



Evaluation of bacterial biosensors to determine chromate bioavailability and to assess ecotoxicity of soils



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HIGHLIGHTS

- Bacterial biosensors pCHRGFP1 and pCHRGFP2 are able to measure chromate in soils.
- Biosensors are alternative methods to EPA 7199 and DPC for chromate measurement.
- Soil properties influence the rates of water-extractable chromate decrease.
- Springtails grazers of bacteria influence the chromate fate in soil.
- Reproduction of springtails correlates with bioavailable chromate in soil.

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ABSTRACT

Chromate can be considered a potent environmental contaminant and consequently, an understanding of chromate availability and toxicity to soil biology is essential for effective ecological assessment of metal impact in soils. This study shows the response of two bacterial bioreporters, pCHRGFP1 *Escherichia coli* and pCHRGFP2 *Ochrobactrum tritici*, to increasing concentrations of chromate in two different soils. The bioreporters, carrying the regulatory gene *chrB* transcriptionally fused to the *gfp* reporter system, exhibited different features. In both, the fluorescence signal and the chromate concentration could be linearly correlated but *E. coli* biosensor functioned within the range of 0.5–2 μM and *O. tritici* biosensor within 2–10 μM chromate. The bioreporters were validated through comparative measurements using the chemical chromate methods of diphenylcarbazide and ionic chromatography. The bacterial sensors were used for the estimation of bioavailable fraction of chromate in a natural soil and OECD artificial soil, both spiked with chromate in increasing concentrations of 0–120 mg Cr(VI) kg^{-1} of soil. OECD soil showed a faster chromate decrease comparing to the natural soil. The toxicity of soils amended with chromate was also evaluated by ecotoxicological tests through collembolan reproduction tests using *Folsomia candida* as test organism. Significant correlations were found between collembolans reproduction and chromate concentration in soil (lower at high chromate concentrations) measured by biosensors. Data obtained showed that the biosensors tested are sensitive to chromate presence in soil and may constitute a rapid and efficient method to measure chromate availability in soils.

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1. Introduction

The rapid development of the industry related with paper production, fertilizers, pesticides and others has conducted to the discharge of large amounts of metal-contaminated residues into the environment, resulting in a serious problem of environmental contamination. Unlike organic contaminants, metals are not

biodegradable and tend to accumulate in living organisms, becoming toxic and carcinogenic. For instance, chromium is a metal with different oxidation states, although only hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)] are stable in the environment (Krishna and Philip, 2005). The hexavalent chromium compounds exist mainly as chromate and dichromate and have high solubility, bioavailability and mobility. These compounds are associated with several diseases such as allergic reactions, contact dermatitis and cancer of the lung (Ramírez-Díaz et al., 2008). The Agency for Toxic Substances & Disease Registry (ATSDR) from USA included the Cr(VI) in hazardous substances list

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(<http://www.atsdr.cdc.gov/spl/index.html>) since 2011. Chromium is commonly used in metal finishing and tanning industries and, therefore, soil may be contaminated with chromium through wastewaters and land disposal of sewage sludges (Zheng et al., 2007).

The soil is a very complex and heterogeneous matrix whose biodiversity supports the provision of several ecosystem services (nutrient cycling and soil formation) of most importance for food production and maintaining socioeconomic activities (Bronick and Lal, 2005). The preservation of soils depends on monitoring soil contamination in order to prevent the dispersion of pollution and avoid drastic consequences. Therefore, it is important to map the concentration of toxic compounds present in soils. The development of cost effective methods to measure soil contamination, namely regarding the contaminant fraction most available for soil organisms, is needed. According to the literature, metal toxicity and particularly, chromate toxicity in soil depends on soil properties such as organic matter content, the concentration of metal ions (iron and manganese) that influence the oxidation–reduction cycle of chromium (Kotaś and Stasicka, 2000), soil texture (percentage of clay, silt and sand) and pH (Dube et al., 2001; Banks et al., 2006).

There are various classical methods for metal detection. These include atomic absorption spectroscopy (AAS), atomic emission spectroscopy (AES), and inductively coupled plasma mass spectrometry (ICP-MS). These methodologies involve expensive instrumentation, require additional chemical compounds, which are pollutants and are unable to detect metal bioavailable concentrations. Nevertheless, the measurement of the bioavailable fraction of metals is a parameter of high interest since it determines the toxicity of metals to the organisms (Bontidean et al., 2004).

