Virginia journal of Science
Volume 53, Number 3
Fall 2002

Chromium Tolerant Microbial Communities from the Chesapeake Bay Watershed

Kristine L. Lowe[1], **Ruth E. Fliflet**[1], **Tony Ly**[1], **Brenda J. Little**[2] and **Joanne Jones-Meehan**[1*], [1]Chemistry Division, Code 6115, Naval Research Laboratory, Washington, DC 20375
[2]Oceanography Division, Code 7303, Naval Research Laboratory, Stennis Space Center, MS 39529.

Key Words: Chromium tolerance, *Shewanella*, contaminant metals
Chesapeake Bay

ABSTRACT

Chromium tolerant bacteria were enumerated from portions of the Chesapeake Bay watershed and examined for their potential to reduce Cr(VI). Water and sediment samples were collected from various locations in Baltimore Harbor and Bear Creek, as well as Sandy Point State Park in Maryland and the Anacostia River in Washington, DC. Samples were spread onto agar plates with $CrO_4$2- (5 ppm) as the sole terminal electron acceptor. Plates were incubated anaerobically and colony forming units (CFU) enumerated. CFU arising on minimal-$CrO_4$2- medium ranged from 10^3-10^4 mL^{-1} or g^{-1} and community estimates from sites in proximity to Baltimore City were approximately 6-30X higher than distal sites. Bacterial identification by BIOLOG™ or 16S rRNA sequencing indicated the presence of bacteria of the genera *Klebsiella*, *Pseudomonas*, *Burkholderia*, *Kluyvera* and others. Typical Cr(VI) reduction rates by these isolates were significantly lower than *Shewanella oneidensis* , a known metal-reducing bacterium. Results suggested that microbial communities in the Chesapeake Bay watershed, particularly in Baltimore Harbor and Bear Creek, had a high tolerance for Cr(VI) and/or could grow slowly with Cr(VI) as a terminal electron acceptor. However, the isolates did not rapidly degrade Cr(VI) in the laboratory.

INTRODUCTION

The Chesapeake Bay is the largest estuary in the U.S. and is fed by a broad watershed that includes six states (New York, Pennsylvania, Delaware, Maryland, Virginia and West Virginia) and the District of Columbia, encompassing an area of approximately 12,000 km^2 (Pritchard and Schubel, 2001). Forests, cultivated and abandoned agricultural land, wetlands and residential areas surround the Bay and its adjacent watershed. It is home to a wide range of aquatic wildlife and has regional economic importance in the fishery and shipping industries (Lippson and Lippson, 1997). In addition, the Chesapeake Bay is a popular site for recreational boaters and tourists.

* To whom correspondence should be addressed. E-mail: jonesmee@ccf.nrl.navy.mil; Telephone: (202)404-6361; Fax: (202)404-8515

As a result of past and recent human activities, pollutants and other contaminants (i.e., pesticides, herbicides, organophosphates, polychlorinated biphenyls [PCBs], petroleum products and heavy metals) have accumulated in the Bay (Lynch, 2001). Pollutants reach the Bay through river drainage, runoff and direct discharge (Curtin, 2001). One of the more problematic contaminants is chromium, which was mined north of the Bay in the 19th and 20th centuries. Chromium is an important industrial metal used in the manufacture of many diverse products, including ferrous and nonferrous alloys, paints, pigments, wood preservatives and corrosion inhibitors (Fendorf et al., 2000). Such manufacturing industries have operated in and around the Bay region during the past two centuries.

Chromium is a redox active transition metal with a wide range of possible oxidation states, although, only two (+6 and +3) are stable in the environment. It is a widespread contaminant in the environment and is recognized as a toxic substance and carcinogen (Kimbrough et al., 1999). Cr(VI) is highly water soluble and is easily transported through aquatic environments. In contrast, Cr(III) is much less soluble and precipitates as a hydroxide above pH 5.5. Due to its lower solubility, Cr(III) is considered less toxic and is, in fact, a necessary micronutrient for humans and other animals (Hamilton and Wetterhahn, 1987).

Chromium tolerance may occur by several potential mechanisms including plasmid-encoded resistance, transport mechanisms and reduction (Wang, 2000; Cervantes et al., 2001). Reduction of soluble (more toxic) Cr(VI) to less soluble (less toxic) Cr(III) is influenced by several factors (e.g., pH, temperature, redox potential) and can be mediated by various chemical species (i.e., Fe(II), S^{2-}), some plants and several microorganisms (Fendorf et al., 2000; Lytle et al., 1998; Wang, 2000). A metal-reducing microorganism, *Shewanella oneidensis*, has been shown in laboratory experiments to reduce Cr(VI) at high rates (Daulton et. al., 2001). Thus, one potential strategy for environmental Cr(VI) removal would be the addition of *S. oneidensis* into contaminated sites. However, it is not known at this time whether *S. oneidensis* can compete with native microflora at Cr(VI) contaminated sites. Therefore, a possible remediation plan would be to stimulate naturally-occurring Cr-tolerant and Cr(VI)-reducing bacteria (CRB) in contaminated environments by fertilization or other environmental manipulation. Alternatively, wastewater treatment schemes could be developed using naturally-occurring CRB in bioreactor systems. *In situ*, naturally-occurring CRB may have Cr(VI) reduction capabilities superior to those of *S. oneidensis*.

