the development

218 of PICT is dependent on soil type. In addition, this bacterial community tolerance to Cr

219 fluctuation in reference soils, together with the natural Cr content in soils (7 – 394 mg·kg$^-$

220   [1], Table S2), highlights the importance of selecting reference soils for PICT studies

221   (Campillo-Cora et al., 2022a; Campillo-Cora et al., 2021b). Likewise, when Cr was added

222   to soils, bacterial community tolerance to Cr varied greatly between soils with the same

223   Cr level. A range from -6.37 (S8) to -3.56 (S6) was determined for soils polluted with the

224   lowest Cr level in soil (31.25 mg $Cr \cdot kg^{-1}$); from -6.27 (S8) to -3.79 (S7) for 62.5 mg

225    $Cr \cdot kg^{-1}$; from -6.26 (S8) to -3.65 (S7) for 125 mg $Cr \cdot kg^{-1}$; from -6.27 (S5) to -3.41 (S7)

226   for 250 mg $Cr \cdot kg^{-1}$; and from -6.09 (S8) to -2.87 (S3) for 500 $mg \cdot kg^{-1}$.

227   Overall, bacterial communities showed two different responses to Cr addition to

228   the soil (Figure 2): (1) bacterial communities of S1, S2, S3, S6, S7, S8 and S10 developed

229   tolerance in response to Cr additions; while (2) bacterial communities of S4, S5 and S9

230   did not develop tolerance following Cr addition to the soil. Based on the PICT hypothesis,

231   the bacterial community is first exposed to the metal (i.e. selection phase of PICT), and

232   if metal exerts toxicity, then the most sensitive organisms of the community will

233   disappear, while the tolerant ones will be favoured. Therefore, whether the microbial

234   community developed tolerance to Cr is a toxicity indicator. Later, the microbial

235   community tolerance is quantified through a second exposition to Cr (i.e. detection phase

236   of PICT) (Blanck, 2002; Tlili et al., 2016). Accordingly, Gong et al. (2002) and Ipsilantis

237   and Coyne (2007) reported an increase in bacterial community tolerance to Cr with

238   increasing Cr levels in soil and rhizosphere. Van Beelen et al. (2004) found that bacterial

239   community tolerance to Cr(VI) increased with increasing Cr in pore water. Ogilvie and

240   Grant (2008) determined a tendency to increase the bacterial community tolerance to Cr

241   when the Cr level increases in estuarine sediments. Our results showed that bacterial

242   community tolerance to Cr increased with increasing Cr levels in soils only in 7 of the 10

243   soils studied (Figure 2). However, our results showed that the Cr level in soil from which

244   bacterial communities developed tolerance to Cr varied depending on the soil ($\Delta log IC_{50}$

245 > 0.3). Bacterial communities from S7 and S10 showed an increased tolerance at 31.25

246 mg Cr·kg^{-1}, bacterial communities from S1 and S3 at 62.5 mg Cr·kg^{-1}, bacterial

247 communities from S2 and S8 at 250 mg Cr·kg^{-1}, and bacterial communities from S6 at

248 500 mg Cr·kg^{-1}. In other words, Cr was more toxic for bacterial communities depending

249 on soil type, following the sequence: S7, S10 > S1, S3 > S2, S8 > S6. In other soils, our

250 results show that microbial communities did not develop tolerance to Cr, even at high Cr

251 levels. For example, bacterial communities of S6 did not show tolerance to Cr even at

252 2000 mg·kg^{-1} (Figure 2). Similarly, Shi et al. (2002b, 2002a) and Ipsilantis and Coyne

253 (2007) did not find tolerant microbial communities to Cr even at high Cr levels, from 447

254 up to 263000 mg Cr·kg^{-1}. Therefore, considering that Cr-pollution sometimes has no toxic

255 effect on microbial communities and that, in other cases, microbial communities are

256 affected by Cr from very low levels of Cr-pollution, including soil properties in the

257 assessment of Cr-pollution is highly recommended, as for other heavy metals (Campillo-

258 Cora et al., 2022b).

259

260 *3.2 Estimation of the increase in bacterial community tolerance to Cr as a function of soil*

261 *properties*

262 The bacterial community tolerance to metals may be influenced by several soil properties,

263 such as soil pH, clay content or organic matter content (Ogilvie and Grant, 2008; Shi et

264 al., 2002b). The effect of soil properties on bacterial community tolerance can occur in

265 soil (selection phase of PICT), or in the determination phase of PICT. The effect of the

266 soil properties in the selection phase occurs in the soil, i.e. the first time bacterial

267 communities are exposed to the metal. For example, Fernández-Calviño and Bååth (2016)

268 found that bacterial community tolerance to Cu was lower in vineyard soils with high pH

269 in comparison to more acid soils, as Cu toxicity was reduced. On the other hand, the effect

270   of soil properties may occur in the detection phase, i.e. confounding factors leading to

271   altered tolerance measures (Lekfeldt et al., 2014). For example, Fernández-Calviño et al.

