

## Natural Attenuation of Cr(VI) Contamination in Laboratory Mesocosms

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*The processes leading to the natural attenuation of hexavalent chromium (Cr(VI)) in marine systems are not well understood. To determine the rate at which Cr(VI) could be reduced and the effect of Cr(VI) on bacterial communities in marine sediments, we performed mesocosm experiments with 37.85 L aquaria containing San Diego Bay sandy sediments and seawater. Constant levels of 0, 0.25 (low), and 1.5 mM (high) Cr(VI) were maintained in the water column for 2 months. Chemical analyses of sediment cores taken from the mesocosms indicated that Cr accumulated in the upper 5 mm of the sandy sediments. In general, the distribution of total Cr did not correlate with Fe, Mn, or total organic carbon. Enrichment cultures of metal (iron and chromium)- and sulfate-reducing bacteria from the upper horizon (0–5 mm) of sediments were performed to look for the potential contributors in the detoxification/removal process. PCR of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE) was used to examine the microbial community structure in sediment depth profiles. When Cr(VI) was present, the number of DGGE bands decreased only in the upper 5 mm of sediments indicating an inhibition of certain bacterial populations and/or a selection for Cr-resistant bacteria in this region. Analysis of the DGGE bands was not especially helpful as most sequences were related to unknown, unidentified, or uncharacterized bacterial cloned sequences.*

**Keywords** hexavalent chromium reduction, marine sediments, metal analysis, metal-reducing bacteria, microbial community analysis

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## Introduction

Chromium (Cr) exists primarily in two oxidation states: Cr(VI), which is soluble, toxic, carcinogenic, and mobile in aqueous environments and Cr(III) which is generally insoluble or particle reactive, nonmobile, and much less toxic. Cr is common in Navy harbors due to its usage in paint and water cooling systems because of its antifoulant and anticorrosive properties (U.S. Environmental Protection Agency 1999). For example, total chromium levels of approximately 4.8 mM have been found in Green Sand Beach at Mare Island Naval Shipyard (San Francisco, CA) (Abu-Saba 1998).

Various methods such as oxygen sparging, physical removal (e.g., dredging), chemical treatment, and burial have been used to try to remediate metal contaminated environments (Fujii et al. 1990; Spindler 1997). Most of these methodologies are not only expensive, but are also often environmentally detrimental. The United States Environmental Protection Agency has determined that it can be more cost effective and feasible to rely on natural attenuation, the biogeochemical processes in sediments driven to a large extent by the activities of indigenous microorganisms, to immobilize metal contaminants (U.S. Environmental Protection Agency 1999). However, little is known regarding the microbial ecology at most sites and, therefore, predicting the fate of contaminants at those sites is challenging.

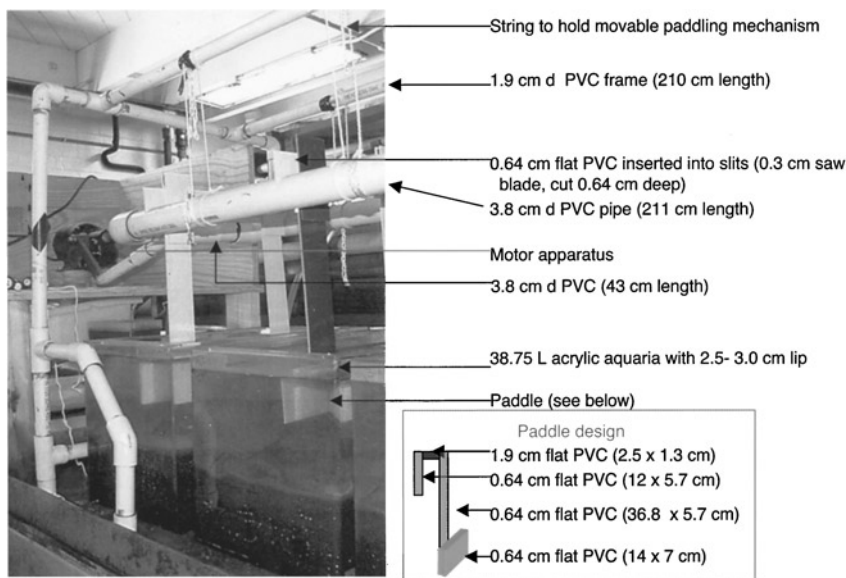
In recent years, much information has been obtained regarding Cr(VI) reduction by pure cultures of bacteria ranging from strict to facultative anaerobes and aerobic microorganisms (Lovley 1993; Silver 1996; Lovley and Coates 1997; Tebo and Obratsova 1998; Turick et al. 1998; Bae et al. 2000; Francis et al. 2000; Lee et al. 2000; Myers et al. 2000; Park et al. 2000; McLean and Beveridge 2001). Even though studies of pure cultures have demonstrated a variety of Cr(VI) reduction mechanisms, whether the organisms and mechanisms studied are representative of those occurring in Cr contaminated environments is unknown. Recently, Marsh et al. (2000) investigated Cr(VI) reducing capacities of microorganisms in natural sediment slurries to determine whether Cr(VI) could be reduced in sandy and clay-like sediments with added electron acceptors such as nitrate, sulfate, and ferric iron. In our laboratory, we have compared marine sulfidogenic and nonsulfidogenic Cr(VI)-reducing consortia using the molecular community analysis technique, PCR and denaturing gradient gel electrophoresis (PCR-DGGE), to determine the changing microbial composition during the reduction of 0.5 and 1.5 mM Cr(VI) (Arias and Tebo 2003). The results of both of these studies indicate that sulfate-reducing bacteria are strongly inhibited by Cr(VI) and that other facultative and strict anaerobes are important agents for Cr(VI) reduction in contaminated environments. More information is required, however, since characteristics such as microbial community structure, pH, temperature, and other environmental factors will influence bacterial capacities for detoxification of heavy metals in polluted environments.