To assess the feasibility of such bioremediation strategies, we evaluated the prevalence of Cr-tolerant and other bacterial communities in the Chesapeake Bay watershed, which includes regions previously shown to contain high levels of contaminants including chromium (Baker et. al., 1997). Environmental isolates were identified and tested for their ability to reduce Cr(VI) in the laboratory.

METHODS

Sampling locations.

Surface water samples (top 2 cm) were collected from five sites in the Chesapeake Bay watershed on July 14, 2000 (Figure 1). They included: two sites, HP (39° 17’ 08" N, 76° 36’ 42" W) and FP (39° 16’ 53" N, 76 35 33 W) in Baltimore City Harbor; one site, FM (39° 15’ 46" N, 76° 34’ 43" W), approximately 2 km downstream; and two sites distal to Baltimore. The distal sites included: one site approximately 40 km further

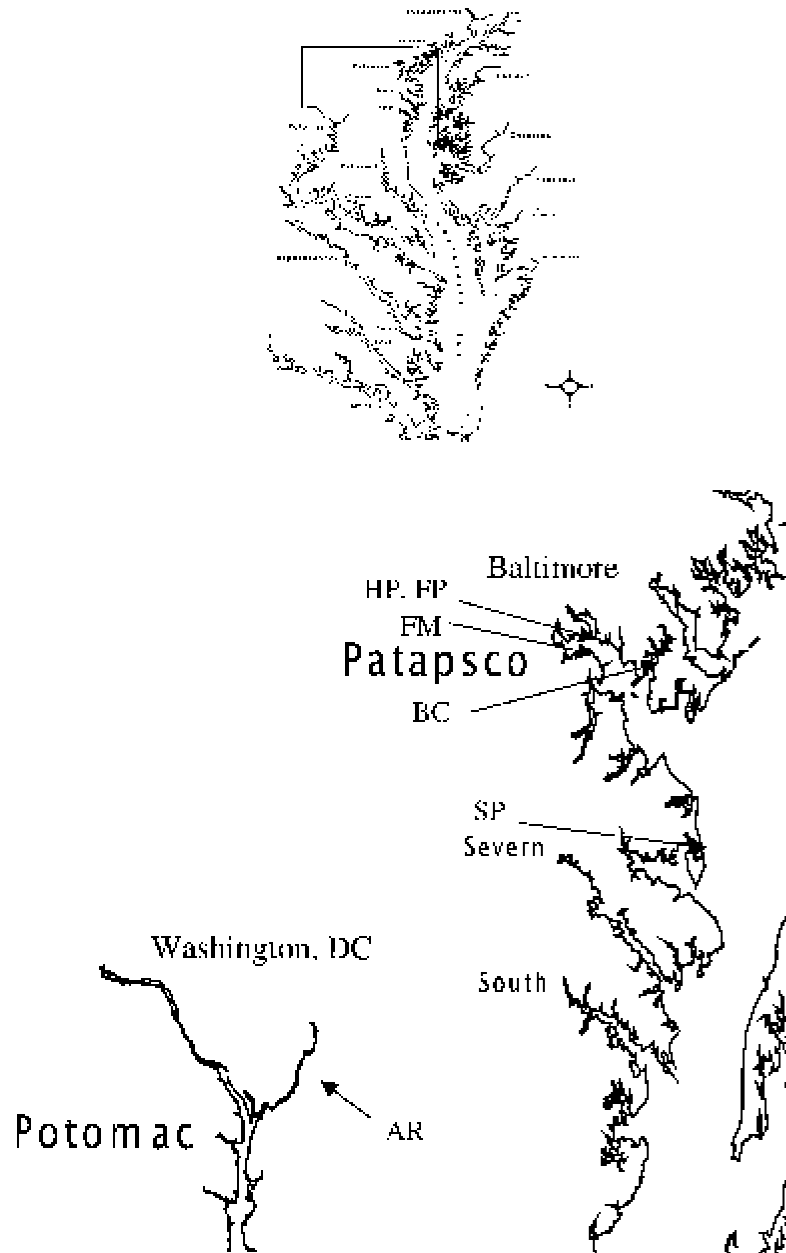

FIGURE 1. Map of sampling locations

downstream at Sandy Point State Park (SP; 39° 00' 44" N, 76° 23' 42" W) near Annapolis, Maryland and a second site in the northwest branch of the Anacostia River near Washington, DC (AR; 38° 53' 17" N, 76° 58' 04" W), approximately 60 km west of the Bay proper. Sediment samples were collected on July 3, 2001 from Bear Creek (BC; 39° 14' 41" N, 76° 29' 40" W), a tributary of the Patapsco River near Dundalk, Maryland. Bear Creek sediment samples were taken in a small channel that is fed by the main creek at high tide. Suction corers were used to remove 18-cm deep columns of sediment, which were stored on ice during transfer to the laboratory. The sediment cores were sectioned into 2-cm deep layers, each layer was homogenized and processed as described below. At all sites, water temperature was 26°C and salinity was approximately 1% (Pritchard and Schubel, 2001).

Sediment Chemical Analysis.

