MICROBIAL ECOLOGY OF PERCHLORATE-REDUCING BACTERIA THAT ACCUMULATE HIGH LEVELS OF CHLORATE

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by

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MICROBIAL ECOLOGY OF PERCHLORATE-REDUCING BACTERIA THAT ACCUMULATE HIGH LEVELS OF CHLORATE

Abstract

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Perchlorate (ClO$_4^-$) is a harmful oxidant found in drinking water sources throughout the US. Recently, several strains of perchlorate-reducing bacteria (PCRB) that accumulate large amounts of chlorate during perchlorate reduction have been isolated and studied. In this study, it was estimated that these novel perchlorate reducers have similar $q_{\text{max}}$ values to conventional PCRB (8.3mgClO$_3^-$/mgX-day and 11.5mgClO$_4^-$ /mgX-day) and much higher $K$ values (58.3mg/L for chlorate and 192.6mg/L for perchlorate). Based on these kinetic parameters, these novel perchlorate reducers are unlikely to play a significant role in perchlorate reduction at concentrations below 200 mg/L, but may contribute to faster degradation at high concentrations. Analysis of the nucleotide and amino acid sequences of the catalytic subunit of the (per)chlorate reductase enzyme (pcrA) of three high chlorate accumulating isolates were highly similar and distinct from conventional PCRB, suggesting that structural differences in the pcrA enzyme could account for differences in microbial activity.
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CHAPTER 1
INTRODUCTION

1.1 Sources and Uses of Perchlorate

Perchlorate (ClO$_4^-$) is an oxyanion commonly manufactured as the solid salts ammonium, potassium, and sodium perchlorate. Ammonium perchlorate was first produced in the United States in the mid-1940s in the munitions industry by the Department of Defense (USEPA, 2002). Today, perchlorate salts are produced for applications such as pyrotechnics, fireworks, lubricating oils, textile dye fixing, nuclear reactors, electronic tubes, aluminum refinishing, automobile air bags, paint and enamel production, and pharmaceuticals, but the most common production of perchlorate is as ammonium perchlorate for use in explosives and rocket propellant. Perchlorate salts are produced and manufactured commonly in all but six states in the U.S. (Motzer, 2001; USEPA, 2002).

Although the perchlorate ion is largely inert, other components in solid rocket fuels have a short shelf life. Therefore, rocket fuel must be replaced regularly, resulting in large amounts of perchlorate being disposed (Motzer, 2001). Approximately 15.9 million kg of perchlorate salts have entered the environment since the 1950’s, mostly from manufacturing, testing, or storing facilities for missiles (Gullick et al., 2001; Xu et al., 2003).

Perchlorate deposits occur naturally in the environment, most notably in the Atacama desert of northern Chile (Motzer, 2001). Perchlorate is contained in the natural
nitrate deposits in the desert, and these deposits are used as fertilizer in the U.S. (Coates and Achenbach, 2004). Other natural sources of perchlorate include natural geologic sources and atmospheric generation (Dasgupta et al., 2005; Jackson et al., 2005; Plummer et al., 2006; Rajagopalan, 2006). While perchlorate exists naturally, the main contributor to perchlorate contamination of water appears to be synthetic sources (Coates and Achenbach, 2004).

Perchlorate contamination in the U.S. is widespread and has been detected in natural water sources (Snyder et al., 2005), in more than 100 groundwater wells in California, Nevada, and Utah (USEPA, 2002), and in 81 of 82 breast milk and dairy milk samples taken throughout the U.S (Kirk et al., 2005). In a recent survey, perchlorate has also been detected in vitamins (Renner, 2006b), lettuce (Sanchez et al., 2005a; Sanchez et al., 2005b), and fruits (Renner, 2006a). Perchlorate found in fruits has been detected at levels over the safe dose recommended by the National Academy of Sciences (Renner, 2006a; Renner, 2006b).

1.2 Toxicity

Perchlorate is harmful to humans and wildlife because it disrupts natural functioning of the thyroid gland, which is responsible for regulating growth, cell differentiation, and metabolism of lipids, proteins, and carbohydrates (Clark, 2000). When perchlorate enters the body, it prevents proper uptake of iodide by the thyroid, disrupting hormone production (Logan et al., 2001; EPA, 2006). Long-term exposure to perchlorate may result in hypothyroidism (Coates and Achenbach, 2004).

Chlorate and chlorite, intermediates in the pathway of biological perchlorate reduction, are also harmful to the environment. Chlorate is toxic to brown algae at levels above 20 μg/L and chlorate and chlorite have caused hemolytic anemia in laboratory animals (Coates et al., 1999).
1.3 Regulations

According to the National Academy of Science (NAS), humans can safely consume perchlorate at a rate of 0.7 μg/kg of body weight per day. This level is calculated from the no-observed-adverse-effect-level (NOAEL) of 7 μg/kg/day and uses a safety factor of 10 for protection of infants and pregnant women. The EPA’s reference dose (RfD) of 0.0007 mg/kg/day for perchlorate is based on this NAS consumption level (EPA, 2006). The drinking water equivalent level (DWEL) of 24.5 μg/L was established based on a 70 kg adult drinking 2L of water per day, assuming that drinking water is the only source of perchlorate consumption (Scharfenaker, 2005). There is no federal drinking water standard for perchlorate currently, although perchlorate is listed on the Drinking Water Contaminant Candidate List or CCL (EPA, 2006). Table 1-1 shows perchlorate standards for some states. “Action Level” is defined by the National Safety Council as the concentration at which a compound warrants action under Superfund Amendments and Reauthorization Act (SARA) and the National Oil and Hazardous Substances Contingency Plan (2005).

TABLE 1-1

STATE PERCHLORATE REGULATIONS FOR ARIZONA, CALIFORNIA, MASSACHUSETTS, AND NEVADA (SALAMONE, 2006)

<table>
<thead>
<tr>
<th>State</th>
<th>Action Level (μg/L)</th>
<th>Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arizona</td>
<td>14</td>
<td>Health-based Guidance Level (HBGL)</td>
<td>(AZDEQ, 2004)</td>
</tr>
<tr>
<td>California</td>
<td>6</td>
<td>Public Health Goal (PHG)</td>
<td>(OEHHA, 2003)</td>
</tr>
<tr>
<td>Massachusetts</td>
<td>2</td>
<td>Maximum Contaminant Level (MCL)</td>
<td>(MassDEP, 2006)</td>
</tr>
<tr>
<td>Nevada</td>
<td>18</td>
<td>Provisional Action Level</td>
<td>(NDEP, 2006)</td>
</tr>
</tbody>
</table>
1.4 Chemistry

Ammonium, potassium, and sodium perchlorate salts are highly mobile in aqueous systems because of their relatively high solubilities (Gingras and Batista, 2002; Xu et al., 2003). Ammonium perchlorate (NH$_4$ClO$_4$) in particular has a solubility of 220 g/L and makes up 90% of all manufactured perchlorate salts (Motzer, 2001). Perchlorate salts disassociate into the perchlorate anion and their corresponding cation (Motzer, 2001). Although highly reactive in their solid states, perchlorate salts are persistent in aqueous phase due to the inert nature of the perchlorate anion and its slow reaction kinetics (Urbansky, 1998; Espenson, 2000). Therefore perchlorate contamination can persist for long periods of time.

1.5 Perchlorate Treatment

Several treatment options exist for perchlorate remediation from groundwater. For example, significant reduction of perchlorate by ultraviolet oxidation has been demonstrated. Ion exchange, a popular treatment option, is capable of separating perchlorate from water, but it creates a waste composed of concentrated perchlorate and the caustic solutions used in the process of separation. Reverse osmosis and electrodialysis are two additional treatment options capable of separating perchlorate from the solution, but are expensive and also produce perchlorate-containing waste streams (Motzer, 2001).

1.6 Biodegradation

1.6.1 Introduction

Among the many treatment options for perchlorate contamination, biodegradation is very promising. (Gingras and Batista, 2002). Biological reduction of perchlorate is able to completely remove perchlorate from the environment, producing the end product of innocuous chloride (Cl$^-$). In addition, perchlorate and chlorate have high reduction
potentials beneficial for microbial metabolism; the reduction potential of perchlorate to chloride at standard conditions (298 K) is 1.38V while the reduction potential for chlorate to chloride at standard conditions is 1.03V (Coates and Achenbach, 2004). Equation 1-1 is the half reaction of the reduction of perchlorate to chloride (Urbansky, 2000).

EQUATION 1-1

\[
\text{ClO}_4^- + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{Cl}^- + 4\text{H}_2\text{O}
\]

Biological reduction is therefore a promising treatment strategy for perchlorate contamination (Urbansky and Schock, 1999; Nerenberg et al., 2002; Xu et al., 2003).

1.6.2 Pathway

Perchlorate can be biologically reduced to chloride by dissimilatory perchlorate-reducing bacteria (PCRB). Perchlorate can also be reduced co-metabolically by denitrifying bacteria, but this is a slow process, does not contribute to microbial growth, and is not believed to be a significant pathway for perchlorate transformation in the environment (Coates and Achenbach, 2004).

Biological perchlorate reduction by PCRB occurs in three phases (Figure 1-1) (Rikken et al., 1996). In the first step, perchlorate (\(\text{ClO}_4^-\)) is reduced to chlorate (\(\text{ClO}_3^-\)) in a two-electron transfer. In the second step, chlorate is reduced to chlorite (\(\text{ClO}_2^-\)) in another two-electron transfer. Finally, chlorite is transformed to chloride (\(\text{Cl}^-\)) and oxygen (\(\text{O}_2\)) without a transfer of electrons (Rikken et al., 1996; Kengen et al., 1999; Nerenberg et al., 2002; Xu et al., 2003). It is thought that the first step, in which perchlorate is reduced to chlorate, is rate-limiting (Rikken et al., 1996; Nerenberg et al., 2002).
Figure 1-1. The perchlorate degradation pathway.

The presence of oxygen inhibits reduction of perchlorate (Wallace et al., 1996; Chaudhuri, 2002; Xu et al., 2003; Xu et al., 2004) and chlorate (Xu et al., 2004). While some oxygen is produced along the degradation pathways of both perchlorate and chlorate, PCRB and CRB quickly reduce it so that oxygen never accumulates to significant levels (Xu et al., 2004).

The metabolic reduction of perchlorate to chloride and oxygen is thought to involve two separate enzymes (Giblin and Frankenberger, 2001; Nerenberg, 2003). The first two steps in the pathway, the reduction of perchlorate to chlorate and the reduction of chlorate to chlorite, are both catalyzed by the enzyme (per)chlorate reductase, a type II member of the microbial dimethyl sulfoxide (DMSO) reductase family of molybdenum enzymes (Kengen et al., 1999; Bender et al., 2005). (Per)chlorate reductase is genetically homologous to other type II members of the DMSO family such as nitrate reductase, selenate reductase, dimethyl dehydrogenase, ethylbenzene dehydrogenase, and chlorate reductase. Type II enzymes have a heterotrimeric structure and have a site for Fe-S binding in the β-subunit (Bender et al., 2005).

The (per)chlorate reductase enzyme is encoded by four distinct genes: pcrA, pcrB, pcrC, pcrD. Of these four genes, pcrA is the longest, at 2,784 base pairs (bp) (Bender et al., 2005). Figure 1-2 illustrates the predicted subunit assembly and pathway by which electrons are transferred during (per)chlorate reduction.
It is thought that a NirT-type cytochrome embedded in the membrane transfers electrons from a quinone pool to the PcrABC reductase enzyme. It is predicted that these electrons are then used in the reduction of (per)chlorate at the active site of PcrA. PcrD is not present in this protein complex and is thought to function in enzyme assembly (Bender et al., 2005).

The third step of (per)chlorate reduction, the dismutation of chlorite, is catalyzed by chlorite dismutase and is coded in the chlorite dimutase gene \( (cld) \) (van Ginkel et al., 1996; Kengen et al., 1999; Nerenberg et al., 2002; Bender et al., 2004). Studies of this gene have yielded important information regarding the evolution of the capability for (per)chlorate reduction. PCRB are not only phylogenetically and phenotypically diverse; the order and transcriptional direction of the genes involved in perchlorate reduction, particularly the \( cld \) gene, are also different among species. A comparison of a phylogenetic tree with a tree based on the \( cld \) gene suggests that the evolution of perchlorate reduction is based on horizontal transfer (Coates and Achenbach, 2004).

Some bacteria are only capable of chlorate reduction and are termed chlorate-reducing bacteria (CRB). Since these bacteria can reduce chlorate but cannot reduce...
perchlorate, it is thought that a separate chlorate reductase enzyme exists (Logan et al., 2001; Xu et al., 2003).

The fact that only one enzyme, (per)chlorate reductase, catalyzes both perchlorate and chlorate reduction in PCRB suggests that it is possible that competitive inhibition between the alternative substrates takes place, as shown in Figure 1-3, resulting in some degree of chlorate accumulation (Nerenberg et al., 2006). Competitive inhibition occurs when two structurally similar chemicals compete for the active site of an enzyme (Rittmann and McCarty, 2001).

Figure 1-3 Perchlorate degradation pathway (Nerenberg et al., 2006). Perchlorate and chlorate compete for the (per)chlorate reductase enzyme.

1.7 Chlorate Accumulation

Chlorate accumulation among perchlorate reducing bacteria (PCRB) was first reported by Nerenberg et al. (2002) and later by Salamone (2006). Figure 1-4 shows an example of chlorate accumulation by conventional PCRB. This experiment was performed in a batch system. With an initial perchlorate concentration of 200 mg/L, chlorate accumulated to a maximum concentration of only 1.3 mg/L, about 0.65% of the initial perchlorate concentration on a mass basis. The low amount of chlorate accumulation may explain why chlorate accumulation has not been more widely reported in the literature.

Chlorate accumulation among these bacteria is especially high, suggesting that the enzyme for perchlorate reduction in these bacteria may be distinct from that of conventional PCRB. The ramifications of this high accumulation have not yet been shown, but it is possible that coupling HCAP with conventional PCRB or CRB may enhance, or hinder, overall perchlorate reduction kinetics.
1.8 Abundance and Diversity of PCRB and CRB

1.8.1 PCRB

PCRB, which can reduce both chlorate and perchlorate [(per)chlorate], are numerous in the environment and isolates can be found in many different locations including wastewater, rivers, sediments, and soils. (Logan, 1998; Coates et al., 1999; Xu et al., 2003). In a study of the abundance of PCRB and CRB, Coates et al. (1999) found concentrations of PCRB ranging from $2.31 \times 10^3$ to $2.4 \times 10^6$ cells per gram of sample in environments such as soils, sediments, and swine waste. In that study, all 13 species isolated from environmental sources were gram-negative bacteria.

