Increase of bacterial community induced-tolerance to Cr in response to soil properties and Cr level in the soil

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ABSTRACT

Chromium (Cr) soil pollution is a pressing global concern that demads thorough assessment. The Pollution Induced Community Tolerance (PICT) methodology serves as a highly sensitive tool capable of directly assessing metal toxicity within microbial communities. In this study, ten soils exhibiting a wide range of properties were subjected to Cr contamination, with concentrations ranging from 31.25 to 2000 mg $Cr \cdot kg^{-1}$, in addition to the control. Bacterial growth, assessed using the [^{3}H]-leucine incorporation technique, was used to determine whether bacterial communities developed tolerance to Cr, i.e., PICT to Cr in response to Cr additions to different soil types. Obtained results revealed that at concentrations of 1000 or 2000 mg $Cr \cdot kg^{-1}$, certain bacterial communities showed inhibited growth, likely attributable to elevated Cr toxicity, while others continued to thrive. Interestingly, with Cr concentrations below 500 mg $Cr \cdot kg^{-1}$, bacterial communities demonstrated two distinc responses depending on soil type: 7 of the 10 studied soils exhibited an increased bacterial community tolerance to Cr, while the remaining 3 soils did not develop such tolerance. Furthermore, the Cr level at which bacterial communities developed tolerance to Cr varies among soils, indicating varying levels of Cr toxicity between studied soils. The Dissolved Organic Carbon (DOC) and the fraction of Cr extracted with distilled water (H_2O-Cr) played an essential role in shaping the impact of Cr on microbial communities (R^2 = 95.6 %). These factors (DOC and H_2O-Cr) contribute to increase Cr toxicity in soil, i.e., during the selection phase of PICT methodology.

Keywords:

PICT, bacterial growth, Cr, dissolved organic carbon, metal bioavailability, risk assessment

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 $mg \cdot kg^{-1}$, but in soils developed from mafic and volcanic rocks can reach up to 10000 $mg \cdot kg^{-1}$ (Gonnelli and Renella, 2013). Cr contents up to 2879 and 3865 $mg \cdot kg^{-1}$ 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 $mg \cdot kg^{-1}$ 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

et al., 2022). Bacterial diversity was negatively correlated with total and available Cr, while microbial community structure was altered (Zhang et al., 2021). However, sometimes differentiating if the microbial response is due to Cr toxicity or to soil properties variation is a difficult task (Liu et al., 2019), in addition to the complex biogeochemical behaviour of Cr in soils (Ao et al., 2022). Therefore, a microbial indicator specifically related to Cr toxicity that reduces interference of other soil properties is needed to assess the Cr toxicity, such as the Pollution Induced Community Tolerance (PICT) methodology. PICT is a sensitive tool that can be used as a direct indicator of metal toxicity in the microbial community (Blanck, 2002). PICT methodology is based on the selective pressure that the metal exerts on a microbial community, which favoured the proliferation of more tolerant species over the more sensitive ones. Thus, the microbial community that was exposed to the pollutant should show higher tolerance than that of the unexposed reference microbial community (Blanck, 2002; Tlili et al., 2016). PICT methodology has been successfully applied to assess Cr pollution in soils or sediments (Gong et al., 2002; Ipsilantis and Coyne, 2007; Ogilvie and Grant, 2008; Santás-Miguel et al., 2021; Shi et al., 2002a, 2002b; Van Beelen et al., 2004). The microbial community tolerance should be quantified in a short-term assay by a sensitive endpoint, such as bacterial growth measured using [^{3}H]-leucine incorporation (Berg et al., 2012; Boivin et al., 2006; Lekfeldt et al., 2014). Despite the high sensitivity and specificity, the PICT methodology might present some difficulties, mainly due to the influence of soil properties (Blanck, 2002; Lekfeldt et al., 2014). Shi et al. (2002b) found similar values of PICT to Cr and Pb both at low and high Cr (263 $g \cdot kg^{-1}$) and Pb (10000 $mg \cdot kg^{-1}$) levels, respectively, suggesting that different soils affected Cr and Pb bioavailability. Similarly, Shi et al. (2002a) did not found bacterial community tolerance to Cr (or Pb), regardless of exposure history to Cr (or Pb), suggesting that several factors (organic matter, pH,

redox potential) might influence metal availability. Boivin et al. (2006), Fernández-Calviño et al. (2012) and Fernández-Calviño and Bååth (2016) also reported different tolerance values to heavy metals in soils with similar values of metals but different soil properties. Soil properties may affect PICT development due to effects on metals speciation, adsorption and bioavailability (Bradl, 2004; Shahid et al., 2017).