A portion of each 2-cm sediment sample was examined for the presence of chromium (Cr), iron (Fe), zinc (Zn) and nickel (Ni). Metals were extracted from sediment using the method of James et al. (1995). Briefly, 5 g of sediment were placed in sterile 125 mL flasks with 25 mL of a carbonate-hydroxide buffer (pH 13). The mixture was shaken until homogeneous and left standing for 1 h. The flasks were transferred to an 80°C water bath for 45 min and were mixed every 15 min. Due to evaporation, sterile distilled water was added as required to maintain a constant volume. After incubation, sediment slurries were transferred to sterile tubes and centrifuged for 30 min at 3,000 rpm. The supernatant was collected, filtered through a 0.2 µm filter and analyzed for heavy metals by Flame Atomic Absorption Spectroscopy (FAAS) using an air-acetylene flame (Model 906; GBC Scientific Equipment, Arlington Heights, Illinois). Standard curves were generated for each metal (Cr, Fe, Zn, and Ni) using purified standards. Sediment metal concentrations were estimated from plotted absorbance values on standard curves. The instrument automatically corrected for background interference by subtracting absorbance measurements from a deuterium lamp operating concurrently with the analyte light source.

Culture incubations.

All incubations were performed in duplicate at 26° C. An aliquot (100 µL) was withdrawn from each water sample and inoculated into liquid enrichment cultures consisting of a defined minimal medium (Daulton et. al., 2001) supplemented with 18 mM lactate and 5 ppm Cr(VI) in the form of K_2CrO_4 (Sigma, St. Louis, Missouri), as the sole terminal electron acceptor (medium hereafter abbreviated NS). Enrichment cultures were incubated anaerobically in glass canisters using an anaerobic gas generating system (BBL Gas Pak Plus; Becton Dickinson Co., Cockeysville, Maryland). After 10 days, 1 mL of each enrichment culture was removed, serially diluted and spread onto solid NS medium. Dilutions spread onto NS plates were immediately placed into anaerobic canisters at 26°C. After 3 weeks, the canisters were opened and colony forming units (CFU) per mL of original water sample estimated.

A 1 g sample from each homogenized sediment layer of Bear Creek was transferred to 9 mL of sterile saline, serially diluted and spread onto commercially-prepared agar media (Nutrient Agar [NA]; Difco Laboratories, Detroit, Michigan) supplemented with NaCl (1.5% w/v) and NS agar plates. One set of NA plates was incubated aerobically for 1 week. A second set of NA plates and the NS plates were placed in anaerobic

canisters at 26° C. After 3 weeks, the canisters were opened and CFU g^{-1} wet sediment were enumerated.

CFU data were determined on NS and other plates by counting any visually-observed colonies arising on the plates and back-calculating the density based on the number of dilutions. With regard to the anaerobic NS plates, since no other electron acceptor was available in the medium other than Cr(VI), the CFU on those plates must have respired with Cr(VI) and were therefore denoted as the chromium-reducing bacteria (CRB) population.

Most probable numbers (MPN).

A 1 g sample of each sediment layer was inoculated in anaerobic growth medium for enumeration of culturable sulfate-reducing bacteria (SRB). SRB liquid growth medium consisted of Postgate's B medium (Postgate, 1984) supplemented with lactate (15 mM) and NaCl (1.5% w/v; designated PB medium). 1 mL of the initial inoculum was sequentially diluted by 10X to a final 10^{-9}-fold dilution. Each series was performed in triplicate. Anaerobic dilution tubes were scored for SRB by noting the presence of a black FeS precipitate after 21 days at 26°C. MPN were determined using the program MOST PROBABLE NUMBER CALCULATOR© Version 4.04 (Klee, 1996).

Microbial Identification.

A set of CFU arising on anaerobic NS plates from water and sediment samples was selected and pure cultures were screened using the BIOLOG™ Microstation System (BIOLOG, Inc., Hayward, California) according to the manufacturer's instructions. One isolate, designated AR-4, was identified by polymerase chain reaction (PCR) amplification and direct sequencing of the 16S rRNA gene. Isolate AR-4 was grown in Luria-Bertani (LB; Difco Laboratories, Detroit, Michigan) broth overnight, an aliquot (0.5 mL) was centrifuged at 12,000 rpm and the supernatant discarded. The cell pellet was resuspended in 50 µL of PCR Core System I (Promega Corp., Madison, Wisconsin) and 100 pmol each of the forward and reverse primers was added. The universal 16S rRNA primers used were: Primer 375 with the sequence (5'-3'): CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC CCG TCA ATT CCT TTG AGT TT (forward) and Primer 371 with the sequence (5'-3'): cct acg gga ggc agc ag (reverse). A 'hot start' cycle was initiated (92°C for 5 min), followed by 40 cycles under the following conditions: 92°C for 30 sec (denaturing), 55°C for 30 sec (annealing) and 72°C for 1 min (extension). This was followed by a final extension cycle (72°C for 3 min). The product was held at 4 ^{o}C and PCR products were visualized on a 1.4 % agarose gel. PCR products were purified using Microcon™ YM-100 filters (Millipore Corp., Bedford, Massachusetts) according to the manufacturer's instructions. The concentration of PCR product was estimated spectrophotometrically by absorbance at 260 nm.