Mesocosm studies are useful for imitating environmental conditions to investigate ecological processes without physically threatening or polluting the environment. Moreover, mesocosms allow for the manipulation of one particular variable and examining its subsequent effect on the system (Ingham et al. 1986). In this study, laboratory mesocosms were created to mimic environmental conditions to elucidate Cr(VI) reduction capacities in marine sediments, to investigate the effect of Cr(VI) on indigenous sediment microorganisms, and possibly to identify potential bacteria involved in the reduction of Cr(VI).

## Materials and Methods

### *Mesocosm Set-Up and Construction*

Approximately 200 L of sandy sediments were collected at low tide from Barrio Logan Park (Coronado Bridge, San Diego Bay, California), a site known to contain organic and



**FIGURE 1** Mesocosm apparatus designed to keep the overlying water column aerobic. Motor driven paddles oscillate back and forth at  $\sim 30 \times \text{min}^{-1}$ . A Grainger motor was used for the mesocosm paddling mechanism. The motor was connected to a piece of PVC ( $5 \times 10 \times 2$  cm) bolted to a PVC lever ( $12.7 \times 1.27 \times 2.5$  cm) which was bolted to a round 2.5 cm diameter, 15 cm PVC pipe. This pipe was attached to a 2.54 cm ‘T’ PVC fitting on the movable rectangular PVC apparatus that served as a guide for the paddles.

heavy metal contaminants (Chadwick et al. 1996). The mud was well mixed and screened to remove large debris (twigs, rocks, etc.) and dispensed into five acrylic semi-open aquaria (38 L, 2.5-cm acrylic border) (Figure 1). The sediment height was approximately 12 cm in each aquaria. The aquaria were placed on a table with continuously running seawater to maintain temperature at 15–16°C. To allow chemical gradients to become established in the sediments, running seawater was flowed into and out of the aquaria. After 2 weeks, the overlying water was siphoned out and 20 L of sand-filtered seawater from the Scripps Institution of Oceanography (SIO) pier was added and the water level marked. To mimic minor environmental wave action and maintain oxic conditions in the overlying water column, the water was continuously mixed with slow-moving paddles. A Grainger motor attached to a polyvinylchloride (PVC) frame made specifically for the 5 aquaria was connected to a flat PVC pipe and served as a movable guide for the paddles on the frame (Figure 1). To compensate for evaporation during the experiment, distilled water was periodically added to maintain a constant water level in the mesocosms.

### ***Cr(VI) Maintenance, Core Sampling, and Analysis***

Visible stratification of the sediments occurred within 2 weeks with the formation of two distinct layers, an approximately 1.0-cm thick orange-brown surficial layer and an underlying black layer. After stratification occurred, Cr(VI) was added at a concentration of either 0.25 mM or 1.5 mM. The “no Cr” controls and 0.25 mM Cr(VI) mesocosms were run in duplicate; circumstances only allowed for one aquarium with 1.5 mM Cr(VI). At specific time intervals, duplicate samples were measured for Cr(VI) colorimetrically using the diphenylcarbazide technique (Urone 1955). Cr(VI) was added when necessary to maintain

the designated concentration for 60 days in the overlying water column. After 60 days, the experiment was terminated and the overlying water was pumped out.

For microbiological and molecular ecological analysis, multiple small cores were randomly taken from flat sites in the aquaria using 10 ml disposable pipettes with the taper tips removed. For geochemical analysis, larger (~5 cm diameter) cores were used. Some cores were manipulated immediately for culturing of iron-, sulfate-, and chromium-reducing bacteria; other cores were frozen at  $-80^{\circ}\text{C}$  for further analysis.

To determine total organic carbon, chromium, manganese, and iron concentrations, the larger cores were sliced in 5-mm increments, oven dried ( $60^{\circ}\text{C}$ ), ground, and homogenized. Organic carbon was determined by weight loss after ignition at  $550^{\circ}\text{C}$  (Páez-Osuna et al. 1984). Metals were measured using a Perkin-Elmer inductively coupled plasma-optical emission spectrometer (ICP-OES). For metal digestion, sediments (0.25 g) were mixed with 0.25 g of lithium tetraborate and 0.5 g of lithium metaborate and melted at  $1100^{\circ}\text{C}$ . After digestion and cooling, the sediment formed a glass that was dissolved in a 5% nitric acid solution (50 ml total). This liquid solution was measured on the ICP-OES and compared to metal standard solutions (Fisher).

### ***Iron, Sulfate, and Chromium-Reducing Enrichments***

To obtain specific enrichments, approximately 1 g of homogenized sediments from the first 5 mm was added to 10 ml of medium for marine bacteria (no sulfate) (Widdel and Bak 1992). The medium contained the following basal salts (g/L): NaCl (20),  $\text{NH}_4\text{Cl}$  (0.25),  $\text{MgCl}_2$  (3.0), KCl (0.5),  $\text{CaCl}_2$  (0.1),  $\text{KH}_2\text{PO}_4$  (0.2),  $\text{NaHCO}_3$  (8.0), and yeast extract (0.1). Vitamin mix (1ml/L) and microelement solution (1ml/L) were added and prepared according to the recipe (Widdel and Bak 1992). Various electron acceptors were used separately and added to the basal medium at final concentrations: 28 mM  $\text{SO}_4^{2-}$  as  $\text{Na}_2\text{SO}_4$ , 30 mmol/L Fe(III) as ferrihydrate, or 0.25 mM Cr(VI) as  $\text{K}_2\text{CrO}_4$ . Lactate (20 mM), acetate (20 mM), or  $\text{H}_2/\text{CO}_2$  (80:20) served as electron donors. Enrichments were cultivated under a  $\text{N}_2$  atmosphere in serum bottles capped with butyl rubber stoppers and aluminum crimped seals. Incubations were performed in duplicate at room temperature. After 1 month the amount of Fe(II) and  $\text{HS}^-$  produced and Cr(VI) reduced were determined as indicators for growth of iron-, sulfate-, and Cr(VI)-reducing bacteria, respectively. Fe(II) and  $\text{HS}^-$  were detected colorimetrically (Stookey 1970; Cord-Ruwish 1985). Cr(VI) reduction was detected visually by the disappearance of the yellow color of Cr(VI) or colorimetrically using diphenylcarbazide (Urone 1955).