Assays using organisms have been developed to evaluate the toxicity of contaminants in the environment, which is directly related to their bioavailable fraction (Girotti et al., 2008). Assays using microorganisms have been seen as an excellent methodology since they have short life cycle, can be easily maintained in laboratory cultures at low cost and are highly sensitive and selective to specific analytes (Tibazarwa et al., 2001). In this way, the development of metal-specific biosensor tools functioning on the basis of a reporter system has been acquiring increasing attention. The use of microbial cells as the biological recognition element may be an important tool in environmental studies to evaluate the extent of contaminated areas and to monitor bioremediation processes. Two examples are the use of MC1061 (p_{znt}RLuc) and AE104 (p_{chr}BLuc) biosensors to detect the bioavailable fraction of cadmium, zinc, mercury and chromium in soil (Ivask et al., 2002). More recently, the use of biosensors constructed through the fusion of the regulatory gene, *chrB* of the *chr* resistance determinant of *Ochrobactrum tritici* 5bv11 (Branco et al., 2008) with the reporter gene, green fluorescence protein (*gfp*), has been reported as an efficient and sensible approach to detect chromate in environmental waters spiked with chromate (Branco et al., 2013). These biosensors, pCHRGFP1 *Escherichia coli* and pCHRGFP2 *O. tritici*, have revealed distinct sensitivity to chromate concentrations. In the present work, these two biosensors were used with the aim to detect and monitor Cr(VI) in different chromate contaminated soils showing the usefulness of the biosensors as an alternative tool for monitoring chromate. Collembolan reproduction tests with *Folsomia candida*, following standard procedures (usually performed to evaluate the habitat function of contaminated soils), were also performed as a way to relate the quantification of Cr(VI) with the toxicity toward this invertebrate species. Additionally, measurements of Cr(VI) in aqueous solutions through biosensors were compared with measurements performed by classic chemical methods (using diphenylcarbazide and ionic chromatography methods) in the same solutions to further validate the usefulness of the biosensors for chromate measurements.

2. Material and methods

2.1. Test soils

In this study two different soil types were tested: (1) a natural soil collected in the campus of Coimbra Agronomic School and (2) an artificial standard soil from the Organization for Economic Cooperation and Development (OECD). The natural soil is characterized by 62.4% of sand, 21.2% of silt, 16.4% of clay (sandy loam texture; LNEC, 1970), pH 6.9, cation exchange capacity of 0.025 cmol g⁻¹ (ISO, 1994), organic matter content of 3.3 ± 0.1% (loss on ignition at 500 °C for 6 h) and water-holding capacity of 36.2% (ISO, 1999). The OECD artificial soil was composed by 69.5% of sand, 10% of *Sphagnum* peat (air dried and sieved at 2 mm), 20% of clay and 0.5% of calcium carbonate to adjust the pH to 6 ± 0.5. Its water-holding capacity was 65.1% (ISO, 1999).

Contamination gradients of sodium chromate were prepared, in natural and OECD artificial soil, immediately before the beginning of the experiments. Portions of 300 g (dry weight equivalent; DW) of the natural and the OECD artificial soils were weighted and different amounts of a sodium chromate stock solution (1 M) were added to each soil to obtain the final gradient of contamination of 0, 10, 20, 40, 60, 80 and 120 mg Cr(VI) kg⁻¹ soil (Table 1).

2.2. Ecotoxicological tests

Collembola reproduction tests were performed using the springtails *F. candida* and following the procedures described in the ISO 11267 (ISO, 1999). The test organisms were taken from the laboratory cultures of the University of Coimbra. Synchronized cultures were prepared following the procedures described by Natal-da-Luz et al. (2009) and only organisms 10–12 d old were used in the reproduction tests. Springtails were exposed to a concentration gradient of Cr(VI) in the natural soil and OECD artificial soil (procedures for soils contamination are described in Section 2.1). Soil moisture was adjusted to 50% of the water-holding capacity before being used in the tests. At the beginning of the experiment the soil moisture and the pH were measured in each test treatment. For each concentration five replicates were prepared. The replicates consisted of glass flasks (4 cm of diameter, 7 cm of height) with 30 g of fresh soil and ten springtails. Two milligrams of granulated dry yeast were added as food at the beginning and after 14 d of test. The test containers were covered with a lid during the test and opened weekly for a few seconds to allow aeration. The experiment was conducted at a constant temperature of 20 ± 2 °C, and under a photoperiod of 16 h light and 8 h dark. At the day 14, the water loss was reestablished by compensating the weigh losses of the test containers with distilled water when the weight loss was higher than 2%. After 28 d of exposure, each test container was emptied into a small vessel, which subsequently was filled with water. After the addition of few drops of blue ink and gentle stirring, the animals floating on the water surface were

Table 1

Natural and OCDE soil treatments used in this study. Soils were spiked with the different concentrations of chromate.

Soil treatments	Final concentration of chromate in soils (mg kg ⁻¹)	μM chromate g ⁻¹ of soil
C0	0	0
C1	10	19.2
C2	20	38.5
C3	40	79.9
C4	60	115.4
C5	80	153.9
C6	120	230.8

photographed and the number of juveniles and surviving adults was determined. Missing adult springtails were considered dead. An additional replicate per test concentration, but without springtails, was prepared and submitted to the same conditions for pH and moisture measurements at the end of the test. Additionally, 3 replicates with springtails and 3 replicates without springtails were prepared for each test concentration and submitted to the same conditions. These replicates were sampled by collecting 1 g of soil twice in each container after 0, 14 and 28 d after the beginning of the test to evaluate the available concentration of Cr(VI) over the experiment in the different test treatments.