We hypothesize that soil pollution with Cr induces the development of bacterial community tolerance to Cr, but the magnitude of the increases depends on soil physicochemical characteristics. Therefore, we aim to determine the induced bacterial community tolerance to Cr in response to the addition of different Cr levels to 10 soils with variable properties. We also aim to assess the importance of soil properties on the increase of bacterial community tolerance to Cr.

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). These concentrations were selected as previously undertaken in Campillo-
130 Cora (2020, 2021a), as they represent a broad exponential range of Cr contamination,
131 which promotes the development of bacterial community tolerance to Cr, despite the
132 considerable variability in soil properties. This facilitates subsequent comparisons of
133 bacterial community tolerance to Cr results between the different soils studied. Once soil
134 samples were spiked with Cr, microcosms were incubated in the dark at 22 ºC for two
135 months, to ensure the reactivation of bacterial communities (Meisner et al., 2013).

136 After the incubation period, bacterial community tolerance to Cr was estimated
137 through the PICT methodology (Blanck, 2002). The homogenization-centrifugation
138 technique was performed to extract soil bacterial communities (Bååth, 1992). The
139 bacterial community tolerance to Cr was determined as previously for Cu (Fernández-
140 Calviño et al., 2011), with modifications based on suggestions by Lekfeldt et al. (2014).
141 For this purpose, each microcosm was distributed in three 50 mL centrifuge tubes and
142 MES buffer was added in a ratio 1:10 soil/buffer (20 Mm pH 6; 4-
143 Morpholineethanesulfonic acid, CAS no: 4432-31-9) (Lekfeldt et al., 2014). The
144 suspensions soil/MES were mixed using a multi-vortex at maximum intensity for 3 min.
145 This step was followed by low-speed centrifugation to remove most of the fungal biomass
146 (1000 x g, 10 min) (Bååth, 1994; Bååth et al., 2001; Rousk and Bååth, 2011). Soil
147 supernatants, i.e. bacterial suspensions, were filtered through glass wool and 1.5 mL

aliquots were transferred into 2 mL micro-centrifugation tubes. A volume of 0.15 mL of different Cr concentrations (made from $K_2Cr_2O_7$) was added to micro-centrifugation tubes, obtaining nine Cr concentrations (3.3×10^{-4} to 10^{-8} M) plus a blank (0.15 mL of distilled water). Then, the ^{3}H-leucine incorporation method was used to estimate bacterial growth (Bååth et al., 2001). A volume of 0.2 µL [^{3}H]Leu (37 MBq mL^{-1} and 5.74 TBq mmol^{-1}. Amersham) with non-labelled Leu (19.8 µL) was added to each tube, resulting in 300 nM Leu in the bacterial suspensions. Bacterial suspensions were incubated for 8 h at 22ºC. Bacterial growth was stopped with 75 µL of 100% trichloroacetic acid. The washing procedure and subsequent radioactivity measurement were carried out according to Bååth et al. (2001). Radioactivity was measured by liquid scintillation counting using a Tri-Carb 2810 TR (PerkinElmer, USA)

2.4 Data analysis

2.4.1 Estimation of bacterial community tolerance to Cr (log IC_{50})

A dose-response curve was obtained for each soil microcosm. To compare the dose-response curves, i.e. inhibition curves, with each other, bacterial growth was expressed as relative bacterial growth. For each inhibition curve, generally, the four lowest added metal concentrations to bacterial suspensions not showed bacterial growth inhibition (Figure 1). Thus, relative bacterial growth was calculated by dividing all bacterial growth data by the average of results from the four lowest added metal concentrations (including blank), obtaining comparable dose-response curves. From each dose-response curve, log IC_{50} was determined as a tolerance index, i.e. Cr concentration resulting in 50% inhibition of bacterial community growth. Higher log IC_{50} values mean higher bacterial community tolerance to Cr, and lower log IC_{50} values mean lower bacterial community tolerance to

Cr. Log IC_{50} was calculated using the following logistic model (Fernández-Calviño et al., 2011):

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

where Y is the measured level of Leu incorporation, c is the bacterial growth rate without added Cr, b is a slope parameter indicating the inhibition rate, X is the logarithm of Cr added, and a is log IC_{50}.

To detect whether bacterial community tolerance increase from different studied soils occurs, Δlog IC_{50} was determined as the difference between log IC_{50} value from each Cr level in soil (2000, 1000, 500, 250, 125, 62.5 or 31.25 mg Cr·kg^{-1}) and the control soil (0 mg Cr·kg^{-1}). A difference of 0.3 was taken as a reference value to determine if bacterial community tolerance increased since it represents twice the Cr concentration in terms of added Cr to bacterial suspensions. If Δlog IC_{50} is higher than 0.3, we will consider an increase in bacterial community tolerance to Cr (Fernández-Calviño and Bååth, 2016, 2013).