### ***Bacterial DNA Analysis***

To obtain representative results regarding the microbial community structure, 3 small pipette cores from each mesocosm were sliced into 5-mm segments while frozen. The segments from similar depth profiles were combined and homogenized. From the homogenized sediments, duplicate DNA extractions per aquarium were performed using the BIO 101 kit with bead-beating in the Fast Prep (BIO101, Vista, CA) instrument for 17 s at 4.5 m/s. Duplicate PCRs were done on each extraction; therefore, a total of 4 PCR products per each 5 mm horizon were obtained. DNA was amplified with eubacterial primers 1055 (*Escherichia coli* position 1055 to 1070; 5'-ATGGCTGTCGTTTCAGCT-3') and the universal ssu rRNA primer 1392 (underlined), which incorporates a 5' GC-clamp: 5'-CGCC CGCCGCGCCCCGCGCCCGGCCCGCCCGCCCCGCCCCACGGGCGGTGTGTAC-3' (Raskin et al. 1995; Ferris et al. 1996). Then, 50 and 100 ng/ $\mu\text{L}$  template DNA was amplified using a standard PCR protocol (1 cycle at  $95^{\circ}\text{C}$  for 3 min; followed by 30 cycles of  $95^{\circ}\text{C}$  for

1 min; 50°C for 1 min; 72°C for 1 min; followed by 1 extension cycle of 72°C for 7 min). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Chatsworth, CA). After purification, PCR products from identical horizons per aquarium were pooled and used for further analysis.

### ***Denaturing Gradient Gel Electrophoresis, Band Sequencing, and Cloning***

Denaturing gradient gel electrophoresis (DGGE) was carried out in a BioRad DGGE system (Dcode Detection System, Hercules, CA). All solutions and run parameters were according to the manufacturer's directions. Equal amounts (~300 ng) of pooled PCR products were loaded on 35–70% denaturant gels and run at 60°C for 6 or 16 h at 200 or 80 V, respectively. Gels were stained with SYBR GOLD (Molecular Probes, Eugene, OR) and visualized under UV light. Gel images (several DGGEs were run to assure reproducibility of the results) were captured with a NucleoVision gel documentation system (NucleoTech Corp, San Mateo, CA).

Dominant bands in the gel were aseptically excised; DNA was extracted using the Qiagen gel extraction kit (Qiagen Inc., Chatsworth, CA). This DNA served as a template for PCR reamplification following the same protocol (primers 1055f and 1392r sans GC clamp) as previously described. The gel fragment PCR products were cloned into the TA cloning vector pCR2.1-TOPO (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Plasmid DNA was purified, and the cloned fragments were sequenced. Sequence data were obtained using primers 1055f and 1392r. Sequence comparisons to known sequences were performed using the NCBI BLAST website (Altschul et al. 1997).

## **Results and Discussion**

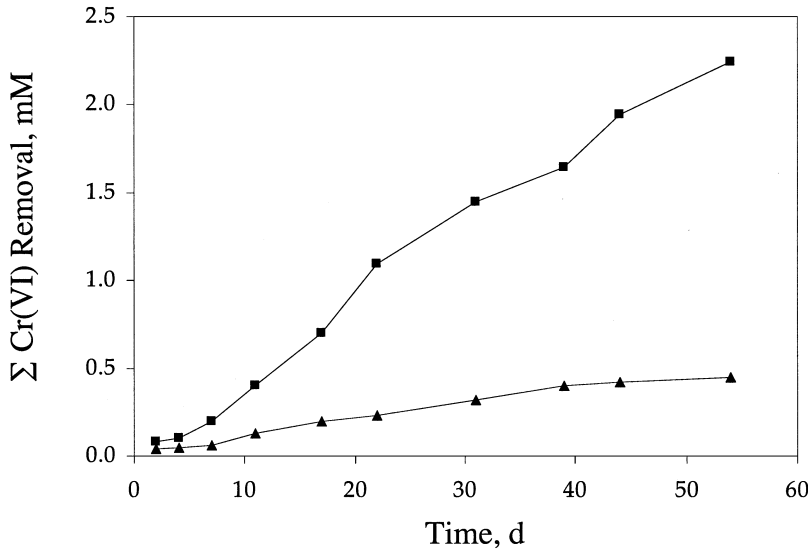
### ***Cr(VI) Reduction/Removal Rate***

To simulate a marine system with a continual input of Cr(VI), laboratory mesocosm experiments were set up to evaluate the rate of Cr(VI) removal from the overlying water and possibly identify the microorganisms that may contribute to the reduction of Cr(VI) in the sediments. The physicochemical parameters of each aquarium were similar and remained constant throughout the study: the pH was 8.2, oxygen in the overlying water column was approximately 82% air saturation, and water temperature was 15–16°C.

