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## RAPID COMMUNICATION

# BIOLOGICAL PERCHLORATE REDUCTION IN HIGH-SALINITY SOLUTIONS

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**Abstract**—Perchlorate ( $\text{ClO}_4^-$ ) has been detected in numerous ground and surface waters, and has recently been added to the drinking water Candidate Contaminant List in the United States. Perchlorate can be removed from drinking water using ion exchange, but this results in the production of highly saline (7–12%) perchlorate-contaminated brines. Perchlorate-degrading microbial enrichments capable of growth in highly saline water were obtained by screening six salt water environments including marine and lake surface waters, salt marshes, subtidal sediments, and a biofilm/sludge from a seawater filter. Perchlorate reduction was obtained in three of these samples (seawater, saline lake water, and biofilm/sludge) at a salinity of 3%. The salinity range of two of these cultures was extended through serial transfers into media having higher salt concentrations (3–7%). Growth rates were measured over a salinity range of 1–15%. The maximum growth rate measured for the saline lake-water enrichment was  $0.060 \pm 0.003 \text{ d}^{-1}$  (doubling time of  $11.6 \pm 0.8 \text{ d}$ ) at a salinity of 5%. Growth rates decreased to  $0.037 \pm 0.002 \text{ d}^{-1}$  at a salinity of 11%, and no growth was observed at salinities of 13 or 15%. These results demonstrate for the first time that biological perchlorate reduction is possible in solutions having a salinity typical of ion exchange brines. © 2001 Elsevier Science Ltd. All rights reserved

**Key words**—ammonium perchlorate, biodegradation, brine, chlorate, drinking water, ion exchange, munitions, perchlorate, rocket propellant

## INTRODUCTION

Perchlorate ( $\text{ClO}_4^-$ ) has recently been added to the US Environmental Protection Agency's drinking water Candidate Contaminant List due to its interference with hormone production by the thyroid gland, and additional toxicity studies are underway (Urbansky and Schock, 1999). A survey of surface and ground waters in the US has indicated that perchlorate contamination is extensive in many locations, particularly in the arid southwestern states (Urbansky, 1998). Perchlorate is used as an oxidizer in solid rocket propellant, but it exists naturally in fertilizers derived from Chilean caliche (Schilt, 1979; Attaway and Smith, 1993; Susarla *et al.*, 1999, 2000; Nzungu *et al.*, 1999; Urbansky *et al.*, 2000).

Perchlorate is used as a terminal electron acceptor by pure and mixed cultures of microorganisms during the oxidation of many different substrates (Logan, 1998; Herman and Frankenberger, 1998). The reduction sequence of perchlorate is  $\text{ClO}_4^- \rightarrow \text{ClO}_3^- \rightarrow \text{ClO}_2^- \rightarrow \text{Cl}^- + \text{O}_2$ , and none of the

intermediates accumulate in solution (Attaway and Smith, 1993; Logan, 1998; Herman and Frankenberger, 1998). Chlorite disproportionation to chlorite and oxygen is a non-energy yielding step catalyzed by chlorite dismutase, but both chlorate and oxygen are used as electron acceptors by perchlorate-respiring microbes (Rikken *et al.*, 1996; Coates *et al.*, 1999).

Several ion exchange systems have been developed to remove perchlorate from contaminated waters (Kawaski *et al.*, 1993; Batista *et al.*, 2000; Gu *et al.*, 2000; Tripp and Clifford, 2000; Venkatesh *et al.*, 2000). Due to the high affinity of perchlorate for these resins, very high salt concentrations (7–12%) are needed to regenerate the column (Venkatesh *et al.*, 2000; Batista *et al.*, 2000). The perchlorate in these brines can be treated through a high-temperature, rare earth metal process (Venkatesh *et al.*, 2000) but costs could be reduced if treatment could be conducted at lower temperatures without expensive catalysts.

Microbiological treatment of perchlorate-containing solutions has not been reported thus far to occur at the high salinities typical of perchlorate-contaminated ion exchange brines. A perchlorate-degrading isolate obtained by Bruce *et al.* (1999) had an optimal

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salinity of 1% and grew only at <2% NaCl. Many perchlorate-degrading bacteria also can degrade perchlorate (Coates *et al.*, 1999; Logan, 1998), but one isolate (identified as *Ideonella dechloratans*) failed to grow at 3% NaCl (Malmqvist *et al.*, 1994). Preliminary tests with two perchlorate-degrading isolates in our laboratory (unpublished data), and acclimation experiments by others (Roberts, 2000) have similarly failed to produce cultures capable of perchlorate reduction at high salinity (> 3%). Using cultures from environments containing high salt concentrations, we report here that it is possible to obtain perchlorate degradation even at a salinity as high as 11%.