2.4.2 Estimation of bacterial community tolerance increase to Cr (multiple linear regression analyses)

A multiple regression analysis, using the backward elimination method, was performed to obtain an equation that allows estimating the increase in bacterial community tolerance to Cr (Δlog IC_{50}) from soil properties (Campillo-Cora et al., 2021b, 2022a, b). As the inhibition curves for some soils did not fit the logistic model (equation 1) for the highest Cr concentrations (1000 and 2000 mg·kg^{-1}), Δlog IC_{50} from 500 mg·kg^{-1} was used for estimations. Once the equation was estimated, determining factors were verified: linearity, error independency, residues homoscedasticity, residuals normality,

196 autocorrelation, collinearity and presence of outliers. All statistics were performed using
197 IBM SPSS Statistics 25 software (IBM, USA).
198

3. Results and discussion

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

Figure 1 shows bacterial growth inhibition curves obtained for each microcosm. Generally, a sigmoid dose-response behaviour is observed in the inhibition curves, indicating that when the added Cr concentration to bacterial suspension was low, relative bacterial growth was close to 1, while decreased when the Cr concentration increased. Most of the bacterial growth data fitted the logistic model, obtaining $R^2 \geq 0.87$, (Table S5). However, some data from 1000 and 2000 mg Cr·kg^{-1} did not fit the logistic model, i.e., bacterial populations were not able to normally grow probably due to high Cr toxicity. In the case of 2000 mg·kg^{-1}, bacterial populations only grew normally in 4 of the 10 studied soils, while at 1000 mg·kg^{-1} they grew normally in 7 soils. These differences in bacterial growth for the same Cr levels may indicate the influence of soil properties on Cr availability, as was previously suggested by Van Beelen et al. (2004). They found tolerant communities to Cr(III) in polluted soils with high Cr levels (2894 mg·kg^{-1}) but also reported that microbial communities from soils polluted with 3935 mg Cr·kg^{-1} did not show tolerance to Cr(III), suggesting the influence of soil properties on metal toxicity. Therefore, in order to determine which properties influence Cr toxicity, the data of 1000 and 2000 mg Cr·kg^{-1} were not considered in the following analysis.

The log IC_{50} values determined from inhibition curves using the logistic model (equation 1) are presented in Table 1. Bacterial community tolerance to Cr (log IC_{50}) greatly varied between soils, even in the reference soils with no added Cr, log IC_{50} oscillated from -6.40 (S8) up to -3.88 (S6) (log units). The variation of bacterial community tolerance to Cr in the reference soils may be an indicator that the development of PICT is dependent on soil type. In addition, this bacterial community tolerance to Cr fluctuation in reference soils, together with the natural Cr content in soils (7 – 394 mg·kg^{-}

[1], Table S2), highlights the importance of selecting reference soils for PICT studies (Campillo-Cora et al., 2022a; Campillo-Cora et al., 2021b). Likewise, when Cr was added to soils, bacterial community tolerance to Cr varied greatly between soils with the same Cr level. A range from -6.37 (S8) to -3.56 (S6) was determined for soils polluted with the lowest Cr level in soil (31.25 mg $Cr \cdot kg^{-1}$); from -6.27 (S8) to -3.79 (S7) for 62.5 mg $Cr \cdot kg^{-1}$; from -6.26 (S8) to -3.65 (S7) for 125 mg $Cr \cdot kg^{-1}$; from -6.27 (S5) to -3.41 (S7) for 250 mg $Cr \cdot kg^{-1}$; and from -6.09 (S8) to -2.87 (S3) for 500 $mg \cdot kg^{-1}$.

Overall, bacterial communities showed two different responses to Cr addition to the soil (Figure 2): (1) bacterial communities of S1, S2, S3, S6, S7, S8 and S10 developed tolerance in response to Cr additions; while (2) bacterial communities of S4, S5 and S9 did not develop tolerance following Cr addition to the soil. Based on the PICT hypothesis, the bacterial community is first exposed to the metal (i.e. selection phase of PICT), and if metal exerts toxicity, then the most sensitive organisms of the community will disappear, while the tolerant ones will be favoured. Therefore, whether the microbial community developed tolerance to Cr is a toxicity indicator. Later, the microbial community tolerance is quantified through a second exposition to Cr (i.e., detection phase of PICT) (Blanck, 2002; Tlili et al., 2016). Accordingly, Gong et al. (2002) and Ipsilantis and Coyne (2007) reported an increase in bacterial community tolerance to Cr with increasing Cr levels in soil and rhizosphere. Van Beelen et al. (2004) found that bacterial community tolerance to Cr(VI) increased with increasing Cr in pore water. Ogilvie and Grant (2008) determined a tendency to increase the bacterial community tolerance to Cr when the Cr level increases in estuarine sediments. Our results showed that bacterial community tolerance to Cr increased with increasing Cr levels in soils only in 7 of the 10 soils studied (Figure 2). However, our results showed that the Cr level in soil from which bacterial communities developed tolerance to Cr varied depending on the soil ($\Delta \log IC_{50}$

