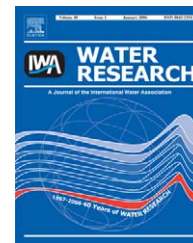


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Kinetics of a hydrogen-oxidizing, perchlorate-reducing bacterium

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ABSTRACT

This paper provides the first kinetic parameters for a hydrogen-oxidizing perchlorate-reducing bacterium (PCRB), *Dechloromonas* sp. PC1. The q_{\max} for perchlorate and chlorate were 3.1 and 6.3 mg/mgDW-day, respectively. The K for perchlorate was 0.14 mg/L, an order of magnitude lower than reported for other PCRB. The yields Y on perchlorate and chlorate were 0.23 and 0.22 mgDW/mg, respectively, and the decay constant b was 0.055/day. The growth-threshold, S_{\min} , for perchlorate was 14 μ g/L, suggesting that perchlorate cannot be reduced below this level when perchlorate is the primary electron-acceptor, although it may be possible when oxygen or nitrate is the primary acceptor. Chlorate accumulated at maximum concentrations of 0.6–4.3 mg/L in batch tests with initial perchlorate concentrations ranging from 100 to 600 mg/L. Furthermore, 50 mg/L chlorate inhibited perchlorate reduction with perchlorate at 100 mg/L. This is the first report of chlorate accumulation and inhibition for a pure culture of PCRB. These Chlorate effects are consistent with competitive inhibition between perchlorate and chlorate for the (per)chlorate reductase enzyme.

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1. Introduction

Perchlorate (ClO_4^-) is an industrial oxidant predominantly used primarily in solid rocket fuels, fireworks, and explosives (Schilt, 1979; Kirk et al., 1991). Perchlorate contamination of water supplies is widespread (Gullick et al., 2001), and in most cases, perchlorate contamination can be traced to former perchlorate waste disposal sites at defense-related facilities (Wallace et al., 1996; Motzer, 2001). Perchlorate is on the US Contaminant Candidate List (Scharfenaker, 2005), and some states, such as New York, California, Maryland, New Mexico, and Massachusetts, have perchlorate advisory levels for drinking water at 6 μ g/L or less (EPA, 2005).

Biological reduction to chloride is a promising treatment approach for perchlorate (Urbansky and Schock, 1999; Xu et al., 2003; Hatzinger, 2005), and hydrogen-based bioreactors are ideal for treating oxidized contaminants, such as perchlorate and nitrate, in drinking water (Giblin et al., 2000; Miller and Logan, 2000; Lee and Rittmann, 2002; Nerenberg et al., 2002). Kinetic parameters are needed to design such systems. This research provides the first kinetic parameters for a hydrogen-oxidizing perchlorate-reducing bacterium (PCRB).

The perchlorate-reduction pathway is thought to consist of the (per)chlorate reductase, which sequentially reduces perchlorate to chlorate, then chlorate to chlorite, via

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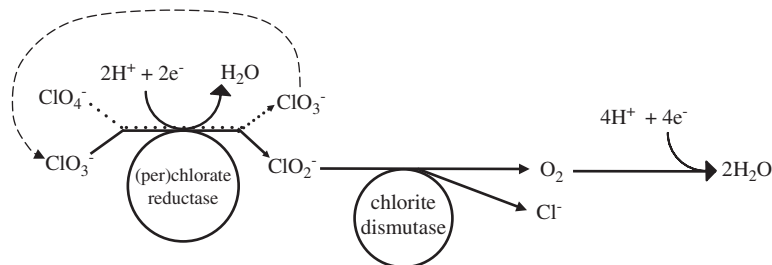


Fig. 1 – Schematic of perchlorate-reduction pathway, based on accepted roles of (per)chlorate reductase and chlorite dismutase enzymes. The dotted and dashed lines illustrate that the (per)chlorate reductase enzyme acts on perchlorate (ClO_4^-) and its reduction product, chlorate (ClO_3^-).