## MATERIALS AND METHODS

### Sample sources

The following samples were obtained at four different locations in the United States: surface water, Great Salt Lake, Utah (GSL); salt marsh water, Delaware Bay estuary (DB); bottom sediments (SBS) and surface water (SBW) from a brackish coastal lagoon and a biofilm/sludge mixture from a seawater filter system sludge (SBB) from the top of the large sand filter of the university seawater system, both near the marine lab of the University of California, Santa Barbara Marine Institute; and muddy sediments from the Texas Gulf Coast (TGC). Samples were sent by overnight mail on ice and stored in the refrigerator prior to use. The original salinity of the SBW sample was measured as 2.2%, but the samples were not otherwise characterized.

### Media

All media were prepared in ultra-pure water (Milli-Q; Millipore Corp., New Bedford, MA) containing a phosphate buffer, acetate as a growth substrate, and a natural salt mixture (SM) to provide most trace metals (Instant Ocean; Aquarium Sciences Inc., OH). Molybdate was used in some media at high concentrations to inhibit sulfate reduction (Taylor and Oremland, 1979). To avoid precipitates that formed during autoclaving, all solutions were sterilized by filtration through 0.2- $\mu$ m-pore diameter membrane filters (Millipore Corp., New Bedford, MA). Media was degassed for at least 2 days by stirring in an anaerobic glove box containing an atmosphere of  $\sim$ 1.5% H<sub>2</sub> and 98.5% N<sub>2</sub> (Coy Laboratory Products, Grass Lake, MI).

Media used for initial acclimation experiments (medium A; pH = 7.6  $\pm$  0.2) contained, per liter of water: 0.5 g NaC<sub>2</sub>H<sub>4</sub>O<sub>2</sub>, 8 mg NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 4.83 g NaMoO<sub>4</sub>. Perchlorate and/or nitrate were added to this medium at SM:NaClO<sub>4</sub>:NaNO<sub>3</sub> mass ratios (as grams per liter of water) of: 29.3:0.3:0 (perchlorate medium, A-P); 26.7:0.5:3.0 (high-nitrate medium, A-HN); and 29.3:0.3:0.01 (low nitrate medium, A-LN). Enrichments were transferred into media (medium T; pH = 7.0) containing NaCl at different concentrations (19.62–59.60 g/L), in order to vary salinity from 3 to 7%, along with the following (per liter of water): 1.55 g K<sub>2</sub>HPO<sub>4</sub>, 0.85 g NaH<sub>2</sub>PO<sub>4</sub>, 0.50 g NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1.0 g NaClO<sub>4</sub>, 1.0 g NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>, 0.48 g NaMoO<sub>4</sub> and 5.0 g SM. Medium G (pH = 7.0) was used to determine mixed culture kinetics over a salinity range of 1–15% (NaCl concentrations of 5.28 to 145 g/L). Medium G contained (per liter of water): 0.78 g K<sub>2</sub>HPO<sub>4</sub>, 0.43 g NaH<sub>2</sub>PO<sub>4</sub>, 0.20 g NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.62 g NaClO<sub>4</sub>, 0.70 g NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub> and 2 g SM.

### Isolation procedures

Water and sediment samples were transferred (5%) into media containing perchlorate (Medium A-P) or high

concentrations of nitrate and perchlorate (medium A-HN). Most perchlorate-respiring isolates are capable of denitrification (Logan, 1998; Herman and Frankenberger, 1999) and therefore it was thought that enriching the denitrifying population might lead to a greater abundance of perchlorate-respiring microorganisms in the sample. When high-nitrate medium samples became turbid, they were transferred into medium A-LN containing perchlorate (300 mg/L) and low concentrations of nitrate (10 mg/L), to allow cells to shift from using nitrate to using perchlorate. When cultures again became turbid ( $\sim$ 10<sup>8</sup> cells/mL by direct count; Hobbie *et al.*, 1977), they were transferred into nitrate-free medium (A-P) containing perchlorate.

The cells of any culture that grew in the perchlorate medium (A-P) were resuspended (5% by volume) in the transfer medium set at a salinity of 3%. If growth continued in this medium, the cells were successively transferred to higher salt concentration (from 4 to 7%). Cultures capable of perchlorate degradation using acetate were also tested for their ability to use two other growth substrates: Tween 80 (5% v/v) and ethanol (100 mg/L).

### Growth kinetics

Growth rates for one of the mixed cultures (GSL) in medium G were determined as a function of salinity (1–15%). Cultures used in this growth rate experiment were first acclimated to perchlorate (medium T) at a salinity of 7%. Samples from a dense cell suspension (1 mL) were transferred into fresh medium (G; 19 mL) contained in 28-mL tubes (BellCo Glass Inc., Vineland, NJ) in the anaerobic glove box and sealed with butyl rubber stoppers (West BioDIRECT<sup>®</sup>, Lionville, PA) and aluminum crimp tops. Growth rates ( $\mu$ ) were determined from absorbance (*A*) measurements on cultures at 600 nm. The growth rate was calculated as the slope of the line based on a linear regression of ln *A* versus *t* using

$$\ln A = \ln A_0 + \mu t \quad (1)$$

where *A*<sub>0</sub> is the initial absorbance, and *t* the sampling time. Only data with absorbances larger than 0.02 were used in the regression analysis.

