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Ioannis Ipsilantis  
*University of Kentucky*

Mark S. Coyne  
*University of Kentucky*, [mark.coyne@uky.edu](mailto:mark.coyne@uky.edu)

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## Soil Microbial Community Response to Hexavalent Chromium in Planted and Unplanted Soil

Ioannis Ipsilantis and Mark S. Coyne\*

### ABSTRACT

Theories suggest that rapid microbial growth rates lead to quicker development of metal resistance. We tested these theories by adding hexavalent chromium [Cr(VI)] to soil, sowing Indian mustard (*Brassica juncea*), and comparing rhizosphere and bulk soil microbial community responses. Four weeks after the initial Cr(VI) application we measured Cr concentration, microbial biomass by fumigation extraction and soil extract ATP, tolerance to Cr and growth rates with tritiated thymidine incorporation, and performed community substrate use analysis with BIOLOG GN plates. Exchangeable Cr(VI) levels were very low, and therefore we assumed the Cr(VI) impact was transient. Microbial biomass was reduced by Cr(VI) addition. Microbial tolerance to Cr(VI) tended to be higher in the Cr-treated rhizosphere soil relative to the non-treated systems, while microorganisms in the Cr-treated bulk soil were less sensitive to Cr(VI) than microorganisms in the non-treated bulk soil. Microbial diversity as measured by population evenness increased with Cr(VI) addition based on a Gini coefficient derived from BIOLOG substrate use patterns. Principal component analysis revealed separation between Cr(VI) treatments, and between rhizosphere and bulk soil treatments. We hypothesize that because of Cr(VI) addition there was indirect selection for fast-growing organisms, alleviation of competition among microbial communities, and increase in Cr tolerance in the rhizosphere due to the faster turnover rates in that environment.

CHROMIUM is widely used in industry and a known toxic element (Nriagu and Nieboer, 1988). Trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)] are the dominant forms in nature (Bartlett, 1991). Chromium's wide use has caused environmental contamination. However, there are relatively few studies of Cr effects on the soil microbial community (Viti and Giovanetti, 2001; Shi et al., 2002a; Shi et al., 2002b).

Low molecular weight organic substances, such as root exudates and citric acid have dual effects on Cr chemistry: reducing Cr(VI) to Cr(III), and complexing Cr(III) and maintaining it in solution (James and Bartlett, 1984). Organic, complexed Cr(III) may subsequently sorb to Mn oxides and undergo oxidation to Cr(VI) (James and Bartlett, 1983). Hexavalent Cr is a bioavailable and soluble Cr form that is toxic to microorganisms, plants, and animals relative to Cr(III) (Nriagu and Nieboer, 1988). Chromium is not regulated in biosolids by the USEPA because it is typically in the Cr(III) form and is believed to form stable complexes with organic matter that are not bioavailable in this kind of waste (Chaney et al.,

1996). However, Gong et al. (2002) showed toxicity by Cr(III) to soil microorganisms.

Experiments targeting the rhizosphere microbial communities in metal-contaminated soils are few (Giller et al., 1998). Kozdrój and van Elsas (2000) used artificial root exudates to study the effects of organic compounds on microbial diversity of heavy metal polluted soils. Carlot et al. (2002) isolated Cd-tolerant, plant growth-promoting rhizobacteria from *Brassica* roots to evaluate potential use in phytoremediation. However, the effects on the whole microbial community were not examined.

Soil microbial communities can be useful bioindicators of soil pollution (Gong et al., 2002), and studying the effects of metals on microbial communities is more direct than measuring metal bioavailability and speciation (Ellis et al., 2002). Soil microbial communities can also indicate the capacity of soil to restore itself and perform certain functions (e.g., organic matter decomposition) after temporary or permanent disturbance (Giller et al., 1998; Griffiths et al., 2000). Babich and Stotzky (1985) indicated that heavy metals reduced microbial biomass and species diversity in soil. In addition, Díaz-Raviña and Bååth (1996) showed that higher growth rates can lead to faster metal tolerance development. However, the rhizosphere, an environment with high microbial growth rates (Soderberg and Bååth, 1998), has not been examined in light of this hypothesis.

The objective of this experiment was to investigate the effects of Cr(VI) on bulk and rhizosphere microbial communities. Based on Babich and Stotzky (1985) and Díaz-Raviña and Bååth (1996) we expected Cr(VI) to reduce soil microbial biomass and diversity in both bulk and rhizosphere soil, and increase microbial community tolerance to Cr(VI) in both soil types, but faster, and therefore to a greater extent in the short-term study we performed for the rhizosphere soil relative to the bulk soil.

