Persistence of Perchlorate and the Relative Numbers of Perchlorate- and Chlorate-Respiring Microorganisms in Natural Waters, Soils, and Wastewater

Jun Wu, Richard F. Unz, Husen Zhang, and Bruce E. Logan*

Dept. of Civil and Environmental Engineering, The Pennsylvania State University, University Park, PA

Abstract: Cell numbers of perchlorate (PRM) - and chlorate (CRM)-reducing microorganisms and the persistence of perchlorate were determined in samples of soils, natural waters, and wastewater incubated under laboratory conditions. Complete perchlorate reduction in raw wastewater and creek water was achieved in 4 to 7 days and 8 to 29 days, respectively, depending on the individual growth substrate (acetate, lactate, citric acid, or molasses) employed. Perchlorate persisted in most mixed cultures developed with 2 g of “pristine” soil, but declined in mixed cultures developed with 100 g of soil. Less than seven days were required to completely reduce perchlorate in cultures started with 10 g of a perchlorate-contaminated soil obtained from a site in Texas. The concentration of PRM was estimated using a 5-tube most probable number (MPN) procedure. To account for discrepancies due to differences in the total number of bacteria (per mass of sample) in the samples, difficulty in removing bacteria from soil samples, and the lack of an unequivocal method to measure total viable cells in these different systems, we normalized our MPN results on the basis of 10^2 or 10^5 total bacteria counted using acridine orange direct counts (AODC). There were more PRM in wastewater samples on a per-cell basis (15 to 350 PRM/10^6-AODC) than in water samples (0.02 to 0.4 PRM/10^6-AODC). There were also more PRM in soils from sites exhibiting direct evidence of perchlorate contamination (100 to 200 PRM/10^6-AODC) than from other sites (nondetectable to 0.77 PRM/10^6-AODC). These results demonstrate that perchlorate-reducing bacteria are present at perchlorate-contaminated sites, and that perchlorate can be degraded by these microorganisms through the addition of different electron donors, such as acetate and lactate.

Introduction

The discovery of extensive perchlorate (ClO₄⁻) contamination of surface and groundwaters has led to a national drinking water crisis; perchlorate contamination of these waters may endanger the drinking water supplies of over 12 million people (Betts, 1999; Logan, 1998; Urbansky, 1998). In 1997, perchlorate was found in over 30% of wells tested by the California Department of Health Services (CDHS) at concentrations up to 280 μg/L (Gullick et al., 2001). Concentrations of perchlorate in groundwater in Nevada have reached 3.7 × 10⁵ μg/L, and as much as 165 μg/L has been found in water samples from Lake Mead (Gullick et al., 2001), which is a major drinking water source for Nevada and a part of the Colorado River that also serves as a water source for Arizona and California. The CDHS established an interim action level of 18 μg/L for drinking water in the state of California, and other states have established similar interim levels (Urbansky, 1998). The U.S. Environmental Protection Agency (EPA) has added perchlorate to the candidate contaminant list (CCL) in order to set a national drinking water concentration under the Safe Drinking Water Act (Urbansky and Schock, 1999).

* Corresponding author: Tel: (814) 863-7908, Fax: (814) 863-7304; Email: blogan@psu.edu

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Despite any known perchlorate- or chlorate-to-chloride natural cycles, bacteria capable of perchlorate and chlorate [(per)chlorate-] respiration exist in nature (Coates et al., 1999c; Logan, 1998; van Ginkel et al., 1995). Perchlorate is microbiologically degraded to chloride via the sequence of $\text{ClO}_4^->\text{ClO}_3^->\text{ClO}_2^->\text{Cl}^-+\text{O}_2$, and none of the intermediates or oxygen accumulate in solution (Rikken et al., 1996; van Ginkel et al., 1996). A single enzyme has been found to catalyze the reduction of both chlorate and perchlorate (van Ginkel et al., 1998). However, chlorate-reducing isolates obtained by Mulvaney (1999) were incapable of perchlorate reduction, demonstrating that not all chlorate-respiring microorganisms (CRM) are perchlorate-respiring microorganisms (PRM) (Logan et al., 2001).