For 60 days, Cr(VI) was maintained at constant levels of either 0, 0.25 (low), or 1.5 (high) mM in the overlying water column. Figure 2 shows the cumulative amount of Cr(VI) removed from the overlying water column which is equivalent to the amount of Cr(VI) added. During the first 7 days, we observed a lag in the metal removal for both low and high Cr(VI) systems, followed by a fairly constant rate of Cr removal. This initial lag followed by the steady Cr removal period is reminiscent of bacterial growth. The rates for the first 7 days were calculated as 0.38 ( $\pm 0.19$ )  $\mu\text{M h}^{-1}$  and 1.98  $\mu\text{M h}^{-1}$  for the two 0.25 mM and single 1.5 mM Cr(VI) mesocosms, respectively (Figure 2). After 7 days, the rates were 0.83 ( $\pm 0.17$ )  $\mu\text{M h}^{-1}$  and 4.98  $\mu\text{M h}^{-1}$ , respectively (Figure 2). These results suggest that initially the bacteria were adapting to their environment and after 7 days, they contributed substantially to the removal of Cr(VI).

### ***Total Metals and Organic Carbon Distributions***

It is very well known that some sediment components such as Fe and Mn oxides, organic matter, and clay minerals bind or adsorb heavy metals (Saleh et al. 1989). Therefore, total



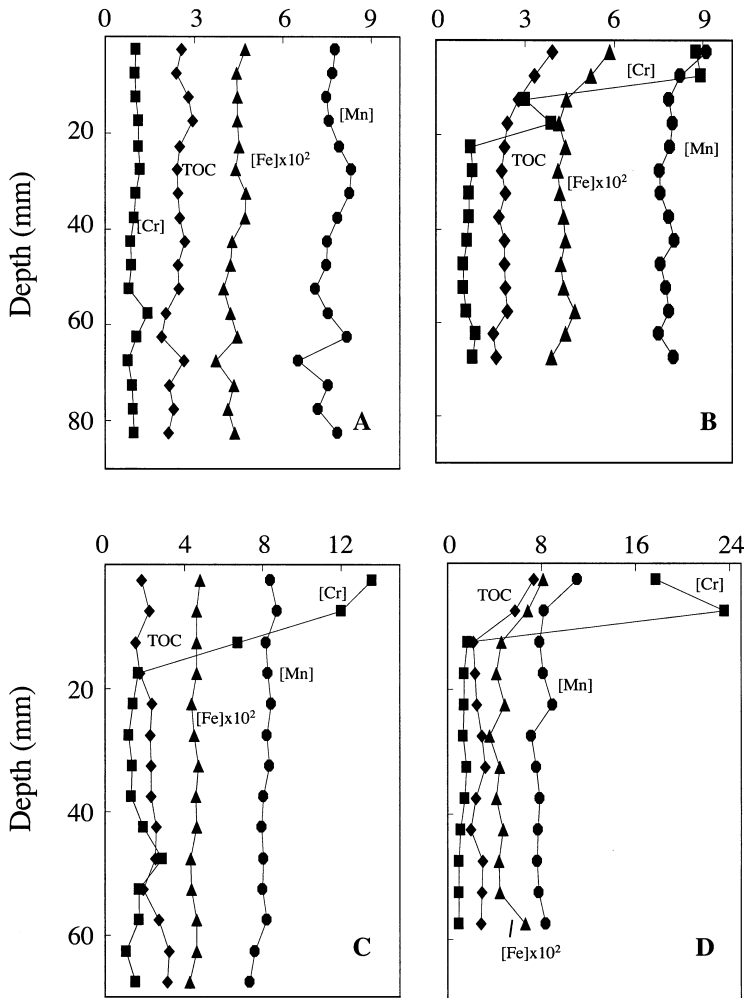
**FIGURE 2** Cumulative Cr(VI) removal in the overlying water column over time (days) in the 0.25 mM (triangles) or 1.5 mM (squares) mesocosms. The values for the 0.25 mM mesocosm represent the average of two separate mesocosms while only a single mesocosm was run with 1.5 mM Cr(VI). As Cr(VI) was removed from the water column, more Cr(VI) was added back to maintain relatively constant levels of Cr(VI) in the mesocosms over the duration of the experiment.

Fe, Mn, Cr, and organic carbon (TOC) were measured in the sediments to determine whether these compounds were correlated to Cr concentrations.

The control mesocosm served as the regional background and the level of total chromium ( $Cr_t$ ) was 1 mM (Figure 3A). This chromium concentration is comparable to levels (0.25 to 1.5 mM) found in other coastal environments (Green-Ruiz 2000). In the experimental mesocosms, added chromium was found to accumulate in the upper 20 mm; below 20 mm, the concentrations returned to background levels (Figure 3B–3D). The highest values for  $Cr_t$  were found in the top 5 mm of the sediment (Figure 3B–3C), and they were approximately 8.9 and 13.5 mM in the low and high Cr(VI) mesocosms, respectively. These levels of chromium have been seen in other contaminated sediments; e.g., in sediments near Barcelona, Spain, Cr ranged from 6–17 mM (Palanques and Diaz 1994).

The accumulation of chromium in the experimental mesocosm sediments is similar to those observed in other natural systems where heavy metals (i.e., lead) have contaminated the sediments (Andrews et al. 1999; Green-Ruiz 2000). According to the contamination factor index, the degree of contamination ( $C_d$ ) is the ratio of the pollutant concentration relative to background levels (Premazzi et al. 1986). If we consider our “no Cr” control mesocosm to be an average coastal sediment background then the  $C_d$  for  $Cr_t$  in the first 5 mm of the sediment was moderate ( $6 \leq C_d \leq 12$ ) to considerable ( $12 \leq C_d \leq 24$ ) for the low and high chromium mesocosms, respectively.