PCRB are facultative anaerobes or microaerophiles, and many are also able to reduce nitrate (Coates and Achenbach, 2004). PCRB are found in the $\alpha$, $\beta$, $\gamma$, and $\varepsilon$ subclasses of the Proteobacteria phylum (Coates and Achenbach, 2004), and most are located in genera *Dechloromonas* and *Azospira* (or *Dechlorosoma*) of the $\beta$ subclass of Proteobacteria (Coates and Achenbach, 2004).

Although most (per)chlorate reducers are genetically similar, close relatives of (per)chlorate reducers do not necessarily share the capability to reduce perchlorate. Therefore, 16s rRNA studies for detection of the potential for perchlorate reduction are useless (Coates and Achenbach, 2004). Instead, perchlorate reduction can only be detected through analysis of functional genes. Unfortunately the (per)chlorate reductase gene has been reported for only two species, *Dechloromonas agitata* and *Dechloromonas aromatica* (Bender et al., 2005).

1.8.2 CRB

CRB are bacteria that are capable of chlorate reduction but not perchlorate reduction. Although it appears CRB are more prevalent in the natural environment than PCRB, fewer species have been isolated (Logan et al., 2001; Xu et al., 2004). Unlike perchlorate reducers, CRB are not expected to experience competition from perchlorate,
because their chlorate reductase enzyme does not reduce perchlorate. Instead, chlorate is reduced to chlorite by a putative chlorate reductase enzyme. As with (per)chlorate reducers, chlorite is then transformed by disproportionation to chloride and oxygen by chlorite dismutase (Wolterink et al., 2003).

Since PCRB accumulate chlorate, it is possible that relationships exist between CRB and PCRB wherein CRB reduce chlorate produced in the perchlorate reduction pathway. There also may be relationships between HCAP and conventional PCRB. CRB may play an even more significant role in the presence of HCAP because HCAP accumulate higher amounts of chlorate while degrading perchlorate. CRB, therefore, would have a greater opportunity for syntrophic (mutually beneficial), commensal (one species benefiting without affecting the other), or parasitic (one species benefiting at the expense of the other) relationships with HCAP. If these relationships exist, and if they affect the overall kinetic behavior of the community, current kinetic parameters obtained based on reduction of perchlorate by single species may be inaccurate for environmental engineering applications.

1.9 Previous Studies on HCAP

This research project expands upon work performed by Anna Salamone (2006). Her work focused on characterizing the kinetics of perchlorate reduction of an HCAP isolate, HCAP-C. She determined kinetic parameters of perchlorate and chlorate reduction of HCAP-C and tested co-cultures of HCAP-C with CRB and conventional PCRB. Salamone found inconsistent maximum specific perchlorate reduction rates, \( q_{p_{\text{max}}} \), for HCAP-C under different initial perchlorate concentrations. This research finds a consistent \( q_{\text{max}} \) value for HCAP-C reduction of perchlorate when the initial perchlorate concentration is 200 mg/L. Salamone also tested co-cultures of HCAP-C with CRB and PCRB to determine the interactions of these isolates and predict kinetic behaviors in the natural environment, but those tests were only run once. This project tests co-cultures of
HCAP-C and CRB in duplicate to establish reliable kinetic behaviors. It also uses computer modeling to back up these experimental findings.

1.10 Research Needs

In order to effectively design treatment systems, accurate kinetic parameters describing microbial perchlorate reduction are needed. Kinetic studies on high chlorate-accumulating PCRB have never been reported, but these novel perchlorate reducers may play important roles in environmental perchlorate reduction. When HCAP are combined with other PCRB or with CRB in the environment, interactions among the isolates may lead to aggregate perchlorate reduction kinetics that are distinct from those of conventional PCRB whose reduction rates have already been characterized. These kinetic differences may affect reactor design, resulting in the need for accurate kinetic parameters for HCAP, especially when combined with PCRB and CRB.

1.11 Objectives

In this research, kinetic parameters for the HCAP isolate HCAP-C will be determined, interactions between HCAP, PCRB, and CRB will be predicted, and causes of chlorate accumulation will be investigated. Interactions between HCAP, PCRB, and CRB in the natural environment will be predicted through modeling co-cultures of HCAP, CRB, and conventional PCRB. The causes of chlorate accumulation in HCAP will be investigated namely through genetic analysis of the pcrA gene which is responsible for the (per)chlorate reductase enzyme.

This thesis first examines the development of kinetic parameters for biological reduction of perchlorate by HCAP and reduction of chlorate by CRB (Chapter 2). It then discusses the possible interactions between HCAP and CRB in the environment through laboratory batch studies of co-cultures of HCAP and CRB, which are supported by computer-generated models. Additionally, computer-generated models of co-cultures of HCAP, PCRB, and CRB, which predict the interactions of these bacteria in the natural
environment under steady-state conditions, suggest reasons why chlorate accumulation has not been reported for mixed-cultures and further predicts that HCAP will be out-competed by PCRB at perchlorate concentrations below 200 mg/L (Chapter 3). Finally, the nucleotide and amino acid sequences of the catalytic subunit of (per)chlorate reductase (PcrA) in several HCAP and PCRB strains are analyzed to determine if chlorate accumulation can be predicted by the pcrA gene (Chapter 4).
2.1 Introduction

In order to predict the interactions of HCAP with PCRB and CRB in the natural environment, kinetic parameters describing the reduction of perchlorate and chlorate by HCAP are needed. In this chapter, estimates of the yields, half maximum specific substrate utilization rates, and maximum specific substrate utilization rates for HCAP-C reduction of both perchlorate and chlorate are determined. Additionally, the half maximum specific substrate utilization rate of CRB reduction of chlorate was estimated.

Most PCRB accumulate small amounts of chlorate during perchlorate reduction. Nerenberg et al. (2006) carried out batch tests on the PCRB *Dechloromonas* sp. PC1, with a 200 mg/L initial perchlorate concentration, and found that the chlorate concentration gradually increased to around 1.5 mg/L, then decreased concurrently with the decrease in perchlorate concentration. Nerenberg (2003) found that the maximum chlorate accumulation for PC1, as a percentage of the initial perchlorate concentration, was relatively constant, at around 0.5 - 1%, on a mass basis. The constant percent of chlorate accumulation is consistent with competitive inhibition between perchlorate and chlorate for the (per)chlorate reductase enzyme (Nerenberg, 2003).

Recently, Salamone (2006) reported that some PCRB accumulate high levels of chlorate. These bacteria, termed HCAP, can accumulate chlorate at concentrations of 10 to 30% of the initial perchlorate concentration when grown in batch tests. In order to
quantify the chlorate accumulation, and possibly explain it via a competitive inhibition model, and also in order to predict potential syntrophic, commensal, or parasitic interactions between PCRB, HCAP, and CRB, kinetic and stoichiometric parameters are needed. In this chapter, chlorate accumulation is shown for three strains of PCRB, two of which behave as HCAP, and kinetic and stoichiometric parameters are presented for these strains as well as for one strain of CRB.

2.2 Source of Isolates

Robert Nerenberg at Northwestern University isolated two strains of bacteria used in this research. Those isolates are *Dechlorosoma* sp. HCAP-C, formerly *Dechlorosoma* sp. PCC (GenBank accession number for 16S rRNA: AY126453) isolated from activated sludge, and *Dechloromonas* sp. PC1 (GenBank accession number for 16S rRNA: AY126452) isolated from a bench-scale, perchlorate-reducing MBfR (Nerenberg et al., 2006). After isolation, strains were stored in 30% glycerol at –80 °C. For this study, the stored isolates were reconstituted and grown in liquid medium. To confirm that the isolates were pure, reconstituted cultures were plated aerobically on R2A agar (Difco). *Dechlorosoma* sp. KJ and *Pseudomonas* sp. PDA were obtained from Bruce Logan (Civil and Environmental Engineering, Penn State University) and reconstituted in the same manner as HCAP-C and PC1.

2.3 Materials and Methods

2.3.1 Chemicals and Medium

Batch tests were conducted in a “high nutrient” growth medium, modified from the growth medium described by Nerenberg et al. (2006), with a pH of approximately 7. It is called “high nutrient” because the concentration of trace minerals, calcium, and iron was twice those used by Nerenberg et al. (2006). The higher nutrient concentrations were used to eliminate potential trace mineral deficiencies that may be accounting for chlorate
accumulation. One liter of medium contained the following: 1.386 g Na$_2$HPO$_4$, 0.849 g KH$_2$PO$_4$, 0.1 g (NH$_4$)$_2$SO$_4$, 0.2 g MgSO$_4$.7H$_2$O, 2 mL trace mineral solution, and 2 mL Ca-Fe solution. The Ca-Fe solution contained, per liter: 1 g CaCl$_2$.2H$_2$O and 1 g FeSO$_4$.7H$_2$O. The trace mineral solution contained, per liter: 100 mg ZnSO$_4$.7H$_2$O, 30 mg MnCl$_2$.4H$_2$O, 300 mg H$_3$BO$_3$, 200 mg CoCl$_2$.6H$_2$O, 10 mg CuCl$_2$.2H$_2$O, 10 mg NiCl$_2$.6H$_2$O, 30 mg Na$_2$MoO$_4$.2H$_2$O, and 30 mg Na$_2$SeO$_3$. Research-grade chemicals and ultra-pure water (Nanopure Diamond, Barnstead) were used for all solutions.

2.3.2 Analytical Methods

Perchlorate, chlorate, chloride, and acetate were analyzed using a Dionex ICS2500 ion chromatograph (IC, Dionex Corporation, Sunnyvale, CA) with a 4-mm Dionex AS-11 column, an AG-11 pre-column, and a conductivity detector. An eluent gradient from 4mM to 50mM sodium hydroxide was used. The program consisted of a 5-minute equilibration time, injection of the sample, a 9-minute isocratic run, and a gradient of eluent over 2 minutes. A Dionex ASRS suppressor was used in internal recycle mode. Injection was performed with a Dionex AS40 Automated Sampler. The injection volume was 200 μL. The detection limit for chlorate was 5 μg/L.

Optical density (OD) at 600 nm was measured with a spectrophotometer (Spec 20, Thermo Spectronics, Rochester, NY) and converted to dry weight using an empirical conversion factor adapted from prior work (Salamone, 2006). The conversion factor used in this research was 575 mg/L DW / OD. This is similar to the conversion factor previously determined for PC1 (Nerenberg et al., 2006).

Epifluorescence micrographs were taken of three isolates, HCAP-C, PC1, and PDA. Cells were extracted during exponential growth and stained with electrophoresis grade acridine orange produced by Fisher Scientific.
2.3.3 Batch Tests

Batch tests were used to analyze chlorate accumulation by PCRB. All tests were conducted in 1-L bottles with 200 mL of media, or in 200-mL serum bottles with 30 mL of media. Bottles were capped with thick, butyl rubber stoppers with an aluminum-crimped seal. In order to create anaerobic conditions, bottles were successively vacuum-degassed to –25 psig and pressurized with pure nitrogen, three times. The final headspace contained pure nitrogen pressurized to 20 psig. Bottles were shaken at 200 rpm at ambient temperature (22°C).

Tests were mainly performed at perchlorate concentrations of approximately 200 mg/L. This concentration was chosen because Salamone (2006) showed that lower \( q_{\text{max}} \) values were obtained when perchlorate concentrations were above 200 mg/L, suggesting substrate inhibition occurs at high perchlorate concentrations.

Previous studies of HCAP by Nerenberg and Salamone did not consider the media type as a cause of the relatively high chlorate accumulation during perchlorate reduction. In order to test the possibility that media type affects the amount of chlorate accumulated during perchlorate reduction, batch tests were performed as described previously in five difference media types with initial perchlorate concentrations of approximately 200 mg/L. The five media types were LB broth, 16mM reactor medium, 16mM reactor medium with LB broth, high nutrient growth medium, and MS medium. These media types were chosen because they are used by labs that study microbial perchlorate reduction, but these labs have not reported chlorate accumulation.

LB broth was prepared according to directions described on the container of LB agar produced by MO BIO Laboratories, Inc. 16mMol reactor medium was prepared according to Nerenberg (2003). 16mMol reactor medium with LB broth is that medium described by Nerenberg with the addition of LB broth at 10% dilution. High nutrient growth medium is described in section 2.3.1. MS medium was prepared according to Logan et al., (2006). All media types had initial pH values between 6.3 and 6.9.
Acetate was used as the electron donor for all batch tests. A literature review of half maximum specific substrate utilization rates for several bacterial strains grown on acetate in anaerobic conditions is shown in Table 2-1. In order to prevent acetate from limiting the growth rate of bacteria during batch tests, initial concentrations were 600 mg/L and the final concentrations were around 400 mg/L, well above most estimates for K values for anaerobic growth on acetate (see section 2.3.5 for further description of K values). Although the estimated K value for KJ growth on acetate is high, most estimates for K values were below 200 mg/L.

### TABLE 2-1

REPORTED K VALUES FOR ISOLATES GROWN ON ACETATE

<table>
<thead>
<tr>
<th>Isolate(s)</th>
<th>K (mg/L)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Desulfo bacter postgatei</em></td>
<td>4.13</td>
<td>(Ingvorsen, 1984)</td>
</tr>
<tr>
<td>Thermophilic butyrate-utilizing bacterium and <em>Methanobacterium thermoautotrophicum</em></td>
<td>23.6</td>
<td>(Ahring, 1987)</td>
</tr>
<tr>
<td>KJ</td>
<td>470</td>
<td>(Logan et al., 2001)</td>
</tr>
<tr>
<td>PDX</td>
<td>45</td>
<td>(Logan et al., 2001)</td>
</tr>
<tr>
<td><em>D. acetoxidans</em></td>
<td>35.4</td>
<td>(Oude-Elferink et al., 1998)</td>
</tr>
<tr>
<td><em>D. amnigenus</em></td>
<td>35.4</td>
<td>(Oude-Elferink et al., 1995; Oude-Elferink et al., 1998)</td>
</tr>
<tr>
<td><em>Methanosarcina sp.</em></td>
<td>177</td>
<td>(Jetten et al., 1990; Oude-Elferink et al., 1994)</td>
</tr>
<tr>
<td><em>Methanosaeta soehngenii</em></td>
<td>23.6-41.3</td>
<td>(Jetten et al., 1990; Ohtsubo et al., 1992; Oude-Elferink et al., 1994)</td>
</tr>
<tr>
<td><em>Methanosaeta concilii</em></td>
<td>16.52-70.8</td>
<td>(Ohtsubo et al., 1992; Oude-Elferink et al., 1994)</td>
</tr>
<tr>
<td><em>Methanosaeta strain MTAS</em></td>
<td>29.5</td>
<td>(Ohtsubo et al., 1991; Ohtsubo et al., 1992)</td>
</tr>
<tr>
<td><em>Methanosaeta strain MTKO</em></td>
<td>69.03</td>
<td>(Ohtsubo et al., 1991; Ohtsubo et al., 1992)</td>
</tr>
</tbody>
</table>
2.3.4 Calculation of Yield for HCAP-C

The cell growth yields for HCAP-C on chlorate and perchlorate were determined from batch experiments performed during this study and in previous work by Anna Salamone (2006). Batch tests were performed as described previously with initial chlorate and perchlorate concentrations of approximately 200 mg/L.