2.3. Extraction of chromate from soil

For chromate extractions, the soil samples (1 g) were adjusted to a 1:10 soil:water ratio (using autoclaved ultrapure water) and placed on an orbital shaker (250 rpm), at 25 °C for incubation. Since during the incubation, Cr(VI) may be reduced to Cr(III) by humic acids (Huang et al., 2012), incubation periods of 1, 3 and 16 h were used. After these incubation times, all soil–water suspensions were centrifuged by 3000 rpm for 10 min at 4 °C and the supernatant recovered.

2.4. Quantification of Cr(VI)

In order to compare the efficiency and sensitivity of different techniques in chromate quantification, aqueous chromate solutions with increasing concentrations of chromate (0–10 µM) were prepared by using a 1 M of a stock solution of sodium chromate (Na₂CrO₄). The concentration of chromate in these solutions was measured by three different methodologies: two chemical analyses (colorimetric method and ionic chromatography) and one biological analysis (chromate biosensors). This last technique was also used to determine the chromate in the soil samples.

2.4.1. Colorimetric diphenylcarbazide (DPC) method and ionic chromatography (EPA 7199 method)

For the colorimetric method, three samples per solution were measured and a diphenylcarbazide solution was used following the procedures described in Standard Methods (1998). The absorbance values obtained at 540 nm were converted to chromate concentrations based on a calibration curve. For the measurements through ionic chromatography, the methods described by EPA-RCA 7199 (<http://www.epa.gov/epaoswer/hazwaste/test/main.htm>) were followed using one sample per concentration. Samples were filtrated through a 0.45 µm filter and the pH adjusted to 9.0–9.5 with a buffer solution of (NH₄)₂SO₄/NH₄O prior to chromatography analysis (Dionex ICS-5000).

2.4.2. Chromate biosensors

The strains pCHRGFP1 *E. coli* and pCHRGFP2 *O. tritici* (Branco et al., 2013) were used as biosensors to measure the chromate concentration in the aqueous chromate solutions used with the chemical methods and in extracts obtained from the soil samples collected over the collembolan reproduction test (chromate extraction from soil samples described in Section 2.3).

Each chromate biosensor was inoculated in Luria Bertani (LB) at 37 °C, overnight in an orbital shaker at 180 rpm to increase cellular mass. After incubation, each culture was diluted in 100 mL of LB medium to an Optical Density (OD 600 nm) of 0.2, and incubated at 37 °C in an orbital shaker at 180 rpm until mid-exponential growth phase (approximately 3 h of incubation). Afterward, cultures were centrifuged at 4000 rpm (Eppendorf 5810 R) for 20 min, and cells were resuspended in TMM medium (6.06 g L⁻¹ Tris, 4.68 g L⁻¹ NaCl, 1.49 g L⁻¹ KCl, 1.07 g L⁻¹ NH₄Cl, 0.43 g L⁻¹ Na₂SO₄, 0.2 g L⁻¹ MgCl₂·6H₂O, 0.03 g L⁻¹ CaCl₂·2H₂O, 0.23 g L⁻¹

Na₂HPO₄·12H₂O) supplemented with 0.3% glucose. These bacterial suspensions were used for biosensors assays.

For each bioassay, 2 mL of the bacterial suspension and the same volume of Cr(VI) standard solutions or soil–aqueous solutions were mixed and incubated in an orbital shaker (180 rpm) at 37 °C for 5 h. This period was chosen based on preliminary works that revealed higher fluorescence signals after 5 h of incubation of biosensors with chromate solutions. Afterward, aliquots of 200 µL (in triplicate) were collected from each solution and transferred to clear 96 wells microplate (Corning). Fluorescence intensity was measured through a fluorescence microplate reader (Infinite M200, Fisher) with excitation and emission wavelengths of 475 and 510 nm, respectively.

A calibration curve for each biosensor was constructed in order to correlate the fluorescence signal emitted by bacteria with Cr(VI) concentration of the solution. Thus, increasing concentrations of chromate (0–20 µM) in aqueous solutions were prepared from a stock solution of Na₂CrO₄ 1 M and submitted to the bacterial suspensions as previously described.

2.5. Statistical analysis

In the calibration curves, one-way ANOVAs followed by Dunnet post-hoc tests were performed to estimate the LOEC (lowest-observed-effect concentration) for each biosensor. In each aqueous solution, the chromate measurements performed by biosensors were compared with chromate measurements by colorimetric diphenylcarbazide through independent samples *t*-tests. The measurements using biosensors were compared with measurements using ionic chromatography method through single sample *t*-tests.