The amount of Fe (400 mmol/L), Mn (8 mmol/L), and TOC (~3%) (Figure 3A) in the control mesocosms were similar to those in natural profiles (Voutsinou-Taliadouri and Varnavas 1995; Green-Ruiz 2000). In the experimental mesocosms, there were no correlations between the Cr and Fe, Mn, or TOC (Figure 3A–C), except for one core (Figure 3D) from the low Cr(VI) mesocosm. Others have also observed a lack of correlation between anthropogenic sources of Cr and Fe, Mn, or organic carbon (Armienta and Quere 1995).

Total C<sub>org</sub> (%) and Metal (mmol/L) Concentrations

**FIGURE 3** Total Cr, Mn, Fe, and organic C concentration profiles from (A) control, (B) 0.25 mM, (C) 1.5 mM, and (D) an outlier core from the 0.25 mM mesocosms. Note: the concentration of Fe is 100 times greater than the values shown on the x-axis.

The core shown in Figure 3D with anomalously high concentrations of Cr (17 mM), Fe (800 mmol/L), Mn (11 mmol/L), and total organic carbon (7.5%) (Figure 3D) is indicative of heterogeneity in our mesocosm system. Most likely, the large amount of organic material in this core complexed the metals present (Provini and Gaggino 1986); hence, correlations between analyzed metals and organic carbon were obtained in this particular subcore only.

### ***Bacterial Enrichments***

According to the geochemical data (Cr profiles), we hypothesized that microbial populations in Cr(VI)-amended treatments would be altered in the surface sediments relative to the controls. Based on previous pure culture studies, we expected that sulfate- and ferric iron-reducing bacteria as well as other Cr(VI)-reducing bacteria would be important

**TABLE 1** Anaerobic bacteria enriched from the mesocosms experiments

Bacteria	Mesocosms								
	No Cr(VI), control			0.25 mM Cr(VI)			1.5 mM Cr(VI)		
	H <sub>2</sub> /CO <sub>2</sub>	ace	lac	H <sub>2</sub> /CO <sub>2</sub>	ace	lac	H <sub>2</sub> /CO <sub>2</sub>	ace	lac
SRB	+	+	+	+	-	+	-	-	-
FeRB	+	+	+	+	+	+	+	+	+
CrRB*:									
0.1	+	-	+	+	+	+	+	+	+
0.25	-	-	-	+	+	+	+	+	+
0.5	n.d.	n.d.	n.d.	-	-	-	+	+	-
1.5	-	-	-	-	-	-	-	-	-

\*CrRB were grown in the range of Cr(VI) concentrations (from 0.1 to 1.5 mM); (+) indicates growth; (-) no growth; (n.d.) not determined.

H<sub>2</sub>/CO<sub>2</sub>, acetate (ace), and lactate (lac) were added as substrates at 20 mM concentration.

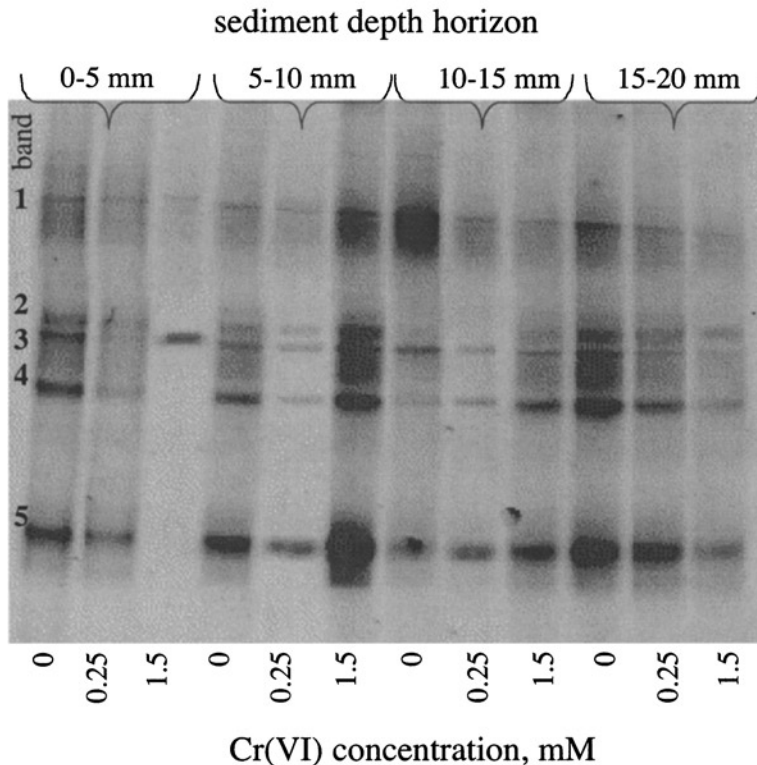
microorganisms in Cr(VI) reduction in our mesocosms. To determine the occurrence of these physiological groups of microorganisms, we used the top sediment horizon (0–5 mm) to enrich for bacteria from each mesocosm using different environmentally relevant substrates, such as acetate, hydrogen, and lactate (Table 1). The microbial populations varied depending on the level of chromium in the mesocosm. For example, sulfate-reducing bacteria (SRB) were found in the control (no added Cr) mesocosm with all tested substrates; in the low Cr(VI) mesocosm, they were detected only with hydrogen and lactate, whereas in the high Cr(VI) mesocosm, they were absent (Table 1). Inhibition of SRB by Cr(VI) at >0.5 mM has been previously observed (Marsh et al. 2000; Arias and Tebo 2003). This toxic effect of Cr(VI) on SRB is reasonable since the chromate ion can easily enter the bacterial cell as it is structurally analogous to phosphate (HPO<sub>4</sub><sup>=</sup>) and sulfate (SO<sub>4</sub><sup>=</sup>) and can short-circuit ATP sulfurylase (Taylor and Oremland 1979; Wetterhahn and Hamilton 1989). An alternate possibility is that the considerable Cr(VI) concentration might be maintaining a higher redox potential in the upper sediment horizons thereby creating conditions thermodynamically unfavorable for the SRB in the 1.5 mM Cr(VI) mesocosms (the E<sub>0</sub>' of the HCrO<sub>4</sub><sup>-</sup>/Cr<sup>3+</sup> is 0.414 V while the E<sub>0</sub>' for SO<sub>4</sub><sup>2-</sup>/HS<sup>-</sup> is -0.217V). Iron-reducing bacteria (FeRB) were found in all mesocosms. They are most likely key bacteria in Cr(VI) removal from the water due to the immobilization of Cr via reduction of Cr(VI) by Fe(II) (Sedlak and Chan 1997) or perhaps direct utilization of Cr(VI) as an electron acceptor (Tebo and Obraztsova 1998; Francis et al. 2000).