### MATERIALS AND METHODS

#### Treatment of Soil with Chromium(VI) and Development of the Plant Rhizosphere

Woolper silt loam (fine, mixed, mesic, Typic Argiudoll, 7% sand, 60% silt, and 33% clay, 120 g kg<sup>-1</sup> organic matter, pH 6.7, 620 g kg<sup>-1</sup> water holding capacity) was used in this study. The soil was collected from the surface 5 cm and sieved moist through a 2-mm sieve. Treatments were: (i) bulk soil; (ii) bulk soil amended with 447 mg kg<sup>-1</sup> Cr(VI); (iii) rhizosphere soil; (iv) rhizosphere soil amended with 447 mg kg<sup>-1</sup> Cr(VI). There were three replicates of each treatment, and all values in this study are reported on a dry-weight soil basis unless specifically noted. The desired Cr(VI) concentration for this experiment was high, but one that would also allow the plants

Dep. of Plant and Soil Sciences, Univ. of Kentucky, Agricultural Science Building, Lexington, KY 40546-0091 USA. Received 22 Nov. 2005. \*Corresponding author (mark.coyne@uky.edu).

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**Abbreviations:** TdR, tritiated thymidine; Bc, biomass C; Ec, extractable C; AWCD, average well color development.

to grow. Each individual replication of 1 kg moist soil (694 g dry weight basis) was treated with a  $K_2Cr_2O_7$  solution by thorough mixing in a rotating shaker, and subsequently dispensed into plastic pots. The control treatments received only water.

Fifteen seeds of Indian mustard were sown in pots intended for rhizosphere soil, and the moisture water content was adjusted to field capacity. After germination, five plants per pot were allowed to grow. All pots were kept in a greenhouse with additional light up to 12 h per day. Deionized water was added to each pot daily by sub-irrigation to minimize leaching and to prevent the soil from drying. After 4 wk, plant shoots were removed, roots with the adhering soil were removed and the rest of the soil (hereafter called rhizosphere soil) was individually mixed for every replicate. In this study the operational definition of the rhizosphere soil was all the soil in the planted pots. This definition differs from the usual terminology, but is supported by James and Bartlett (1984), who indicate that the effect of the rhizosphere on Cr extends to the rest of soil in a pot. Bulk and rhizosphere soil were stored at 4°C for several weeks until further analysis.

### pH, Chromium Analysis, and Biomass Measurements

The methods described by Bartlett and James (1996) were used for Cr analysis. Duplicate samples were measured for each replicate. Total extractable Cr was measured after extraction of one g of soil by 50 mL of 10 mM monobasic di-potassium citrate buffer pH 7.2 for 72 h, and measured by atomic adsorption spectroscopy. Exchangeable Cr(VI) was measured colorimetrically at 540 nm after an adjustment of the *s*-diphenylcarbazide method for use with a microplate autoreader. Labile Cr(III) was determined by difference. The pH of each pot was measured in triplicate by glass electrode on air-dried soil in a 1:1 soil/water paste.

The method of Vance et al. (1987), as modified by Wu et al. (1990), was used for fumigation-extraction. Moist soil was adjusted to 55% of water holding capacity and incubated in darkness at room temperature for 7 d in air-tight plastic bags to remove the effects of initial disturbance. Samples were fumigated with  $CHCl_3$  vapor for 24 h and 20 g were extracted with 0.5 M  $K_2SO_4$ . Organic C in the extracts was determined with an automated total organic C analyzer (Shimadzu 5000A, Kyoto, Japan). Soil biomass C (Bc) was calculated from the extractable C (Ec) by the equation:  $Bc = 2.22Ec$  (Wu et al., 1990).

For ATP analysis we extracted microorganisms from soil by diluting 10 g of soil in 100 mL sterile distilled water in sterile plastic bags, homogenizing for 5 min in a Stomacher lab blender (Brinkman Instruments, Inc., Westbury NY), and centrifuging for 10 min at 750 g at 5°C. Fifty  $\mu$ L of the extract were placed into polypropylene cuvettes, along with 50  $\mu$ L of ATP releasing agent (Turner Designs, Sunnyvale CA), 50  $\mu$ L HEPES buffer pH 7.0, and 100  $\mu$ L luciferine-luciferase (Turner Designs). Standards and blanks were prepared in water or in the extractant. A luminometer (Turner Designs 20/20) was used to measure ATP. The basic concept of this approach is similar to that of the tritiated thymidine procedure modified for soil by Bååth (1992); instead of working with soil itself, the method measures part of the microbial community by working with soil extract and homogenization-centrifugation.

### Tritiated Thymidine Incorporation Measurements

We used the method of Bååth (1992) for tritiated thymidine incorporation (TdR). Tolerance measurements were made in bulk and rhizosphere soil as defined by the working definitions previously mentioned. Six g of soil were homogenized with 60 mL of sterile distilled water in a plastic bag for 5 min and

centrifuged for 10 min at 700 g and 5°C in sterile plastic centrifugation tubes. The supernatant was poured through sterile glass wool, and 1.8 mL was added to plastic vials. Cr(VI) solutions of different concentrations were added (0.2 mL) after filter sterilization through 0.22- $\mu$ m polycarbonate filters (Millipore, Bedford, MA). After 15 to 20 min at room temperature, 100 nM [methyl- $^3H$ ] thymidine (1 mCi  $mL^{-1}$ , Amersham, Little Chalfont, England) was added to each vial. Incorporation was stopped after 2 h by adding 1 mL of 5% ice-cold formalin. The formalin was added immediately before the thymidine for zero-time controls. The suspensions were incubated for 15 min in an ice bath, and filtered through 25-mm cellulose-acetate filters (0.45- $\mu$ m pore size; Osmonics, Minnetonka, MN). The filters were previously soaked in unlabeled thymidine solution for 1 h to reduce sequestration of radioactive thymidine on the filters of the control with zero time incubation, which was used to account for non-biotic sequestration of label on the filters. The filters were washed three times with 5 mL of ice-cold 80% ethanol to remove thymidine bound to lipids, and three times with 5 mL of ice-cold 5% trichloroacetic acid to precipitate the macromolecules.