The chromate measurements performed in the soils from the extra replicates of the collembolans reproduction tests with and without organisms after 0, 14 and 28 d of test were compared in each test concentrations through repeated measures ANOVAs (using the test containers as subject) followed by a post-hoc Newman–Keuls test. In collembolans reproduction test the number of surviving adults and juveniles observed in each replicate of the test concentrations was compared to that of the control replicates by means of a one-way ANOVAs followed by a Dunnet's post-hoc test. Spearman correlations were used to evaluate the association between the average number of juveniles found in each test concentration and the average of chromate concentrations measured over the collembolans reproduction tests (at 0, 14th and 28th d) in the replicates with and without springtails. The reproduction EC50 values for springtails were estimated considering the average of the chromate concentrations, measured over the experiment with the biosensors, in the replicates with and without collembolans, by using nonlinear regressions following the Hormesis model (EC, 2004). Data normality and homogeneity were previously checked by Kolmogorov–Smirnov and Bartlett tests, respectively, for ANOVAs and *t*-tests. For regressions, normality and homogeneity of variances were verified graphically by analyzing regression residuals. The statistical analyses of data were performed using STATISTICA, version 7.

3. Results

3.1. Optimization of chromate extraction from soil

Three shaken times (1, 3 and 16 h) for chromate amended soils were tested and the values of chromate extracted were compared. As 3 h of water–soil shaken resulted in higher chromate extraction level, this condition was chosen to perform all the chromate extractions performed for this study (data not shown).

The best results were obtained when biosensor strains were grown in LB medium until exponential phase, and then maintained on MMT medium supplemented with 0.3% glucose. Moreover, the highest fluorescence for chromate quantification by the biosensors method was achieved when using 96 well transparent microplates, excitation wavelength of 475 nm, and 5 h of incubation of mixtures (bacteria sensor and water–soil samples).

3.2. Calibration of the biological responses

The sensitivity of individual biosensors can be compared by assessing the lowest observable effective concentration (LOEC) that is the lowest chromate concentration which allowed a significant increased of fluorescence intensity, with twice of signal, compared to zero control. Moreover, the operational range of each biosensor was defined starting from the LOEC up to the chromate concentration which induced the maximum linear fluorescence. In these calibration experiments, the LOEC for pCHRGFP1 *E. coli* and pCHRGFP2 *O. tritici*, for standard chromate solutions, were 0.5 μM and 2 μM , respectively (Fig. 1). The chromate concentrations which induced the maximum fluorescence achieved in the linear interval also varied between the two biosensors. The pCHRGFP1 *E. coli* displayed maximum linear response when exposed to 2 μM of chromate and pCHRGFP2 *O. tritici* had the maximum linear signal induced at 10 μM of chromate. Considering the linear range behavior of biosensors, pCHRGFP1 *E. coli* and pCHRGFP2 *O. tritici*, showed a different operating range. Moreover pCHRGFP1 *E. coli* exhibited a high degree of sensitivity at low chromate concentrations but worked in a narrow concentrations range (0.5–2 μM) compared to pCHRGFP2 *O. tritici* (2–10 μM). Considering these calibration curves, the biosensor *E. coli* pCHRGFP1 was used to analysis the chromate concentrations $\leq 2 \mu\text{M}$ and the biosensor *O. tritici* pCHRGFP2 chromate concentrations between 2 μM and 10 μM .

3.3. Assessment of sensitivity of biosensors

To confirm the responsiveness of biosensors to detect chromate, the chromate measurements by biosensors and chemical methods (colorimetric diphenylcarbazide and ionic chromatography) were compared (Fig. 2). The diphenylcarbazide methodology was only able to detect concentrations of chromate greater than 3 μM . Among the methods tested, the diphenylcarbazide technique

generally showed measurements significantly different to those obtained by biosensors (except for the 4 μM concentration).

The measurements obtained using the EPA method 7199 showed high similarity to those obtained using the biosensors (in the most aqueous chromate solutions the measurements were not significantly different, $p > 0.05$). Therefore, bacterial sensors and EPA method 7199 were the most efficient to quantify the chromate from aqueous solutions.

3.4. Detection of chromate in soils

The available Cr(VI) concentration in the soil–water suspensions was measured with the biosensors, the dilution used in every assay and the detection limit or range of linearity of each sensor were taken into account for these assays. In both natural, and OECD artificial soils, the chromate concentration significantly decreased over the 28 d of the reproduction test, in the generality of the test concentrations, in both replicates with and without organisms. The exceptions were in test with natural soil on the C5 concentration (80 mg Cr(VI) kg^{-1}) in the replicates with and without organisms and on the C4 concentration (60 mg Cr(VI) kg^{-1}) in the replicates with organisms. In these cases, the chromate concentration after 14 d of the assay was significantly higher than that at the beginning of the test (Figs. 3 and 4). In the natural soil, the decrease of chromate over the 28 d was relatively slow but significant after 14 d of the experiment, in the most concentrations. The OECD soil exhibited a rapid decrease of chromate in all treatments (C0–C6) where short periods (less than 14 d) were enough to bring to zero μM of chromate.