272   (2011) reported that the measurement of PICT to Cu was altered because of the presence

273   of the finer soil fraction in the bacterial suspensions when Cu concentrations were added.

274   That is, the finer particles will bind part of the Cu added to bacterial suspensions, resulting

275   in lower available Cu, so higher Cu concentrations will be necessary to inhibit the

276   bacterial growth leading to apparent higher tolerance, i.e. overestimated bacterial

277   community tolerance to Cu.

278   The equation presented in Table 2 related the increase of bacterial community

279   tolerance to Cr ($\Delta \log IC_{50}$) with soil properties, explaining 95.6 % of the data variance (p

280   < 0.001). Only $\Delta \log IC_{50}$ for 500 mg Cr·kg^{-1} were used. The increase of bacterial

281   community tolerance to Cr was estimated by using soil properties (p < 0.05): DOC and

282   extracted Cr using distilled water (H$_2$O-Cr). Figure 3 shows estimated $\Delta \log IC_{50}$ versus

283   measured $\Delta \log IC_{50}$, with a homogeneous distribution around the line 1:1 ($R^2 = 0.95$).

284   DOC showed a significant positive relationship with $\Delta \log IC_{50}$ (p < 0.05; Table

285   2), i.e. when DOC increases, the bacterial community tolerance to Cr also increases. This

286   DOC effect might be a confounding factor in the detection phase of PICT, as was

287   previously reported for Cu (Campillo-Cora et al., 2021b; Lekfeldt et al., 2014). When

288   bacterial communities are extracted from soil, DOC is extracted too. Later, when Cu is

289   added to bacterial suspensions, Cu and DOC may bind together (Beesley et al., 2010),

290   reducing Cu bioavailability and altering bacterial community tolerance to Cr

291   (overestimation). Bérard et al. (2016) reported a similar effect for microbial community

292   tolerance to Pb measurements. However, in a previous study (Campillo-Cora et al.,

293   2022c), we found that when dissolved organic matter (DOM) increases on bacterial

294   suspensions, then bacterial community tolerance to Cr decreases, i.e. when DOM

295   increases in bacterial suspensions, Cr becomes more toxic to bacteria. Hence, the DOC

296   effect in Cr bioavailability in the detection phase should be discarded because of the

297   positive relationship with $\Delta \log IC_{50}$ (Table 2) and attributed to an effect in the selection

298   phase in soil. In the soil, however, when DOC is present, Cr(VI) may be reduced to

299   Cr(III), i.e. Cr toxicity decreases when DOC is present (Ao et al., 2022). If fact, the use

300   of organic amendments to reduce Cr toxicity in soils is very common (Abou Jaoude et

301   al., 2020; Mitchell et al., 2018; Yang et al., 2021). A hypothesis is that the presence of

302   DOC in soil enhanced the reduction of Cr(VI) to Cr(III) (Wittbrodt and Palmer, 1997),

303   but during this process free radicals may also be formed (Kotaś and Stasicka, 2000),

304   increasing general toxicity for bacterial communities (Campillo-Cora et al., 2022c). In

305   response to increased toxicity in soil, then bacterial communities showed tolerance to Cr.

306   Another hypothesis might be the ability of Cr(III) to coordinate various organic

307   compounds, leading to the inhibition of some metalloenzyme systems (Kotaś and

308   Stasicka, 2000), which might result in a more tolerant bacterial community.

309   The Cr fraction extracted with distilled water (H_2O-Cr) showed a positive

310   relationship with $\Delta \log IC_{50}$ ($p < 0.001$, Table 2). Usually, the soluble form of heavy metals

311   represents the soil solution metal content, which is the most mobile and bioavailable form

312   (Kabata-Pendias, 2011). In the vase of Cr, probably Cr(VI) (Ao et al., 2022). Thus, H_2O-

313   Cr exerts its effect in soil, during the selection phase. H_2O-Cr content in soil increases as

314   added Cr level in soils increases (Campillo-Cora et al., 2021a). Whether Cr exerts

315   toxicity, the most sensitive bacterial species were removed, while the tolerant ones

316   survived, resulting in a more tolerant community to Cr. Later, in the detection phase,