The labeled filters were placed in plastic scintillation vials containing 0.5 mL of 0.1 M NaOH and incubated in a 90°C water bath for 2 h to solubilize macromolecules. The vials were allowed to cool, and 5 mL of Bio-safe II scintillation cocktail was added. The radioactivity was quantified by counting in a Packard 1900 TR liquid scintillation analyzer (Meridian, CT). An external standards method was used to correct for quenching.

Higher TdR incorporation rates can result from higher numbers of bacteria. Therefore, the data were expressed as specific TdR incorporation (STdR), on the basis of TdR incorporation per unit of biomass carbon or ATP using the fumigation-extraction and ATP data. We used only 10% of the fumigation-extraction biomass carbon, assuming it to be the percentage of soil microbes extracted with the homogenization-centrifugation method (Bakken, 1985), and the TdR incorporation of the control Cr concentration.

### Community Substrate Use Analysis (BIOLOG GN Plates)

Six g of soil were diluted in 60 mL sterile deionized water in a plastic bag and extracted in a laboratory blender for 5 min. Ten mL of the extract were further diluted into 90 mL of 0.85% NaCl. The diluent was inoculated into BIOLOG GN plates, which were incubated at 25°C. The color developed was measured using a microplate autoreader (BIO-TEK instruments EL 311) at 630 nm, every 4 h, up to 72 h. One plate for each pot was inoculated.

The average well color development (AWCD) was calculated for each plate for each reading time as the arithmetic average of absorbance values for all 95 wells, after subtracting the value of the blank well from each substrate (Garland and Mills, 1991). If the subtraction gave a negative value, zero was used for that individual well. The AWCD for each treatment and time was the average of the AWCD of the three corresponding plates.

To normalize for different inoculum densities we calculated the Gini coefficient and performed principal component (PC) analysis at 0.75 AWCD (Garland, 1996; Harch et al., 1997), for which the reading of each plate with a value closest to 0.75 AWCD was used. The Gini coefficient was calculated using the formula:

$$G = \frac{\sum_{i=1}^N \sum_{j=1}^N |x_i - x_j|}{2N^2 \bar{x}}$$

where  $x_i$ ,  $x_j$  refer to an absorbance value for each of the carbon source wells (1 to 95),  $i$  and  $j$  are the wells,  $N$  is the total number of carbon wells sources (95), and  $x$  is the AWCD (Harch et al., 1997).

### Statistical Analysis

The logarithm of the inhibition concentration that caused 50% reduction in thymidine incorporation ( $IC_{50}$ ) was calculated by least squares fitting to a logistic model:  $A = 100/[1 + e^{b(S-a)}]$  (Doelman and Haanstra, 1989; Díaz-Raviña et al., 1994; Shi et al., 2002a), where  $A$  is thymidine incorporation expressed as a percentage of the control,  $b$  is a slope parameter indicating inhibition rate,  $S$  is the logarithm of Cr concentration, and  $a$  is  $IC_{50}$ . Instead of zero, a very low Cr(VI) concentration ( $10^{-5} M$ ), that did not cause inhibition of incorporation, was used as the control (Díaz-Raviña et al., 1994). The  $IC_{50}$  and  $b$  values were estimated by using nonlinear regression with the equation above using the SAS MODEL procedure (SAS Institute, Inc., 1989). Principal component analysis was performed using the SAS PRINCOMP procedure. A  $2 \times 2$  ANOVA was performed for the major effects (metal and plant) and their interaction, with multiple comparisons using an experiment wise error rate of  $\alpha = 0.05$  and least significant means with the Tukey adjustment.

## RESULTS

### Effects of Chromium on Indian Mustard, Chromium Analysis, and Biomass Measurements

Metal hydrolysis in soil is an acidifying reaction and the Cr(VI) treatment stunted the plants, but there was no significant effect on the pH by the end of the experiment. After 4 wk the extractable Cr was low (Table 1),  $12 \mu\text{g Cr(VI) kg}^{-1}$  soil, and  $91 \text{ mg Cr(III) kg}^{-1}$  soil. In both rhizosphere treatments Cr (VI) levels were below detection.

Fumigation-extraction and ATP (Table 2) measurements were positively correlated ( $R^2 = 0.55$ ), and both methods showed that Cr addition resulted in lower soil microbial biomass, with no difference in this effect between bulk and rhizosphere soil, as the interaction of the metal with the plant was not significant. The unfumigated controls had significantly higher background microbial carbon in the Cr treatments, compared with non-Cr-treated soil (data not shown).