For DNA sequencing, purified PCR product (75 ng) was amplified using the Big Dyeä Terminator Kit (PE Applied Biosystems, Foster City, California) and 3.2 pmol of 371 Primer according to the manufacturers' protocol. The reaction conditions were: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min (25 cycles). The reaction mixture was held at 4°C, followed by purification of the PCR product through isopropanol precipitation. Samples were loaded into an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California). Approximately 450 bp were sequenced and aligned. Obtained sequences were compared to known sequences in the National

Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1990).

Cr(VI) reduction assays.

Selected CFU arising on NS plates were grown aerobically in LB broth in 250 mL flasks on a benchtop stirplate. Upon reaching an A_{600} of 0.5 (approximately 10^9 cells mL^{-1}), the culture was purged with N_2, treated with chloramphenicol (100 µg mL^{-1} final concentration) and supplemented with 5 ppm Cr(VI) in the form of K_2CrO_4. Samples were aseptically removed and Cr(VI) concentration estimated via the diphenylcarbazide method (Clesceri et al., 1998). Cr(VI) reduction rates by the Chesapeake Bay bacterial isolates were compared to those obtained from sterile (uninoculated) media controls and a known metal-reducing bacterium, *Shewanella oneidensis*.

A second set of CFU arising on NS plates was grown in NS broth supplemented with 5 ppm Cr(VI) in the form of K_2CrO_4, in glass flasks on a bench-top shaker for 1 week. Samples were aseptically removed each day and the Cr(VI) concentration was estimated by the diphenylcarbazide method as described previously. Cr(VI) reduction rates by Chesapeake Bay bacterial isolates were compared to those obtained from sterile (uninoculated) media controls and *Shewanella oneidensis*.

Cultures of *S. oneidensis* were incubated with sediment from the upper 2 cm of Bear Creek to evaluate any effect that sediment microorganisms might have on Cr(VI) reduction by this organism. Sediment was mixed with LB broth to a concentration of 10% (w/v) and stirred for 1 h prior to the introduction of Cr(VI) or *S. oneidensis*. Due to the presence of observable sulfides in the sediments, a total of 5 ppm of Cr(VI) was added slowly over a period of 2-3 hours to saturate any S^{2-} that might potentially reduce Cr(VI), and thus compete with consortia microorganisms or *S. oneidensis*, during experiments. After the saturation period, 10 ppm Cr(VI) and a 2% inoculum of *S. oneidensis* was added to the sediment/LB slurry. Levels of Cr(VI) were monitored as before. Controls consisted of sediment slurries lacking *S. oneidensis* and slurries using autoclaved sediment.

RESULTS AND DISCUSSION

Metal Analysis of Bear Creek Sediment.

Concentrations of Cr, Fe, Zn and Ni were determined from sediment using a hot carbonate-hydroxide extraction procedure (James et al, 1995). Recovery of Cr(VI) with this method is greater than 90% based on studies using Cr(VI)-spiked loam soil and sand (James et al., 1995). Experiments in our lab with Cr(VI)-spiked illite clay and organic-rich soil also exhibited recoveries of greater than 90% (unpublished data). Estimates of sediment metal concentration (Table 1) were determined with flame atomic absorption spectroscopy (FAAS). Values for the various metals ranged from a high of greater than 17 ppm to below detection limits. Low level detection limits of FAAS are metal-specific but in general range from 0.001 ppm (Zn) to 0.02 ppm (Fe) (Thompson et al., 1978; Slavin, 1978; Cantle, 1982). With the exception of Ni, all metal concentration estimates for Bear Creek sediments were within detection limits for the method.

Iron is important in the transport of Cr, as Fe(II) can chemically reduce soluble Cr(VI) to insoluble Cr(III), and zinc and nickel are often co-contaminants in chromium-

TABLE 1. Depth concentration profile for total chromium and other metal contaminants in a representative Chesapeake Bay sediment from Bear Creek (Dundalk, Maryland).

Sediment Sample[a] Depth (cm)	Cr	Fe	Zn	Ni
		Parts per million (ppm)		
0-2	0.265	14.485	5.000	0.200
2-4	0.156	17.030	2.886	0.040
4-6	0.385	14.149	2.643	0.000
6-8	0.260	17.812	2.914	0.760
8-10	0.328	2.604	5.200	0.000
10-12	0.338	15.050	3.400	0.120
12-14	0.302	9.307	4.771	0.000
14-16	0.603	8.931	2.157	0.000
16-18	0.270	12.742	2.314	1.080

[a]Sediment cores were sectioned and extracted using a carbonate-hydroxide buffer procedure (James et al., 1995). Aqueous extracts were analyzed by Flame Atomic Absorption Spectroscopy (FAAS) using an air-acetylene flame. Measurements were compared to standard curves of purified standards. Values are the mean of duplicate measurements.