> 0.3). Bacterial communities from S7 and S10 showed an increased tolerance at 31.25 mg $Cr \cdot kg^{-1}$, bacterial communities from S1 and S3 at 62.5 mg $Cr \cdot kg^{-1}$, bacterial communities from S2 and S8 at 250 mg $Cr \cdot kg^{-1}$, and bacterial communities from S6 at 500 mg $Cr \cdot kg^{-1}$. In other words, Cr was more toxic for bacterial communities depending on soil type, following the sequence: S7, S10 > S1, S3 > S2, S8 > S6. In other soils, our results show that microbial communities did not develop tolerance to Cr, even at high Cr levels. For example, bacterial communities of S6 did not show tolerance to Cr even at 2000 $mg \cdot kg^{-1}$ (Figure 2). Similarly, Shi et al. (2002b, 2002a) and Ipsilantis and Coyne (2007) did not find tolerant microbial communities to Cr even at high Cr levels, from 447 up to 263000 mg $Cr \cdot kg^{-1}$. Therefore, considering that Cr-pollution sometimes has no toxic effect on microbial communities and that, in other cases, microbial communities are affected by Cr from very low levels of Cr-pollution, including soil properties in the assessment of Cr-pollution is highly recommended, as for other heavy metals (Campillo-Cora et al., 2022b).

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

The bacterial community tolerance to metals may be influenced by several soil properties, such as soil pH, clay content or organic matter content (Ogilvie and Grant, 2008; Shi et al., 2002b). The effect of soil properties on bacterial community tolerance can occur in soil (selection phase of PICT), or in the determination phase of PICT. The effect of the soil properties in the selection phase occurs in the soil, i.e. the first time bacterial communities are exposed to the metal. For example, Fernández-Calviño and Bååth (2016) found that bacterial community tolerance to Cu was lower in vineyard soils with high pH in comparison to more acid soils, as Cu toxicity was reduced. On the other hand, the effect

of soil properties may occur in the detection phase, i.e. confounding factors leading to altered tolerance measures (Lekfeldt et al., 2014). For example, Fernández-Calviño et al. (2011) reported that the measurement of PICT to Cu was altered because of the presence of the finer soil fraction in the bacterial suspensions when Cu concentrations were added. That is, the finer particles will bind part of the Cu added to bacterial suspensions, resulting in lower available Cu, so higher Cu concentrations will be necessary to inhibit the bacterial growth leading to apparent higher tolerance, i.e. overestimated bacterial community tolerance to Cu.

The equation presented in Table 2 related the increase of bacterial community tolerance to Cr ($\Delta\log IC_{50}$) with soil properties, explaining 95.6 % of the data variance ($p < 0.001$). Only $\Delta\log IC_{50}$ for 500 mg $Cr \cdot kg^{-1}$ were used. The increase of bacterial community tolerance to Cr was estimated by using soil properties ($p < 0.05$): DOC and extracted Cr using distilled water (H_2O-Cr). Figure 3 shows estimated $\Delta\log IC_{50}$ versus measured $\Delta\log IC_{50}$, with a homogeneous distribution around the line 1:1 ($R^2 = 0.95$).

DOC showed a significant positive relationship with $\Delta\log IC_{50}$ ($p < 0.05$; Table 2), i.e. when DOC increases, the bacterial community tolerance to Cr also increases. This DOC effect might be a confounding factor in the detection phase of PICT, as was previously reported for Cu (Campillo-Cora et al., 2021b; Lekfeldt et al., 2014). When bacterial communities are extracted from soil, DOC is extracted too. Later, when Cu is added to bacterial suspensions, Cu and DOC may bind together (Beesley et al., 2010), reducing Cu bioavailability and altering bacterial community tolerance to Cr (overestimation). Bérard et al. (2016) reported a similar effect for microbial community tolerance to Pb measurements. However, in a previous study (Campillo-Cora et al., 2023), we found that when dissolved organic matter (DOM) increases on bacterial suspensions, then bacterial community tolerance to Cr decreases, i.e. when DOM increases in bacterial