two-electron transfers (Kengen et al., 1999; Bender et al., 2005), and the chlorite dismutase, which transforms chlorite into chloride and oxygen by disproportionation (van Ginkel et al., 1996; Giblin and Frankenberger, 2001; Bender et al., 2002). As shown Fig. 1, chlorate produced by perchlorate reduction should compete with perchlorate for the catalytic site of the (per)chlorate-reductase enzyme, presumably slowing the perchlorate reduction rate. While some amount of chlorate accumulation is possible, it only has been reported for a mixed culture growing on nitrate and perchlorate (Nerenberg et al., 2002). This research is the first to show chlorate accumulation for a pure culture growing on perchlorate.

2. Materials and methods

2.1. Chemicals and media

The growth medium, adapted from Aragno and Schlegel (1992), was prepared using ultra-pure water (Nanopure, Barnstead/Thermolyne, Dubuque, Iowa) and research-grade chemicals. The growth medium contained, per liter: 1.386 g Na_2HPO_4 , 0.849 g KH_2PO_4 , 0.1 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL trace mineral solution, and 1 mL Ca-Fe solution. The Ca-Fe solution contained, per liter: 1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The trace mineral solution contained, per liter: 100 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 30 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 300 mg H_3BO_3 , 200 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 30 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 30 mg Na_2SeO_3 . The pH was adjusted using 1 M NaOH, for a final pH of 7.0, after three repetitions of vacuum degassing and equilibrating with a gas mixture of 95% H_2 and 5% CO_2 added to the headspace. The non-growth medium was a 4-mM phosphate buffer in deionized water at pH 7, with 100% hydrogen in the headspace. The temperature for all experiments was 22 °C.

2.2. Perchlorate-reducing isolate

Dechloromonas sp. PC1 was isolated from a hydrogen-based, autotrophic hollow-fiber membrane biofilm reactor that was actively reducing 5 mgN/L nitrate and around 1 mg/L perchlorate (Nerenberg et al., 2002). The reactor's effluent perchlorate concentration was less than 4 $\mu\text{g/L}$ and effluent nitrate below 50 $\mu\text{g/L}$. PC1 grows autotrophically with hydro-

gen, but also can grow heterotrophically with organic electron donor such as acetate. A 1-mL aliquot of reactor effluent was added to a serum bottle containing 25 mL of growth medium with perchlorate and placed on a shaker table at room temperature. After growth was observed, via an increase in the optical density (OD) at 600 nm, 100 μL of bacterial suspension was plated aerobically on R2A agar (DIFCO, Detroit, MI) and then re-grown in fresh medium. Purity was confirmed by serial plating and regrowing in batch culture, as well as by sequencing the 16S rDNA.

2.3. Batch experiments

The OD at 600 nm was measured with a spectrophotometer (Spec 20, Thermo Spectronics, Rochester, NY) and converted to dry weight (DW) using an empirical conversion factor determined for PC1. The conversion factor was determined by growing bacteria to exponential phase on perchlorate to an OD of around 0.2, filtering a known volume on a 0.45- μm cellulose filter paper (Gelman Sciences, Ann Arbor, MI), drying to constant weight at 104 °C, and measuring the net DW, i.e., the DW of the sample minus the DW of the filter paper. The conversion factor was 588-mgDW/OD unit.

Batch tests were carried out in 1-L bottles filled with 200 mL of media or 160-mL serum bottles filled with 25 mL of sterile media. All bottles were capped with butyl rubber stoppers and filled with a gas mixture of 95% hydrogen and 5% CO_2 (for the determination of q_{max} and Y) or with pure hydrogen (for the determination of K). The bottles were shaken on their sides at 200 rpm. The experiments were carried out at least in triplicate. For non-growth tests, the OD was monitored to ensure no growth took place. Growth on acetate was conducted with growth medium with 100 mg/L acetate and 100 mg/L perchlorate, and with a nitrogen headspace.