bearing wastewaters (Germain and Patterson, 1974). In addition, studies in our laboratory and others indicate that toxic metals inhibit chromium reduction and growth of chromium-tolerant microorganisms in the laboratory (Lowe et al., 2002; Garbisu et al., 1997; Hardoyo et al., 1991). In Bear Creek sediments, chromium concentrations ranged from 0.156 ppm (2-4 cm depth) to 0.603 ppm (14-16 cm). These levels were significantly lower than previous measurements (Baker et al., 1997). In that report, Cr levels as high as 1,800 ppm were measured. However, Baker et al. utilized different methods of analysis than those used here and sampled in different locations. The samples in the present study were taken more than 1 km upstream from those in the previous study, were located near-shore rather than in the center of the creek and were colleted 4 and 5 years later. Baker et al. (1997) contend that significant spatial differences in contaminant concentrations exist within the Bay. Other investigators agree with this conclusion (Pritchard and Schubal, 2001). Tidal currents, river input, seasons, vertical and horizontal mixing, salt gradients, winds and proximity to contaminant sources all contribute to differential dispersal and accumulation patterns of contaminants in the Bay and thus could account for the large variances in contaminant concentrations from this study to that of Baker et al. However, the values for Cr(VI) found at the sites sampled are higher than natural chromium levels found in aquatic environments, which typically range from 0.5 to 2 ppb (Shiller and Boyle, 1987).

Iron is often a limiting nutrient in aquatic environments (Sunda, 2000). Concentrations of Fe were highest in the upper regions of the sediment and declined in the lower regions of the sediment column. The highest value for Fe was obtained at a depth of 6-8 cm while the lowest value for Fe corresponded to a depth of 8-10 cm. Concentrations of Zn fluctuated in the sediment and ranged from approximately 2 to 5 ppm. Concentrations of Ni were low or below detection in most of the sediment samples. The highest Ni value (1 ppm) was obtained at the bottom of the sediment core (16-18 cm).

TABLE 2. Depth profiles of aerobic and anaerobic microbial populations in Chesapeake Bay sediments (Bear Creek, Dundalk, Maryland).

Depth (cm)	Total Aerobic ($CFU\ g^{-1}$)[a]	Total Anaerobic ($CFU\ g^{-1}$)[a]	SRB ($Cells\ g^{-1}$)[b]	CRB ($CFU\ g^{-1}$)[c]
0-2	3.6×10^5	2.6×10^4	1.1×10^5	6.3×10^4
2-4	1.9×10^5	1.7×10^4	4.2×10^2	2.6×10^4
4-6	1.6×10^5	0.3×10^4	2.4×10^4	2.3×10^4
8-10	0.9×10^5	0.2×10^4	9.3×10^2	0.7×10^4
14-16	2.8×10^5	0.8×10^4	1.9×10^4	0.4×10^4
16-18	3.4×10^5	0.3×10^4	2.4×10^2	0.9×10^4
Average	2.3×10^5	1.0×10^4	2.3×10^4	2.2×10^4

[a]Total aerobic and anaerobic community estimates were generated from incubations on Nutrient agar (NA) plates supplemented with 1.5% NaCl (w/v).
[b]Sulfate Reducing Bacteria (SRB) were estimated by Most Probable Number (MPN) analysis using PB medium.
[c]Chromium (VI) Reducing Bacteria (CRB) populations were estimated from 21 day anaerobic incubations on NS medium plates.

Aerobic and Anaerobic Microbial Populations from the Bear Creek sediment.

After aerobic incubations of the Bear Creek sediment on NA plates, colony counts were obtained (Table 2). Concentrations of aerobically-grown microbial colonies were relatively constant throughout the sediment column with the exception of a section corresponding to a depth of 8-10 cm, in which the concentration of culturable bacteria was 2 to 4 times lower. The highest concentration of anaerobic CFU arising on NA was obtained in the uppermost layer (0-2 cm) of the sediment. The mean concentration of culturable aerobic bacteria for the entire sediment column was 2.33×10^5 CFU g^{-1} and the microbial community was estimated to be on average 38% Gram-positive and 62% Gram-negative (data not shown). It should be noted that microbial populations are underestimated by cultivation techniques, which probably represent less than 1% of the native microbial community in marine/estuarine environments (Amann et al., 1995). This is due to an inability in the laboratory to duplicate the *in situ* environmental conditions for cultivating microbes (i.e., laboratory media may lack a required nutrient; microscale changes in temperature, pH or O_2 concentration may be significant; microorganisms may have symbiotic or commensal relationships with other biota, etc.)

CFU from anaerobically-grown NA incubations were highest in the upper 4 cm and declined with depth (Table 2). As in the aerobic population, the highest CFU estimate was obtained in the uppermost regions of the sediment column and the lowest value was located at approximately 8-10 cm. The decline in microbial population at 8-10 cm, both aerobically and anaerobically may be due in part to iron limitation (Table 1). The mean of the anaerobically-grown population for the entire length of the sediment core was 9.6×10^3 CFU g^{-1}, which was 2 orders of magnitude lower than that for the aerobic community.

Many sulfate-reducing bacteria (SRB) are difficult to cultivate on solid media. Therefore, SRB population estimates were made in liquid cultures using Most Probable Number (MPN) analysis. MPN were in the range of 10^2 to 10^5 CFU g^{-1} (Table 2). SRB were observed at every depth tested with two spatially-distinct primary populations:

TABLE 3. Population estimates of Chromium (VI) Reducing Bacteria (CRB) from water samples taken from the Chesapeake Bay watershed.