299 suspensions, Cr becomes more toxic to bacteria. Hence, the DOC effect in Cr
300 bioavailability in the detection phase should be discarded because of the positive
301 relationship with $\Delta\log IC_{50}$ (Table 2) and attributed to an effect in the selection phase in
302 soil. In the soil, however, when DOC is present, Cr(VI) may be reduced to Cr(III), i.e. Cr
303 toxicity decreases when DOC is present (Ao et al., 2022). If fact, the use of organic
304 amendments to reduce Cr toxicity in soils is very common (Abou Jaoude et al., 2020;
305 Mitchell et al., 2018; Yang et al., 2021). A hypothesis is that the presence of DOC in soil
306 enhanced the reduction of Cr(VI) to Cr(III) (Wittbrodt and Palmer, 1997), but during this
307 process free radicals may also be formed (Kotaś and Stasicka, 2000), increasing general
308 toxicity for bacterial communities (Campillo-Cora et al., 2023). In response to increased
309 toxicity in soil, then bacterial communities showed tolerance to Cr. Another hypothesis
310 might be the ability of Cr(III) to coordinate various organic compounds, leading to the
311 inhibition of some metalloenzyme systems (Kotaś and Stasicka, 2000), which might
312 result in a more tolerant bacterial community.

313 The Cr fraction extracted with distilled water (H_2O-Cr) showed a positive
314 relationship with $\Delta\log IC_{50}$ ($p < 0.001$, Table 2). Usually, the soluble form of heavy metals
315 represents the soil solution metal content, which is the most mobile and bioavailable form
316 (Kabata-Pendias, 2011), and in the case of Cr in soils is usually Cr(VI) (Ao et al., 2022).
317 Thus, H_2O-Cr exerts its effect in soil, during the selection phase. H_2O-Cr content in soil
318 increases as added Cr level in soils increases (Campillo-Cora et al., 2021a). Whether Cr
319 exerts toxicity, the most sensitive bacterial species were removed, while the tolerant ones
320 survived, resulting in a more tolerant community to Cr. Later, in the detection phase,
321 when bacterial growth is measured and Cr is added to bacterial suspensions, tolerant
322 bacteria allow greater Cr concentrations, leading to a higher tolerant community. Van
323 Beelen et al. (2004) found a significant increase in microbial community tolerance to

Cr(VI) with Cr(VI) pore-water concentration. Similarly, Fernández-Calviño and Bååth (2016) reported a positive relationship between bacterial community tolerance increase (Δlog IC_{50}) to Cu versus water-soluble Cu concentrations logarithm ($R^2 = 0.79$). Kunito et al. (1999) also determined a positive correlation between IC_{50} values and soluble-exchangeable Cu ($r = 0.76$), while total Cu did not show any significant relationship ($r = 0.013$, $p > 0.05$).

3.3 Concluding remarks

In the present study, we aimed to improve the PICT methodology for the assessment of soil pollution, using bacterial growth as the endpoint. Dissolved organic carbon (DOC) and the fraction of Cr extracted with distilled water (H_2O-Cr) were the main factors controlling the Cr effect on microbial communities, determined by the increase of bacterial community tolerance to Cr. The main selection pressure of Cr on the microbial community presumably occurs in soil, i.e. the selection phase of PICT. In the case of DOC, Cr became more toxic to bacterial communities as DOC increased in soil, leading to an increase in bacterial community tolerance to Cr in response to toxicity. Secondly, H_2O-Cr is related to the toxic and active form of Cr, probably Cr(VI), and the higher the H_2O-Cr content in the soil, the higher the tolerance to Cr developed by bacterial communities. The outcomes of this study may be helpful for normalising Cr toxicity thresholds for soil with different properties. In addition, overestimations or underestimations of Cr toxicity based on total or bioavailable Cr content may be avoided, since soil properties should be considered during risk assessment.

Acknowledgements

This study has been funded by the Spanish Ministry of Economy and Competitiveness through the project CTM2015-73422-JIN (FEDER Funds). David Fernández-Calviño holds a Ramón y Cajal contract (RYC-2016-20411) financed by the Spanish Ministry of Economy, Industry and Competitiveness. Daniel Arenas-Lago thanks the Ministerio de Ciencia e Innovación of Spain and the University of Vigo for the postdoc grant Juan de la Cierva Incorporación 2019 (IJC2019-042235-I). Claudia Campillo-Cora holds a Predoctoral fellowship financed by Xunta de Galicia (ED481A-2020/084). Funding for open access charge: Universidade de Vigo/CISUG.