Biomass measurements with ATP by homogenization-centrifugation showed the same trend as fumigation-extraction. In contrast to the fumigation-extraction procedure, the effect of Cr on extractable ATP was more pronounced for the rhizosphere soil, where there was an approximately 10-fold decrease in ATP with Cr addition (Table 2).

**Table 1. Citrate-extractable (labile) Cr(III) and phosphate-exchangeable Cr(VI) analysis, 1 mo after the Cr(VI) amendment.†**

Parameter	Cr amendment		Cr(III) mg kg <sup>-1</sup> soil	Cr(VI) μg kg <sup>-1</sup> soil
	0	447		
Rhizosphere	0	447	0.5 (0.07)	ND‡
Bulk soil	0	447	20.6 (2.5)	ND
	0	447	2.0 (0.7)	1.9 (0.8)
			91.1 (9.3)	12.2 (2.5)

† Values are the mean of three replications. Standard deviation given in parentheses.

‡ Below detection limit.

### Tritiated Thymidine Incorporation

Overall TdR incorporation in the rhizosphere soil tended to be higher than in the bulk soil, and higher for the Cr treatments, but because of high variability there were no significant differences (Fig. 1, Table 2). When the data were expressed as a percent of the control to measure tolerance, there was no difference in the rate at which TdR incorporation declined as Cr concentration increased (Fig. 1). The bulk soil  $IC_{50}$  was higher than that of the Cr-treated bulk soil and the non-treated rhizosphere. The Cr-treated rhizosphere soil  $IC_{50}$  tended to be higher, but was not significantly different than that of the non-treated rhizosphere (Table 2). The variability in the rate of TdR incorporation was high overall.

The specific thymidine incorporation rate (STdR) measured in terms of fumigation-extraction C revealed that there was a higher incorporation rate in the presence of Cr for both rhizosphere and bulk soil (Table 2). Expressing STdR per unit fumigation-extraction-C gave incorporation rates four times higher in the Cr-treated rhizosphere soil. Variability was very high for ATP, and although the Cr treatments had much higher STdR, they were not significantly different from the unamended treatments.

### Community Substrate Use Analysis (BIOLOG Plates)

The AWCD reflected the biomass results, because inoculum density is known to affect color development (Garland and Mills, 1991; Haack et al., 1995). The AWCD was slower and lower in samples from Cr treatments (Table 2, Fig. 2). *L*-pyroglutamic acid was the only substrate in which there was faster and higher well color development for the Cr treatments.

The Gini coefficient, an indicator of microbial evenness, for which higher values reflect less evenness (i.e., less diversity), showed that evenness was higher with Cr addition, and that there were no differences in evenness between bulk soil and rhizosphere soil (Table 2).

Principal component analysis showed a partitioning of the treatments (Table 2, Fig. 3). The first principal component accounted for 66% of the variability and was strongly associated with Cr effects, while the interaction with the plant was also significant (Table 2). The Cr treatments were on the positive part of the PC1 axis, while the non-Cr-treated bulk and rhizosphere soil were associated with the negative part of PC1 (Fig. 3). The plant effect was stronger for PC2, where rhizosphere treatments had a tendency to associate with positive values relative to the PC2, and the bulk soil treatments with negative values relative to PC2.

## DISCUSSION

### Chromium Toxicity

*Brassica juncea* (Indian mustard) was used, because the *Brassica* family accumulates heavy metals and also rhizofiltrates Cr(VI) (Dushenkov et al., 1995). Metal hydrolysis is an acidifying reaction but at the concentration used, Cr had no permanent effect on lowering pH, which has been previously observed (Gong et al., 2002).

**Table 2. Summary of results of: biomass carbon measured by fumigation-extraction (FE), ATP, tritiated thymidine (TdR) incorporation at control ( $10^{-5}$  M) Cr(VI) concentration, logarithm of Cr(VI) concentration ( $M$ ) that caused 50% reduction in TdR incorporation ( $IC_{50}$ ), the slope-rate of inhibition of TdR incorporation with increasing Cr(VI) concentration, specific TdR incorporation (STdR) expressed with FE and ATP data, average BIOLOG well color development (AWCD), Gini coefficient at 0.75 AWCD, principal components 1 and 2 (PC-1, PC-2), and  $2 \times 2$  ANOVA with interaction table showing  $P$  values. Numbers in the same row followed by different letters are significantly different at  $\alpha = 0.05$ , as determined by multiple pair-wise comparisons with the Tukey adjustment and experiment-wise error of 0.05,  $n = 3$ .**