3.5. Evaluation of chromate toxicity using springtails

The average number of adults and juveniles found in replicates of each test concentration of the collembolans reproduction test, with natural and OECD soils, is shown in Fig. 5. In natural soil, the number of surviving adults and juveniles was significantly lower than in control replicates (0 mg kg^{-1}), for concentrations higher or equal to 60 mg Cr(VI) kg^{-1} (C4). On the other hand, in artificial soil the number of adults and juveniles was not significantly different from control in any of the concentrations tested. The EC50 values estimated for natural soil were 4.60 (3.95–5.25) and 6.79 (5.19–8.38) mg Cr(VI) kg^{-1} , considering the chromate measurements using biosensors in soil from the replicates with and without springtails, respectively. The EC50 values could not

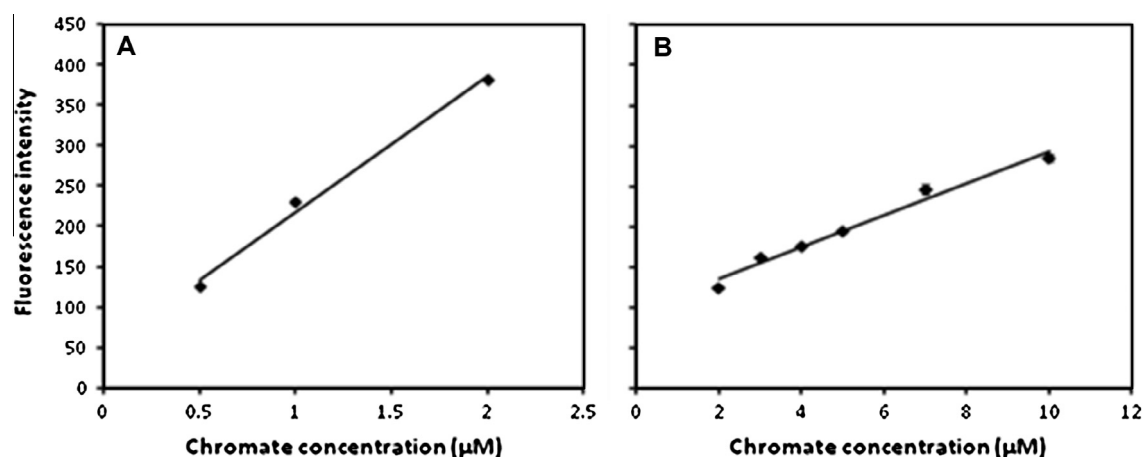


Fig. 1. Induction of fluorescence (expressed as fluorescence intensity; average, $n = 3$) of biosensors by increasing concentrations of chromate in aqueous solutions. Graph A shows the linear range of the calibration curve of pCHRGFP1 *E. coli* and the graph B shows the linear range of the calibration curve of pCHRGFP2 *O. tritici*. All results have standard error, calculated from triplicate measurements, but may not be visible due their small size.

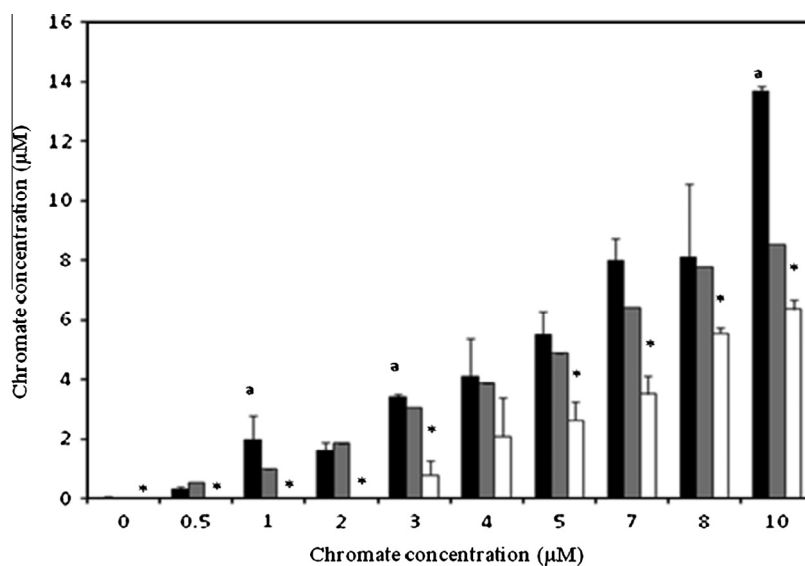


Fig. 2. Chromate concentrations measured in metal aqueous solutions with increasing contamination of chromate. The measurements were performed by biosensors (black bars), EPA 7199 method (EPA; gray bars) and diphenylcarbazide method (DCP; white bars). Each bar of the biosensors and EPA methods represent the mean value of three independent samples (+ standard deviation). * – means significant difference compared to the measurements through biosensors within the same concentration; a – means significant difference compared to the measurements through EPA method.