317   when bacterial growth is measured and Cr is added to bacterial suspensions, tolerant

318   bacteria allow greater Cr concentrations, leading to a higher tolerant community. Van

319   Beelen et al. (2004) found a significant increase in microbial community tolerance to

320 Cr(VI) with Cr(VI) pore-water concentration. Similarly, Fernández-Calviño and Bååth

321 (2016) reported a positive relationship between bacterial community tolerance increase

322 (Δlog IC_{50}) to Cu versus water-soluble Cu concentrations logarithm ($R^2 = 0.79$). Kunito

323 et al. (1999) also determined a positive correlation between IC_{50} values and soluble-

324 exchangeable Cu ($r = 0.76$), while total Cu did not show any significant relationship ($r =$

325 0.013, $p > 0.05$).

326

327 *3.3 Concluding remarks*

328 In the present study, we aimed to improve the PICT methodology for the assessment of

329 soil pollution, using bacterial growth as the endpoint. Dissolved organic carbon (DOC)

330 and the fraction of Cr extracted with distilled water (H_2O-Cr) were the main factors

331 controlling the Cr effect on microbial communities, determined by the increase of

332 bacterial community tolerance to Cr. The main selection pressure of Cr on the microbial

333 community presumably occurs in soil, i.e. the selection phase of PICT. In the case of

334 DOC, Cr became more toxic to bacterial communities as DOC increased in soil, leading

335 to an increase in bacterial community tolerance to Cr in response to toxicity. Secondly,

336 H_2O-Cr is related to the toxic and active form of Cr, probably Cr(VI), and the higher the

337 H_2O-Cr content in the soil, the higher the tolerance to Cr developed by bacterial

338 communities. The outcomes of this study may be helpful for normalising Cr toxicity

339 thresholds for soil with different properties. In addition, overestimations or

340 underestimations of Cr toxicity based on total or bioavailable Cr content may be avoided,

341 since soil properties should be considered during risk assessment.

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556

557 **Tables**

558 **Table 1**

559 Bacterial community tolerance (expressed as log IC_{50}) to different levels of Cr pollution

560 in the 10 studied soils (average ± SE)

Cr (mg·kg⁻¹) Soil	2000 Log IC_{50}±error	1000 Log IC_{50}±error	500 Log IC_{50}±error	250 Log IC_{50}±error	125 Log IC_{50}±error	62.5 Log IC_{50}±error	31.25 Log IC_{50}±error	0 Log IC_{50}±error
S1	-5.34±0.03	-5.35±0.05	-5.28±0.03	-5.30±0.03	-5.33±0.03	-5.30±0.04	-5.83±0.06	-5.82±0.05
S2	-4.04±0.24	-4.55±0.42	-4.61±0.21	-4.68±0.41	-4.78±0.43	-4.70±0.21	-4.81±0.19	-5.02±0.13
S3	*	*	-2.87±0.51	-4.38±0.15	-4.62±0.16	-4.70±0.18	-5.46±0.03	-5.38±0.05
S4	-5.85±0.08	-5.76±0.05	-5.80±0.07	-5.69±0.05	-5.66±0.04	-5.68±0.04	-5.90±0.08	-5.66±0.07
S5	*	-4.47±0.11	-5.80±0.19	-6.27±0.07	-5.86±0.10	-5.98±0.06	-6.02±0.10	-6.09±0.07
S6	*	-3.47±0.06	-3.38±0.08	-4.48±0.13	-4.18±0.16	-3.97±0.12	-3.56±0.23	-3.88±0.11
S7	*	-3.44±0.09	-3.35±0.07	-3.41±0.09	-3.65±0.11	-3.79±0.07	-3.85±0.05	-4.32±0.12
S8	-3.63±0.13	-6.03±0.06	-6.09±0.09	-5.90±0.09	-6.26±0.04	-6.27±0.03	-6.37±0.07	-6.40±0.15
S9	*	*	-4.32±0.27	-4.37±0.39	-4.70±0.23	-4.43±0.13	-3.82±0.05	-4.11±0.04
S10	*	*	-4.75±0.13	-4.64±0.09	-4.48±0.09	-4.69±0.09	-4.76±0.04	-5.16±0.07

561 *Unadjusted data

562

25

Table 2

The equation for estimating bacterial community tolerance increase to Cr ($\Delta\log IC_{50(500-0)}$) was obtained by multiple regression analysis using all soil samples (n=10).