Sample[a]	Cr(VI) Reducing Bacteria ($CFU\ g^{-1}$ wet sediment)[b]
HP	3.5×10^4
FP	2.5×10^4
FM	4.4×10^4
SP	4.3×10^3
AR	1.5×10^3

[a]Locations HP, FP and FM were near Baltimore City; samples SP and AR were farther away. See text for detailed descriptions of sampling locations.
[b]Water samples were diluted, spread onto NS media plates and incubated anaerobically for 21 days. CFU, colony forming units.

one located in the upper layer of the sediment and one at a depth of 14-16 cm (Table 2). SRB are potentially important members of the Chesapeake Bay microbial community with respect to chromium contamination. Reduction products of sulfate ($SO_4$2-) respiration by SRB can chemically reduce Cr(VI) (Beukes et al., 1999; Fendorf et al., 2000). In addition, data suggests that certain SRB can directly reduce Cr(VI) (Tebo and Obraztsova, 1998; Tucker et al., 1998; Smith and Gadd, 2000; Michel et al., 2001). $CrO_4$2- ions are structurally similar to $SO_4$2-. Passage of $CrO_4$2- into cells may occur via sulfate transport pathways (Nies et al., 1998).

Populations of Cr Tolerant Bacteria from the Chesapeake Bay watershed.

Chromium reducing bacteria (CRB) community estimates were highest in the upper 6 cm of Bear Creek sediment and decreased with depth (Table 2). CFU in the upper regions of Bear Creek were comparable to those obtained for water samples from Baltimore City. CRB from the anaerobic incubations of water samples ranged in concentration from 1.5×10^3 to 4.4×10^4 cell mL^{-1}. Locations in Baltimore City, on average, showed CRB counts 15 times higher than those farther away (Table 3). The highest values were obtained from sites FM and HP in Baltimore City.

Identification and Chromium (VI) Reduction Capacity of Cr Tolerant Isolates from the Chesapeake Bay.

To test the Cr(VI) reducing capacity of CRB isolates, cultures of selected CRB were initially grown to a density of approximately 10^9 cells mL^{-1} in LB then treated with chloramphenicol to inhibit additional protein production. The media was supplemented with 5 ppm Cr(VI) and sampled every 10 min for Cr(VI) concentration. Most CRB isolated from water samples displayed little or no Cr(VI) reduction ability in short term experiments (data not shown). The best reduction was observed for isolates FP-5 (6 ppb min^{-1}), AR-4 (6.5 ppb min^{-1}) and SP-4 (16 ppb min^{-1}) corresponding to 7%, 9% and 16% reduction of added Cr(VI), respectively (Figure 2A). Previous studies in our lab indicate that the experimental error of the diphenylcarbazide method is between 10 and 15% (data not shown). Therefore, the values obtained for these isolates probably do not represent significant Cr(VI) reduction. Cr(VI) reduction rates for isolates from Sandy Point Park and Anacostia River water were similar to those of isolates from water samples in Baltimore City.