2.4. Analytical methods

Perchlorate, chlorate, chlorite, chloride, and acetate were analyzed using a Dionex ion chromatograph (IC) (4000i or ICS 2500) with conductivity detection using a 4-mm AS-11 column and an AG-11 guard column. For low perchlorate concentrations, a 4-mm AS-16 column and an AG-16 guard column were used. Both columns were used with a sodium hydroxide eluent. The AS-11 column was used with a 7-min equilibration time at 4 mM, a gradient from 4 to 60 mM over

8 min, then 5 min at 60 mM. For the AS-16 column, a 50-mM isocratic program was used. The injection loop volume was 500 μ L. A Dionex ASRS suppressor was used in chemical suppression mode with a 75 mM H₂SO₄ suppressant at 5 mL/min or in a recycle mode. Samples were injected with a Dionex autosampler.

2.5. Estimation of kinetic parameters

Batch tests were used to determine kinetic parameters under autotrophic conditions with hydrogen as the electron donor. Kinetic parameters for perchlorate and chlorate were determined in separate experiments using the standard Monod substrate-utilization and biomass-accumulation equations for batch growth (Rittmann and McCarty, 2001):

Substrate mass balance

$$\frac{dS}{dt} = -\frac{q_{\max}S}{S+K}X, \quad (1)$$

Biomass mass balance

$$\frac{dX}{dt} = \frac{Yq_{\max}S}{S+K}X - bX, \quad (2)$$

where Y is the biomass true yield (M_X/M_S) under autotrophic conditions for perchlorate or chlorate with hydrogen as a donor, b the endogenous decay rate ($1/T$), q_{\max} the maximum specific substrate utilization rate [M_S/M_X-T], S the perchlorate or chlorate concentration [M_S/V], and K the concentration of the half-maximum substrate-utilization rate [M_S/V] for perchlorate or chlorate. The standard unit system is days for time (T), mg DW for biomass (X), mg perchlorate or chlorate for substrate (S), and liters for volume (V). When chlorate was the substrate, $Y = Y_C$, the yield obtained for ClO₃⁻ reduction to Cl⁻. When ClO₄⁻ was the substrate, $Y = Y_P$, the yield obtained when ClO₄⁻ was reduced to Cl⁻. Perchlorate reduction to chloride was considered to occur in a single step.

The q_{\max} and Y values were determined concurrently from batch growth tests with high initial perchlorate or chlorate concentrations. The K parameters were determined independently from non-growth batch tests with constant biomass; hence, the substrate concentration was fitted using Eq. (1) only. Because K for chlorate, K_C , appeared to be well below the 0.01 mg/L chlorate detection limit by our IC method, it was not determined experimentally.

The endogenous decay rate, b , was determined by growing cells to densities above 0.2 with hydrogen as an electron donor, purging the hydrogen with oxygen-free nitrogen, and measuring the decrease in OD over several days. Tests were carried out in 1-L bottles filled with 200 mL of media or 160-mL serum bottles filled with 25 mL of sterile media. Curve fitting was carried out using the biomass mass balance equation (Eq. (2)).

The kinetic parameters for perchlorate reduction were used to determine the growth threshold concentration, S_{\min} , which defines the lowest concentration of a growth substrate that provides a non-negative growth rate (Rittmann and McCarty, 2001). S_{\min} is computed as

$$S_{\min} = \frac{Kb}{Yq_{\max} - b}. \quad (3)$$

2.6. Chlorate accumulation and inhibition

Chlorate accumulation was documented for PC1 during special growth tests in which the chlorate concentration was monitored. Initial perchlorate concentrations were 100, 200, 300, or 600 mg/L. For the inhibition tests, a growth test with perchlorate was initiated, and around 65 mg/L of chlorate was added to the bottle after around 5 h.

2.7. Parameter-fitting

Non-linear least-square error minimization was used to estimate best-fit values for the kinetic parameters (Sáez and Rittmann, 1992). In this technique, the modeling equations are solved numerically, and parameters are selected to minimize the sum of the relative least-square residuals. The equations were solved by finite differences in a Microsoft Excel spreadsheet.