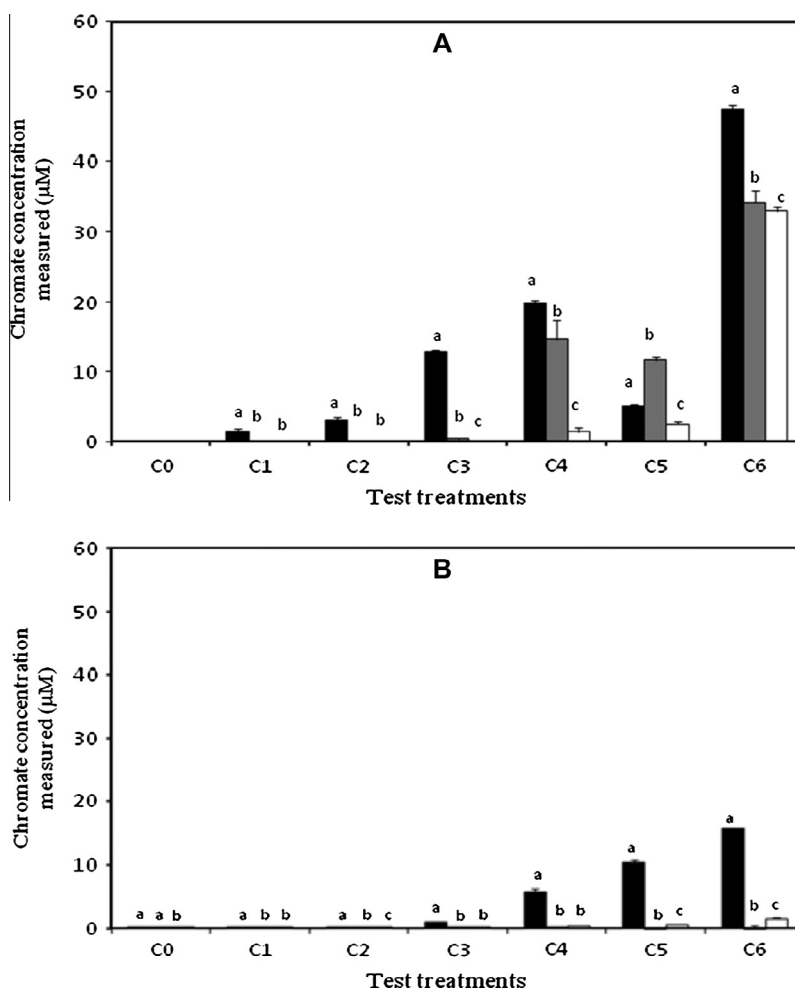


Fig. 3. Quantification of chromate (average + standard deviation, $n = 3$) present in natural (graph A) and OECD (graph B) soils without springtails by using biosensors at different times: 0 d (black column), 14 d (gray column) and 28 d (white column). Test treatments correspond to the following nominal chromate concentrations: C0 – 0 mg kg⁻¹; C1 – 10 mg kg⁻¹; C2 – 20 mg kg⁻¹; C3 – 40 mg kg⁻¹; C4 – 60 mg kg⁻¹; C5 – 80 mg kg⁻¹; C6 – 120 mg kg⁻¹. Different letters within the same treatment means significant differences along time.