To determine the yield on chlorate by HCAP-C, data from three batch tests performed by Anna Salamone were used. Total biomass produced during the test was divided by the total chlorate reduced to obtain an experimental yield. Total biomass produced is the difference between the final biomass and the initial biomass. Similarly, data from four batch tests performed during this study were used to determine HCAP-C yield on perchlorate. Again, total biomass produced during the test was divided by the total perchlorate reduced to obtain the yield.

2.3.5 Calculation of \( K \) and \( q_{\text{max}} \) for HCAP-C

Biodegradation of chlorate and perchlorate can be described by the Monod equation. Equation 2-1 describes the specific substrate utilization rate, \( q \) (mgS/mgX-day) (Rittmann and McCarty, 2001).

EQUATION 2-1

\[
q = q_{\text{max}} \frac{S}{S + K}
\]

In this equation, \( S \) is the rate-limiting substrate concentration (i.e., perchlorate) (mgS/L), \( K \) is the half maximum specific substrate utilization rate (mgS/L), and \( q_{\text{max}} \) is the maximum specific substrate utilization rate (mgS/mgX-day).

The Monod equation for perchlorate and chlorate reduction can be changed to account for competitive inhibition as shown in Equations 2-2 and 2-3 (Rittmann and McCarty, 2001).
In these equations, $S_p$ and $S_c$ are perchlorate and chlorate concentrations, respectively (mgS/L), $q_p$ is the specific perchlorate reduction rate (mgS/mgX-day), $q_{p\text{max}}$ is the maximum specific perchlorate reduction rate (mgS/mgX-day), $q_c$ is the specific chlorate reduction rate (mgS/mgX-day), $q_{c\text{max}}$ is the maximum specific chlorate reduction rate (mgS/mgX-day), $K_p$ and $K_c$ are the half-maximum-rate concentrations for perchlorate and chlorate (mg/L), and $X$ is the biomass concentration (mgX/L).

**AQUASIM.** The computer program AQUASIM version 2.1f was used to fit kinetic parameters to collected data. The Swiss Federal Institute for Environmental Science and Technology (EAWAG) designed AQUASIM for simulation and identification of aquatic systems (Reichert, 1995). The reduction of perchlorate and chlorate by HCAP-C was monitored and data were collected during this research project and during previous work performed by Anna Salamone. AQUASIM estimates kinetic parameters by minimizing the sum of the squares of the weighted deviations between
actual data and results of the calculated model. This is performed according to the following equation.

EQUATION 2-4

\[ \chi^2(p) = \sum_{i=1}^{n} \left( \frac{y_{\text{meas},i} - y_i(p)}{\sigma_{\text{meas},i}} \right)^2 \]

In Equation 2-4, \( y_{\text{meas},i} \) is the \( i \)\(^{th} \) measurement, \( \sigma_{\text{meas},i} \) is the standard deviation of that measurement, \( y_i(p) \) is the model variable corresponding to the \( i \)\(^{th} \) measurement, \( p = (p_1, \ldots, p_m) \) are the model parameters, and \( n \) is the number of data points.

2.3.6 Calculation of K for PDA

The half-maximum specific substrate utilization rate, K, was measured by monitoring the degradation rate of PDA on chlorate and 600 mg/L acetate in a non-growth medium of 16mM phosphate buffer. The buffer is the medium described in section 2.3.1 without the addition of trace mineral or Ca-Fe solution. Tests were run in an anaerobic glove box to prevent oxygen contamination. Non-growth medium was left in the glove box for a week before the tests were performed to ensure the absence of dissolved oxygen in the medium.

Three tests were run successively with initial concentrations of 10 mg/L, 5 mg/L, and 1 mg/L of chlorate. AQUASIM was again used to fit a half maximum specific substrate utilization rate to the data. Chlorate degradation was again modeled by Monod kinetics according to Equation 2-1. According to Monod kinetics, substrate is degraded according to the following equation.
In Equation 2-5, the rate of substrate degradation depends on the biomass concentration X, the half maximum specific substrate utilization rate K, and the maximum specific substrate utilization rate $q_{\text{max}}$. When the substrate concentration is much larger than the half maximum specific substrate utilization rate, K can be neglected and the equation reduces to Equation 2-6.

EQUATION 2-6

$$\frac{dS}{dt} = Xq_{\text{max}}$$

Because degradation is occurring under non-growth conditions, X is a constant and therefore substrate degradation should be linear with respect to time when S is much greater than K. In a batch reactor, substrate concentrations are initially high and K can be neglected. As the substrate concentration decreases during biological reduction and approaches the value of K, degradation will no longer be linear. By keeping X a constant, K can be approximated as the substrate concentration at which degradation of the substrate is no longer linear. In the model, the yield on chlorate was 0.34 mgX/mgClO$_3^-$-day.

2.4 Results

Epifluorescence micrographs of isolates HCAP-C, PC1, and PDA are shown in Figures 2.1, 2.2, and 2.3 below. HCAP-C appear to be rods approximately 1μm in
length. PC1 also appear rod-shaped, but are about 0.2μm in length. PDA appear to be spherical cocci and are about 0.5μm in diameter.

Figure 2-1 Epifluorescence micrograph of *Dechlorosoma* sp. HCAP-C

Figure 2-2 Epifluorescence micrograph of *Dechloromonas* sp. PC1
2.4.1 Chlorate Accumulation among PCRB

Figures 2.4, 2.5 and 2.6 shown below are results of batch tests described with conditions stated previously. Initial perchlorate concentrations were approximately 200 mg/L. Isolates HCAP-C and PC1 behave as HCAP, accumulating maximum chlorate concentrations 24% and 10.4% of the initial perchlorate concentration. Although previous tests suggest PC1 behaves as a conventional PCRB (Nerenberg et al., 2006; Salamone, 2006), this study found the strain to behave as an HCAP. The discrepancy between this study and previous research is still under investigation. Species KJ behaves as a conventional PCRB accumulating a maximum concentration of chlorate 0.65% of the initial perchlorate concentration.
Figure 2-4 Batch growth of *Dechlorosoma* sp. HCAP-C. Maximum chlorate accumulation was 42.2 mg/L, approximately 24% of the initial perchlorate concentration.

Figure 2-5 Batch growth of *Dechloromonas* sp. PC1. Maximum chlorate accumulation was 18.6 mg/L, approximately 10.4% of the initial perchlorate concentration.
Figure 2-6 Batch growth of *Dechlorosoma* sp. KJ. Maximum chlorate accumulation was 1.3 mg/L, approximately 0.65% of the initial perchlorate concentration.

### 2.4.2 Effect of Growth Medium on Chlorate Accumulation

HCAP-C accumulated chlorate to levels over 20% of the initial perchlorate concentration in all five media types tested. Table 2-2 below lists the compiled results of the test. It appears that media type does not cause chlorate accumulation among HCAP, and therefore the cause may be attributed to other factors such as the genetic makeup of the (per)chlorate reductase gene.
TABLE 2-2

RESULTS OF A MEDIA TEST PERFORMED ON HCAP-C.

<table>
<thead>
<tr>
<th>Media</th>
<th>Maximum ClO₃⁻ Accumulation [a]</th>
<th>% Accumulation [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB broth</td>
<td>36.4 mg/L</td>
<td>21.6%</td>
</tr>
<tr>
<td>16mM Reactor Medium</td>
<td>38.3 mg/L</td>
<td>20.7%</td>
</tr>
<tr>
<td>LB+16mM Reactor Medium</td>
<td>37.1 mg/L</td>
<td>22.0%</td>
</tr>
<tr>
<td>MS</td>
<td>40.4 mg/L</td>
<td>22.4%</td>
</tr>
<tr>
<td>High Nutrient Medium</td>
<td>42.2 mg/L</td>
<td>23.9%</td>
</tr>
</tbody>
</table>

[a] Initial perchlorate concentrations were approximately 200 mg/L.
[b] Percent accumulation is relative to initial perchlorate concentration.

2.4.3 Determining Kinetic Parameters of HCAP-C

HCAP appear to have slower perchlorate reduction kinetics than conventional PCRB (Salamone, 2006). To determine the reduction kinetics of HCAP, perchlorate and chlorate reduction by HCAP-C was studied. Results of batch tests performed on HCAP-C were used to determine parameters. Batch tests were performed as described in section 2.3.3.

Calculation of Yield.

Results of the calculation of yield on chlorate and perchlorate by HCAP-C are shown below in Tables 2-3 and 2-4. In the tables, the yield on perchlorate is measured in mgX/mgS-day, where X is dry weight biomass concentration measured in mg/L. The plots used to obtain the yields are provided in Appendix A.
### TABLE 2-3

**EXPERIMENTAL YIELD ON CHLORATE BY HCAP-C**

<table>
<thead>
<tr>
<th>Batch Test</th>
<th>Initial Biomass (mg/L)</th>
<th>Final Biomass (mg/L)</th>
<th>Chlorate reduced (mg/L)</th>
<th>Yield (mgX/mgClO$_3^-$-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClO$_3^-$ Test 1</td>
<td>1.73</td>
<td>74.2</td>
<td>213.5</td>
<td>0.34</td>
</tr>
<tr>
<td>ClO$_3^-$ Test 2</td>
<td>5.46</td>
<td>92.6</td>
<td>209.7</td>
<td>0.42</td>
</tr>
<tr>
<td>ClO$_3^-$ Test 3</td>
<td>3.45</td>
<td>69.0</td>
<td>238.8</td>
<td>0.27</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
</tbody>
</table>
TABLE 2-4
EXPERIMENTAL YIELD ON PERCHLORATE BY HCAP-C

<table>
<thead>
<tr>
<th>Batch Test</th>
<th>Initial Biomass (mg/L)</th>
<th>Final Biomass (mg/L)</th>
<th>Perchlorate reduced (mg/L)</th>
<th>Yield (mgX/mgClO$_4^-$-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ClO$_4^-$ Test 1</td>
<td>5.75</td>
<td>66.7</td>
<td>167.7</td>
<td>0.36</td>
</tr>
<tr>
<td>ClO$_4^-$ Test 2</td>
<td>2.88</td>
<td>109.3</td>
<td>184.0</td>
<td>0.58</td>
</tr>
<tr>
<td>ClO$_4^-$ Test 3</td>
<td>1.73</td>
<td>77.6</td>
<td>197.1</td>
<td>0.39</td>
</tr>
<tr>
<td>ClO$_4^-$ Test 4</td>
<td>4.03</td>
<td>75.9</td>
<td>226.2</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Average | 0.41 |
Standard Deviation | 0.12 |

The yield determined for perchlorate is based on the complete reduction of perchlorate to chloride, but in the competitive inhibition models used in this study shown below in section 2.5.2, the yield on perchlorate is based on the partial reduction of perchlorate to chlorate. To determine this partial yield, the experimental yield determined for the complete reduction of perchlorate was converted on a molar basis to the yield of partial reduction of perchlorate to chlorate. One mole of perchlorate is reduced to one mole of chlorate during perchlorate reduction. Therefore, the yield determined for complete reduction of perchlorate was converted to a molar yield by multiplying by the molecular weight of perchlorate (99.5 mg/mol) resulting in a yield of 40.8 mgX/mol ClO$_4^-$. Similarly, the yield for the reduction of chlorate was converted to a molar yield by multiplying by the molecular weight of chlorate (83.5 mg/mol) resulting in a yield of 28.4 mgX/mol ClO$_3^-$. To find the yield for incomplete reduction of perchlorate, the molar yield for chlorate was subtracted from the molar yield for complete reduction.
reduction of perchlorate, 12.4 mgX/mol ClO₄⁻. This molar yield was then converted back to a mass yield by dividing by the molecular weight of perchlorate. The resulting yield for the incomplete reduction of perchlorate to chlorate was 0.1 mgX/mgClO₄⁻-day.

The determined yield on the reduction of perchlorate to chlorate was consistent with the theoretical yield determined from the yield on chlorate and the number of electrons transferred. This theoretical yield was also 0.1 mgX/mgClO₄⁻-day. This theoretical yield was based on the experimental yield on chlorate, 0.34 mgX/mgClO₃⁻-day. The calculations are shown in Appendix B.

**Calculation of $K$ and $q_{\text{max}}$**

**Chlorate.** The reduction kinetics of chlorate for HCAP-C are much simpler than those of perchlorate, and were therefore the first to be estimated. This relative simplicity is due to the absence of competitive inhibition during chlorate reduction. Equation 2-1 was used in the program to estimate kinetic parameters. Data taken from three batch experiments performed by Anna Salamone were used in the program as measured data, and output kinetic parameters were averaged to obtain final reduction kinetics for chlorate. All batch tests were performed with conditions described previously with initial chlorate concentrations of approximately 200 mg/L.

The results of the estimation of chlorate reduction kinetics by AQUASIM are shown in Table 2-5 below. AQUASIM graphs of the results are presented in Appendix C.
Perchlorate. Two sets of perchlorate reduction parameters were found for HCAP-C. The first set neglects competitive inhibition and uses the standard Monod equation, Equation 2-1. The second set of parameters accounts for competitive inhibition, as described in section 2.5.1, and uses Equations 2-2 and 2-3. Estimated parameters of chlorate reduction (shown above) were used in both models for perchlorate reduction. Data taken from three batch experiments performed during this research project were used in the models, and output kinetics were again averaged. All batch tests were performed with conditions described previously with initial perchlorate concentrations of approximately 200 mg/L.