Parameter	Rhizosphere	Rhizosphere + Cr	Bulk soil	Bulk soil + Cr	Metal	Plant	Metal $\times$ plant
FE biomass carbon (mg C kg <sup>-1</sup> soil dry wt.)	803a	278c	562b	90d	<0.0001	<0.0001	0.13
ATP (ng ATP g <sup>-1</sup> soil dry wt.)	10.5a	1.1b	7.0ab	2.4ab	0.005	0.5	0.2
TdR (mol TDR mL <sup>-1</sup> h <sup>-1</sup> ) $\times$ ( $10^{-14}$ )	29.0a	42.1a	6.9a	22.0a	0.2	0.099	0.9
$IC_{50}$	-3.51b	-3.27ab	-3.15a	-3.52b	0.5	0.4	0.0043
Slope	2.89a	3.25a	3.96a	2.91a	0.5	0.5	0.2
STdR-FE <sup>†</sup>	0.6b	2.4ab	0.2b	4.3a	0.005	0.3	0.18
STdR-ATP <sup>‡</sup>	46.1a	2220a	16.1a	226.7a	0.2	0.3	0.3
AWCD at 48 h	1.21a	0.81b	1.06ab	0.45c	0.0001	0.009	0.19
Gini at 0.75 AWCD	0.41a	0.25b	0.42a	0.24b	0.001	0.9	0.7
PC-1	-5.8bc	2.0b	-7.0c	10.8a	<0.0001	0.067	0.024
PC-2	0.9a	3.7a	-2.2a	-2.4a	0.4	0.017	0.3

<sup>†</sup> With 10% of biomass C to reflect biomass of the soil solution, mol Tdr mL<sup>-1</sup> h<sup>-1</sup> mg<sup>-1</sup> biomass C  $\times$   $10^{-18}$ .

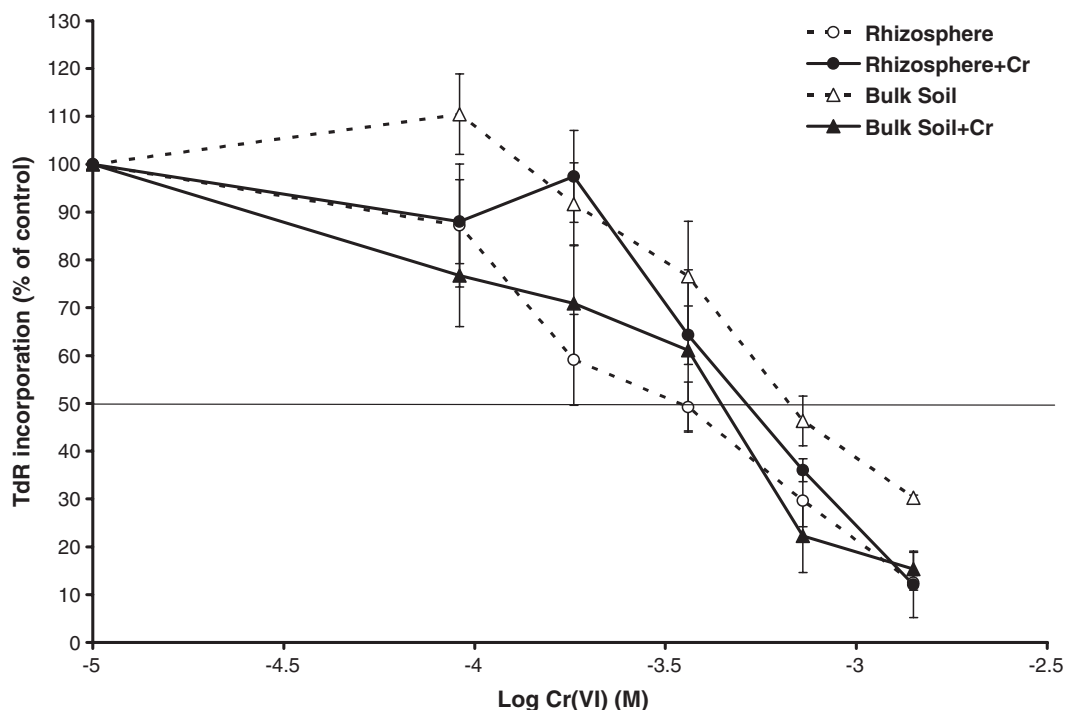
<sup>‡</sup> mol TdR  $\times$   $10^{-14}$  mL<sup>-1</sup> h<sup>-1</sup> ng<sup>-1</sup> ATP.

The extractable Cr was considered the biologically available Cr. One month after the Cr application the level of Cr(VI), the highly toxic form, was low, and for this reason it appears that the results represent an ‘echo’ of the impact of the Cr application to the soil. The non-treated soil Cr(III) and (VI) concentrations show natural background Cr levels, while levels of Cr(VI) of the treated bulk soil are close to natural levels (Katz and Salem, 1994). The average total Cr concentration in Kentucky soils is 108 mg kg<sup>-1</sup> soil (Karathanasis and Seta, 1993). Adsorption and precipitation reactions mediated by clay and organic matter were the most probable reasons why Cr was no longer plant available, and the non-alkaline soil pH did not favor the presence of Cr(VI)

(Bartlett and Kimble, 1976). The perturbation caused by Cr was therefore considered transient, much like heat, flood, or fumigation (Griffiths et al., 2000; Ranneklev and Bååth, 2001), rather than persistent, as is the case with other heavy metals (McGrath et al., 1995; Giller et al., 1998). This would not have been the case if the soil used had a capacity to re-oxidize Cr(III) to Cr(VI), as did the vegetated tannery soil used by Viti and Giovanetti (2001).