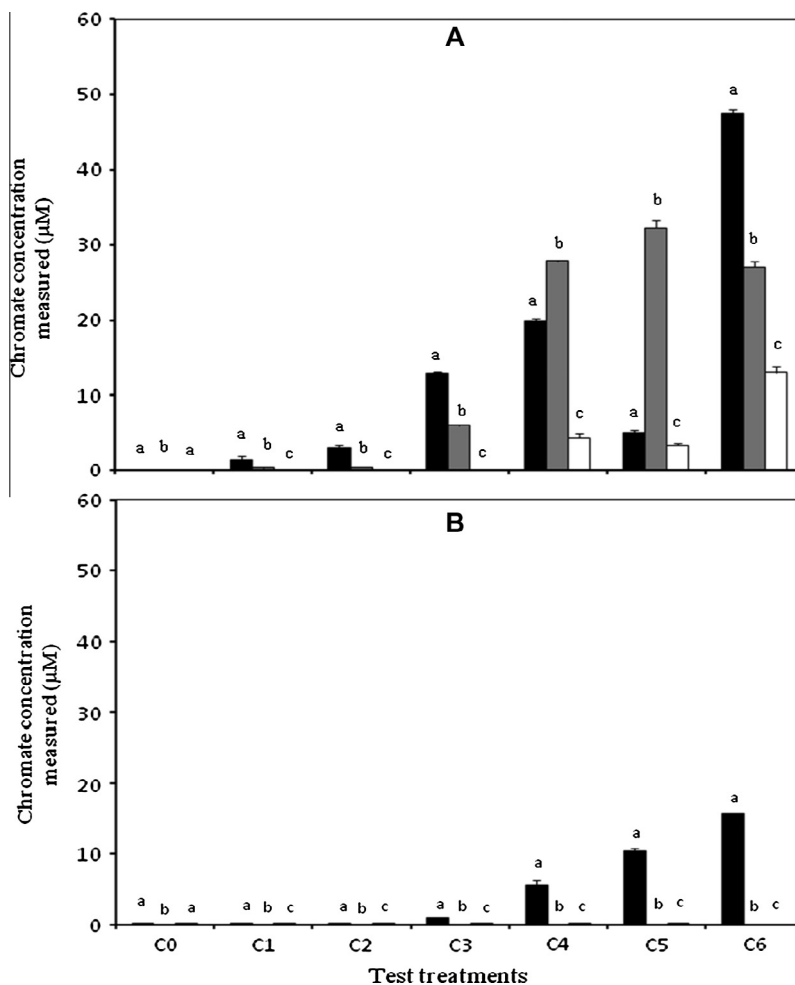


Fig. 4. Quantification of chromate (average + standard deviation, $n = 3$) present in natural (graph A) and OECD (graph B) soils with springtails by using biosensors at different times: 0 d (black column), 14 d (gray column) and 28 d (white column). Test treatments correspond to the following nominal chromate concentrations: C0 – 0 mg kg⁻¹; C1 – 10 mg kg⁻¹; C2 – 20 mg kg⁻¹; C3 – 40 mg kg⁻¹; C4 – 60 mg kg⁻¹; C5 – 80 mg kg⁻¹; C6 – 120 mg kg⁻¹. Treatment codes as in Fig. 3. Different letter within the same treatment means significant differences along time.

be estimated for OECD artificial soil since no toxicity was found for collembolans. The average number of juveniles in the replicates of the concentration gradient was significantly correlated with the average chromate measurements over the tests of natural soil with springtails ($R = -0.900$, $p = 0.006$) and in replicates of OECD soil with ($R = -0.767$, $p = 0.026$) and without ($R = -0.760$, $p = 0.029$) springtails. No significant correlation was found between juveniles production and chromate concentrations of replicates of natural soil without springtails ($R = -0.704$, $p = 0.078$).

4. Discussion

In the environmental, the portion of metal available to biota does not corresponds to a measure of total soil metal concentration. The evaluation of the bioavailable fraction of the metals in environment by different biosensors can involve a range of organisms with different characteristics and functions but their performance in complex environmental samples is sometimes affected (Maderova et al., 2011). Consequently, the successful environmental application of the biosensors developed up to now has been relatively limited (Ivask et al., 2007; Kohlmeier et al., 2008). Considering the limitations observed by using biosensors in soils, the objective of this work was to demonstrate the usefulness and adequacy of the use of two biosensors in the measurement of

chromate in soils with different physical and chemical properties. These biosensors, pCHRGFP1 *E. coli* and pCHRGFP2 *O. tritici*, were previously demonstrated to be very sensitive and very specific for chromate detection in river waters (Branco et al., 2013). However, in soil, its composition and physicochemical properties determine the binding capacity of metals as well as their oxidation state, and these two factors dictate the bioavailability of metals in this complex matrix. Furthermore, this study intended to relate the chromate bioavailability with its ecotoxicity in two soils with contrasting properties. This later parameter was determined using *F. candida* in standard reproduction tests, and determining the reproduction EC50 values based on the bioavailable concentration of chromate measured in the soil by biosensors.

Calibration curves performed with pCHRGFP1 *E. coli* and pCHRGFP2 *O. tritici* cells in standard aqueous chromate solutions confirmed that the biosensors had different sensitivities to chromate. The construction pCHRGFP1 *E. coli* showed higher sensitivity for chromate than pCHRGFP2 *O. tritici*. Other analytical methodologies, such as diphenylcarbazide and EPA 1799 methods, were also employed in order to compare the efficiency of the different approaches in chromate measurement. Biosensors and EPA 1799 methods demonstrated to be very sensitive and very accurate, whereas the colorimetric method showed a large discrepancy between the quantity measured and the chromate concentration in solution. The low sensitivity of DPC method to

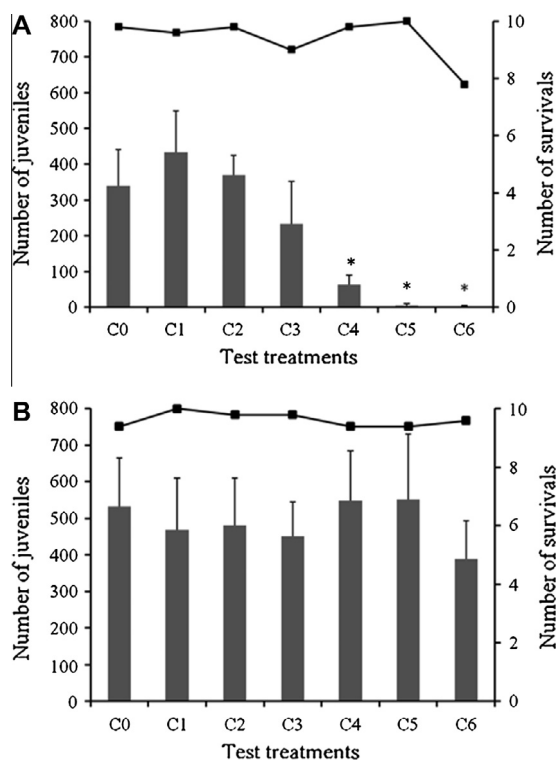


Fig. 5. Reproduction tests with *Folsomia candida*. Survival (black line; average, $n = 5$) and reproduction (gray bars; average + standard deviation, $n = 5$) when exposed to a natural soil (graph A) and OECD artificial soil (graph B) contaminated with increasing concentrations of chromate. Treatment codes as in Fig. 4. * means that the number of juveniles is significantly different from control ($p \leq 0.05$).