Aslander (1928) is generally credited for the first observation of microbiological degradation of chlorate in soils. Much later, it was shown that CRM could be easily enriched in wastewater and that the concentration of biodegradable organic matter in the wastewater was proportional to the production of chloride from chlorate (Bryan, 1966; Bryan and Rohlch, 1954). van Ginkel et al. (1995) demonstrated qualitatively that CRM were present in a variety of environments such as wastewaters, rivers, sediments, and soils (2.5 mL liquid samples, 2- to 10-mg solid samples), but did not address the concentration of CRM in these systems. Coates et al. (1999c) used a three-tube most probable number (MPN) technique with acetate and chlorate and found that CRM ranged from $2.3 \times 10^3$ to $2.4 \times 10^4$ CRM/g in samples obtained from a variety of environments, including soils, sediments, and a swine waste lagoon. The 13 isolates obtained from these samples were all Gram-negative, nonfermenting facultative anaerobes, many of which were not closely related to established genera. Only chloride was used in MPN and isolation tests, but all CRM were found to be capable of perchlorate respiration even though none of the sample inocula were known to be contaminated with either chlorate or perchlorate (Coates et al., 1999b).

Although these data suggest that (per)chlorate-respiring bacteria are widely distributed in nature, perchlorate persists in environments such as groundwater aquifers used for drinking water sources. A variety of ex situ physical, chemical, and biological systems have been proposed for treating drinking water to remove perchlorate (Brown et al., 2000; Earley et al., 1999; Logan and Kim, 1998; Urbansky, 1998; Venkatesh et al., 1999), but fewer systems have been devised for in situ remediation. Intrinsic remediation is possible if perchlorate-degrading microorganisms exist naturally in the system and can compete for existing sources of organic matter. Otherwise, perchlorate reduction must be enhanced through addition of suitable nutrients (biostimulation) or microorganisms (bioaugmentation) to contaminated sites. The time required for effective biostimulation is a function of the concentration of perchlorate-respiring bacteria at these sites. The abundance and growth rates of (per)chlorate-respiring bacteria in these natural systems is therefore critical to understanding factors that affect in situ bioremediation.

In order to evaluate the potential for soil bioremediation and treatment of perchlorate-contaminated water, we measured the abundance of both chlorate- and perchlorate-respiring microorganisms in a variety of pristine samples, a sample from a monitoring well in the vicinity of perchlorate-contamination, and from a perchlorate contaminated soil. It has been proposed that CRM may be suitable candidates for remediation of hydrocarbon-contaminated soils (Coates et al., 1999a). Therefore, we also tested for the concentration of CRM in a soil sample known to be contaminated with gasoline and solvents such as perchloroethylene (PCE). Perchlorate degradation was determined in enrichment cultures containing individual electron donors, including acetate, lactate, citric acid, molasses, and a poly lactate release compound (HRC™, Regenesis Corp.). The polylactate compound is especially formulated for the slow release of lactic acid on hydrolysis in water and is used to stimulate hydrogen production via fermentation of the lactate for the in situ degradation of compounds such as trichloroethylene (TCE) (Koenigsberg and Norris, 1999). Our results indicate that the abundance of PRM at perchlorate-contaminated sites is higher than those in pristine soils, suggesting intrinsic remediation is occurring at perchlorate-contaminated sites, but at rates too slow to fully eliminate perchlorate contamination at these sites.

**Methods**

**Sources of Samples**

Water samples were collected from a natural, cold water creek (Spring Creek, State College, PA). Wastewater samples were obtained at the State College Wastewater Treatment Plant from the primary clarifier effluent (defined here as raw wastewater), and the overflow (treated wastewater) from the second of two activated sludge reactors operated in series for nitrate removal. All water samples were kept in clean, 1-L plastic jars in the refrigerator (<2 days) prior to use.

Soil samples were obtained from sites in State College Pennsylvania (two sites), Cosa Mesa, California, and MacGregor, Texas. The Pennsylvania samples were a near-surface (depth of approximately 0.1 m) public park soil (PA-top), and a deep (approximately 2 m) soil (PA-deep) obtained from a site excavated to
remove PCE- and gasoline-contaminated soils. The soil sample from California (CA) was taken at a site where a monitoring well was being installed to monitor perchlorate contamination, although no perchlorate was detected in this soil. The Texas soil sample (TX) was obtained at a site contaminated with perchlorate (Perlmuter et al., 2000). The CA and TX soil samples were received on ice via overnight mail and stored at 8°C prior to use.