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Tables

Table 1

Bacterial community tolerance (expressed as log IC_{50}) to different levels of Cr pollution in the 10 studied soils (average ± SE)

Cr (mg·kg^{-1})	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**0**
Soil	Log IC_{50}±error	Log IC_{50}±error	Log IC_{50}±error	Log IC_{50}±error	Log IC_{50}±error	Log IC_{50}±error	Log IC_{50}±error	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

*Unadjusted data

570 **Table 2**

571 The equation for estimating bacterial community tolerance increase to Cr (Δlog $IC_{50(500-0)}$)
572 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) **H_2O-Cr** *(p<0.001)*	87.309	<0.001	0.956

573 DOC is dissolved organic carbon ($g \cdot kg^{-1}$); H_2O-Cr is Cr extracted using H_2O. Values associated with the
574 independent variables are shown together with the standard errors (±). P-values associated with each
575 independent variable are shown below variables (in brackets)
576
577
578

Figures

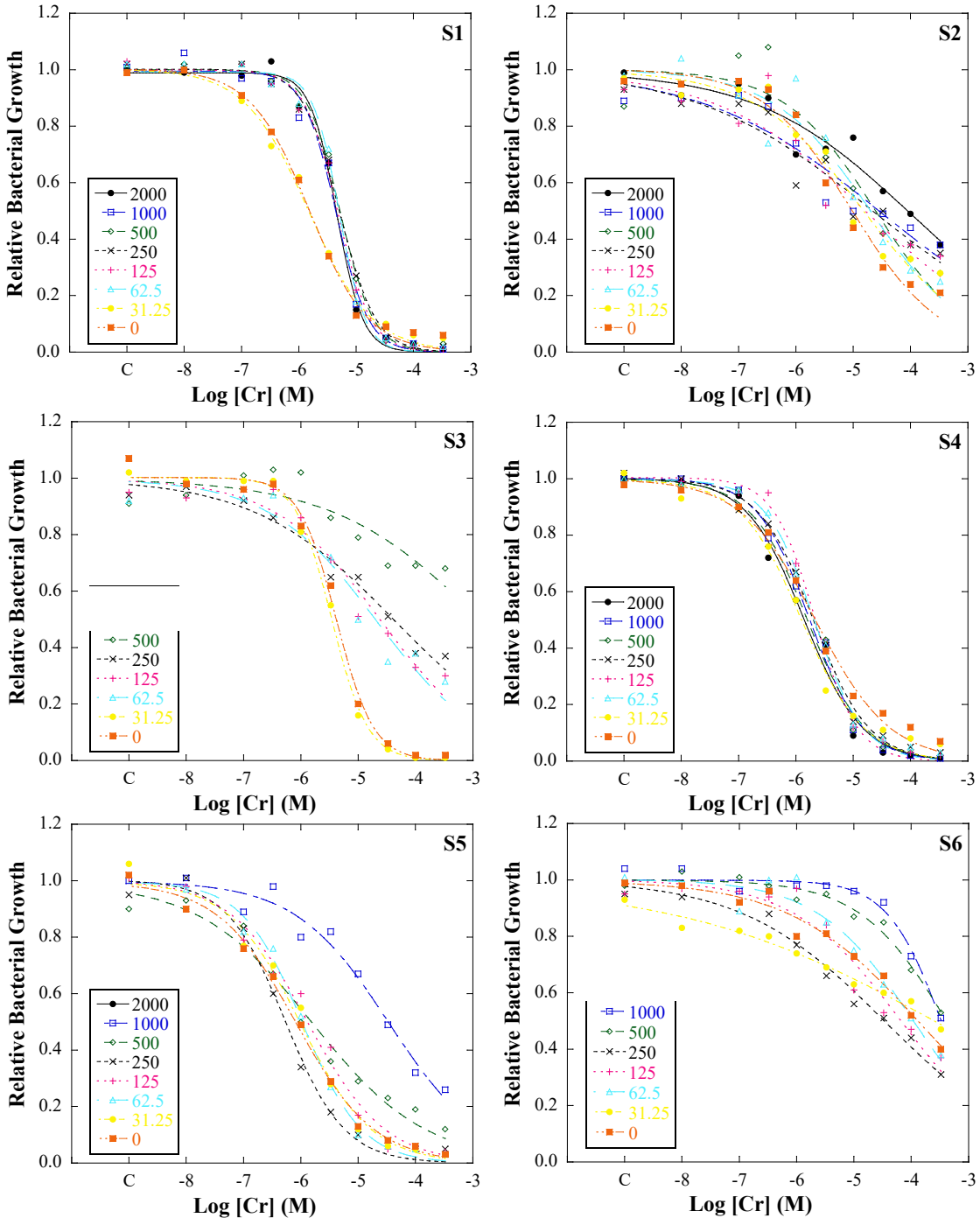
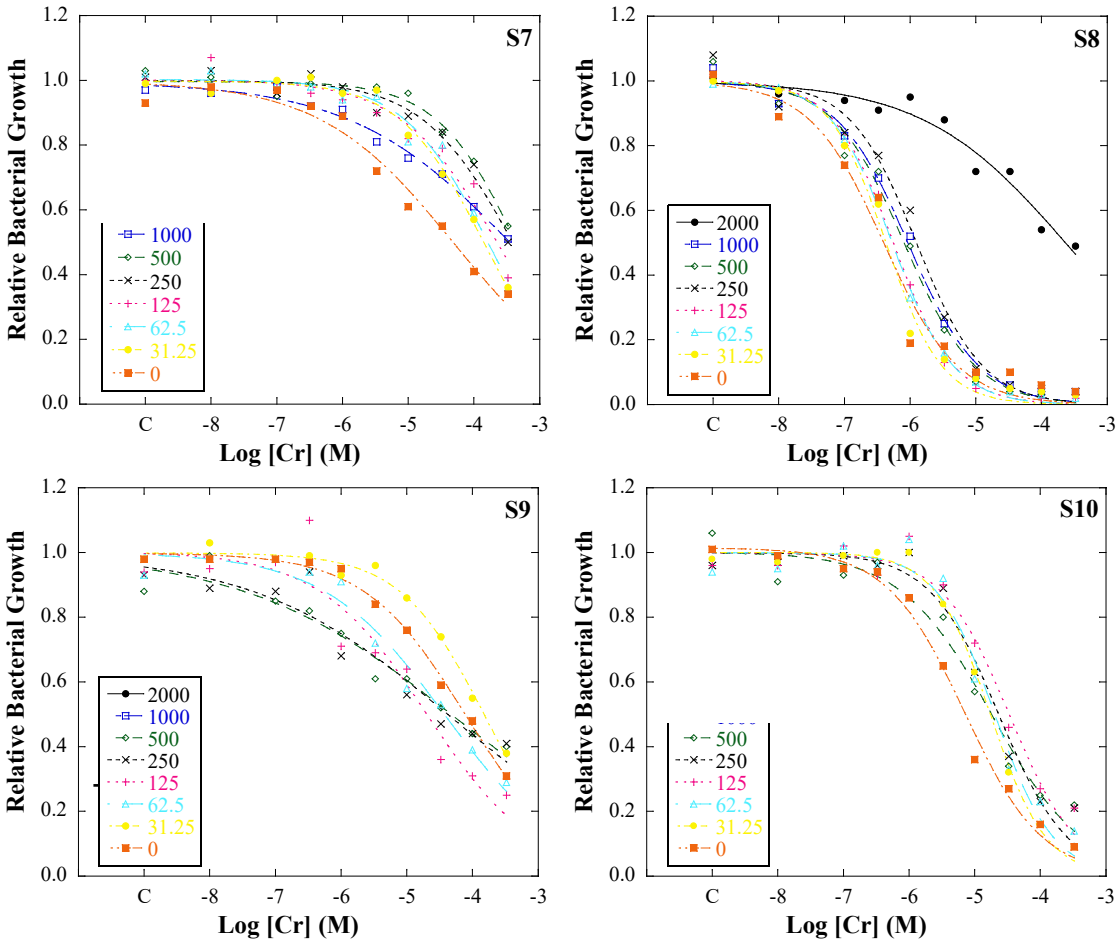