The two parameters K and $q_{\text{pmax}}$ were found simultaneously by the AQUASIM program. According to Monod kinetics, the rate of substrate utilization, $q$, depends on both K and $q_{\text{pmax}}$ according to Equation 2-1. When the substrate concentration, S, is high, K may be neglected if it is small enough relative to S. The model uses these data points to find the value of $q_{\text{pmax}}$. At low substrate concentrations, K is inversely proportional while $q_{\text{pmax}}$ is directly proportional to q. These points are used to estimate K. When there are not enough data points at high S concentrations, the program cannot find $q_{\text{pmax}}$ and
there may exist multiple sets of \( q_{\text{max}} \) and \( K \) that fit the same data set. It is possible that because batch test 3 had few data points at high S concentrations, the AQUASIM model accounting for competitive inhibition was unable to estimate both \( q_{\text{max}} \) and \( K \). Therefore, an average \( K \) value from batch tests 1 and 2 was used in the model to solve for \( q_{\text{prmax}} \).

The results of the estimation of perchlorate reduction kinetics by AQUASIM neglecting competitive inhibition are shown in Table 2-6 below and those accounting for competitive inhibition are shown in Table 2-7. AQUASIM graphs are presented in Appendix C.

### TABLE 2-6

PERCHLORATE REDUCTION KINETICS FOR HCAP-C USING STANDARD MONOD EQUATIONS

<table>
<thead>
<tr>
<th></th>
<th>( q_{\text{prmax}} ) (mgS/mgX-day)</th>
<th>( K ) (mgS/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{ClO}_4^- ) Batch Test 1</td>
<td>4.9</td>
<td>64.4</td>
</tr>
<tr>
<td>( \text{ClO}_4^- ) Batch Test 2</td>
<td>4.7</td>
<td>88.2</td>
</tr>
<tr>
<td>( \text{ClO}_4^- ) Batch Test 3</td>
<td>3.6</td>
<td>77.3</td>
</tr>
<tr>
<td>Average</td>
<td>4.4</td>
<td>76.6</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.7</td>
<td>11.9</td>
</tr>
</tbody>
</table>
The model neglecting competitive inhibition produced a K value 39.8% of the K produced by the competitive inhibition model and a \( q_{\text{pmax}} \) value 38.3% of the \( q_{\text{pmax}} \) produced by the competitive inhibition model. This large difference in \( q_{\text{pmax}} \) was expected and also found by Salamone (2006) in her estimations of \( q_{\text{pmax}} \) for HCAP-C. According to the theory of competitive inhibition, chlorate interferes with the reduction of perchlorate, slowing the overall reduction rate. If competitive inhibition were neglected but chlorate accumulated, this reduced rate would be interpreted as a smaller \( q_{\text{pmax}} \) and possibly a larger K. Because it appears competition is taking place between chlorate and perchlorate for the (per)chlorate reductase enzyme, it is important to account for that competition in order to arrive at accurate estimations of \( q_{\text{pmax}} \) and K.

Table 2-8 below is a compilation of parameters from various PCRB found in the literature by Salamone (2006). Parameters are based on basic Monod kinetics neglecting competitive inhibition. Results of kinetic parameter estimations neglecting competitive inhibition from this study on HCAP-C have also been included.
TABLE 2-8

COMPARISON WITH PARAMETERS FROM LITERATURE FOR PCRB

[ADAPTED FROM SALAMONE (2006)]

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Acceptor</th>
<th>$q_{\text{max}}$ mg acceptor mgDW^{-1} day^{-1}</th>
<th>$K$ mg/L</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio dechloratans</em></td>
<td>Perchlorate</td>
<td>1.68</td>
<td>-</td>
<td>(Korenkov et al., 1976)</td>
</tr>
<tr>
<td><em>Wolinella succcinogenes</em> HAP-1</td>
<td>Perchlorate</td>
<td>2.57</td>
<td>-</td>
<td>(Wallace et al., 1996; Wallace et al., 1998)</td>
</tr>
<tr>
<td>GR-1</td>
<td>Perchlorate</td>
<td>5.65</td>
<td>-</td>
<td>(Rikken et al., 1996)</td>
</tr>
<tr>
<td>KJ</td>
<td>Perchlorate</td>
<td>24</td>
<td>33</td>
<td>(Logan et al., 2001)</td>
</tr>
<tr>
<td>PDX</td>
<td>Perchlorate</td>
<td>7.5</td>
<td>12</td>
<td>(Logan et al., 2001)</td>
</tr>
<tr>
<td>SN1A</td>
<td>Perchlorate</td>
<td>4.60</td>
<td>2.2</td>
<td>(Waller, 2004; Waller et al., 2004)</td>
</tr>
<tr>
<td>ABL1</td>
<td>Perchlorate</td>
<td>5.43</td>
<td>4.8</td>
<td>(Waller et al., 2004)</td>
</tr>
<tr>
<td>INS</td>
<td>Perchlorate</td>
<td>4.35</td>
<td>18</td>
<td>(Waller et al., 2004)</td>
</tr>
<tr>
<td>RC1</td>
<td>Perchlorate</td>
<td>6.00</td>
<td>12</td>
<td>(Waller et al., 2004)</td>
</tr>
<tr>
<td>PC1</td>
<td>Perchlorate</td>
<td>3.1</td>
<td>0.14</td>
<td>(Nerenberg et al., 2006)</td>
</tr>
<tr>
<td>HCAP-C</td>
<td>Perchlorate</td>
<td>3.0</td>
<td>42</td>
<td>(Salamone, 2006)</td>
</tr>
<tr>
<td>HCAP-C</td>
<td>Perchlorate</td>
<td>4.4</td>
<td>76.6</td>
<td>This Study</td>
</tr>
<tr>
<td>GR-1</td>
<td>Chlorate</td>
<td>7.48</td>
<td>-</td>
<td>(Rikken et al., 1996)</td>
</tr>
<tr>
<td>PC1</td>
<td>Chlorate</td>
<td>6.3</td>
<td>&lt;0.014</td>
<td>(Nerenberg et al., 2006)</td>
</tr>
<tr>
<td>HCAP-C</td>
<td>Chlorate</td>
<td>6.5</td>
<td>21</td>
<td>(Salamone, 2006)</td>
</tr>
<tr>
<td>HCAP-C</td>
<td>Chlorate</td>
<td>8.3</td>
<td>58.3</td>
<td>This Study</td>
</tr>
</tbody>
</table>

*Kinetic parameters were determined neglecting competitive inhibition*

While the maximum specific substrate utilization rates, $q_{\text{max}}$, for HCAP-C found in this study and in a previous study by Salamone (2006) are similar to other PRCB, $K$ values for HCAP-C on both chlorate and perchlorate appear to be relatively high compared to other PCRB. A high $K$ value usually correlates to high growth thresholds, and HCAP-C would therefore need a higher perchlorate concentration to compete effectively with common PCRB (Rittmann and McCarty, 2001). This is important for remediation purposes when the perchlorate concentrations are low and suggests that HCAP would not play a significant role at concentrations below 200 mg/L.

The $K$ and $q_{\text{max}}$ values found in this study for HCAP-C grown on chlorate and perchlorate were high relative to those found by Salamone (2006). This difference can be
attributed to the fact that different methods were used to acquire estimations of kinetic parameters. Salamone (2006) found parameters by visually fitting a model to experimental data. In this study, the computer program AQUASIM used a mathematical solver to determine the best fit of a model to the experimental data.

2.4.4 Determining Half Maximum Specific Substrate Utilization Rate of PDA

Results of three tests performed on PDA as described previously are shown below in Table 2-9. AQUASIM models are shown in Appendix D.

<table>
<thead>
<tr>
<th>Test</th>
<th>Initial ClO$_3^-$ concentration</th>
<th>K (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 mg/L</td>
<td>0.40</td>
</tr>
<tr>
<td>B</td>
<td>5 mg/L</td>
<td>5.0E-6</td>
</tr>
<tr>
<td>C</td>
<td>10 mg/L</td>
<td>7.5E-5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td>0.23</td>
</tr>
</tbody>
</table>

The results of AQUASIM estimations for K varied greatly among the three tests. An average value of 0.13 mg/L was obtained, but the standard deviation of this value was 0.23 mg/L. Since the models could not give a consistent result for a K value, it is estimated that K for PDA on chlorate is lower than 0.4 mg/L, but an actual value could not be obtained from the results of this study.
2.5 Conclusions

According to the estimated kinetic parameters for HCAP-C based on AQUASIM modeling, it appears that the $q_{\text{max}}$ and $K$ values for HCAP-C are higher than conventional PCRB. Most significantly, $K$ for growth on perchlorate was extremely high, 192.6 mg/L, more than four times higher than any other reported $K$ values for PCRB. A high $K$ for HCAP-C was also found in a previous study by Salamone (2006). High $K$ values likely result in high growth thresholds, meaning HCAP-C may not survive under low perchlorate concentrations. Therefore, HCAP-C may not play a major role at contamination sites with perchlorate concentrations below 200 mg/L. However, the high $q_{\text{max}}$ and $K$ values also suggest that when coupled with CRB or PCRB so that competitive inhibition can be ignored, HCAP may be more efficient at reducing perchlorate than PCRB at very high concentrations, e.g. at the g/L scale.

An accurate $K$ value for PDA, a CRB, was not determined during this study. It is likely that the $K$ value for PDA is below 0.4 mg/L.
CHAPTER 3
CO-CULTURES WITH HCAP-C

3.1 Introduction

Although pure-culture isolates of HCAP accumulate large amounts of chlorate during batch degradation of perchlorate, chlorate accumulation has not been reported in the field. Although this may be simply because investigators were not measuring for chlorate at the appropriate concentrations, another potential explanation is that syntrophic relationships exist among HCAP, PCRB, and CRB, where these relationships prevent accumulation of chlorate. If these relationships exist, they could also alter the overall reaction kinetics of perchlorate, with respect to that observed in pure-culture isolates. Although typically it is beneficial to determine pure-culture kinetics for the design of engineering systems, these kinetic parameters may not be suitable for direct use if such relationships exist among different species of perchlorate and chlorate reducers.

3.2 Previous Co-Culture Experiments in Batch

To determine potential relationships among HCAP, PCRB, and CRB, Salamone (2006) performed two separate studies of co-cultures of HCAP with (a) PCRB and (b) CRB. This preliminary study found that in a co-culture of HCAP and PCRB, slightly faster reduction kinetics of perchlorate were observed than in a pure-culture of PCRB, and much higher kinetics were observed than with a pure-culture of HCAP. The study also found that in a co-culture of HCAP with CRB, slightly faster reduction kinetics of perchlorate were again seen as compared with pure-culture HCAP. A weakness of these
studies was that the tests were only run once, so results could not be checked for reproducibility.

3.2.1 Co-Culture Experiments

To check the reproducibility of results from Salamone (2006), a series of co-cultures grown on perchlorate were monitored for reduction time of perchlorate and for accumulation of chlorate during that reduction.

Batch tests were prepared in high growth medium as described in Chapter 2 with initial perchlorate concentrations of 200 mg/L. Inoculate biomass was in exponential growth, and initial biomass concentrations were approximately equal for each batch test, ranging from 1.7 to 2.9 mg/L. Bottles were inoculated in an anaerobic glove chamber so that bacteria were not exposed to atmospheric oxygen. Biomass concentration was monitored throughout the experiments. Analytical methods were those described in Chapter 2.

Three scenarios were tested for the co-culture of HCAP-C and PDA and all three scenarios were run twice. The scenarios were the following:

1. A co-culture with a biomass ratio of HCAP-C to PDA of 7:1 (12.5% PDA)
2. A co-culture with a biomass ratio of HCAP-C to PDA of 1:1 (50% PDA)
3. A co-culture with a biomass ratio of HCAP-C to PDA of 1:3 (75% PDA)

Six batch tests were prepared with ratios of initial biomass concentrations as described by the three scenarios listed above. Additionally, pure-culture HCAP-C was grown in batch with an initial biomass concentration approximately equal to that of the co-cultures with an initial perchlorate concentration of 200 mg/L.
3.2.2 Results of Co-Culture Experiments

Figure 3-1 below is a graph of perchlorate reduction by pure-culture HCAP-C and by co-cultures of HCAP-C and PDA.

![Co-Cultures HCAP-C and PDA](image)

**Figure 3-1 First round of perchlorate reduction tests on Co-Cultures**

When the test was run a second time, the results were slightly different. Figure 3-2 shows results of a second run of co-cultures with initial conditions identical to those of the first run.
The amount of chlorate accumulated by the different co-cultures was inconsistent between the first and second rounds of tests. In the first test, the co-culture with a ratio of 7:1 HCAP-C to PDA accumulated the most chlorate, a maximum of 21.4 mg/L. However, in the second test, this co-culture only accumulated a maximum of 0.2 mg/L chlorate. This result is inconsistent with the previous test on the 7:1 co-culture, and aside from experimental error, a possible explanation is the long lag time before significant reduction of perchlorate occurred. During this lag time, PDA may have had enough time to grow to a more significant concentration than in the previous test. Another inconsistency is the co-culture of equal parts HCAP-C and PDA that accumulated 4.8 mg/L in test 1 and 11.2 mg/L in test 2.
Table 3-1 is a compiled list of the maximum amount of chlorate accumulated during the tests as well as the initial amount of biomass for each test.

**TABLE 3-1**

**MAXIMUM OBSERVED CHLORATE AND INITIAL BIOMASS CONCENTRATIONS FOR CO-CULTURES**

<table>
<thead>
<tr>
<th>Test</th>
<th>HCAP-C Control</th>
<th>7:1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1:1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1:3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Salamone&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Maximum Observed Chlorate (mg/L)</td>
<td>47.0</td>
<td>29.8</td>
<td>0.2</td>
<td>4.8</td>
<td>11.2</td>
</tr>
<tr>
<td>% of Initial Perchlorate</td>
<td>22.2</td>
<td>14.1</td>
<td>0.1</td>
<td>2.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Initial Biomass (mg/L)</td>
<td>2.9</td>
<td>1.7</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ratio of HCAP-C to PDA in a co-culture

<sup>b</sup>Results of Salamone (2006) test on a co-culture of approximately equal parts of HCAP-C and PDA

Co-culture tests run by Salamone (2006) were performed with approximately equal amounts of initial PDA and HCAP-C. Results of those tests suggested that a co-culture of HCAP-C and PDA have a faster reduction rate of perchlorate than HCAP-C alone. In the research presented here, bottles were inoculated with different initial amounts of HCAP-C and therefore the time for complete reduction of perchlorate could not be compared among the tests to determine which had the fastest rate of perchlorate reduction. To compare the specific rates of perchlorate reduction, kinetic parameters were determined for each co-culture test using the AQUASIM program. Since the goal was simply to compare the global perchlorate reduction rates, the program was used with
standard Monod equations for perchlorate reduction to chloride, neglecting competitive inhibition. These kinetic parameters obtained by the program for the co-cultures were compared to those of pure culture HCAP-C obtained in a similar fashion. Table 3-2 below shows the results of those parameter estimations. AQUASIM plots of parameter estimations are presented in Appendix E.