**Growth Medium**

All experiments were conducted using a mineral salts medium (pH = 6.9) prepared in ultra-pure water (Milli-Q; Millipore Corp., New Bedford, MA) containing per liter: 1.55 g K₂HPO₄, 0.98 g NaH₂PO₄·H₂O, and 0.5 g NH₄H₂PO₄ and either chloride or perchlorate (0.5 g). This solution was autoclaved at 121°C for 15 min. A concentrated mineral salts supplement was added aseptically to the medium such that, after dilution, the medium contained per liter: 75 mg MgSO₄·7H₂O, 3 mg EDTA, 2 mg ZnSO₄·7H₂O, 1 mg CaCl₂·2H₂O, 4 mg FeSO₄·7H₂O, 0.4 mg NaMnO₄·2H₂O, 0.2 mg CuSO₄·5H₂O, 0.4 mg CoCl₂·6H₂O, 1 mg MnCl₂·2H₂O, 0.1 mg NiCl₂·6H₂O, 0.1 mg NaSeO₃, and 0.6 mg H₃BO₃. The medium was de-oxygenated by stirring in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI) for at least 2 days resulting in minimal dissolved oxygen concentrations (approximately 0.15 mg/L). Electron acceptors (perchlorate or chloride) and one of three different electron donors (acetate, citric acid, lactate) were added from concentrated solutions to produce final concentrations ranging from 100 to 300 mg/L. Molasses was added directly from a syrupy solution and measured on the basis of total organic carbon (TOC; approximately 300 mg/L).

In one batch soil microcosm experiment, perchlorate degradation was examined using a poly lactate release compound (HRC™, Regenesis Corp., San Clemente, CA) developed for soil bioremediation (Koenigsberg and Norris, 1999). The growth medium was prepared as above, except HRC™ was used as the electron donor. HRC™ was supplied to microcosms by placing a small mass (40 mg) of the gel-like compound onto a small piece of aluminum foil that was placed directly into the sample bottle.

**Microcosms**

Water and soil microcosms were prepared using the growth medium and incubated in an anaerobic chamber. All samples were incubated at 25°C. Duplicate creek water or wastewater samples (100 mL) were combined with equal volumes of medium in 250-mL Erlenmeyer flasks and continuously mixed on a stir plate. Smaller (2 g) soil samples were added to 250-mL flasks containing 200 mL of medium, mixed on a stir plate for 1 day, and stored (static) in the chamber. Larger masses of soil (100 g) were added to 150 mL of medium in 250-mL bottles (Wheaton Science Products, Millville, NJ) and the bottles sealed (I-Loc™ closures, Wheaton Science Products). The 250-mL bottles were removed from the anaerobic glove box, shaken (horizontally) at 150 rpm (Lab-line Instrument Inc., model 3520), and then transferred back to the glove box during sampling.

In perchlorate degradation experiments involving HRC™ and Texas soil, 10 g of soil were placed in duplicate 150-mL serum bottles (Wheaton Science Products) containing the polylactate compound. Separate bottles were prepared without polylactate (negative control) and with lactate at a mass concentration (267 mg/L) equal to mass of polylactate compound for comparison of perchlorate degradation times. The bottles were filled completely with mineral salts medium, crimp sealed with butyl rubber tops (West BioDIRECT®, Lionville, PA), and incubated and analyzed as above.

**MPN Experiments**

The PRM and CRM were enumerated using a five-tube MPN method (APHA et al., 1998). All serial dilutions (1:10) were prepared in the anaerobic glove box using sterile crimp-top tubes (Bellco Glass Company, Vineland, New Jersey) sealed with the butyl rubber stoppers and aluminum seals. Inoculated tubes were then removed and incubated in the dark for 60 days, except as noted. Creek water and raw wastewater were used directly in MPN dilutions. Bacteria from soil samples in MPN tests were first desorbed from the soil by placing a sample (approximately 4 g) in a surfactant solution (200 mL of 0.1% Tween 20) and manually agitating the sample for 3 min. Samples were then stored overnight in a refrigerator to permit settling of larger particles. The supernatant was used as the inoculum. Evidence of growth indicated by increased turbidity (A₆₅₀) was confirmed by demonstrating >10% (per)chlorate reduction at the end of the experiment. The extraction of cells with surfactants such as Tween (Kuwae and Hosokawa, 1999), Triton-X (Zwifel and Hagström, 1995), and sodium PPI (Yu et al., 1995) was done to increase the recovery and enhance the dispersion of cells in water and soil samples. Sodium PP₃ (0.1%) is most often used for soil samples (van Elsas and Smalla, 1997) and for MPN analysis of cell concentrations (Beloin et al., 1988), but Tween is also used for the same purposes (Mochizuki et al., 1999). Although Tween is not as widely used as so-
The samples analyzed clearly differed in the numbers of total bacteria (e.g., creek water versus wastewater), but there is no universally accepted method to determine viable cell concentrations. Furthermore, even measuring the total number of cells in a sample is difficult because the extraction of bacteria from soil samples is known to be incomplete (Lindahl and Bakken, 1995). The abundance of (per)chlorate respiring microorganisms therefore was expressed as a fraction of total bacterial numbers based on acridine orange direct counts (AODC; Hobbie et al., 1977). Samples were prepared for cell counts by mixing three times on a vortexer (Glas-Col Comp., Terra Haute, Indiana; motor setting 9) for 20 sec. Cells were filtered onto 0.2-µm-pore-diameter polycarbonate filters (Poretics Corp., Livermore, California), and counted (>20 fields, 20 to 50 cells per field) on an epifluorescence microscope (model BH2, Olympus American Incorporated, Melville, New York).