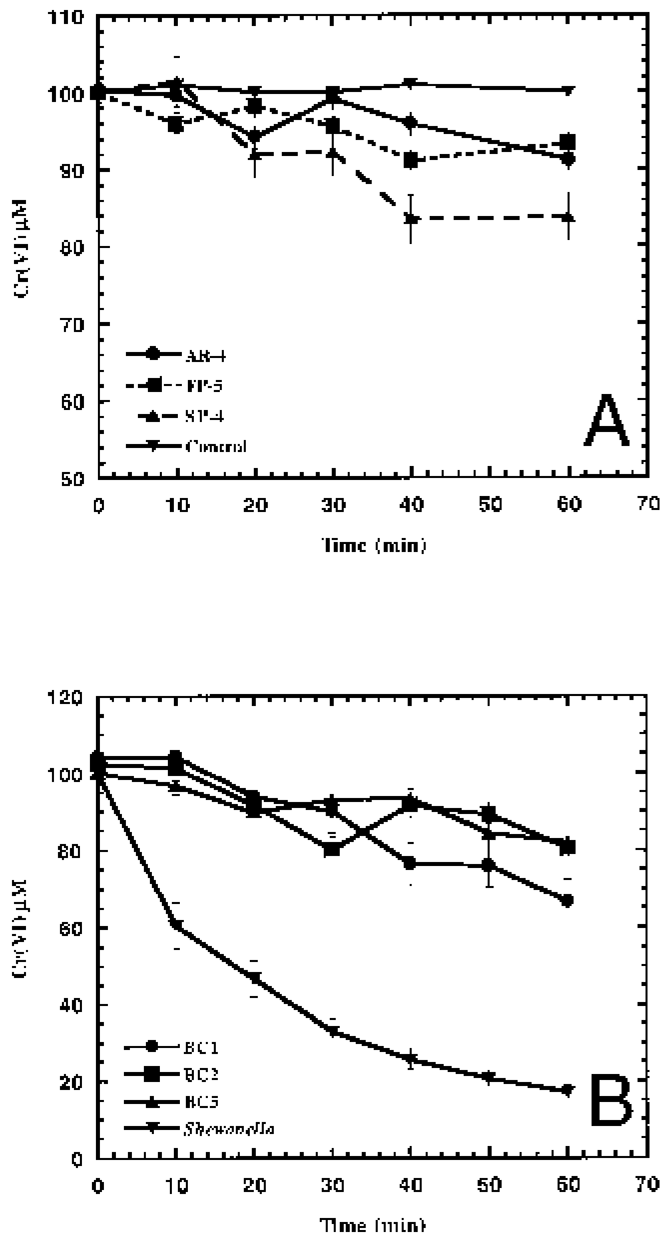

FIGURE 2. Chromium (VI) reduction by isolated Chesapeake Bay chromium reducing bacteria (CRB) and *Shewanella oneidensis*. Cultures were grown aerobically in Luria-Bertani (LB) broth to a density of approximately 10^9 cells mL^{-1}, treated with chloramphenicol and amended with 5 ppm Cr(VI). Samples were aseptically removed and assayed for Cr(VI) reduction over a 1 h time period. Control cultures consisted of sterile medium. The Cr(VI) concentration was determined using the diphenylcarbazide method (Clesceri et al., 1998). Cr(VI) values are the mean of triplicate experiments with standard error. A) CRB isolates from water; B) sediment CRB isolates and *S. oneidensis*, known metal-reducer

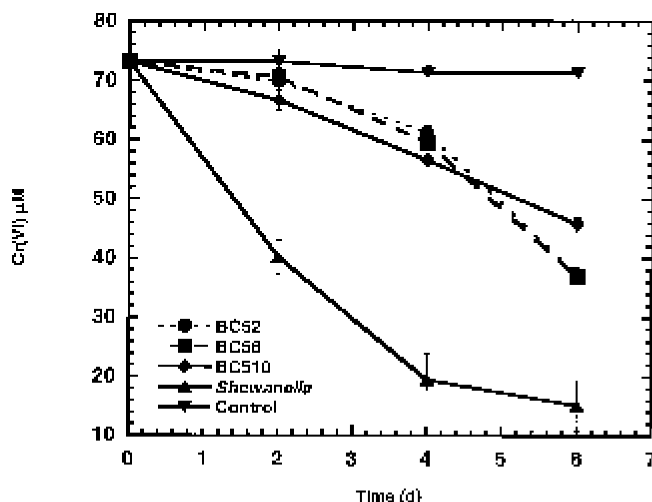

FIGURE 3. Chromium reduction by isolated Chesapeake Bay sediment CRB in a 1-week culture incubation. Isolates were inoculated directly into minimal (NS) medium amended with 3.75 ppm Cr(VI) and incubated aerobically with shaking at room temperature. Samples were aseptically removed and assayed for Cr(VI) reduction. Control cultures consisted of sterile medium. The Cr(VI) concentration was determined using the diphenylcarbazide method (Clesceri et al., 1998). Cr(VI) values are the mean of triplicate experiments with standard error.

Water-borne isolate SP-4 was identified as *Klebsiella pneumoniae* and isolate FP-5 was identified as *Pseudomonas putida* by BIOLOG^{TM} analysis. Several other pseudomonads have been reported to reduce Cr(VI). They include *P. ambigua* (Suzuki et al., 1992), *P. stutzeri* (Badar et al., 2000), and *P. synxantha* (McLean et al., 2000). Park et al. (2000) reported the isolation of a chromium (VI) reductase from *P. putida*, however, optimal enzymatic activity for their protein was achieved at pH 5.0 and 80°C. Isolate AR-4 from the Anacostia River was identified as *Kluyvera georgiana* by 16S rRNA sequencing (98% similarity). Members of the genus *Kluyvera* are found in synergistic relationships with terrestrial plants where they are believed to provide the plant with protection from heavy metal toxicity (Burd et al., 1998).

In short-term experiments, Cr(VI) reduction rates by Bear Creek sediment isolates were higher than those for CRB isolates from water samples (Figure 2B). The highest rates were observed in sediment isolates BC-1 (3.5 ppb min^{-1}), BC-2 (1.5 ppb min^{-1}) and BC-5 (1.5 ppb min^{-1}). These were identified by BIOLOGä analysis as being most similar to *Burkholderia* sp., although, the percent similarity to known organisms was less than 50%. Overall, these isolates degraded 36%, 29% and 21% of added Cr(VI) in one hour, respectively. No isolate reduced Cr(VI) at a rate equal to that of *Shewanella oneidensis*, a known metal reducing bacterium (Figure 2B). Rates for *S. oneidensis* were approximately 60 ppb min^{-1}, which resulted in an 84% decrease in the Cr(VI) concentration in 1 h. No Cr(VI) reduction was observed in sterile (uninoculated) controls, indicating that Cr(VI) reduction was biologically mediated.