### Microbial Biomass Measurements

Fumigation-extraction and ATP measurements indicated that Cr significantly reduced microbial biomass.



**Fig. 1. Thymidine incorporation in a range of Cr concentrations of extracts of bulk soil, bulk soil treated with Cr, rhizosphere, and rhizosphere soil treated with Cr, expressed as percent of the control-tolerance indicator (error bars represent standard error,  $n = 3$ ). The line at 50% incorporation relative to the control is to assist in visualizing  $IC_{50}$ .**

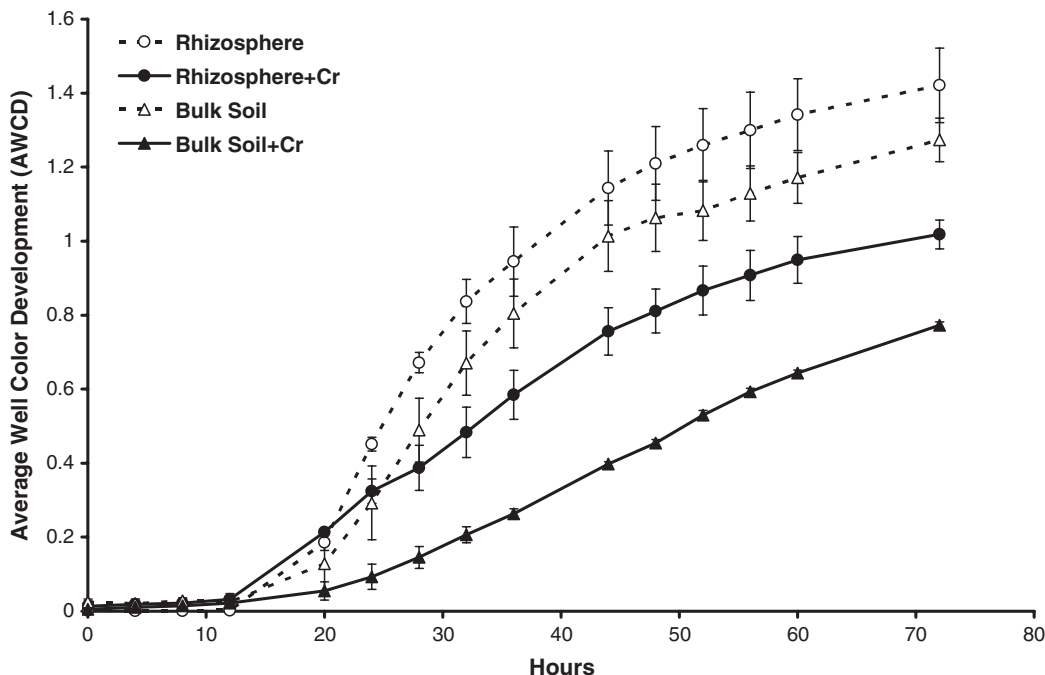


Fig. 2. Average well color development (AWCD) in BIOLOG plates for Cr-amended and unamended bulk and rhizosphere soil (error bars represent standard error,  $n = 3$ ). Symbols: open circles (○), rhizosphere, closed circles (●), rhizosphere + Cr, open triangles (△), bulk soil, closed triangles (▲), bulk soil + Cr.

One of the explanations for lower biomass in metal-contaminated soils is lower efficiency of conversion of carbon into biomass (McGrath et al., 1995). However, because Cr(VI) was no longer present at significant concentrations, lower biomass could probably be explained by an initial killing impact. The higher background carbon in the non-fumigated controls of biomass mea-

surements of the Cr-treated soil is evidence of such an event. The rhizosphere apparently moderates Cr effects; this can be due to greater substrate availability through root exudates, as well as to reduction or complexation of Cr(VI) by exudates. Rhizobia, for example, are protected by the host plant in metal-contaminated soils (Giller et al., 1998), and root exudates increased counts