One experiment was performed without cell desorption using different masses of soils (rather than aqueous dilutions) to confirm the low concentration of perchlorate-respiring bacteria. PA-top and CA soils (5 g, 1 g, 0.5 g, 0.1 g, 0.05 g, 0.01 g, and 0.001 g) were added directly to crimp top tubes containing medium as described above. Evidence of (per)chlorate degradation was determined by IC analysis after a 70-day incubation period.

**Analytical Methods**

All anions (perchlorate, chloride, acetate, lactate, and nitrate) were measured by ion chromatography (IC; model DX500, Dionex Corp., Sunnyvale, CA) using an IonPac AS11 column (Dionex, 1998). Chlorite was not measured because numerous studies have verified that perchlorate and chlorate are completely converted to chloride by bacteria without the accumulation of intermediates in solution (Herman and Frankenberger, 1998; Coates et al., 1999c; Miller and Logan, 2000).

Sample turbidity (absorbance) was measured directly in sealed anaerobic tubes (BellCo Glass Company) at 600 nm (A_{600}) using a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, New York). Tubes were shaken before each measurement. Total organic carbon (TOC) concentrations were measured in triplicate using a TOC 5000A analyzer (Shimadzu Scientific, Columbia, Maryland) after acidification (1N HCl) and gas (ultra zero air with 90% N₂ and 10% O₂) sparging to remove carbon dioxide. Perchlorate has not been found to strongly sorb to sand (Kim and Logan, 2000); therefore, background concentrations of perchlorate in the TX sample were estimated by a single equilibrium desorption test by dissolving approximately 1g of soil in ultrapure water, stirring and heating (80°C) for 2 h, and then leaving the sample overnight (12 h) in a water bath (60°C). Water was then analyzed for perchlorate using IC.

**Results**

**Water and Wastewater Samples**

Perchlorate reduction by water and wastewater samples was supported by all four substrates (Figure 1). Complete perchlorate removal was achieved in 4 to 7 days by raw wastewater, but took 8 to 29 days with creek water. The longer degradation times by the creek water sample were presumably due to the much lower total cell concentration in the creek water sample (0.34 x 10^{9}/mL) than in the wastewater sample (59 x 10^{9}/mL). There was no consistent ordering of the different electron donors for complete perchlorate degradation times between the two samples. Perchlorate degradation times decreased in the order of citric acid > molasses > lactate > acetate for water samples, and lactate = citric acid > acetate > molasses for the wastewater samples. The possibility of lactate and molasses fermentation prior to perchlorate degradation was not tested.

High concentrations of CRM and PRM were found in wastewater, but not creek water samples based on MPN analysis of samples using acetate as a growth substrate. For example, CRM concentrations were 5.0 x 10^{9}/mL and 7.0 x 10^{9}/mL in raw and treated wastewater (Table 1), but were only 0.04/mL in water samples. The higher concentrations of both CRM and PRM in the wastewater than in the water samples (Table 1) appeared to be related to the total number of bacteria in the two sample types. AODC in treated and raw wastewater samples were one and three orders of magnitude larger than in the water samples (Table 1).

On a per-cell (AODC) basis, the concentrations of (per)chlorate reducing microorganisms were higher in wastewater than in creek water. CRM and CRM were always present in wastewater samples at >14 PRM or CRM per million microorganisms (range 14 to 1130 CRM/10^6-AODC, 15-350 PRM/10^6-AODC; Table 1). In contrast, there was less than 0.02 CRM or PRM per 10^6-AODC in the creek water sample (range 0.02 to 0.4/10^6 AODC).

Concentrations of CRM were always equal to or greater than PRM in the water and wastewater samples,
with a ratio of 1.0 to 3.2 CRM/PRM (Table 1). The greater abundance of CRM than PRM suggests that not all chloride-respiring bacteria are capable of perchlorate respiration. However, the higher concentration of CRM than PRM could also be due to some unknown attribute of the bacteria such as a greater susceptibility to perchlorate toxicity than to chloride toxicity at concentrations used in the MPN test.