### TABLE 3-2

**KINETIC PARAMETERS OF CO-CULTURES**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>HCAP-C Control</th>
<th>7:1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1:1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>1:3&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>K (mg/L)</td>
<td>39</td>
<td>10</td>
<td>43</td>
<td>0.2</td>
</tr>
<tr>
<td>q&lt;sub&gt;max&lt;/sub&gt; (mgS/mgX-d)</td>
<td>2.1</td>
<td>1.6</td>
<td>3.3</td>
<td>2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Kinetic parameters were determined based on basic Monod kinetics neglecting competitive inhibition

<sup>b</sup>Ratio of HCAP-C to PDA in a co-culture

The average K for the co-cultures was 21 mg/L with a standard deviation of 15. This is slightly lower than the K value obtained for the pure-culture HCAP-C control, 39 mg/L. The average q<sub>max</sub> for the co-cultures was 2.7 mgS/mgX-d with a standard deviation of 0.88. This is slightly higher than the q<sub>max</sub> obtained for the control, 2.1 mgS/mgX-d, but lower than the q<sub>max</sub> obtained from previous experiments, 4.4 mgS/mgX-d. A lower K and higher q<sub>max</sub> generally correlates to a faster overall reduction rate.

### 3.2.3 Modeling Co-Cultures of HCAP-C and PDA in Batch

AQUASIM modeling was used to confirm HCAP-C and PDA co-culture test results presented above. Three scenarios were tested, all of which had initial perchlorate
concentrations of 200 mg/L and initial biomass concentrations of 2 mg/L. The scenarios were:

1. A co-culture with a biomass ratio of HCAP-C to PDA of 7:1 (12.5% PDA)
2. A co-culture with a biomass ratio of HCAP-C to PDA of 1:1 (50% PDA)
3. A co-culture with a biomass ratio of HCAP-C to PDA of 1:3 (75% PDA)

Additionally, pure-culture HCAP-C grown in batch was also modeled with AQUASIM. The initial perchlorate concentration was 200 mg/L and the initial biomass concentration was 2 mg/L.

3.2.4 AQUASIM Parameters

The computer program AQUASIM version 2.1f was used in this modeling test (Reichert, 1995). Model variables, parameters, and processes are listed in Tables 3-3, 3-4, and 3-5 respectively. An example of the model compartments is listed in Table 3-6 and is a list of the model compartments for a co-culture of HCAP-C and PDA in a batch reactor with a 7:1 ratio of HCAP-C to PDA.

### TABLE 3-3

**MODEL VARIABLES**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Chloride concentration</td>
<td>mg/L</td>
</tr>
<tr>
<td>C3</td>
<td>Chlorate concentration</td>
<td>mg/L</td>
</tr>
<tr>
<td>C4</td>
<td>Perchlorate concentration</td>
<td>mg/L</td>
</tr>
<tr>
<td>Xc</td>
<td>CRB biomass</td>
<td>mg/L</td>
</tr>
<tr>
<td>XH</td>
<td>HCAP biomass</td>
<td>mg/L</td>
</tr>
</tbody>
</table>
### TABLE 3-4

**MODEL PARAMETERS**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>Specific decay rate</td>
<td>0.05</td>
<td>mgX/mgX-day</td>
</tr>
<tr>
<td>Kc_C3</td>
<td>K for chlorate for CRB</td>
<td>1</td>
<td>mg/L</td>
</tr>
<tr>
<td>KH_C3</td>
<td>K for chlorate for HCAP</td>
<td>58.3</td>
<td>mg/L</td>
</tr>
<tr>
<td>KH_C4</td>
<td>K for perchlorate for HCAP</td>
<td>192.6</td>
<td>mg/L</td>
</tr>
<tr>
<td>qc_C3</td>
<td>q for chlorate for CRB</td>
<td>9</td>
<td>mg C3/mgX-day</td>
</tr>
<tr>
<td>qH_C3</td>
<td>q for chlorate for HCAP</td>
<td>8.3</td>
<td>mg C3/mgX-day</td>
</tr>
<tr>
<td>qH_C4</td>
<td>q for perchlorate for HCAP</td>
<td>11.5</td>
<td>mg C4/mgX-day</td>
</tr>
<tr>
<td>Y_C3</td>
<td>Yield on chlorate</td>
<td>0.34</td>
<td>mgX/mgC4-day</td>
</tr>
<tr>
<td>Y_C4</td>
<td>Yield from perchlorate to chlorate</td>
<td>0.1</td>
<td>mgX/mgC3-day</td>
</tr>
</tbody>
</table>

### TABLE 3-5

**MODEL PROCESSES**

<table>
<thead>
<tr>
<th>Process Name</th>
<th>Description</th>
<th>Rate</th>
<th>Stoichiometric Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorate</td>
<td>Chorate degradation</td>
<td>qH_C3<em>XH</em>C3/(C3+KH_C3*(1+C4/KH_C4)) + qc_C3<em>Xc</em>C3/(C3+Kc_C3) - b*Xc</td>
<td>C3 C4 Xc XH Xp</td>
</tr>
<tr>
<td>Perc</td>
<td>Perchlorate reduction to chlorate</td>
<td>qH_C4<em>XH</em>C4/(C4+KH_C4*(1+C3/KH_C3))</td>
<td>86.5/99.5 -1</td>
</tr>
<tr>
<td>Xc</td>
<td>CRB biomass formation</td>
<td>qc_C3<em>Xc</em>Y_C3<em>C3/(C3+Kc_C3)-b</em>Xc</td>
<td>1</td>
</tr>
<tr>
<td>XH</td>
<td>HCAP biomass formation</td>
<td>qH_C4<em>XH</em>Y_C4<em>C4/(C4+KH_C4)+qH_C3</em>XH<em>Y_C3</em>C3/(C3+KH_C3)-b*XH</td>
<td>1</td>
</tr>
</tbody>
</table>
The kinetic parameters used in the AQUASIM program for HCAP-C were those gathered during this study and presented in Chapter 2. The $q_{\text{max}}$ for PDA was estimated from $\mu_{\text{max}}$ and $Y$ from Logan et al. (2001) from the equation $q_{\text{max}} = \mu_{\text{max}} / Y$ (Rittmann and McCarty, 2001) as performed previously by Salamone (2006).

### 3.2.5 Results of HCAP-C and PDA Co-Culture Modeling

Results of AQUASIM modeling are presented in Appendix E. There appeared to be a trend between the ratio of HCAP-C to PDA and the time required to reduce
perchlorate to 0.1 mg/L, an arbitrary value. The ratio of 7:1 HCAP-C to PDA reduced perchlorate to 0.1 mg/L in 7.5 days while the ratio of 1:1 and 1:3 did the same in 9.2 days and 10.6 days respectively. This trend was expected because it should take longer for the perchlorate to be reduced when HCAP-C starts at a lower concentration.

Less chlorate was accumulated in batch reactors with high initial concentrations of PDA. This was also expected because PDA reduces the chlorate accumulated by HCAP-C. The reactor with an initial ratio of HCAP-C to PDA of 7:1 accumulated approximately 6.9 mg/L chlorate while the reactors with initial ratios of 1:1 and 1:3 HCAP-C to PDA accumulated approximately 0.9 mg/L and 0.4 mg/L chlorate respectively.

3.2.6 Conclusions

Initial studies by Salamone (2006) found that a co-culture of HCAP-C and PDA had faster reduction kinetics for perchlorate than HCAP-C alone. This study finds that $q_{\text{max}}$ appears to increase with increasing amounts of PDA, but in all cases the $q_{\text{max}}$ values were lower than those found for pure-culture HCAP-C in Chapter 2. The range of maximum observed chlorate accumulation was lower than that of the control pure-culture HCAP-C, suggesting that there is a syntrophic relationship between HCAP-C and PDA whereby PDA reduces chlorate accumulated by HCAP-C during perchlorate degradation.

Models of these experiments confirm results of laboratory tests on co-cultures of PDA and HCAP-C, namely that complete reduction of 200 mg/L perchlorate occurs between 8 and 15 days and chlorate accumulation ranges between 0.2 and 21.4 mg/L.

3.3 Modeling Co-Cultures with HCAP-C in a CSTR

Models of co-cultures using AQUASIM were conducted by Salamone (2006). However, these models only predicted the relationships among HCAP, PCRB, and CRB in a batch reactor. Predicting co-cultures in a CSTR may be significant for several reasons. First, if CRB are able to degrade the ClO$_3^-$ accumulated by HCAP in a co-
culture, HCAP growth would suffer because it would not be able to benefit from growth on the conversion of ClO$_3^-$ to Cl$^-$. This step involves a six-electron transfer as opposed to the conversion of ClO$_4^-$ to ClO$_3^-$, which is only a two-electron transfer. In a batch growth co-culture, HCAP would be at a disadvantage because it has a slightly slower conversion rate of ClO$_3^-$ (8.3mgClO$_3$/mgX-day) (this study) than does CRB (9mgClO$_3$/mgX-day) (Salamone, 2006), and a much higher K (58.3 mgS/L)(this study) than does CRB (1 mgS/L)(this study). However, in a CSTR, perchlorate would be constantly fed to the reactor giving HCAP a chance to reach a steady-state biomass concentration growing on the conversion of ClO$_4^-$ to ClO$_3^-$ alone. The same is true of a co-culture of PCRB and CRB, which may have faster kinetics of perchlorate degradation than PCRB alone because CRB may be able to grow on ClO$_3^-$ faster than PCRB whose (per)chlorate reductase enzymes must reduce both ClO$_4^-$ and ClO$_3^-$. Models of co-cultures were performed to test a few hypotheses pertaining to the syntrophic relationships that may exist between HCAP, PCRB, and CRB at steady-state conditions in a CSTR. These hypotheses were the following:

1. High accumulation of ClO$_3^-$ has not yet been observed in the natural environment because PCRB and CRB grow on the ClO$_3^-$ accumulated by HCAP.

2. CRB can accelerate reduction of ClO$_4^-$ in a CSTR by removing the ClO$_3^-$ from the degradation pathway of PCRB or HCAP, preventing competitive inhibition of the (per)chlorate reductase enzyme.

3. HCAP grown in the presence of CRB have faster perchlorate reduction kinetics than PCRB alone because of the syntrophic relationship that occurs between CRB and HCAP.

4. Given their high K for perchlorate and chlorate, HCAP will not play a significant role in the environment at low perchlorate concentrations.
To test these hypotheses, several models were constructed in which HCAP, PCRB, and CRB were grown in a CSTR co-culture with an influent concentration of 200 mg/L perchlorate.

3.3.1 AQUASIM Parameters

AQUASIM was again used to model the hypotheses presented above. Model variables, parameters, and processes are listed in Tables 3-7, 3-8, and 3-9 respectively. An example of the model compartments is listed in Table 3-10 and is a list of the model compartments for a co-culture of HCAP, PCRB, and CRB. The hydraulic retention time (HRT) for the reactors was 10 days and total biomass in the CSTR was 10 mg/L.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Chloride concentration</td>
<td>mg/L</td>
</tr>
<tr>
<td>C3</td>
<td>Chlorate concentration</td>
<td>mg/L</td>
</tr>
<tr>
<td>C4</td>
<td>Perchlorate concentration</td>
<td>mg/L</td>
</tr>
<tr>
<td>Xc</td>
<td>CRB biomass</td>
<td>mg/L</td>
</tr>
<tr>
<td>XH</td>
<td>HCAP biomass</td>
<td>mg/L</td>
</tr>
<tr>
<td>Xp</td>
<td>PCRB biomass</td>
<td>mg/L</td>
</tr>
</tbody>
</table>
### TABLE 3-8
MODEL PARAMETERS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>Specific decay rate</td>
<td>0.05</td>
<td>mgX/mgX-day</td>
</tr>
<tr>
<td>Kc_C3</td>
<td>K for chlorate for CRB</td>
<td>1</td>
<td>mg/L</td>
</tr>
<tr>
<td>KH_C3</td>
<td>K for chlorate for HCAP</td>
<td>58.3</td>
<td>mg/L</td>
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<tr>
<td>KH_C4</td>
<td>K for perchlorate for HCAP</td>
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<td>mg/L</td>
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<tr>
<td>Kp_C3</td>
<td>K for chlorate for PCRB</td>
<td>0.0012</td>
<td>mg/L</td>
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<td>Kp_C4</td>
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<td>mg/L</td>
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<td>qc_C3</td>
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<td>mg C3/mgX-day</td>
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<td>qH_C3</td>
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<tr>
<td>qH_C4</td>
<td>q for perchlorate for HCAP</td>
<td>11.5</td>
<td>mg C4/mgX-day</td>
</tr>
<tr>
<td>qp_C3</td>
<td>q for chlorate for PCRB</td>
<td>5</td>
<td>mg C3/mgX-day</td>
</tr>
<tr>
<td>qp_C4</td>
<td>q for perchlorate for PCRB</td>
<td>8</td>
<td>mg C4/mgX-day</td>
</tr>
<tr>
<td>Y_C3</td>
<td>Yield on chlorate</td>
<td>0.34</td>
<td>mgX/mgC4-day</td>
</tr>
<tr>
<td>Y_C4</td>
<td>Yield from perchlorate to chlorate</td>
<td>0.1</td>
<td>mgX/mgC3-day</td>
</tr>
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### TABLE 3-9
MODEL PROCESSES

<table>
<thead>
<tr>
<th>Process Name</th>
<th>Description</th>
<th>Rate</th>
<th>Stoichiometric Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorate</td>
<td>Chorate degradation</td>
<td>qH_C3<em>XH</em>C3/(C3+KH_C3*(1+C4/KH_C4))+qe_C3<em>Xc</em>C3/(C3+Kc_C3)+qp_C3<em>Xp</em>C3/(C3+Kp_C3*(1+C4/Kp_C4))</td>
<td>-1</td>
</tr>
<tr>
<td>Perc</td>
<td>Percihate reduction to chlorate</td>
<td>qH_C4<em>XH</em>C4/(C4+KH_C4*(1+C3/KH_C3))+qp_C4<em>Xp</em>C4/(C4+Kp_C4*(1+C3/Kp_C3))</td>
<td>86.5, 99.5</td>
</tr>
<tr>
<td>Xc</td>
<td>CRB biomass formation</td>
<td>qe_C3<em>Xe</em>Y_C3<em>C3/(C3+Kc_C3)-b</em>Xc</td>
<td>1</td>
</tr>
<tr>
<td>XH</td>
<td>HCAP biomass formation</td>
<td>qH_C4<em>XH</em>Y_C4<em>C4/(C4+KH_C4)+qh_C3</em>XH<em>Y_C3</em>C3/(C3+KH_C3)-b*XH</td>
<td>1</td>
</tr>
<tr>
<td>Xp</td>
<td>PCRB biomass formation</td>
<td>qp_C4<em>Xp</em>Y_C4<em>C4/(C4+Kp_C4)+qp_C3</em>Xp<em>Y_C3</em>C3/(C3+Kp_C3)-b*Xp</td>
<td>1</td>
</tr>
<tr>
<td>Type:</td>
<td>Mixed Reactor Compartment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compartmen Index:</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active Variables:</td>
<td>C4, C3, C1, KH_C3, KH_C4, qH_C3, qH_C4, XH, b, Xc, Kc_C3, qc_C3, Kp_C3, Kp_C4, qp_C3, qp_C4, Xp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active Processes:</td>
<td>Perc, Chlorate, Xh, Xc, Xp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial Conditions:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Variable(Zone) :</td>
<td>Initial Condition</td>
<td></td>
<td></td>
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<tr>
<td>C4(Bulk Volume) :</td>
<td>200 mg/L</td>
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<tr>
<td>C3(Bulk Volume) :</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xc(Bulk Volume) :</td>
<td>3.33 mg/L</td>
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<td></td>
</tr>
<tr>
<td>Xh(Bulk Volume) :</td>
<td>3.33 mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xp(Bulk Volume) :</td>
<td>3.33 mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflow:</td>
<td>0.1 L/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loadings:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Variable :</td>
<td>Loading</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4 :</td>
<td>200 mg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume:</td>
<td>1 L</td>
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<td>Accuracies:</td>
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<td>Rel. Acc. Q:</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abs. Acc. Q:</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rel. Acc. V:</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abs. Acc. V:</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The kinetic parameters used in the AQUASIM program were based on three bacterial isolates. Parameters for HCAP were those of HCAP-C, and PCRB were those of PC1 (Nerenberg, 2003). The \( q_{\text{max}} \) for PDA was estimated from \( \mu_{\text{max}} \) and \( Y \) from Logan et al. (2001) from the equation \( q_{\text{max}} = \frac{\mu_{\text{max}}}{Y} \) (Rittmann and McCarty, 2001) as performed previously by Salamone (2006).