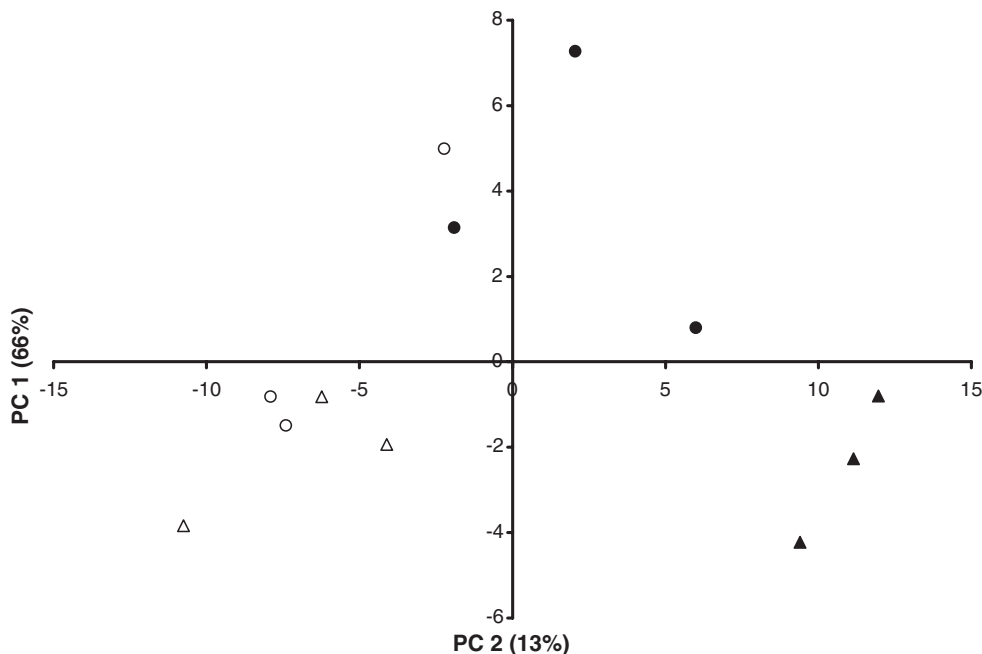


Fig. 3. Principal component analysis of BIOLOG plates data. Each point represents one replicate. Symbols: open circles (○), rhizosphere, closed circles (●), rhizosphere + Cr, open triangles (△), bulk soil, closed triangles (▲), bulk soil + Cr. Values in parentheses show the variation explained by the principal component.

of culturable bacteria from metal-contaminated soils (Kozdrój and van Elsas, 2000).

### Tritiated Thymidine Incorporation

The TdR incorporation results ( $IC_{50}$ ) weakly indicated that prior exposure to Cr increased tolerance in the rhizosphere. However, the response in the bulk soil was surprisingly the opposite. It may be that in bulk soil the initial Cr application eliminated part of the microbial community without selection for tolerance, rendering the microbial community more sensitive to additional Cr application. Another explanation may have to do with the low levels of TdR incorporation in bulk soil.

Shi et al. (2002a), measuring  $^3H$ -leucine incorporation in a series of Cr(VI) concentrations of a microbial community extract from a site with Cr, Pb, and hydrocarbon pollution, found  $IC_{50}$  values of 2.5 mM Cr(VI) and no differences in Cr tolerance regardless of exposure history. However, a non-vegetated Cr-contaminated soil from a tannery gave an  $IC_{50}$  value of 3.68 mM (Shi et al., 2002b). A possible explanation for non-development of Cr tolerance is that tolerance is widespread among soil microorganisms, and higher concentrations may be required to demonstrate such a difference (Giller et al., 1998). Riemann and Lindgaard-Jørgensen (1990) exposed natural seawater and fresh water samples to a range of Cr(VI) concentrations and found  $IC_{50}$  values ranging between 0.4 and 2.36 mM. Viti and Giovanetti (2001), using vegetated tannery soil, found no difference in viable counts between control and Cr-contaminated soil when low (0.15 mM) Cr(VI) levels were added to the medium, but increased counts for the Cr-contaminated sites at 1.5 mM Cr(VI). However, their soil could oxidize Cr(III) to (VI), thus longer term exposure might be needed, as shown for ammonia-oxidizing bacteria (Gong et al., 2002), although in that case copper and arsenate were co-contaminants in addition to Cr.

In our case, the concentration of 447 mg kg<sup>-1</sup> Cr(VI) used might not have been sufficiently high, or present sufficiently long at high concentration to induce tolerance in the bulk soil. On the other hand, Díaz-Raviña and Bååth (2001) showed that microbial communities pre-exposed to heavy metals lost tolerance within 8 d when they were inoculated into sterilized unpolluted soil and therefore, in this study, if there was some selection for a Cr-tolerant microbial community in the Cr-treated bulk soil near the time of impact, this may have disappeared as Cr concentration decreased. This would concur with the hypotheses that decreased metal tolerance and decreased metal toxicity occur with time (Díaz-Raviña and Bååth, 1996).

The specific TdR incorporation expressed on a per mg C basis, shows that there was a higher rate of thymidine incorporation rate in the Cr-treated rhizosphere and bulk soil. Higher rates of thymidine incorporation are associated with higher growth rates (Robarts and Zohary, 1993). Chromium could select for fast growing organisms indirectly. Díaz-Raviña and Bååth (1996) hypothesized that after applying heavy metals, the microorganisms that survive can thrive on the flash event

derived from the C and other nutrients released from the metal-killed organisms. Indeed, this was evidenced by the non-fumigated Cr-treated controls, which had higher biomass C than the untreated soils. Thus, one type of selection can be the ability of some microorganisms to quickly utilize the C released after perturbation, and by means of high growth rates out-compete slow-growing species. This difference in growth rates suggests a physiological difference of the Cr-treated microbial communities, as was suggested for chloroform-fumigated soil (Griffiths et al., 2000). Direct selection for fast-growing organisms could also be possible if fast growth rates are associated with Cr tolerance, which should also be accompanied by an increase in metal tolerance. However, fast growth rates were not associated with an increase in Cr tolerance for the Cr-treated bulk soil.