**Soil Samples**

Perchlorate persisted in all but one soil microcosm test when 2 g of CA or PA soil (top or deep) was used in the test, implying a minimum concentration of PRM and CRM of less than 1 microbe per 2 g of soil (0.5/g-soil) in these soils. Of the three soils (PA-top, PA-deep, and CA) and four different substrates tested, only the CA soil (Figure 2) incubated using citric acid demonstrated measurable perchlorate reduction (complete after 50 days). It is believed that a low concentration of PRM in the soil sample produced the single positive result in this test. At low concentrations of PRM in soil samples, one 2-g sample might contain a PRM and another 2-g sample might not. When the single microcosm that was positive (using citric acid) was split and incubated along with each of the other individual electron donors (acetate, lactate, and molas-
Table 1. Abundance of chlorate-respiring (CRM) and perchlorate-respiring (PRM) microorganisms in water and wastewater samples (using MPNs with acetate or lactate) normalized per million cells (using acridine orange direct counts). Each line represents a different sampling date. CRM/PRM ratios are based on concentration (#/mL) and are calculated only for same-day samples with the identical substrate. All samples were incubated for 60 days.

<table>
<thead>
<tr>
<th>Source</th>
<th>AODC (#/mL)</th>
<th>CRM Acetate (#/mL)</th>
<th>CRM Lactate (#/10^3)</th>
<th>PRM Acetate (#/mL)</th>
<th>PRM Lactate (#/10^3)</th>
<th>CRM/PRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creek water</td>
<td>8.8 x 10^6</td>
<td>0.04</td>
<td>—</td>
<td>0.02</td>
<td>0.35</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>3.6 x 10^6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Raw Wastewater</td>
<td>1.3 x 10^6</td>
<td>5.0 x 10^4</td>
<td>390</td>
<td>3.0 x 10^4</td>
<td>240</td>
<td>1.7</td>
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<tr>
<td></td>
<td>1.2 x 10^6</td>
<td>—</td>
<td>0.14</td>
<td>1.7 x 10^3</td>
<td>14</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Treated wastewater</td>
<td>6.2 x 10^6</td>
<td>7.0 x 10^3</td>
<td>1130</td>
<td>2.2 x 10^3</td>
<td>350</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^6</td>
<td>—</td>
<td>0.14</td>
<td>1.3 x 10^3</td>
<td>1.0 x 10^3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4.8 x 10^6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>70</td>
<td>15</td>
</tr>
</tbody>
</table>

* Number (#) of CRM or PRM per 10^6 cells as measured by AODC.

b Experiment not performed.
Figure 2. Microbial perchlorate degradation in the CA soil sample (2 g soil in 200 mL medium) using one of four different substrates (acetate, lactate, or citric acid, 300 mg/L; molasses, 300 mg/L as TOC).

all samples exhibited perchlorate reduction, indicating it was not the type of electron donor that limited the positive or negative result.

The CA and PA soil samples were reexamined for perchlorate reduction using larger masses of soil (100 g versus 2 g used above) in microcosm tests with acetate or lactate. Even at these higher soil masses, some of the PA and CA microcosm samples did not demonstrate perchlorate reduction (Figure 3). Perchlorate was reduced in the PA-top soil under acetate-oxidizing conditions, but reduction was slow and took nearly 150 days to complete. When lactate was used, perchlorate reduction occurred in both PA-top soil samples (only one replicate is shown in Figure 3). Perchlorate reduction was not consistently obtained for the other two soils in lactate-amended microcosms: one of the soil microcosms for the PA-deep and CA-soil demonstrated perchlorate reduction, while the other soil microcosm did not (Figure 3b).

MPN analysis of the PA soils confirmed that if PRM were present in the soil samples, they existed at extremely low concentrations. In six tests using acetate or lactate as an electron donor and chlorate or perchlorate as the electron acceptor, the concentration of PRM was only found in one of the PA soil tests (0.77 PRM/10^9 AODC) (Table 2). The lack of perchlorate reduction in MPN tests with many PA and CA soils was not due to insufficient extraction of PRM. In separate batch tests using seven different PA-top and CA soil masses, ranging between 0.001 g and 5 g, and no cell extraction, perchlorate reduction was not observed even after a 70-d incubation period (data not shown). This confirmed that low PRM concentrations obtained using the MPN test was not a result of our cell extraction technique. The long lag times observed in soil samples cannot be explained solely by the possible presence of alternate electron acceptors in the sample because there was no comparable lag when the same medium was used for water and wastewater samples (Figure 1).