2.8. Organism identification

The 16S rDNA genes from bacteria isolates were sequenced by polymerase chain reaction (PCR) amplification using PCR Master kits (Roche Applied Science, Indianapolis, IN) and a thermal cycler (PCR Express, Hybaid, Ulm, Germany) using a “touch-down” PCR method (Giovannoni 1991). The partial 16S rDNA sequence was amplified with universal primer combination U27F (5'-AGAGTTTGATCCTGGCTCAG-3'), and U1525R (5'-AAAGAGGTGATCCAGCC-3'). The DNA sequence was sequenced using an ABI 3100 DNA sequencer, after purification of cycle sequence-product using an UltraClean PCR Clean-up Kit (Mo Bio laboratories, Carlsbad, CA). Cycle sequenced was carried out using seven universal primers: U27F, GM5F (5'-ACGGGGGGCC-TACGGGAGGCAGCAG-3'), U805F (5'-GATTAGATACCCTGG-TAGTC-3'), U518R (5'-GTATTACCGCGGCTGCTGG-3'), U1093R (5'-TTGCGCTCGTTGCGGGACT-3'), U1112F (5'-GTCCCGCAAC-GAGCGCAAC-3'), and U1525R.

3. Results and discussion

3.1. PC1 physiology and metabolism

PC1 is a Gram-negative, motile rod. It can grow autotrophically with hydrogen as an electron donor and heterotrophically with acetate. Based on a BLAST search for similar nucleotide sequences, PC1 had 99% similarity to *Dechloromonas* strain JJ (Coates et al., 2001), which is not a PCB, and a 98% similarity to *Dechloromonas* strain HZ, which is a hydrogen-oxidizing, perchlorate-reducing autotroph (Zhang et al., 2002). PC1's 16S rDNA partial sequence was deposited with GenBank (accession no. AY126452).

3.2. Kinetic parameters for perchlorate

Kinetic parameters for PC1 and for other PCB are summarized in Table 1. Typical growth and K_p experiments for perchlorate are shown in Figs. 2 and 3, respectively. The total yield on perchlorate, Y_p , was 0.23 gDW/gClO₄⁻, or 2.9 gDW/eq e⁻ on a per electron-equivalent basis. The “McCarty

method” (Rittmann and McCarty, 2001) estimates the theoretical microbial yield based on thermodynamics. Based on this method, the theoretical true yield for perchlorate is 2.7 gDW/eq e⁻, similar to the experimental value.

The q_{Pmax} was 0.25 e⁻ eq/mgDW-day or 3.1 mgClO₄⁻/mgDW-day. This value is in the middle of previously reported q_{Pmax} values of 1.7–24 mgClO₄⁻/mgDW-day (Korenkov et al., 1976; Wallace et al., 1996, 1998; Rikken et al., 1996; Logan et al., 2001; Waller et al., 2004).

From Table 1, the K_p values for PCRB range from a high of 33 mg/L (Logan et al., 2001) to a low of 2.2 (Waller et al., 2004). The K_p value for PC1 is 0.14 mgClO₄⁻/L, around one order of magnitude lower than the lowest K_p value from the literature. This suggests that PC1 may have a competitive advantage over other species when perchlorate concentrations are very low. However, K_p is high with respect to preliminary treatment goals, which range from 1 to 24 µg/L, suggesting that perchlorate reduction rates will be slowed when the concentrations reach these levels. This may be an important design consideration.

Based on the decay tests with perchlorate as an electron acceptor, the b value is 0.055/day (data not shown). This is within the range expected for oligotrophic bacteria (Rittmann and McCarty, 2001).

3.3. Kinetic parameters for chlorate

Fig. 4 presents the results of a typical growth experiment with chlorate. The true yield for chlorate, Y_C , was 0.22 gDW/gClO₃⁻. Expressed in terms of biomass per electron transferred to the acceptor, Y_C was 2.8-gDW/eq e⁻. For autotrophic growth with hydrogen as an electron donor and ammonium as a nitrogen source, the theoretical true yield for chlorate was 2.6 gDW/eq e⁻ (Rittmann and McCarty, 2001). Thus, the experimentally determined Y_C for PC1 is consistent with the theoretical value.