To determine whether the presence of Cr(VI) in the mesocosms selected for Cr(VI)-reducing bacteria (CrRB) in the sediments and whether organisms from sediments exposed to higher levels of Cr(VI) would be more resistant to Cr(VI) toxicity, enrichments were made from surficial sediments from each mesocosm with 0.1, 0.25, 0.5, and 1.5 mM concentrations of Cr(VI) as the sole electron acceptor (Table 1). Results indicated that microbes enriched from the control mesocosm (no Cr) were capable of reducing Cr(VI) at no more than 0.1 mM whereas enrichments from the low and high Cr mesocosms reduced Cr(VI) up to 0.25 and 0.5 mM Cr(VI), respectively. No enrichments were found to reduce 1.5 mM Cr(VI) from the high Cr mesocosms. It appears that, after Cr(VI) exposure, the bacteria can either resist the toxic effects of Cr(VI) and grow to detectable numbers or adapt and reduce Cr(VI); however, 1.5 mM is probably too great a concentration for these enrichments.

### Bacterial DNA Analysis

The effect of Cr(VI) input on the sedimentary microbial community was analyzed using PCR-DGGE. Figure 4 illustrates a typical DGGE image of the bacterial community in 5 mm sediment increments under Cr(VI) exposure vs. the control. The profiles were similar to the control and not very different under all conditions and depths (>5 mm) except in the upper horizon (0 to 5 mm) where the most Cr accumulation occurred. The lack of major differences between the bacterial communities at depths >5 mm may be due to the fact that the Cr(VI) concentrations were not sufficiently toxic to affect the major bacterial populations. In the image, five major bands were observed that supposedly represent individual species. Relative to the control profile, the mesocosms exposed to 0.25 mM Cr(VI) had some minor differences, specifically, the slight diminution in the intensity of bands 2 and 3 (Figure 4). More extreme differences were observed in the DGGE profile of the sediments exposed to 1.5 mM Cr(VI) as compared to the control: all but two bands, 1 and 3, are not present (Figure 4). This may indicate either a potential toxicity effect on the organisms represented by the other bands or a selection for CrRB whose DNA would overwhelm the signal from the other organisms.

Examining the major bands from the gel by cloning and sequencing, we found that the dominant bacteria in the surface sediments were not significantly related to known bacteria



**FIGURE 4** 16S rDNA fragments on a denaturing gel (35% to 70% denaturant; SYBR gold-stained). Each lane represents the eubacterial microbial population profile under Cr(VI) exposure from specific depths in the sediment for 0, 0.25, and 1.5 mM Cr(VI) conditions. Then, 300 ng DNA was loaded onto each lane.

**TABLE 2** Sequence identification and percent similarity of bacterial 16S rRNA genes from the DGGE gel

Best matched organism	Band #	% ID	# of bases
<i>Clostridium</i> group ( <i>Tissierella</i> spp.)	1	94	194
Unknown bacterium	2		309
Unidentified bacterium ZF2 (TCP dehalogenating enrichment)	3	98	325
<i>Solemya</i> sulfur-oxidizing symbiont	4	95	313
Mixed sequence:	5		
Unidentified bacterium, Wb1_CO5		95	229
Uncultured gamma proteobacterium, MERTZ		90	300

in the database within the 200–300 bp of sequence analyzed (Table 2). The DGGE was repeated twice with 300 ng total bacterial DNA and similar results were obtained. Band 1 is most closely related to the *Tissierella* sp. (95% similarity), which is in the *Clostridium* group and capable of acetogenic metabolism. This band was always present and did not appear to be adversely affected in either depth or Cr(VI) exposure. Band 2 was not related to any bacterium in the database in the sequence obtained and therefore could not be characterized. Band 3 was related to an unidentified bacterium (98% similarity) strain ZF2, isolated from a trichlorophenol (TCP) dehalogenating anaerobic enrichment culture (Breitenstein et al. 2001). The bacterium represented by band 3 was not present in the 0.25 mM Cr(VI) mesocosm; however, it becomes the dominant band under 1.5 mM Cr(VI) exposure. This particular bacterium would be interesting to culture to determine whether it plays a role in dehalogenation or Cr(VI) reduction.