low concentrations have been already reported and explained by the presence of other metals [Mo(VI), Cu(II), Fe(III), Hg(II), V(V)] in solution that could react with diphenylcarbazide resulting in complexes that absorb at the same wavelength of DPC-Cr(VI) (Pettine and Capri, 2005; Unceta et al., 2010). Additionally, the acidic conditions ($\text{pH } 1.0 \pm 0.3$) that DPC method implies, in the presence of Fe(II), sulfide, sulfite and organic compounds, may enhance the reduction of Cr(VI) to Cr(III) which decreases the Cr(VI) amount in assays (Pettine and Capri, 2005; Unceta et al., 2010). Therefore, and since the chemical EPA 1799 method is very expensive and time consuming, the application of these biosensors seems to represent an advance in chromate detection and quantification.

Despite the high water solubility of chromate, the largest amount of the ion in soil is adsorbed to the solid phase and, therefore, become not available to the organisms. The techniques for metal extraction from soil, namely for chromate, influence metal recuperation and, consequently, the amount of metal determined. Extraction periods of 2–3 h were suggested in order to maximize chromate extraction from soil with minimal reduction to Cr(III) (Ivask et al., 2004; Sethunathan et al., 2005). In this work, 3 h of extraction showed higher chromate recuperation values than all other times tested.

In this study two different soils contaminated with concentrations of chromate between 0 and $120 \text{ mg Cr(VI) kg}^{-1}$ of soil were tested. The chromate concentration in soil decreased over the test duration, independently of the soil tested. Chromate in the OECD soil was almost not detected at the end of the experiments. The chromate disappearance observed in both soils can be related to the presence of certain chemical elements, for instance, iron and manganese, low pH, granulometry (sand, clay and silt percentage) and mostly organic matter that promotes chromate reduction to

Cr(III) (Kotaš and Stasicka, 2000). Studies with other metals such as copper have supported that dissolved organic carbon in soil solution strongly influences the complexation of metals. Thus, soils with high organic matter content have shown quick decrease in metal's bioavailability over time as shown by the biosensors (Brandt et al., 2008). The different microbial communities of soils may also interfere with oxidation state of metals (Giller et al., 1998).

In the present study, the natural soil showed a slower rate of disappearance of chromate compared to the concentration range in OECD soil. Furthermore, in natural soil the rate of chromate disappearance seemed lower at concentrations higher than $40 \text{ mg Cr(VI) kg}^{-1}$ (C3) for both soils with and without springtails (Figs. 4 and 5). This can be explained by the fact that high concentrations of metal lead to decreasing microbial communities and consequently, to low ability of microorganisms to reduce Cr(VI). In addition, chromate concentrations could exceed the reductive capacity of the microorganisms present in soils (Viti et al., 2006). In case of OECD soil, the chromate decrease was quick and almost complete for all concentrations tested. These results lead to assume that the chemical and structural properties of OECD soil promote chromate reduction, which consequently reduces chromate toxicity.

The slower disappearance of chromate in natural soil compared to that in OECD soil agrees to the reproductive output of collembolans in the reproduction tests. While in natural soil a clear dose response was observed, no toxicity was reported in the OECD soil. According to the available literature, the toxicity of Cr(III) was tested in soil invertebrates by Lock and Janssen (2002). These authors used OECD soil as substrate and reported a reproduction EC_{50} of $604 \text{ mg Cr(III) kg}^{-1}$ for *F. candida*. This value is considerably higher than the reproduction EC_{50} values estimated in the present study [4.60 and $6.79 \text{ mg Cr(VI) kg}^{-1}$]. These were estimated based on chromate concentrations measured in replicates with and without springtails and did not differ considerably between each other (the 95% confidence intervals overlap). On the other hand, the reproduction of collembolans over the gradient of contamination in natural soil was significantly correlated with chromate measurements of replicates with springtails. These data lead to assume that the collembolans activity influences the concentration of chromate in soil. This can be explained by the fact that springtails use bacteria as nutritional source, thus, limiting the bacterial capacity in the chromate reduction process.

In conclusion, this study showed that the biosensors *E. coli* pCHRGFP1 and *O. tritici* pCHRGFP2 are sensitive and may comprise a rapid method for the measurement of chromate availability/toxicity in contaminated soils. In the present work, it was possible to correlate the soil chromate concentrations measured by the biosensors and the ecotoxicity of chromate to collembolans in different types of soils. However, since the collembolans *F. candida* represent one specific route of exposure to chromate, further tests could not be excluded to verify if the same correlation can be observed with other soil key-species having different routes of exposure (earthworms, enchytraeids, mites).

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