### 3.3.2 Results of Modeling Co-Cultures

The results of AQUASIM models are shown below in Table 3-11. The final column lists the time required to reduce 200 mg/L of perchlorate to a concentration below the DWEL of 24.5 \( \mu \text{g/L} \). All of the corresponding plots referred to in Table 3-11 can be found in Appendix F. Figure 3-3 shows an example of the model results.

<table>
<thead>
<tr>
<th>Figure #</th>
<th>Model</th>
<th>Steady State Chlorate (mg/L)</th>
<th>Time Req'd to Reduce 200 mg/L Perchlorate (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>HCAP + PCRB</td>
<td>8.6E-5</td>
<td>1.8</td>
</tr>
<tr>
<td>3-2</td>
<td>HCAP + CRB</td>
<td>0.05</td>
<td>Never (Steady-state conc. of 27.6 mg/L after 50 days)</td>
</tr>
<tr>
<td>3-3</td>
<td>PCRB + CRB</td>
<td>8.4E-5</td>
<td>1.8</td>
</tr>
<tr>
<td>3-4</td>
<td>HCAP + PCRB + CRB</td>
<td>8.4E-5</td>
<td>1.9</td>
</tr>
<tr>
<td>3-5</td>
<td>HCAP</td>
<td>2.4</td>
<td>Never (Steady-state conc. of 6.5 mg/L after 40 days)</td>
</tr>
<tr>
<td>3-6</td>
<td>PCRB</td>
<td>8.4E-5</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Figure 3-3. Perchlorate reduction and biomass growth of a co-culture of HCAP, PCRB, and CRB in a CSTR. Steady state chlorate accumulation was 8.4E-5 mg/L.

**Hypothesis 1.** According to the models of co-cultures of HCAP + CRB and HCAP + PCRB, CRB and PCRB will immediately reduce chlorate accumulated by HCAP so that there is little accumulation of chlorate. The model predicts that HCAP grown alone will accumulate 2.4 mg/L of chlorate while reducing perchlorate, but in a co-culture with CRB, accumulation reaches a maximum of just 0.05 mg/L. Also, in a co-culture with PCRB, HCAP accumulates chlorate to a maximum of 8.6E-5 mg/L. Therefore, the model upholds the hypothesis that in the natural environment, CRB and PCRB prevent the accumulation of chlorate by HCAP by immediately reducing any chlorate that accumulates.

**Hypothesis 2.** While the presence of CRB prevents chlorate accumulation, overall kinetics of perchlorate reduction are slowed. When HCAP are grown alone, a steady state concentration of 6.5 mg/L perchlorate is reached after 40 days, but in a co-culture of HCAP + CRB, it would take 50 days to reach a steady state concentration of...
27.6 mg/L. When PCRB are grown alone, perchlorate is reduced to a concentration below the DWEL of 24.5 μg/L in just 1.6 days, but in a co-culture of PCRB + CRB, that level is reached in 1.8 days. Therefore according to the model, hypothesis two is incorrect, and CRB slow the overall kinetics of perchlorate reduction of PCRB and HCAP.

One explanation for the decrease in the perchlorate reduction rate when a CRB is added to a CSTR of HCAP is that HCAP do not have enough time to reduce perchlorate in the given HRT. Because CRB have a faster rate of chlorate reduction than HCAP (PDA has a lower K and higher q_{max} than HCAP-C), HCAP are only able to grow on the reduction of perchlorate to chlorate, a process with a lower yield than the reduction of chlorate to chloride. Because HCAP have less substrate on which to grow, it takes more time than is allowed by a 10 day HRT to reach a high enough concentration to reduce the total 200 mg/L of perchlorate. When the HRT is increased to 20 days, a steady state concentration of 17.7 mg/L perchlorate is reached in approximately 37 days. When the HRT is increased to 100 days, a steady state concentration of 10.3 mg/L perchlorate is reached in approximately 25 days. Ultimately, CRB slow the reduction of perchlorate, requiring a longer HRT to reduce perchlorate to the DWEL.

Similarly, an explanation for the decrease in the perchlorate reduction rate when a CRB is added to a CSTR of PCRB is that the K for CRB on chlorate, 1 mg/L, is much higher than the K for PCRB on chlorate, 0.0012 mg/L. K corresponds to a slower reduction rate according to Equation 2-1. Therefore, the kinetic parameters of a CRB are such that a co-culture of CRB + PCRB are less efficient at degrading perchlorate than pure-culture PCRB.

**Hypothesis 3.** As stated before, the addition of CRB to a culture of HCAP does not increase the rate of perchlorate reduction. Because CRB reduce chlorate produced by HCAP, HCAP are unable to grow on that chlorate and the amount of HCAP in the solution is insufficient to reduce the 200 mg/L perchlorate during the given HRT.
Therefore, according to the model, hypothesis three is incorrect; a co-culture of CRB + HCAP is not faster at reducing perchlorate than pure-culture PCRB.

**Hypothesis 4.** Analysis of the model of a co-culture of HCAP + PCRB reveals that at steady state, PCRB reach a concentration of 550 mg/L while HCAP are completely washed out of the system. This is due to the slower kinetics of HCAP. The half maximum specific substrate utilization rates of HCAP for both chlorate and perchlorate are three and four orders of magnitude higher than those of PCRB, which corresponds to slower reduction rates according to Equation 2-1. Additionally, the maximum specific substrate utilization rates for HCAP are slower than those of PCRB, also corresponding to slower reduction rates of perchlorate. In just 20 days, the HCAP concentration falls below 1 mg/L while the PCRB concentration reaches 550 mg/L. Therefore, hypothesis four is correct; in the presence of PCRB at a low perchlorate concentration, HCAP do not play a significant role in perchlorate reduction because of the more favorable kinetics of PCRB.

### 3.3.3 Conclusions

Modeling reveals that in a CSTR, co-cultures of HCAP with CRB and PCRB are less efficient at reducing perchlorate than pure-culture PCRB. This is due mainly to the faster kinetics of PCRB that dominate HCAP when in co-culture. Additionally, a co-culture of HCAP + CRB are less efficient at reducing perchlorate than pure-culture HCAP even though less chlorate accumulates. This is because HCAP do not have enough time to reduce perchlorate in the given HRT.

Two factors may explain why chlorate accumulation has not yet been observed in the natural environment. First, according to the models, chlorate accumulation by HCAP is mitigated by the presence of both CRB and PCRB. In a co-culture of HCAP + CRB, chlorate accumulation reaches a maximum of 0.05 mg/L, less than the amount accumulated by pure-culture PCRB. Second, HCAP are washed out of the system when
grown in the presence of PCRB because of the more favorable perchlorate reduction parameters of PCRB than HCAP. Therefore, it is predicted that at perchlorate concentrations below 200 mg/L in the natural environment where all three types of bacteria are naturally present, HCAP do not play a significant role in perchlorate reduction.
4.1 Introduction

Reduction of perchlorate to chlorate and the reduction of chlorate to chlorite is catalyzed by the enzyme (per)chlorate reductase. A recent study has suggested that this enzyme has a heterotrimeric structure and is encoded by a transcriptional unit \textit{pcrABCD} composed of four genes, \textit{pcrA}, \textit{B}, \textit{C}, and \textit{D}, with \textit{pcrA} encoding the structural \(\alpha\)-subunit of the full enzyme. This \(\alpha\)-subunit is thought to contain the molybdopterin active site where perchlorate reduction takes place (Bender et al., 2005). The \textit{pcrA} genes of three perchlorate-reducing bacteria, including that of \textit{Dechlorosoma} sp. HCAP-C, were sequenced and compared to determine if functional differences in the activity of these bacteria, i.e. chlorate accumulation in HCAP-C, could be attributed to differences in \textit{pcrA} gene sequences or in the inferred amino acid sequence of the \textit{pcrA} polypeptide. The three strains sequenced were a conventional PCRB, \textit{Dechlorosoma} sp. KJ, and two HCAP, \textit{Dechlorosoma} sp. HCAP-C and \textit{Dechloromonas} sp. PC1.

The \textit{pcrA} gene sequences of two PCRB, \textit{Dechloromonas agitata} and \textit{Dechloromonas aromatica}, were used to design primers for sequencing the three isolates of interest. The GenBank accession number for the \textit{pcrA} gene of \textit{D. agitata} is AY180108 and the accession number for the complete genome of \textit{D. aromatica} is CP000089. These sequences are listed in Appendix G. \textit{D. aromatica} behaves as an HCAP accumulating...
chlorate to approximately 16.8% of the initial perchlorate concentration (Coates, 2007). It is not known whether *D. agitata* behaves as an HCAP or as a conventional PCRB.

**4.2 Materials and Methods**

**4.2.1 Collecting DNA sequences of *pcrA***

The first step in investigating the genetic sequence of the functional gene perchlorate reductase of the three isolates HCAP-C, PC1, and KJ was to compile known nucleic acid sequences of the *pcrA* gene of other perchlorate reducing bacteria. This was performed by first searching the NCBI database under the GenBank accession number AY180108 to collect the entire nucleotide sequence for *D. agitata* perchlorate reductase gene subunit A. Once the entire sequence was collected, a BLAST search was performed to find other species that also carried this gene. Currently, *D. aromatica* is the only other species whose *pcrA* gene has been sequenced.

**4.2.2 Primer Design**

The *pcrA* sequence of *D. aromatica* was aligned with that of the *pcrA* sequence of *D. agitata* to find conserved regions of the *pcrA* gene in order to design general *pcrA* gene primers and internal sequencing primers. For this purpose, four criteria were established: the length of the primers be between 15 and 30 bases, the GC content of the primers be approximately 50%, the annealing temperature of the forward and reverse primers be approximately equal, and the *pcrA* sequences to which to primer set anneal be nearly or entirely identical. To determine the approximate annealing temperature, CLC Gene Workbench version 2.2 was used. All designed primers were ordered through Invitrogen™.

The first set of primers was designed to amplify nearly the entire *pcrA* gene. The forward primer was composed of bases 1 through 30 of the *D. agitata pcrA* gene, starting
with the start codon. The reverse primer was composed of bases 2758 through 2784 of \textit{D. agitata pcrA}. These primers are listed in Table 4-1 as Set 1.

**TABLE 4-1**

**PRIMERS USED FOR AMPLIFICATION OF GENE \textit{PCRA} OF GENOMIC DNA**

<table>
<thead>
<tr>
<th>Name</th>
<th>Region^a</th>
<th>Sequence^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCRA_1F</td>
<td>1-30</td>
<td>5’ ATG GCA CGA CTG AGT CGA AGA GAT TTT TTG 3’</td>
</tr>
<tr>
<td>PCRA_2784R</td>
<td>2784-2758</td>
<td>5’ ATC GGC GCA ACT CCG ATC AGT CTC TAG 3’</td>
</tr>
<tr>
<td>PCRA_145F</td>
<td>145-167</td>
<td>5’ CAG TGG TCN TGG GAC AAG AAA AC 3’</td>
</tr>
<tr>
<td>PCRA_2504R</td>
<td>2504-2486</td>
<td>5’ ACC CAN TCG TTG TCC TTG A 3’</td>
</tr>
<tr>
<td>PCRA_1364F</td>
<td>1364-1378</td>
<td>5’ GGT ATT ACA GCG AYG 3’</td>
</tr>
<tr>
<td>PCRA_2504R</td>
<td>2504-2486</td>
<td>5’ ACC CAN TCG TTG TCC TTG A 3’</td>
</tr>
</tbody>
</table>

^a The region for each primer was based on the sequence of \textit{D. agitata}

^b \textit{N} = A, C, G, or T; \textit{Y} = C or T

These primers did not amplify the gene from genomic DNA extracts of HCAP-C, PC1, or KJ, even under non-stringent PCR conditions (e.g. low annealing temperatures and increased magnesium concentrations). Therefore, new primers were designed that were able to anneal to both HCAP-C and PC1. These primers are listed in Table 4-1 as Set 2.

Primer Set 2 amplified a fragment of the \textit{pcrA} gene from all tested organisms except strain KJ. This was indicated by a PCR yield of the correct size and by sequence analysis. A third primer set, Set 3, listed in Table 4-1 was used for strain KJ. This primer set was composed of the reverse primer of Set 2 with a newly designed forward primer. PCR with this primer set, however, yielded a smaller fragment of the \textit{pcrA} gene, approximately 1000 base pairs in length.