The sequence analysis of band 4 was related to a symbiotic sulfur-oxidizing bacterium (96% similarity); this bacterium appeared to be less abundant under 0.25 mM Cr(VI) conditions and completely absent under 1.5 mM Cr(VI) conditions. This suggests that, like SRB, the sulfur-oxidizing bacteria may also be sensitive to Cr(VI) toxicity. Conceivably, the inhibition of the SRB at the 1.5 mM Cr(VI) concentration may also indirectly inhibit growth of these sulfur oxidizers due to the lack of sulfide production. Unfortunately, the sulfur oxidizers are not in culture so it remains to be tested whether they are sensitive to Cr(VI), or were inhibited because SRB were inhibited.

Cloned sequences for band 5 revealed mixed bacterial sequences. One bacterium was most closely related to an unidentified environmental clone from Nullarbor caves in Australia (95% similarity). The other bacterium was related to an uncultured  $\gamma$ -proteobacterium, MERTZ (90% similarity), found in continental shelf sediments collected off Antarctica (Bowman et al. 2001). The low sequence similarity of band 5 sequences to known bacteria, as well as other cloned gel fragment examples with low similarity (<98%) to known bacteria, indicate that we may have several new species of bacteria in our mesocosms or that greater than 300 bp is necessary to better identify these bacteria.

No known SRB, FeRB, or other bacteria that have been previously recognized to be involved in Cr(VI) reduction processes were detected by DGGE in any of the mesocosms. This result is in contrast to the culture work which indicated that the above mentioned bacterial groups were present. An explanation of this may be that there was not a sufficient abundance of sulfate and metal respirers relative to other bacterial groups occurring in the top sediment horizons. In addition, cloned environmental 16S rDNA sequences or bacteria visualized by DGGE are often different than those bacteria that can be cultured (Tiedje et al. 1999). These results imply that the microorganisms identified may be associated with and

resistant to Cr(VI) contamination, but unfortunately, their role in Cr(VI) reduction, if any, is not known.

## Conclusions

Mesocosms served as a useful tool for biogeochemical experiments to study the effects of Cr contamination on marine sediments. The results indicate that a constant input of Cr(VI) into a sandy marine sedimentary system will result in eventual removal of Cr(VI) from the overlying water column. The chemical data showed that most of the Cr removed accumulated and was immobilized in the upper surficial sediments. The initial lag for the reduction of Cr(VI), under both 0.25 and 1.5 mM concentrations, may be due to a biological response. In general, there was a lack of correlation between Cr accumulation in the sediments and Fe, Mn, or organic carbon. The Cr immobilization in the upper horizon, the lag in the time for Cr(VI) removal, and the microbial differences in the first 5 mm of sediment is evidence that, in addition to abiotic factors, indigenous bacteria may play a key role in the removal of Cr(VI) from the overlying water.

Using PCR-DGGE to analyze the bacterial community allowed for visualization of specific microorganisms from the natural sedimentary communities that tolerate Cr(VI) and may be involved in Cr(VI) reduction. The sequences obtained from the DGGE via cloning were reliable; however, the low sequence similarity to known bacteria in the database suggests that the bacteria present in our mesocosms (initially from San Diego Bay) are currently unidentified. Clearly not enough is known about the identity and activities of bacteria in marine sediments. Therefore, further efforts to culture the bacteria must be made to understand the physiology and dynamics of bacteria present, especially if we wish to establish natural attenuation as a feasible remediation strategy for Cr(VI) pollution.

## References

- Abu-Saba KE. 1998. Spatial and temporal variability in the aquatic cycling of chromium. Ph.D. dissertation, University of California, Santa Cruz.
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402. (<http://www.ncbi.nlm.nih.gov/BLAST/>)
- Andrews JE, Greenaway AM, Bigg GR, Webber DF, Dennis PF, Guthrie GA. 1999. Pollution history of a tropical estuary revealed by combined hydrodynamic modeling and sediment geochemistry. *J Mar Syst* 18:333-343.
- Arias YM, Tebo BM. 2003. Cr(VI) reduction by sulfidogenic and non-sulfidogenic microbial consortia. *Appl Environ Microbiol* 69:1847-1853.
- Armienta MA, Quere A. 1995. Hydrogeochemical behavior of chromium in the unsaturated zone and in the aquifer of Leon Valley, Mexico. *Water Air Soil Poll* 84(1-2):11-29.
- Bae W, Kang T, Jung J, Park C, Choi S, Jeong B. 2000. Purification and characterization of NADH-dependent Cr(VI) reductase from *Escherichia coli* ATCC33456. *J Microbiol Biotech* 10:580-586.
- Bowman JP, McCammon SA, McCuaig RD, Gibson JAE, Nichols PD. 2001. Characterization of continental shelf sediments collected off Antarctica: Microbial metabolic activity, community structure and biogeography. unpublished.
- Breitenstein A, Saano A, Salkinoja-Salonen M, Andreesen JR, Lechner U. 2001. Analysis of a 2,4,6-trichlorophenol-dehalogenating enrichment culture and isolation of the dehalogenating member *Desulfitobacterium frapperi* strain TCP-A. *Arch Microbiol* 175(2):133-142.
- Chadwick B, Leather J, Richter K, Apitz S, Lapota D, Duckworth D, Caballero M, Patterson A, Koon G, Valkirs A, Meyers-Shulte K, Stallard M, Montee R, Strei R, Sutton D, Skinner L, Germano