Equation	F	p-value	Adjusted R^2
$\Delta\log IC_{50}$ = - (0.435 ± 0.148) + (1.445 ± 0.320) **DOC** *(p=0.026)* *(p=0.004)* + (0.018 ± 0.001) **H₂O-Cr** *(p<0.001)*	87.309	<0.001	0.956

DOC is dissolved organic carbon ($g \cdot kg^{-1}$); H_2O-Cr is Cr extracted using H_2O. Values associated with the independent variables are shown together with the standard errors (±). P-values associated with each independent variable are shown below variables (in brackets)
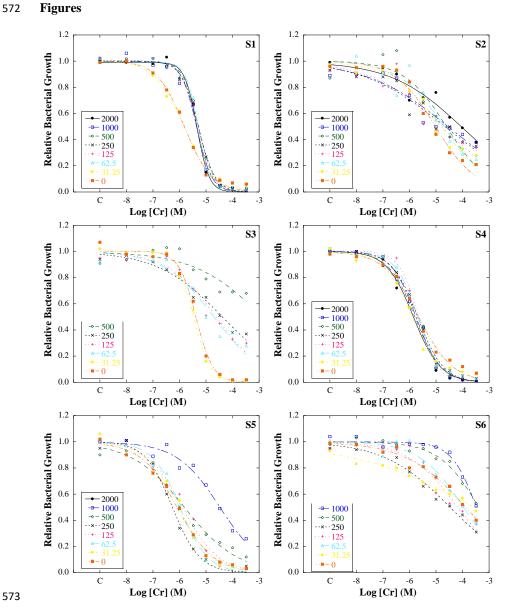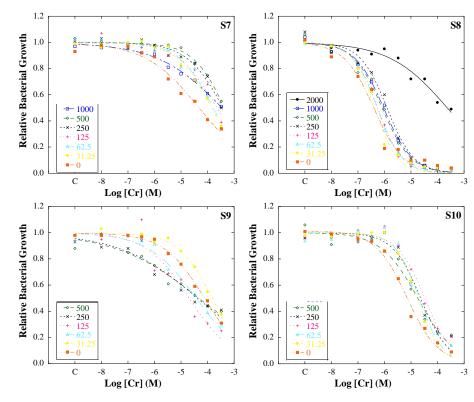
Figures

Figure 1. Bacterial growth inhibition curves for bacterial suspensions extracted from 10 soils artificially polluted with a range of Cr concentrations: 2000, 1000, 500, 125, 62.5, 31.25 and 0 mg·kg^{-1}. Dots indicate real data measured, while the lines represent the fit of the data to the logistic model used.

579

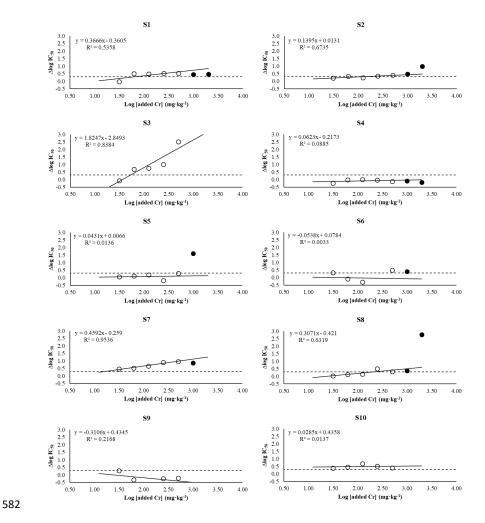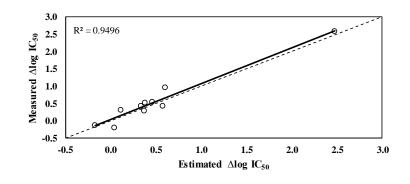580   **Figure 1** (continued)
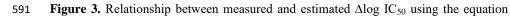
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Figure 2 Bacterial community tolerance variation (expressed as $\Delta \log IC_{50}$ concerning

unpolluted soil) to a range of added Cr to soil (in logarithm scale). White dots represent

data from $\Delta \log IC_{50(31.25-0)}$, $\Delta \log IC_{50(62.5-0)}$, $\Delta \log IC_{50(125-0)}$, $\Delta \log IC_{50(250-0)}$ and $\Delta \log$

$IC_{50(500-0)}$. Black dots represent data from $\Delta \log IC_{50(1000-0)}$ and $\Delta \log IC_{50(2000-0)}$. Continuous

lines represent linear regression fit. The discontinuous line represents the value (0.3) from

which it is considered that the bacterial community has developed tolerance.

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Figure 3. Relationship between measured and estimated $\Delta\log \mathrm{IC}_{50}$ using the equation from Table 2. The stippled line indicated a 1:1 relationship.