The q_{Cmax} value for chlorate was 6.3 gClO₃⁻/gDW-day. On the basis of electrons transferred to the acceptor, q_{Cmax} was 0.31 e⁻ eq/gDW-day. The previously reported value for q_{Cmax} is similar, 7.5 mgClO₃⁻/mgDW-day (Rikken et al., 1996).

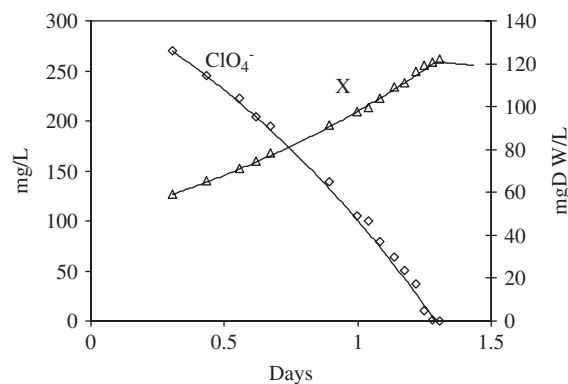


Fig. 2 – Typical growth experiment for perchlorate. The symbols are experimental data, and the curves are the model simulations with the best-fit parameters $q_p = 3.1$ gClO₄⁻/gDW-d, $Y_p = 0.23$ gDW/gClO₄⁻, and $K_p = 0.15$ mgClO₄⁻/L.

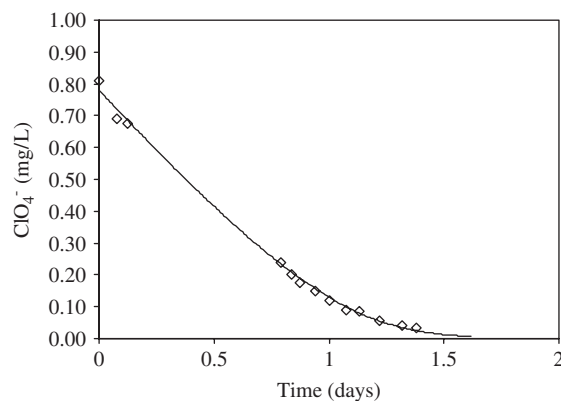


Fig. 3 – Typical K experiment for perchlorate (non-growth conditions). The symbols are experimental data, and the curves are the model simulations with the best-fit parameter $K_p = 0.14$ mgClO₄⁻/L.

Table 1 – Kinetic parameters for PCRB from the literature and this study

Isolate	Acceptor	q_{max} mg acceptor (mgDW ⁻¹ day ⁻¹)	K (mg/L)	Reference
<i>Vibrio dechloratans</i>	Perchlorate	1.68	—	Korenkov et al. (1976)
<i>Wolinella succinogenes</i> HAP-1	Perchlorate	2.57	—	Wallace et al. (1996, 1998)
GR-1	Perchlorate	5.65	—	Rikken et al. (1996)
KJ	Perchlorate	24	33	Logan et al. (2001)
PDX	Perchlorate	7.5	12	Logan et al. (2001)
SN1A	Perchlorate	4.60	2.2	Waller et al. (2004)
ABL1	Perchlorate	5.43	4.8	Waller et al. (2004)
INS	Perchlorate	4.35	18	Waller et al. (2004)
RC1	Perchlorate	6.00	12	Waller et al. (2004)
PC1	Perchlorate	3.1	0.14	This study
GR-1	Chlorate	7.48	—	Rikken et al. (1996)
PC1	Chlorate	6.3	<0.014	This study