Perchlorate reduction occurred rapidly in a separate set of experiments using the TX soil (Figure 4). Perchlorate was degraded to <1 mg/L within 4 days, and to nondetectable concentrations in 7 days with either lactate or polylactate as an electron donor. The rapid degradation of perchlorate in the TX soil was likely due to a substantially greater concentration of perchlorate-reducing bacteria in the TX soil sample than in the other soil samples. We found 1.0 × 10^2 and 2.0 × 10^3 PRM/10^9 AODC and 4.7 × 10^3 and 5.5 × 10^3 CRM/10^9-AODC in the TX soil using acetate and lactate in MPN tests (Table 2). The high concentrations of PRM and CRM in the TX soil (relative to the CA soil) is likely due to the natural enrichment of (per)chlorate reducing bacteria as a result of the existing perchlorate contamination of this soil (500 mg perchlorate/kg-soil, as received). CRM concentrations in the TX soil were larger than those for PRM using acetate (0.021 PRM/CRM) or lactate (0.036 PRM/CRM) as an electron donor.

Discussion

Perchlorate- and chloride-respiring microorganisms are widely distributed in nature. although at highly variable concentrations depending on the source (water or soil) and whether there is evidence of previous perchlorate contamination. In microcosm tests using
Figure 3. Microbial perchlorate degradation in 100 g soil samples. The initial substrate concentration was 500 mg/L of (A) acetate, and (B) lactate. All tests done in duplicate. However, data on both samples are shown (using numbers 1 and 2) only when different results were observed for the duplicate samples.
Table 2. Most probable numbers of (per)chlorate reducers in water samples extracted from soils and incubated for 60 days except as noted

<table>
<thead>
<tr>
<th>Source</th>
<th>Substrate</th>
<th>AODC (#/mL)</th>
<th>CRM (#/10^6)</th>
<th>PRM (#/10^6)</th>
<th>CRM/PRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-top</td>
<td>Lactate</td>
<td>8.0 × 10^6</td>
<td>nda (&lt;2.5)</td>
<td>nd (&lt;1.0)</td>
<td>nd (&lt;2.5)</td>
</tr>
<tr>
<td>PA-deep</td>
<td>Acetate</td>
<td>2.6 × 10^7</td>
<td>nd (&lt;0.77)</td>
<td>nd (&lt;0.3)</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>7.9 × 10^5</td>
<td>nd (&lt;25)</td>
<td>nd (&lt;4)</td>
<td>nd (&lt;25)</td>
</tr>
<tr>
<td>TXd</td>
<td>Acetate</td>
<td>2.5 × 10^6</td>
<td>4700</td>
<td>52</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>2.5 × 10^6</td>
<td>5500</td>
<td>61</td>
<td>200</td>
</tr>
</tbody>
</table>

a Number (#) of CRM or PRM per 10^6 cells as measured by AODC.
b nd = not detected. Numbers in parentheses indicate the lowest detectable value in that experiment.
c Cannot be calculated (—).
d Samples incubated for only 30 d due to a rapid increases in optical density after 10 to 14 d.

Figure 4. Perchlorate reduction in a soil microcosm (TX soil) from a site contaminated with perchlorate using a lactate solution and HRC (a poly-lactate dissolving compound) versus a control (no addition of substrate). Symbol sizes are larger in most cases than the error bars (duplicate samples).
soils with no known perchlorate contamination (pristine soil), perchlorate reduction was absent in all but one of twelve separate 2-g samples even after 110 days of incubation. When a larger soil mass (100 g) was used, we observed perchlorate reduction in many of the samples indicating low concentrations of (per)chlorate-reducing organisms. Soil microcosms from a perchlorate-contaminated site, however, demonstrated complete perchlorate degradation in only 7 days. MPN tests corroborate our speculations concerning low concentrations of (per)chlorate reducing microorganisms in the pristine soil microcosms. CRM were nondetectable in the two PA soils and PRM were only detected in the CA soil at a concentration of 0.3 PRM/g. In contrast, the TX soil contaminated with 500 mg-perchlorate/kg contained 52 to 61 CRM/g-soil and 0.9 to 1.7 PRM/g-soil depending on the electron donor (acetate or lactate) (Table 2). While some microbes may not have been measured in our MPN procedure, the accuracy of the 5-tube method used here is an improvement over the 3-tube MPN procedure utilized in a previous study (Coates et al., 1999c) to measure CRM abundance in different environmental samples.

The abundance of CRM found in our samples agree with estimates by others (Coates et al., 1999c), for some types of water samples, but are substantially lower for most soil and sediment samples. Total cell counts were not reported in the study by Coates et al. (1999c) and therefore a comparison can only be made based on the mass of the original samples used for our cell extraction step. Coates et al. (1999c) reported on the order of approximately $10^3$ CRM/g for three sediments, two soil, and one water sample, approximately $10^4$ for a swamp-water sample, and approximately $10^6$ for a wastewater sample (Table 3). We found $10^3$ to $10^4$ CRM/g in some wastewater samples, but substantially fewer microorganisms in creek water and most soil samples. Data presented in Table 3 for our study results are given as a range of cell concentrations based on the 95% confidence intervals using APHA et al. (1998) for a 5-tube MPN and the results listed in Tables 1 and 2. The lower concentrations of CRM in our samples versus those of Coates et al. (1999c) is due in part to differences in total cell concentrations in the various samples. For example, our wastewater samples had two orders of magnitude more bacteria than our creek water samples. All of our MPN determinations were based on direct measurement of perchlorate or chloride reduction in each tube using ion chromatography. In the study by Coates et al. (1999c), positive tubes were assayed only by turbidity. Counting tubes by turbidity, and not verifying chloride reduction, could overestimate CRM abundance.