Figure 1. Bacterial growth inhibition curves for bacterial suspensions extracted from 10 soils artificially polluted with a range of Cr concentrations: 2000, 1000, 500, 125, 62.5, 31.25 and 0 $mg \cdot kg^{-1}$. Dots indicate real data measured, while the lines represent the fit of the data to the logistic model used. S1, S2, S3, S5, S6, S7, S8, S9 and S10 are referred to studied soil 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.



586 **Figure 1** (continued)

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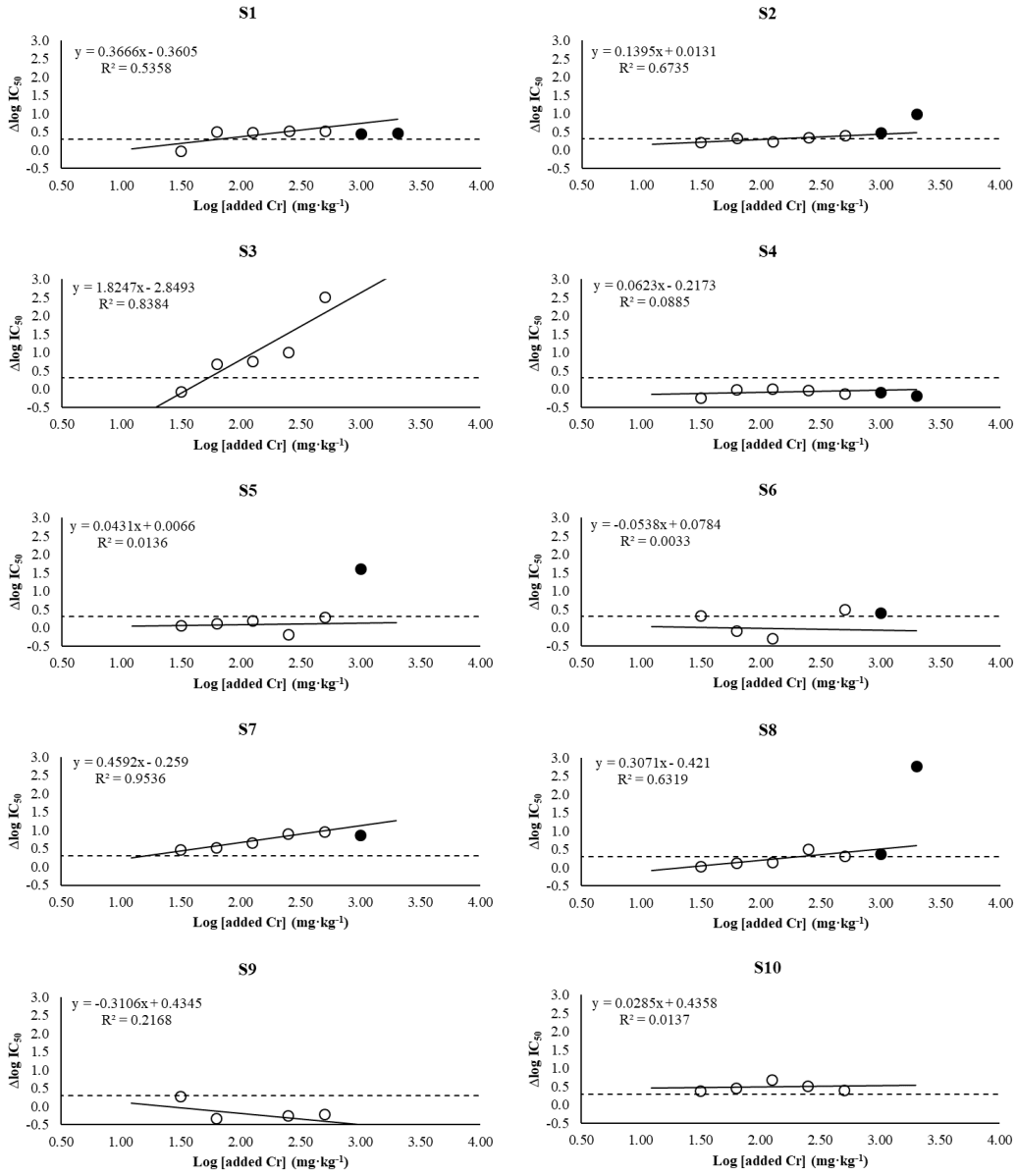

588

589 **Figure 2** Bacterial community tolerance variation (expressed as Δlog IC_{50} concerning
590 unpolluted soil) to a range of added Cr to soil (in logarithm scale). White dots represent
591 data from Δlog $IC_{50(31.25-0)}$, Δlog $IC_{50(62.5-0)}$, Δlog $IC_{50(125-0)}$, Δlog $IC_{50(250-0)}$ and Δlog
592 $IC_{50(500-0)}$. Black dots represent data from Δlog $IC_{50(1000-0)}$ and Δlog $IC_{50(2000-0)}$. Continuous
593 lines represent linear regression fit. The discontinuous line represents the value (0.3) from
594 which it is considered that the bacterial community has developed tolerance. S1, S2, S3,
595 S5, S6, S7, S8, S9 and S10 are referred to studied soil 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.

596

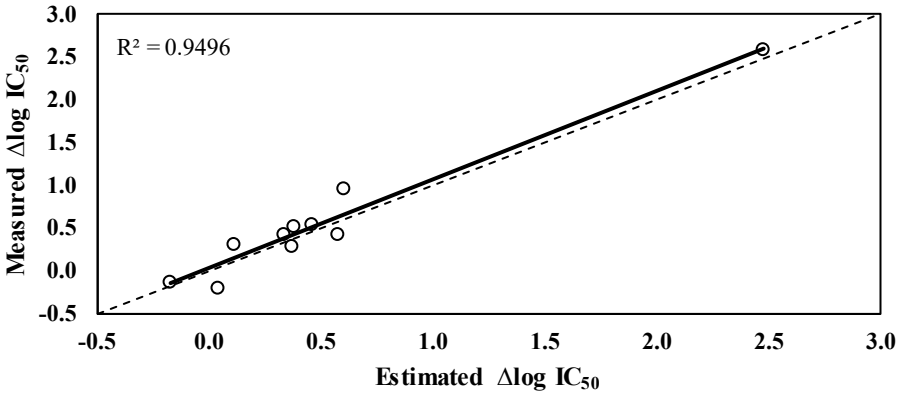

Figure 3. Relationship between measured and estimated Δlog IC_{50} using the equation from Table 2. The stippled line indicated a 1:1 relationship.