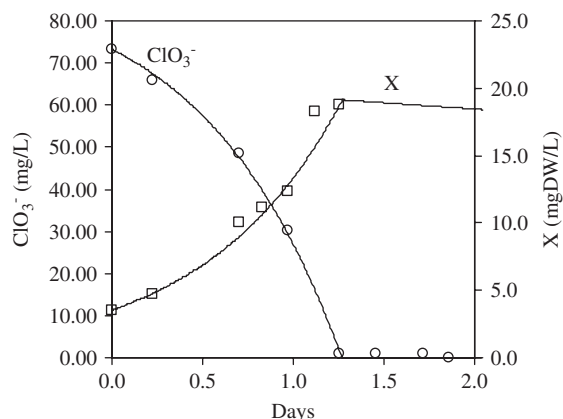


Fig. 4 – Typical growth experiment for chlorate. The symbols are experimental data, and the curves are the model simulations with the best-fit parameters $q_c = 6.3 \text{ gClO}_3^-/\text{gDW}\cdot\text{d}$ and $Y_c = 0.22 \text{ gDW/gClO}_3^-$.

K_C for chlorate was too low to determine from the non-growth batch tests. Based on the lack of curvature at a concentration of 0.027 mg/L, K_C is less than 0.014 mg/L. This is much lower than the K_p . There are no K_C values for PCRB in the literature.

3.4. Growth threshold concentration, S_{\min} , for perchlorate

S_{\min} represents the minimum concentration at which growth can occur on perchlorate as the sole electron acceptor. Using the experimentally determined kinetic parameters ($b = 0.055/\text{day}$, $q_{p\max} = 3.1 \text{ mg/L}$, and $K_p = 0.14 \text{ mg/L}$, and $Y = 0.23 \text{ mgDW/mgClO}_4^-$), the resulting S_{\min} for perchlorate is 0.012 mg ClO_4^-/L . For a biofilm process, the b should be replaced by b' , which includes the specific detachment rate, and endogenous respiration. The MBfR has low detachment values, around 0.01/day (Lee and Rittmann, 2002). Thus, for an MBfR the total b' is around 0.065/day, providing a S_{\min} for perchlorate of $\sim 0.014 \text{ mg/L}$. For a reactor with a more typical detachment rate of 0.1/day (Rittmann et al. 2001), b' would be 0.155/day, and the resulting S_{\min} would be 0.039 mg/L. PC1's S_{\min} is likely to be lower than that for other PCRB, even heterotrophic PCRB, given its low K_p . This suggests that it is not possible to achieve perchlorate standards with perchlorate as the sole electron acceptor. On the other hand, PC1 can use oxygen and nitrate as electron acceptors, and these may serve as primary electron acceptors supporting PC1 biomass, allowing perchlorate to be reduced to below S_{\min} .

3.5. Chlorate accumulation and inhibition

Batch growth tests were used to assess chlorate accumulation during perchlorate reduction. A typical plot is shown in Fig. 5. With an initial perchlorate concentration of 200 mg/L, the chlorate concentration increased from zero to around 1.4 mg/L in 1 day, and then it slowly decreased to zero over 3 days as the perchlorate concentration decreased. Similar patterns were obtained for growth with 100, 300, and 600 mg/L perchlorate, with chlorate accumulating up to 0.6, 2.4, and

4.3 mg/L, respectively (data not shown). This is the first report of chlorate accumulation for a pure culture of PCRB.

Fig. 6 shows a batch growth experiment in which a PC1 culture was grown on perchlorate. Chlorate (50 mg/L) was added when the perchlorate concentration had decreased to around 100 mg/L (at 0.21 day). Perchlorate reduction was inhibited until chlorate was almost completely reduced. Biomass increased more quickly during the chlorate reduction, which is consistent with the higher specific utilization rate for chlorate.