Bacteria capable of chlorate reduction appear to be more abundant than those able to degrade perchlorate. This is based on two observations. First, CRM consistently represented a much larger fraction of cells than PRM in the water and soils examined. As shown in Tables 1 and 2, the CRM/PRM ratio ranged from 1.0 to 47. Second, chloride-reducing bacteria have been isolated that are not capable of perchlorate degradation (Logan et al., 2001; Mulvaney, 1999). Although the observation of greater abundance of CRM than PRM may be due to differences in their culturability on the medium, the finding that there are bacteria capable of reducing chloride, and not perchlorate, suggests otherwise. As ours is the first study to relate the abundance of CRM to total cells, or to directly examine for perchlorate versus chlorate degradation in a variety of samples, it is not possible to compare our results with other studies on the relative abundance of these bacteria. Common points of agreement between this and the findings of Coates et al. (1999c) and van Ginkel et al. (1995) are that PRM and CRM exist in nature, and that their concentrations vary widely.

**Potential for Soil Bioremediation**

We infer from these studies on soil samples that bioaugmentation of perchlorate-degrading bacteria is probably not necessary for soil bioremediation at sites which have been contaminated with perchlorate for some time. It is common at sites contaminated with chemical pollutants that the microbial community becomes enriched with bacteria able to degrade those pollutants (Baker and Herson, 1994). The presence of perchlorate in the TX soil appears to have enriched the number of PRM and CRM in that soil relative to other soils tested. PRM were present at sufficient numbers (200 PRM/10^6 AODC based on MPN) to achieve complete perchlorate degradation using lactate in soil microcosms in only 7 days. It is known that oxygen and nitrate serve as competing electron acceptors for (per)chlorate reduction (Rikken et al., 1996). Therefore, it is likely that intrinsic perchlorate remediation remains limited by a lack of suitable environmental conditions such as complete anoxia and a lack of sufficient electron donor, and not by microorganisms capable of perchlorate degradation.

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Table 3. Comparison of chlorate reductor abundance in different environments (#/g), assuming all bacteria on soils were extracted in tests performed in this study. Data from this study are given as a range based the 95% confidence intervals in APHA et al. (1998) for the 5-tube MPN data listed in Tables 1 and 2.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Source</th>
<th>Acetate</th>
<th>Lactate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater</td>
<td>Swine waste lagoon</td>
<td>$2.4 \times 10^6$</td>
<td>$-^{a}$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>Raw wastewater</td>
<td>$0.2-1.7 \times 10^4$</td>
<td>$0.7-4.8 \times 10^3$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Treated wastewater</td>
<td>$0.3-2.1 \times 10^4$</td>
<td>$0.5-3.9 \times 10^3$</td>
<td>This study</td>
</tr>
<tr>
<td>Sediments</td>
<td>Petroleum-contaminated soil</td>
<td>$9.3 \times 10^3$</td>
<td>$-^{a}$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>Pristine aquatic sediment</td>
<td>$4.6 \times 10^3$</td>
<td>$-^{a}$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>Mississippi river sediment</td>
<td>$4.3 \times 10^3$</td>
<td>$-^{a}$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>Gold mine drainage sediment</td>
<td>$4.3 \times 10^3$</td>
<td>$-^{a}$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td>Soils</td>
<td>Pristine soil</td>
<td>$2.3 \times 10^3$</td>
<td>$-^{a}$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>TX soil</td>
<td>$22-130$</td>
<td>$26-160$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PA-top soil</td>
<td>$&lt;10^{6}$</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt; (&lt;1.0)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>PA-deep soil</td>
<td>nd (&lt;0.3)</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt; (&lt;0.4)</td>
<td>This study</td>
</tr>
<tr>
<td>Natural waters</td>
<td>Florida swamp</td>
<td>$2.3 \times 10^7$</td>
<td>$-$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>Pohic Bay</td>
<td>$1.5 \times 10^4$</td>
<td>$-$</td>
<td>Coates et al. (1999c)</td>
</tr>
<tr>
<td></td>
<td>Creek Water</td>
<td>0.01-0.17</td>
<td>0.06-0.35</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experiment not performed ($-$).