- J, Cheng R, Wang PF. 1996. Sediment quality characterization Naval Station San Diego. San Diego, CA, NRAD. Report: 20-81.
- Cord-Ruwish R. 1985. A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *J Microbiol Meth* 4:33-36.
- Ferris MJ, Muyzer G, Ward DM. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* 62(2):340-346.
- Francis CA, Obratsova AY, Tebo BM. 2000. Dissimilatory metal reduction by the facultative anaerobe *Pantoea agglomerans* SP1. *Appl Environ Microbiol* 66(2):543-548.
- Fujii E, Toda K, Ohtake H. 1990. Bacterial reduction of toxic hexavalent chromium using a fed-batch culture of *Enterobacter cloacae* strain HO1. *J Ferment Bioeng* 69:365-367.
- Green-Ruiz C. 2000. Geochemistry of heavy metals and mineralogy of sedimentary clay minerals from California Gulf. National University of Mexico. Doctoral Thesis.
- Ingham ER, Trofymow JA, Ames RN, Hunt HW, Morley CR, Moore JC, Coleman DC. 1986. Trophic interactions and nitrogen cycling in a semi-arid grassland soil. II. System responses to removal of different groups of soil microbes or fauna. *J Appl Ecol* 23:615-630.
- Lee DC, Park CJ, Yang JE, Jeong YH, Rhee HI. 2000. Screening of hexavalent chromium biosorbent from marine algae. *Appl Microbiol and Biotechnol* 54:597-600.
- Lovley DR. 1993. Dissimilatory metal reduction. *Ann Rev Microbiol* 4:263-290.
- Lovley DR, Coates JD. 1997. Bioremediation of metal contamination. *Curr Opin Biotechnol* 8(3): 285-289.
- Marsh TL, Leon NM, McInerney MJ. 2000. Physiochemical factors affecting chromate reduction by aquifer materials. *Geomicrobiol J* 17:291-303.
- McLean J, Beveridge TJ. 2001. Chromate reduction by a pseudomonad isolated from a site contaminated with chromated copper arsenate. *Appl Environ Microbiol* 67(3):1076-1084.
- Myers CR, Carstens BP, Antholine WE, Myers JM. 2000. Chromium (VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *J Appl Microbiol* 88(1):98-106.
- Páez-Osuna F, Fong-Lee ML, Fernández H. 1984. Comparison of three techniques for analyzing organic matter in sediments. Scientific note. Institution of Marine Science and Limnology. Mexico City. 11(1):233-239.
- Palanques A, Diaz J. 1994. Anthropogenic heavy metal pollution in the surface sediments on the Barcelona continental shelf (Northwestern Mediterranean). *Marine Environ Res* 38:17-31.
- Park C-H, Keyhan M, Wielinga B, Fendorf S, Matin A. 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl Environ Microbiol* 66:1788-1795.
- Pettine M, Barra I, Campanella L, Millero FJ. 1998. Effect of metals on the reduction of chromium (VI) with hydrogen sulfide. *Water Res* 32:2807-2813.
- Premazzi G, Provini A, Gaggino GF, Parise G. 1986. Geochemical trends in sediments from 13 Italian subalpine lakes. In: Sly PG, editor. *Sediments and Water Interactions*. 1 ed. New York: Springer-Verlag. p 157-165.
- Provini A, Gaggino GF. 1986. Depth profiles of Cu, Cr, and Zn in Lake Orta sediments (Northern Italy). In: Sly PG, editor. *Sediments and Water Interactions*. 1 ed. New York: Springer-Verlag. p 167-174.
- Raskin L, Amann RI, Poulsen LK, Rittmann BE, Stahl DA. 1995. Use of ribosomal RNA-based molecular probes for characterization of complex microbial communities in anaerobic biofilms. *Water Sci Technol* 31:261-272.
- Saleh FY, Parkerton TF, Lewis RV, Huang JH, Dickson KL. 1989. Kinetics of chromium transformations in the environment. *Sci Total Environ* 86:25-41.
- Sedlak DL, Chan PG. 1997. Reduction of hexavalent chromium by ferrous iron. *Geochim Cosmochim Acta* 61(11):2185-2192.
- Silver S. 1996. Bacterial resistances to toxic metal ions: a review. *Gene (Amsterdam)* 179(1):9-19.
- Spindler NE. 1997. Metals at midvale. *Soil and groundwater cleanup*. 11:6-9.
- Stookey LL. 1970. Ferrozine, a new spectrophotometric reagent for iron. *Anal Chem* 42:779-781.

- Taylor BF, Oremland RS. 1979. Depletion of adenosine triphosphate in *Desulfovibrio* by oxyanions of Group VI elements. *Current Microbiol* 3:101–103.
- Tebo BM, Obratsova AY. 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. *FEMS Microbiol Lett* 162(1):193–198.
- Tiedje JM, Asuming-Brempong S, Nusslein K, Marsh TL, Flynn SJ. 1999. Opening the black box of soil microbial diversity. *Appl Soil Ecol* 13:109–122.
- Turick CE, Graves C, Apel WA. 1998. Bioremediation potential of Cr(VI)-contaminated soil using indigenous microorganisms. *Bioremed J* 2:1–6.
- Urone PF. 1955. Stability of colorimetric reagent for chromium, S-diphenylcarbazides, in various solvents. *Anal Chem* 27:1354–1355.
- U.S. Environmental Protection Agency. 1999. Natural attenuation for groundwater remediation. (<http://www.epa.gov/OUST/oswermna/summary.pdf>)
- Wetterhahn KE, Hamilton JW. 1989. Molecular-basis of hexavalent chromium carcinogenicity: effect on gene-expression. *Sci Total Environ* 86(1–2):113–129.
- Voutsinou-Taliadouri F, Varnavas SP. 1995. Geochemical and sedimentological patterns in the Thermaikos Gulf, northwest Aegean Sea, formed from a multisource of elements. *Estuar Coast and Shelf Sci* 40:295–320.
- Widdel F, Bak F. 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-H, editors. *The Prokaryotes*. 2nd ed. New York: Springer-verlag. p 3352–3378.