Chlorate's low accumulation and strong inhibitory effect on perchlorate are consistent with the determined kinetic parameters. The following is the expression for the perchlorate specific reduction rate considering competitive inhibition from chlorate (Rittmann and McCarty, 2001):

$$q_p = q_{p\max} \frac{S_p}{S_p + K_p(1 + (S_c/K_c))} \quad (4)$$

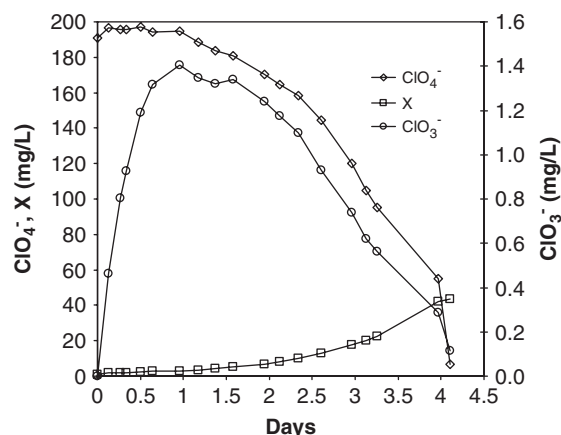


Fig. 5 – Batch test shows reduction of perchlorate, transient accumulation of chlorate, and biomass growth. Note the different concentration scale for ClO_3^- .

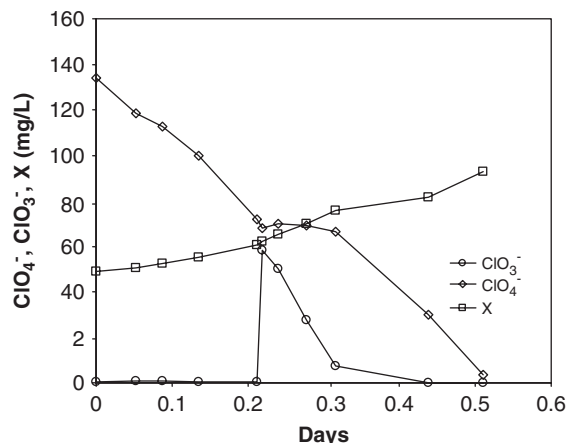


Fig. 6 – Batch test shows reduction of perchlorate before and after a chlorate spike of 50 mg/L. Chlorate inhibits perchlorate reduction. Note the different concentration scale for biomass X.

where S_p and S_c are the perchlorate and chlorate concentrations, K_p and K_c are the half maximum rate concentrations for perchlorate and chlorate, and q_p and q_{pmax} are the actual and maximum perchlorate specific reduction rates. When K_c is very low, as is the case for PC1, a small chlorate concentration can significantly slow the actual perchlorate reduction rate. This explains the low amount of chlorate accumulation, as well as the powerful inhibition when high amounts of chlorate are added.

4. Conclusions

We provide the first kinetic parameters for a hydrogen-oxidizing PCRB. The K_p is much lower than reported for other PCRB. The low S_{min} for perchlorate suggests that other acceptors, such as oxygen and nitrate, are needed to sustain PCRB when the perchlorate concentration is below the S_{min} , as is the case in many perchlorate-contaminated water supplies. Since high concentrations of oxygen and nitrate in the reactor can inhibit perchlorate reduction (Coates, 2002; Coates and Achenbach, 2004), ideal treatment systems may include some influent oxygen and nitrate, but provide low oxygen and low nitrate environments within the reactor. Examples include completely mixed systems, plug-flow systems, and biofilm systems.

The observed chlorate accumulation and chlorate inhibition are consistent with competitive inhibition for the (per)chlorate reductase enzyme. While chlorate inhibition may appear insignificant due to its low levels of accumulation, it still may have a significant effect. This is illustrated by Eq. (4), where a very low value for K_c allows small chlorate concentrations to have a large impact on q_p . Given that K_c for PC1 is much lower than K_p , the competitive inhibition model predicts that chlorate inhibition diminishes at perchlorate concentrations below K_p (Nerenberg, 2003). Therefore, rates determined at high perchlorate concentrations may underestimate actual rates at concentrations below K_p , as chlorate inhibition is relieved.

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