<sup>b</sup> The indicated samples were positive in batch tests using 100 g of soil as shown in Figure 3, although MPN tests were not conducted using masses of soil that large.

<sup>c</sup> nd = not detected. Numbers in parentheses indicate the lowest detectable value in that experiment.

References


Herman, D.C. and W.T. Frankenberger Jr. 1998. Microbial-
Mediated Reduction of Perchlorate in Groundwater. J.
Environ. Qual. 27:750-754.

Filters for Counting Bacteria by Fluorescence Microscopy.

Kim, K. and B.E. Logan. 2000. Fixed-Bed Bioreactor Treat-
Sci. 7:257-265.

Bioremediation Using Slow Release Compounds.
Regenesis Bioremediation Products, San Clemente, CA.

Kuwae, T. and Y. Hosokawa. Determination of Abundance and
Biovolume of Bacteria in Sediments by Dual Stain-
ing with 4′, 6-Diamidino-2-Phenylindole and Acridine
Orange: Relationship to Dispersion Treatment and Sediment
Characteristics. Appl. Environ. Microbiol. 65:3407-
3412.

for Extraction of Bacteria From Soil. FEMS Microbiol.
Ecology. 16:135-142.

Logan, B.E. 1998. A Review of Chlorate- and Perchlorate-

Logan, B.E. and K. Kim. 1998. Microbiological Treatment
of Perchlorate Contaminated Ground Waters. In:
Proceedings of the Southwest Focused Groundwater Con-
ference: Discussing the Issue of MTBE and Perchlorate
in the Ground Water, pp. 87-90. National Ground Water
Association.

Logan, B.E., H. Zhang, P. Mulvaney, M.G. Milner, I.M.
Head, and R.F. Unz. 2001. Kinetics of Perchlorate- and
67(6):2499-2506.

Degradation In An Autotrophic, Gas Phase, Packed Bed

Mochizuki, M., T. Hayashi, K. Nakayama, and T. Masuda.
1999. Studies of Biodegradable Poly(Hexano-6-Lac-
Chem. 71:2177-2188.

Mulvaney, P. 1999. Perchlorate and Chlorate Reduction by
Axenic Cultures. M.S. Thesis, Environmental Pollution
Control, The Pennsylvania State University, University
Park, PA.

Perlmutter, M., J.R. Britto, J.D. Cowan, A. Jacobs, M. Patel,
M. Craig, and B.E. Logan. 2000. Bioremediation of
Perchlorate-Contaminated Groundwater at Naval Weap-
ons Industrial Reserve Plant McGregor, Texas. In: Pro-
ceedings of the 26th Environmental Symposium & Ex-
hibition, National Defense Industrial Association, Long
Beach, CA, March 27-30, pp. 792-797.

Transformation of (Per)Chlorate into Chloride by a
Newly Isolated Bacterium: Reduction and Dismutation.


the Risks Associated with Perchlorate in Drinking Water.

Soil Microbes. In: Manual of Environmental Microbi-
ology, pp. 383-390. (C.J. Hurst, G.R. Knudsen, M.J.
McInerny, L.D. Stetzenbach, and M.V. Walter, Eds.)
ASM Press: Washington, D.C.

van Ginkel, G.C., A.G.M. Kroon, G.B. Rikken, and S.W.M.
Kengen. 1998. Microbial Conversion of Perchlorate,
Chlorate, and Chlorite. In: Proceedings of the South-
west Focused Groundwater Conference: Discussing the
Issue of MTBE and Perchlorate in the Ground Water,
pp.92-95. National Ground Water Association: Colum-
bus, OH.

van Ginkel, G.C., C.M. Plugge, and C.A. Stroo. 1995. Re-
duction of Chlorate with Various Energy Substrates and
31: 4057-4066.

van Ginkel, G.C., G.B. Rikken, A.G.M. Kroon, and S.W.M.
Kengen. 1996. Purification and Characterization of
Chlorite Dismutase: A Novel Oxygen Generating En-

1999. Removal and Destruction of Perchlorate and Other
Anions From Ground Water Using ISEP™ System. In:
Proceedings of the 218th ACS Conference, New Or-
leans, LA. Aug. 22-26. pp. 80-84. American Chemical
Society

Yu, W., W.K. Dodds, M.K. Banks, J. Skalsky, and R.A.
Strauss. 1995. Optimal Staining and Sample Stora-
age Time for Direct Microscopic Enumeration of
Total and Active Bacteria in Soil with Two Flu-
orescent Types. Appl. Environ. Microbiol. 61:3367-
3372.

Bacteria Include a Large Fraction of Non-Nucleoid-
61:2180-2185.