### Analytical methods

Perchlorate was measured by ion chromatography (IC; model DX500, Dionex Corp., Sunnyvale, CA) using a Dionex IonPac AS11 analytical and AG11 guard column (Dionex, 1996; Jackson *et al.*, 2000). Samples were diluted (1000–5000 times) with deionized water to reduce salt concentrations. A calibration curve was developed using the same medium also diluted with deionized water. Sample turbidity (*A*<sub>600</sub>) was measured directly in sealed anaerobic tubes (BellCo Glass Company) using a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY). Tubes were shaken before each measurement. Cell dry weights were calculated (duplicate samples) from membrane filters (25 mm, 0.2  $\mu$ m pore diameter; Osmonics Corp., Minnetonka, MN) rinsed with deionized water. Filters were dried at 105°C for 4 h and cooled in a desiccator prior to being weighed (Mettler Toledo model UMT2, Greifensee, Switzerland).

## RESULTS

After three months of incubation, inocula from all sources tested produced evidence of growth on high- and low-nitrate concentrations with perchlorate in the enrichment medium A (Table 1). Growth was observed on perchlorate with inoculum from only three sources: GSL, SBW, and SBB. When these

Table 1. Growth observed using different natural samples in medium A (initial enrichment) and medium T (transfer media) as a function of salinity (+, growth; -, no growth; nd, not done)

Samples	Growth in enrichment medium A			Growth in medium T as a function of salinity (%)				
	A-HN (high nitrate)	A-LN (low nitrate)	A-P (perchlorate)	3	4	5	6	7
GSL (water)	+	+	+	+	+	+	+	+
SBS (sediment)	+	+	-	nd	nd	nd	nd	nd
SBW (water)	+	+	+	+	+	+	+	+
SBB (biofilm)	+	+	+	nd	nd	nd	nd	nd
DB (water)	+	+	-	nd	nd	nd	nd	nd
TGC (sediment)	+	nd	-	nd	nd	nd	nd	nd

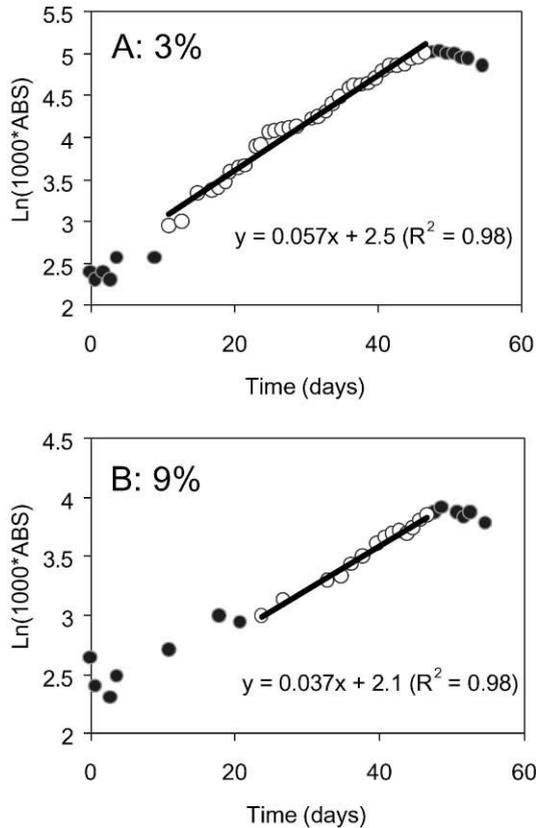


Fig. 1. Examples of absorbance data (duplicates) taken to determine cell growth rates of GSL mixed cultures in medium G at (A) 3% and (B) 9% salinity. Only the data in open circles were used in the regression analysis to determine growth rates.

enrichments were transferred to fresh medium (T), inocula from the GSL and SBW enrichments grew over a salinity range of 3–7%. However, growth of the SBB enrichment was not sustained on medium T and this culture was discarded. Perchlorate reduction (> 10% change in perchlorate concentration) was confirmed following transfers on a non-routine basis for samples demonstrating growth in Table 1. The GSL enrichment was also supported by ethanol or Tween 80 as growth substrates at 7% salinity.