**4.2.3 Polymerase Chain Reaction (PCR)**

Gene amplification reactions (PCR) were prepared by combining extracted genomic DNA, Eppendorf® MasterMix (2.5x), forward and reverse primers, and PCR-
pure water. DNA was extracted from bacterial cells using a PowerSoil™ DNA Isolation Kit produced by MO BIO Laboratories, Inc. The PCR reaction was prepared with final parameter concentrations listed in Table 4-2. A total volume of 50 μL was prepared.

TABLE 4-2
FINAL PCR PARAMETER CONCENTRATIONS

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Approximately 3 ng/μL</td>
</tr>
<tr>
<td>Master Mix (2.5X)</td>
<td>1X (0.024 units/μL)</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>200 nM</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>200 nM</td>
</tr>
</tbody>
</table>

The mixture was then placed in an Eppendorf® Mastercycler ep gradient S thermocycler. The following thermocycler parameters were used.

 Initialization 95°C---5 min

 Denaturation 94°C---30 sec

 Annealing 55-65°C---45 sec 40 cycles

 Elongation 72°C---3 min

 Final Elongation 72°C---7 min

 Hold 10°C

A negative control reaction, containing no added genomic DNA, was included. No amplification was detected in the negative control reaction.

4.2.4 Gel Electrophoresis

After the PCR process was complete, electrophoresis was performed in order to determine if the appropriate gene sequence was amplified. A gel composed of 1.5% agarose in 1X Bionic Buffer (produced by Sigma) containing GelStar® Nucleic Acid Gel
Stain (produced by Cambrex Bio Science) was cast. PCR products were then electrophoresed along the gel at 150 V and 100 mA for approximately 45 minutes.

4.2.5 Sequencing

PCR products were purified prior to sequencing using an UltraClean™ PCR Clean-up™ Kit also produced by MO BIO Laboratories, Inc. The product of the polymerase chain reaction was then sent to SeqWright DNA Technology Services for sequencing. The following primers were used to sequence HCAP-C and PC1. Reverse primer 1364R is the inverse complement of primer 1364F ($R = A$ or $G$).

Forward primer 145F: 5’ CAG TGG TCN TGG GAC AAG AAA AC 3’
Forward primer 1361F: 5’ ACT GGT ATT ACA GCG ACG TGC T 3’
Reverse primer 1364R: 5’ CRT CGC TGT AAT ACC 3’
Reverse primer 2504R: 5’ ACC CAN TCG TTG TCC TTG A 3’

The following primers were used to sequence KJ.
Forward primer 1364F: 5’ GGT ATT ACA GCG AYG 3’
Reverse primer 2504R: 5’ ACC CAN TCG TTG TCC TTG A 3’

4.3 Results

Nucleotide sequences were submitted to GenBank. The accession numbers for the sequences are EU022025 (Dechlorosoma sp. KJ), EU022026 (Dechloromonas sp. PC1), and EU022027 (Dechlorosoma sp. PCC, referred to in this study as HCAP-C).

4.3.1 Alignments

Alignments of the pcrA nucleotide and amino acid sequences of Dechloromonas aromatica, Dechloromonas agitata, Dechloromonas sp. PC1, and Dechlorosoma sp. KJ are presented in Appendix H. Alignments were constructed using CLC Gene Workbench version 2.2 with an open gap cost of 10 and an extension cost of 1. An open gap cost is the penalty applied when a gap is inserted between base pairs in order for sequences to
align. An extension cost is the penalty applied when that gap is extended to more than one base pair. Comparisons of the three strains, HCAP-C, PC1, and KJ, are listed in Tables 4-3, 4-4, and 4-5. Because the \textit{pcr}A KJ sequence obtained in this study was significantly shorter than those of the other isolates, comparisons were only made in the region over which KJ was sequenced. This region is homologous to positions 1466 through 2448 of the sequence of strain \textit{D. agitata}. Amplified 16S rRNA gene sequences contained nearly the entire gene and were greater than 1500 bases in length.

**TABLE 4-3**

**COMPARISON OF 16S rRNA GENE NUCLEOTIDE SEQUENCE SIMILARITY AMONG SPECIES HCAP-C, PC1, AND KJ**

<table>
<thead>
<tr>
<th></th>
<th>HCAP-C</th>
<th>PC1</th>
<th>KJ</th>
<th>\textit{D. aromatica}</th>
<th>\textit{D. agitata}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCAP-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC1</td>
<td>93%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KJ</td>
<td>99%</td>
<td>93%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{D. aromatica}</td>
<td>93%</td>
<td>96%</td>
<td>93%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{D. agitata}</td>
<td>93%</td>
<td>97%</td>
<td>92%</td>
<td>96%</td>
<td>-</td>
</tr>
</tbody>
</table>

**TABLE 4-4**

**COMPARISON OF \textit{pcr}A NUCLEOTIDE SEQUENCE SIMILARITY AMONG SPECIES HCAP-C, PC1, AND KJ.**

<table>
<thead>
<tr>
<th></th>
<th>HCAP-C</th>
<th>PC1</th>
<th>KJ</th>
<th>\textit{D. aromatica}</th>
<th>\textit{D. agitata}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCAP-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC1</td>
<td>99%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KJ</td>
<td>78%</td>
<td>79%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{D. aromatica}</td>
<td>77%</td>
<td>77%</td>
<td>79%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>\textit{D. agitata}</td>
<td>75%</td>
<td>75%</td>
<td>76%</td>
<td>76%</td>
<td>-</td>
</tr>
</tbody>
</table>
The 16S rRNA gene nucleotide sequences of strains KJ and HCAP-C were 99% similar meaning they are more phylogenetically similar to each other than to strain PC1, which is only 93% similar to these strains. Even though HCAP-C and KJ are highly similar phylogenetically, strains HCAP-C and PC1 both behave as HCAP while KJ behaves as a conventional PCRB. The high similarity of the 16S rRNA genes of strains KJ and HCAP-C indicate that the physiological capacity to accumulate large amounts of chlorate is not conserved within lineages. Thus, analysis of 16S rRNA genes alone is likely insufficient for determining chlorate accumulation capacity.

Interestingly, the nucleotide sequences of the functional gene \( pcrA \) of HCAP-C and PC1 were 99% similar while the \( pcrA \) nucleotide sequence of KJ was only 79% similar to that of PC1 and 78% similar to that of HCAP-C. The amino acid sequence of \( D. aromatica \) (an HCAP) is 88% similar to both HCAP-C and PC1, again showing that the functional genes of HCAP are similar. Two HCAP strains, HCAP-C and PC1, represent significantly divergent organisms, based on rRNA gene analysis (93% similarity). However, the \( pcrA \) gene sequences from these two strains are nearly identical. These data are consistent with the hypothesis that a specific allele (or alleles) of the \( pcrA \) gene is responsible for the presence of chlorate accumulation, since these organisms are both capable of chlorate accumulation.

### TABLE 4-5

COMPARISON OF \( pcrA \) AMINO ACID SEQUENCE SIMILARITY AMONG SPECIES HCAP-C, PC1, AND KJ

<table>
<thead>
<tr>
<th></th>
<th>( pcrA ) Amino Acid Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCAP-C</td>
</tr>
<tr>
<td>HCAP-C</td>
<td>-</td>
</tr>
<tr>
<td>PC1</td>
<td>99%</td>
</tr>
<tr>
<td>KJ</td>
<td>85%</td>
</tr>
<tr>
<td>( D. aromatica )</td>
<td>88%</td>
</tr>
<tr>
<td>( D. agitata )</td>
<td>82%</td>
</tr>
</tbody>
</table>
The fact that these organisms are divergent by phylogenetic analysis of rRNA genes may be an indication that the \textit{pcrA} gene can be laterally transferred among bacteria. This is indicated because the phylogeny of the 16S rRNA gene (largely thought to be consistent with the phylogeny of the organism, since the gene is not readily laterally transferred) is not consistent with the phylogeny of the \textit{pcrA} genes. More gene sequences from perchlorate and chlorate reducing bacteria are needed to support this hypothesis.

4.3.2 Phylogenetic Trees

Figure 4-1 below is a phylogenetic tree based on the 16S rRNA gene nucleotide sequences of strains \textit{Dechloromonas aromatica}, \textit{Dechloromonas agitata}, \textit{Dechlorosoma} sp. HCAP-C, \textit{Dechloromonas} sp. PC1, and \textit{Dechlorosoma} sp. KJ. Figures 4-2 and 4-3 are neighbor-joining phylogenetic trees based on nucleotide and amino acid alignments of the functional gene \textit{pcrA} of these same isolates. Trees were constructed based on nucleotide sequence positions 1466 through 2448, relative to the sequence of strain \textit{D. agitata}. Phylogenetic trees were produced using the phylogenetic software package MEGA version 3.1 (Kumar et al., 2004). The scales at the bottom of the figures indicate changes per nucleotide position, and are a reflection of the similarity of the recovered sequences to those sequences found in a sequence database (genbank; \url{www.ncbi.nih.gov}). Values adjacent to nodes are boot-strap values (100 replicates), and are a statistical measure of the robustness of the tree topology. Only values greater than 70% are shown.
Figure 4-1 Neighbor-joining phylogenetic tree of 16S rRNA gene sequences recovered from strains *D. aromatica*, *D. agitata*, HCAP-C, PC1, and KJ. The phylogenetic tree shown here was produced from nearly full length 16S rRNA gene sequences of greater than 1500 bases in length.

Figure 4-2 Neighbor-joining phylogenetic tree of *pcrA* nucleotide sequences recovered from strains *D. aromatica*, *D. agitata*, HCAP-C, PC1, and KJ. The phylogenetic tree shown here was produced from nucleotide sequences of approximately 1000 positions in length.
4.3.3 Conserved Domains

When genetic mutations occur within a functional gene of a bacterium they can result in changes to the amino acid sequence of the respective protein. If these mutations do not interfere with the functioning of a protein that in turn causes cell death or lower competitiveness, they may easily persist through a bacterium’s progeny. However, mutations will not normally propagate through progeny if they are in regions that are important for the proper functioning of a protein and disrupt the functioning of such a protein. Therefore, structurally important regions of essential genes tend to be highly conserved. Highly conserved regions of a gene, or regions of a sequence shared between multiple species with little or no differences in amino acids, generally indicate structural importance. These regions are termed conserved domains.

Any differences in conserved domains among species may indicate structural differences that exist among proteins coded by those domains. For example, the tendency of HCAP to accumulate high levels of chlorate may be encoded in a conserved domain of the \textit{pcrA} gene. Alternatively, these conserved regions may be critical for the initial reduction of perchlorate, and therefore well conserved among all PCRB. Currently, it is not clear if the accumulation of chlorate is encoded in the \textit{pcrA} gene.
alone. Certainly, the nearly identical amino acid sequences of two HCAP strains, HCAP-C and PC1, is suggestive that the *pcrA* gene allele is responsible for chlorate accumulation, but the mode of activity is unknown, making specific prediction difficult. However, comparing the conserved domains of the *pcrA* gene of HCAP and that of conventional PCRB may indicate regions of interest, potentially responsible for chlorate accumulation activity.

Conserved domains of the amino acid sequences of the functional gene *pcrA* of *Dechloromonas aromatica*, *Dechloromonas agitata*, *Dechlorosoma* sp. HCAP-C, *Dechloromonas* sp. PC1, and *Dechlorosoma* sp. KJ were found using the National Center for Biotechnology Information conserved domains database. The major domains of the *pcrA* amino acid sequence of species HCAP-C are shown below in Figure 4.4 and those of *D. aromatica*, *D. agitata*, HCAP-C, PC1, and KJ are listed in Appendix I.

![Figure 4-4 Major conserved domains of the pcrA amino acid sequence of Dechlorosoma sp. HCAP-C.](image)

The major conserved domains of the sequences come primarily from the molybdopterin binding superfamily of proteins or MopB genes, bind molybdopterin, and are mutually homologous. Of these MopB genes, the MopB_Nitrate-R-NarG-Like domain is the binding site for the molybdopterin responsible for reduction of nitrate to nitrite. This enzyme is a cytoplasmic membrane-bound quinol-nitrate oxidoreductase and forms a redox loop to generate a proton-motive force. Regions with high homology to this domain are present at four locations along this segment of the amino acid sequence of the *pcrA* gene of HCAP-C. This domain is also present in the amino acid sequences of *D. aromatic*, *D. agitata*, PC1, and KJ.
Tables 4-6 and 4-7 show a comparison of two of the regions of the MopB_Nitrate-R-NarG-Like domain (cd02750). The region of the MopB domain shown in Table 4-6 is outside of the region obtained for species KJ. Therefore, KJ was not included in this table. In the larger area of the domain represented in Table 4-6, isolates PC1 and HCAP-C were identical and highly similar to *D. aromatica* while other species’ sequences were similar but not exact. However, the domain of *D. agitata* was also more similar to HCAP strains than to their entire amino acid sequences. The entire amino acid sequence of *D. agitata* was 82-85% similar to HCAP sequences while the MopB_Nitrate-R-NarG-Like domain was 85-87% similar to HCAP domains. Therefore, it is unclear whether significant structural differences exist between HCAP and non-HCAP strains in this domain.

### TABLE 4-6

**COMPARISON OF THE MOPB DOMAIN CD02750 OVER THE REGION OF AMINO ACIDS 31 THROUGH 259**

<table>
<thead>
<tr>
<th></th>
<th>HCAP-C</th>
<th>PC1</th>
<th><em>D. aromatica</em></th>
<th><em>D. agitata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MopB cd02750 (31-259)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCAP-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>100%</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>D. aromatica</em></td>
<td>96%</td>
<td>96%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>D. agitata</em></td>
<td>85%</td>
<td>85%</td>
<td>87%</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 4-7

COMPARISON OF THE MOPB DOMAIN CD02750 OVER THE REGION OF AMINO ACIDS 334 THROUGH 452

<table>
<thead>
<tr>
<th>MopB Domain cd02750 (334-452)</th>
<th>HCAP-C</th>
<th>PC1</th>
<th>KJ</th>
<th>D. aromatica</th>
<th>D. agitata</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCAP-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC1</td>
<td>100%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KJ</td>
<td>89%</td>
<td>89%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D. aromatica</td>
<td>90%</td>
<td>90%</td>
<td>91%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D. agitata</td>
<td>91%</td>
<td>91%</td>
<td>94%</td>
<td>93%</td>
<td>-</td>
</tr>
</tbody>
</table>

The MopB_DmsA-EC domain encodes the binding site of molybdopterin responsible for anaerobic dimethylsulfoxide reductase (DMSOR) of *E. coli* and a region homologous to it is found only once in this amino acid sequence segment. It also appears in the amino acid sequences of *D. aromatica*, *D. agitata*, and PC1, but does not appear in the segment of the sequence of isolate KJ probably because only a small portion, roughly half the size of that obtained for HCAP-C and PC1, of the KJ amino acid sequence could be obtained.