In the Cr(VI) reduction experiments incubated for 1 week, CRB from Bear Creek reduced more Cr(VI) than in short-term experiments (Figure 3). In the short-term experiments, cultures were grown in rich media to a high cell density before the addition of Cr(VI). In the 1 week experiments, cells were inoculated directly into NS medium supplemented with Cr(VI) and allowed to grow in the presence of the metal. Cultures

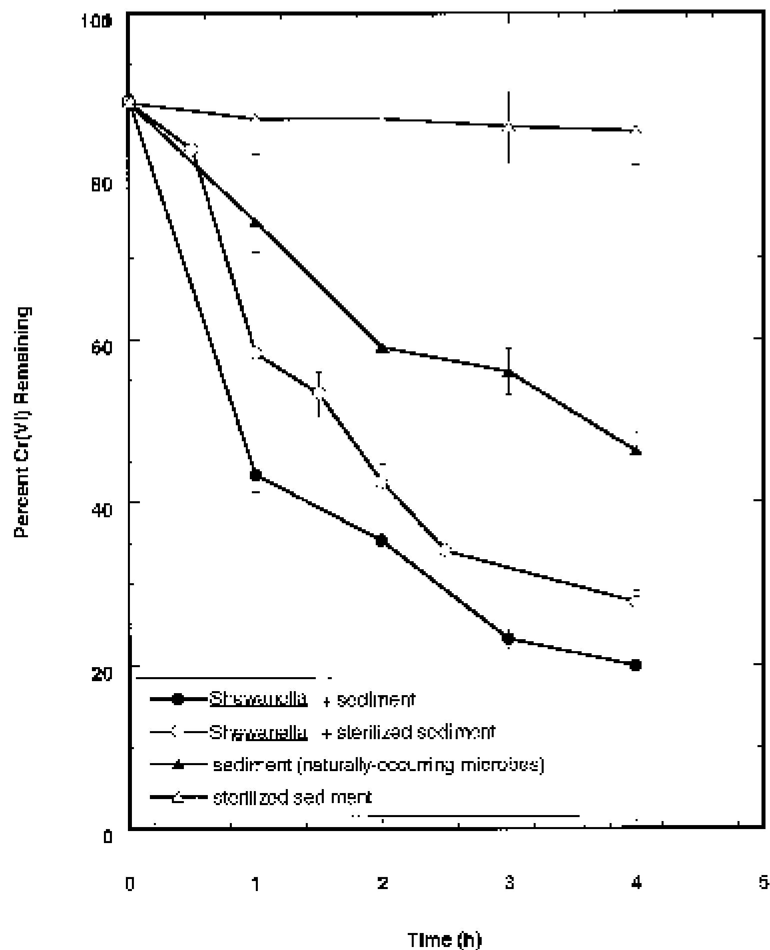

FIGURE 4. Cr(VI) reduction by Bear Creek sediment microbial consortia with and without *S. oneidensis*. Sediment was mixed with LB broth to a concentration of 10% (w/v) and 5 ppm Cr(VI) was initially added to saturate any S^{2-} that might potentially compete with consortia microorganisms or *S. oneidensis* during Cr(VI) reduction experiments. After S^{2-} saturation, 10 ppm Cr(VI) and/or a 2% inoculum of *S. oneidensis* was added to the sediment/LB slurry. Levels of Cr(VI) were monitored as before. Controls consisted of sediment slurries lacking *S. oneidensis* and slurries using autoclaved sediment.

grew to increasingly high concentrations throughout the first 2 days of incubation, then remained constant at or near concentrations of 10^4 cell mL^{-1} (data not shown). The highest Cr(VI) reduction by CRB was observed with isolates BC56 (0.3 ppm d^{-1}), BC52 (0.29 ppm d^{-1}) and BC510 (0.23 ppm d^{-1}). These rates were still less than those for *S. oneidensis* (0.485 ppm d^{-1}). The amount of Cr(VI) reduced in the week-long cultures was typically about 40%, despite the fact that the cultures were one half the concentration of short-term cultures and were growing in a less rich medium.

Slurries of Bear Creek sediment amended with Cr(VI) reduced approximately 48% of the Cr(VI) in 4 h (Figure 4) compared to less than 10% reduction by autoclaved sediment controls. Sterilized sediment inoculated with *S. oneidensis* reduced 69% of the Cr(VI) (Figure 4). When *S. oneidensis* was added to non-sterilized sediment, the

amount of Cr(VI) reduced approached 80%, indicating that *S. oneidensis* enhanced the Cr(VI) reduction by the native consortia (Figure 4).

In conclusion, it would appear that the presence of Cr(VI) is necessary to induce Cr(VI) reduction pathways for some Chesapeake Bay bacteria. By contrast, *S. oneidensis* does not require Cr(VI) induction since *S. oneidensis* cultures rapidly reduce Cr(VI) regardless of prior exposure. We estimated that approximately 80% percent of the Chesapeake Bay microbial community that could be cultured was Cr(VI) tolerant and was found in greater proportion in sediments than in the water column (data not shown). While native Chesapeake Bay microflora are not strong individual candidates for Cr(VI) bioremediation strategies, they do exhibit Cr(VI) tolerance and Cr(VI) reduction. Future work will be necessary to determine the Cr(VI) tolerance strategies (i.e., precipitation at cell surfaces, biosorption, metal-binding protein, etc.) employed by microorganisms in this environment.

ACKNOWLEDGEMENTS

Chromium reduction assays and BIOLOGä analyses were performed by K. Lowe. Culture incubations and DNA sequencing were performed by R. Fliflet. Sediment collection and chemical analyses were performed by T. Ly. This work was funded by the NRL 6.2 Research Program (JJM, BJL), the ONR-ASEE Postdoctoral Fellowship Program (KLL) and the George Washington University Science and Engineering Apprenticeship Program (TL). We thank John Kidwell for assistance in sediment collection and Dr. William Straube for technical assistance.

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