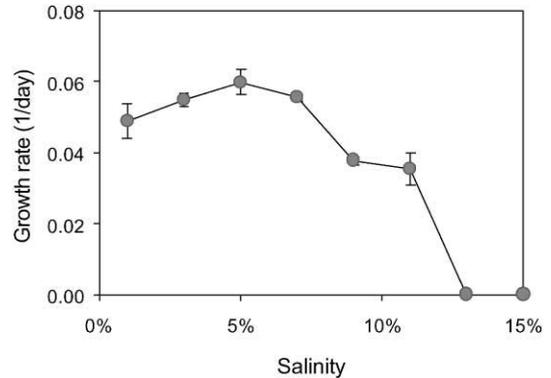


Fig. 2. Growth rates of mixed cultures (GSL) in medium G as a function of solution salinity. (Error bars  $\pm$  SD, duplicate samples.)

#### Growth rates

The GSL biomass appeared to have faster growth rates than the SBW culture (data not shown) based upon the rate at which samples became turbid following serial transfers from the 3% salinity medium to the 7% salinity medium. Subsequently, growth rates of the GSL mixed cultures grown at a salinity of 7% were measured over a salinity range of 1–15% using medium G. The results from two individual trials at 3 and 9% salinity are presented in Fig. 1. Cells grew exponentially but at low rates. Cell growth rates were  $0.057 \text{ d}^{-1}$  ( $R^2 = 0.98$ ,  $n = 33$ ) at a salinity of 3%, and  $0.037 \text{ d}^{-1}$  ( $R^2 = 0.98$ ,  $n = 14$ ) at a salinity of 9%, based on maximum observed growth rates and data for absorbances larger than 0.02. Duplicate trials exhibited similar rates. For example, the duplicate at 3% salinity had a growth rate of  $0.039 \text{ d}^{-1}$  ( $R^2 = 0.98$ ,  $n = 12$ ).

The combined growth data for a salinity range of 1–15% appeared in Fig. 2. The highest growth rate of  $0.060 \pm 0.003 \text{ d}^{-1}$  (doubling time of  $11.6 \pm 0.8 \text{ d}$ ) was obtained at a salinity of 5%. Growth rates decreased to  $0.037 \pm 0.002 \text{ d}^{-1}$  at a salinity of 11%, and no growth observed in the samples at 13 or 15% salinity (Fig. 2).

Perchlorate concentrations and cell dry weights were not sampled due to the small sample volume. However, as an example of the perchlorate reduction

and growth, a 3% salinity tube was sampled after 8 weeks of growth. Perchlorate was reduced from 592 to 45 mg/L, and 71 mg/L of cell dry weight was produced from bacterial growth in this tube.

#### DISCUSSION

Perchlorate-respiring microorganisms (PRM) have been isolated from a variety of fresh waters, waste waters, soils and sediments (Coates *et al.*, 1999; Logan *et al.*, 2000; Miller and Logan, 2000). However, the ability of such microorganisms to grow under high-salinity conditions appears to be limited. Despite the range of sample types and locations, we found that perchlorate reduction was observed in only two of our samples at 3% salinity or higher. To our knowledge, this is the first time microbiological perchlorate reduction has been demonstrated at a salinity of 11%.

Acclimation of cultures to nitrate was not a successful strategy for developing perchlorate degrading enrichments in high-salinity solutions. It is known from previous work that most PRM are capable of denitrification (Logan, 1998; Coates *et al.*, 1999). Although denitrifying populations were developed in enrichment cultures with all inoculum sources, such populations did not ensure subsequent growth when perchlorate was supplied as an electron acceptor. Only when perchlorate was provided as the sole electron acceptor did perchlorate-utilizing enrichments develop in three of the inoculum sources.

The growth of the GSL enrichment in high-salinity media may indicate potential for continuously treating perchlorate-contaminated ion exchange brines, providing growth rates of these bacteria in brine solutions can be increased. Maximum growth rates in batch cultures were only  $0.060 \text{ d}^{-1}$  (doubling time of 11.2 d) at a salinity of 5%. In order to improve growth rates, we are continuing serial transfers of the GSL culture using high-salinity (7 and 11%) media.

Although this work demonstrates the potential for treatment of perchlorate contaminated brine solutions, additional work needs to be done to better develop a combined ion exchange/microbiological treatment processes. We are investigating treatment of brines from actual ion exchange experiments, and the effects of competing anions such as nitrate and sulfate. Nitrate is degraded along with perchlorate (Herman and Frankenberger, 1998, 1999), and sulfate has not been found to inhibit perchlorate reduction (Attaway and Smith, 1993; Logan, 1998; Coates *et al.*, 1999). Continuous treatment of brines using packed beds or suspended cultures, as has been done for direct water treatment, should also be investigated to decrease reactor detention times. In addition, the direct regeneration of resins through addition of acclimated cultures should be investigated as this has proven to be possible for the

regeneration of ion exchange resins used for ammonium removal (Lahav and Green, 1998).

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