### Community Substrate Use Analysis (BIOLOG GN Plates)

The Cr treatment caused overall lower and slower color development in plate wells, which can be attributed to lower microbial biomass, because AWCD is known to be influenced by the initial inoculum density. This limitation of the BIOLOG method can account for the discrepancy between slower growth rates, as indicated with BIOLOG plates, and faster growth rates, as indicated with the STdR data. In other metal toxicity studies in which BIOLOG was used without biomass differences (Kelly and Tate, 1998), there was a lag period for the metal treatments, caused by metal toxicity. In the present study, although there was lower microbial biomass with metal addition, overall there was no lag period, which was another indication that Cr was no longer toxic. However, increased lag time is not always a characteristic of metal-stressed microorganisms (Giller et al., 1998).

When different inoculum densities are involved, multiple plate readings and a set value of AWCD (Garland, 1996), or the area under the curve (Guckert et al., 1996; Hackett and Griffiths, 1997), can be used for normalization. The Gini coefficient at 0.75 AWCD indicates that evenness, or functional diversity, was higher with Cr treatment, showing that Cr application not only decreased microbial biomass, but also had a qualitative impact by increasing diversity (Harch et al., 1997). If Cr, directly or indirectly, selected for fast-growing organisms (as suggested by the STdR results) a decrease in diversity would be expected. However, the initial killing impact of Cr could release survivors from competition, at least temporarily. Giller et al. (1998) proposed extending to microorganisms and heavy metal stress a hump-backed relationship between diversity and stress that applies to plants and animals. In that model, moderate stress increases diversity by limiting predominance of highly competitive species, thus allowing more species to proliferate. Therefore, the effects of Cr could decrease microbial biomass, induce Cr tolerance or select for Cr-tolerant microorganisms, select indirectly for fast-growing organisms, and increase diversity by decreasing competition (i.e., the evenness of the population would increase



because one or more highly competitive and dominant microbial species were eliminated by the treatment).

Other transient impacts, such as fumigation, have caused a decrease in diversity by selecting for fast-growing organisms (Griffiths et al., 2000). For example, heat caused a switch in microbial community from mesophylic to thermophylic, with the mesophylic activity not recovering within the 20-d period measured (Ranneklev and Bååth, 2001). In the current study, microbial biomass was higher in the rhizosphere, and the bulk soil treatments had the same evenness as their respective rhizosphere treatments. The higher biomass in the rhizosphere most likely was because of greater nutrient availability than in the bulk soil.

Rhizosphere and bulk soil communities were distinctively separated, because they had distinct characteristics. We deduce that as an effect of the Cr addition, communities differentiated even more, with increased Cr tolerance (rhizosphere), selection for fast growing organisms, and increased diversity. The two parameters, Cr addition and the rhizosphere, resulted in a partitioning of the soil microbial communities almost on four different quartiles in the principal component analysis. It seems that Cr addition had a greater effect on the bulk soil than on the rhizosphere microbial community. The Cr-treated bulk soil microbial community was further from the untreated soil population in the two-dimensional principal component space than the Cr-treated rhizosphere soil from the respective untreated soil. However, this was not supported by differences in diversity between bulk and rhizosphere soil.

## CONCLUSIONS

Our goal was to test current theories on the effects of heavy metals on soil microorganisms in the rhizosphere using Cr, a metal with rather complex chemistry. One of the limitations of the study was the use of a single sample point and Cr concentration. Had there been no treatment differences we would have been unable to adequately test the hypothesis. Population differences might have developed early, then receded beyond detection, which could not have been determined without more frequent sampling. Population differences may have required more time than our sampling interval to manifest themselves, or required higher or lower Cr concentrations. However, there were observable and statistically significant differences between samples based on the types of analyses we conducted. When these differences developed, and how long they persisted, would be separate issues to investigate. We found that Cr(VI) was rendered biologically unavailable, and therefore we believe that the results represent the echo of Cr toxicity on the microbial community. We expected, and saw, a reduction in soil microbial biomass with Cr treatment. We also expected that Cr would decrease diversity, and increase microbial community tolerance to Cr, especially in the rhizosphere. Ultimately, Cr tolerance was marginally increased in the rhizosphere, but surprisingly decreased in the bulk soil, and Cr application actually increased diversity (as measured by evenness of the popu-

lation) and growth rates. This leads us to hypothesize indirect selection for fast-growing organisms accompanied with alleviation of competition among microbial communities, at least temporarily. In turn, it is tempting to assume that the system was recovering from the Cr application, possibly faster in the rhizosphere. But for that hypothesis to be tested, more frequent sampling of longer duration would be required than what we employed in this study. Following a transient perturbation, systems can regain their original level of function, but recovery is impaired by loss of diversity (Griffiths et al., 2000). Further studies should investigate the ability of Cr-impacted rhizosphere microorganisms to function (such as the ability to degrade organic matter), as well as longer term effects of Cr application.

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