Table 4-8 shows a comparison of the regions of the MopB_DmsA-EC domain (cd02770). In this area of the domain, isolates PC1 and HCAP-C were again identical while other species’ sequences were much less similar. Comparing the domains to the entire amino acid sequence, other isolates maintained their level of similarity to HCAP, 82-88% similar, while two HCAP strains increased their similarity from 99% to 100%.
Finally, a third domain is the NarG segment, which is the nitrate reductase alpha subunit responsible for energy production and conversion. This domain is found in all sequences other than KJ, again probably due to the fact that only a small portion of the KJ sequence was obtained. This domain is not part of the MopB superfamily.

Table 4-9 shows a comparison of the regions of the NarG domain (COG5013). This region was outside of the KJ sequence obtained in this study. In this area of the domain, isolates PC1 and HCAP-C were 99% similar and 90 to 91% similar to *D. aromatica* while *D. agitata*’s sequence was less similar to these. However, as with the MopB-Nitrate-R-NarG-Like domain, all species’ NarG domains were more similar to each other than their entire amino acid sequences were. While the entire amino acid sequence of *D. agitata* was 82-85% similar to HCAP, its NarG domain was 84-86% similar to HCAP.
TABLE 4-9

COMPARISON OF THE NARG DOMAIN COG5013 OVER THE REGION OF
AMINO ACIDS 956 THROUGH 1110

<table>
<thead>
<tr>
<th>NarG Domain COG5013 (956-1110)</th>
<th>HCAP-C</th>
<th>PC1</th>
<th>D. aromatica</th>
<th>D. agitata</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCAP-C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC1</td>
<td>99%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D. aromatica</td>
<td>90%</td>
<td>91%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D. agitata</td>
<td>84%</td>
<td>85%</td>
<td>86%</td>
<td>-</td>
</tr>
</tbody>
</table>

4.4 Conclusions

After analyzing the 16S rRNA gene nucleotide sequences of three perchlorate-reducing isolates, it appears that the tendency to accumulate high levels of chlorate is not shared among phylogenetically similar isolates. However, isolates with highly similar pcrA gene sequences do share the tendency to accumulate high levels of chlorate, suggesting that the functional gene pcrA may be responsible for this tendency of HCAP.

Analysis of several conserved domains of these five isolates also indicated that HCAP bacteria are more similar than conventional PCRB. In general, regions homologous to conserved domains were more similar than were comparison of amino acid sequences for the entire gene, which was expected. Even though conserved domains of HCAP were 88 to 100% similar, all species’ conserved domains were more similar to HCAP than were their amino acid sequences. Therefore, it is unclear whether the tendency to accumulate chlorate is coded in one of the domains analyzed in this study. However, the still significant divergence of the conserved regions between the HCAP and non-accumulating PCRB is significant (6-20% divergence), a result which could indicate that changes in these conserved regions do cause differences in chlorate accumulation.

Because only portions of one gene from three strains of bacteria were sequenced and the specific mode of chlorate accumulation has not yet been identified, these conclusions are preliminary. It will be necessary to sequence the pcrA gene of more
isolates in order to confirm whether HCAP tendencies are coded within this gene, and to perform genetic manipulations of PCRB and HCAP strains to definitively show that changes in this gene are responsible for chlorate accumulation. Additionally, \textit{pcrB} and \textit{pcrC} genes should be sequenced and compared for heightened homology among chlorate accumulators.
The contaminant perchlorate is an extremely soluble and dangerous chemical found in drinking water sources around the US. Even at trace levels, it is a risk to humans and wildlife. The preferred removal method is biological reduction because it is relatively inexpensive and its byproducts, innocuous chloride and oxygen, are harmless. During biological reduction, it is thought that the perchlorate molecule is transformed in a series of three steps from perchlorate to chlorate, then to chlorite, and finally chloride. Both perchlorate and chlorate are thought to be reduced by the (per)chlorate reductase enzyme. Because the first perchlorate reduction step is thought to be rate-limiting, subsequent steps are not usually studied. Recently, it was found that in a few isolates, significant amounts of chlorate accumulate during the reduction pathway of perchlorate, and this accumulation may result in competitive inhibition with (per)chlorate reductase. This has prompted our investigation of chlorate accumulation in these strains. In this study, it was found that these high chlorate accumulators accumulate chlorate regardless of environmental conditions such as media type.

Kinetic parameters for HCAP-C were estimated during this study from standard Monod equations. Biological yields on chlorate and perchlorate were determined for HCAP-C both experimentally and theoretically, and both estimates produced yields of 0.34 mgVSS/mgClO\textsuperscript{3\textsuperscript{-}} and 0.1 mgVSS/mgClO\textsuperscript{4\textsuperscript{-}}. AQUASIM modeling estimated the kinetic parameters $K$ and $q_{\text{max}}$ for both chlorate and perchlorate based on experimental data. Kinetic parameters for HCAP-C were estimated for chlorate: $K$=58.3mg/L and
$q_{\text{max}} = 8.3 \text{mgS/mgX-day}$, and for perchlorate: $K = 192.6 \text{ mg/L}$ and $q_{\text{max}} = 11.5 \text{mgS/mgX-day}$.

A $K$ value for CRB was also estimated with AQUASIM modeling from experimental data, but a reasonably accurate value could not be obtained. It is estimated that $K$ for the CRB *Pseudomonas* sp. PDA grown on chlorate with acetate as the electron donor is less than 0.4 mg/L.

Co-cultures grown in batch were examined for a relationship between HCAP and CRB that could increase the efficiency of biological perchlorate reduction. The experiments suggested that CRB grow on chlorate accumulated by HCAP, and this syntrophy results in an increase to the rate of perchlorate reduction. AQUASIM models of those batch tests confirmed that a syntrophy does exist whereby CRB reduce chlorate accumulated by HCAP. However, models also predicted that unlike experimental findings, HCAP-C grown in pure-culture would be faster at reducing perchlorate, a similar finding to that of Salamone (2006).

AQUASIM models were used to predict relationships among HCAP, PCRB, and CRB grown in a CSTR. It was predicted that pure-culture PCRB are the most efficient at reducing perchlorate at a concentration of 200 mg/L of perchlorate. When HCAP is grown in co-culture with PCRB at this concentration, they are washed out of the system due to their slower kinetics. HCAP grown in co-culture with CRB are less efficient than pure-culture HCAP and require a much longer HRT to reduce the total perchlorate.

In a genetic analysis of the gene coding for (per)chlorate reductase, it was found that the gene sequence of the catalytically active alpha subunit, *pcrA*, is significantly different in HCAP relative to PCRB strains. It, therefore, may play a part in the accumulation of chlorate during perchlorate reduction. However, only five sequences were obtained. Of these five, three are known HCAP, one is a known PCRB, and one is not yet characterized. Before concluding that *pcrA* plays a role in chlorate accumulation among HCAP, a broader range of isolates should be characterized and sequenced.
REFERENCES


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EPA (2006), 'www.epa.gov'.


V. Korenkov, V. Romanenko, S. Kuznetsov and J. Voronov (1976), 'Process for purification of industrial waste waters from perchlorates and chlorates', in.


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Yield estimates were based on experimental data from batch tests of chlorate and perchlorate reduction by HCAP-C. Figures A-1 through A-3 are graphs of results of chlorate batch experiments. Figures A-4 through A-7 are graphs of results of perchlorate batch experiments.

Figure A-1. Batch test 1 of HCAP-C growth on chlorate.
Figure A-2. Batch test 2 of HCAP-C growth on chlorate.

Figure A-3. Batch test 3 of HCAP-C growth on chlorate.
Figure A-4. Batch test 1 of HCAP-C growth on perchlorate.

Figure A-5. Batch test 2 of HCAP-C growth on perchlorate.
Figure A-6. Batch test 3 of HCAP-C growth on perchlorate.

Figure A-7. Batch test 4 of HCAP-C growth on perchlorate.
APPENDIX B

THEORETICAL CALCULATION OF YIELD ON PERCHLORATE

The theoretical yield of perchlorate reduction to chlorate using acetate as the electron donor was calculated based on the electrons exchanged during reduction of perchlorate to chlorate and the reduction of chlorate to chloride. The steps are outlined below.

First, the yield of chlorate to chloride determined experimentally was converted to a molar yield by multiplying by the molecular weight of chlorate, 83.5 g/mol.

\[
0.34mgX / mg\text{ClO}_3^- \times 83.5g\text{ClO}_3^- / mol = 28.39gX / mol\text{ClO}_3^-
\]

Next, the molar yield of chlorate was converted to an electron equivalent basis by dividing by the number of electron equivalents per mole of chlorate, 6 e^-eq/mol.

\[
\frac{28.39gX / mol\text{ClO}_3^-}{6e^-eq / mol\text{ClO}_3^-} = 4.73gX / e^-eq\text{ClO}_3^-
\]

Because the Gibbs free energies for chlorate reduction (-103.125kJ/e^-) (Salamone, 2006) and perchlorate reduction (102.75kJ/e^-) (Rikken et al., 1996) with acetate as an electron donor are approximately equal, the yield for chlorate based on electron equivalents can be converted directly to the yield for perchlorate based on electron equivalents. Therefore, the yield for chlorate was multiplied by the number of electron equivalents per mole of perchlorate when it is reduced to chlorate, 2 e^-eq/mol.
Finally, the molar yield for perchlorate was converted to yield for perchlorate on a mass per mass basis by dividing by the molecular weight of perchlorate, 99.5 g/mol.

\[
\frac{9.46 \text{ g} \text{X/molClO}_4^-}{99.5 \text{ gClO}_4^- / \text{mol}} = 0.095 \text{ mgX/mgClO}_4^-
\]

Therefore, if the yield for reduction of chlorate to chloride is 0.34 mgX/mg ClO\text{\textsubscript{3}}, the yield for reduction of perchlorate to chlorate is 0.1 mgX/mg ClO\text{\textsubscript{4}} based on electron equivalents exchanged during the degradation pathways.
APPENDIX C

KINETIC PARAMETER ESTIMATIONS FOR HCAP-C

The results of AQUASIM models shown in Figures C-1 through C-9. C-1, C-2, and C-3 are estimations of chlorate reduction kinetics. C-4, C-5, and C-6 are estimations of perchlorate reduction kinetics neglecting competitive inhibition. C-7, C-8, and C-9 are estimations of perchlorate reduction kinetics accounting for competitive inhibition.

Figure C-1. HCAP-C reduction of ClO$_3^-$ modeled by AQUASIM, Batch Test 1. $K$ was estimated to be 45.5 mgS/L and $q_{cmax}$ was estimated to be 5.2 mgS/mgX-day.
Figure C-2. HCAP-C reduction of ClO$_3^-$ modeled by AQUASIM, Batch Test 2. K was estimated to be 13.1 mgS/L and $q_{cmax}$ was estimated to be 8.6 mgS/mgX-day.
Figure C-3. HCAP-C reduction of ClO$_3^-$ modeled by AQUASIM, Batch Test 3. K was estimated to be 116.2 mgS/L and q$_{c_{\text{max}}}$ was estimated to be 11.1 mgS/mgX-day.
Figure C-4. HCAP-C reduction of ClO$_4^-$ modeled by AQUASIM, Batch Test 1 using standard Monod equations. K was estimated to be 64.4 mgS/L and $q_{cmax}$ was estimated to be 4.9 mgS/mgX-day.
Figure C-5. HCAP-C reduction of ClO$_4^-$ modeled by AQUASIM, Batch Test 2 using standard Monod equations. $K$ was estimated to be 88.2 mgS/L and $q_{c_{\text{max}}}$ was estimated to be 4.7 mgS/mgX-day.
Figure C-6. HCAP-C reduction of $\text{ClO}_4^-$ modeled by AQUASIM, Batch Test 3 using standard Monod equations. $K$ was estimated to be 77.3 mgS/L and $q_{\text{cm}}$ was estimated to be 3.6 mgS/mgX-day.
Figure C-7. HCAP-C reduction of ClO$_4^-$ modeled by AQUASIM accounting for competitive inhibition, Batch Test 1. $K$ was estimated to be 159.0 mgS/L and $q_{cmax}$ was estimated to be 10.3 mgS/mgX-day.
Figure C-8. HCAP-C reduction of ClO$_4^-$ modeled by AQUASIM neglecting competitive inhibition, Batch Test 2. K was estimated to be 226.2 mgS/L and q$_{cmax}$ was estimated to be 14.9 mgS/mgX-day.
Figure C-9. HCAP-C reduction of ClO$_4^-$ modeled by AQUASIM neglecting competitive inhibition, Batch Test 3. $q_{c_{\text{max}}}$ was estimated to be 9.2 mgS/mgX-day.
APPENDIX D

AQUASIM ESTIMATIONS OF HALF-SATURATION COEFFICIENT FOR PDA REDUCTION OF CHLORATE

The AQUASIM results for each batch test on PDA reduction of chlorate are shown in Figures D-1 through D-3.

![Graph showing PDA on Chlorate (10mg/L)](image)

**Figure D-1.** PDA reduction of chlorate with an initial chlorate concentration of approximately 10mg/L. The estimated K value is 7.5E-5 mgS/L.
Figure D-2. PDA reduction of chlorate with an initial chlorate concentration of approximately 5mg/L. The estimated K value is 5.0E-6 mgS/L.

Figure D-3. PDA reduction of chlorate with an initial chlorate concentration of approximately 1mg/L. The estimated K value is 0.4 mgS/L.
APPENDIX E

RESULTS OF HCAP-C AND PDA CO-CULTURE MODELING IN BATCH

AQUASIM modeling results of parameter estimations for co-culture batch experiments are presented below in Figures E-1 through E-6. Modeling results of batch tests of co-cultures of HCAP-C and PDA are presented in Figures E-7 through E-10.

Figure E-1. AQUASIM model of co-culture test 1 with a 7:1 ratio of HCAP-C to PDA. The predicted K value was 10.9 mg/L and the predicted $q_{max}$ was 1.58 mgS/mgX-d.
Figure E-2. AQUASIM model of co-culture test 1 with a 1:1 ratio of HCAP-C to PDA. The predicted K value was 0.19 mg/L and the predicted $q_{\text{max}}$ was 2.06 mgS/mgX-d.
Figure E-3. AQUASIM model of co-culture test 1 with a 1:3 ratio of HCAP-C to PDA. The predicted $K$ value was 36.83mg/L and the predicted $q_{\text{max}}$ was 3.93mgS/mgX-d.
Figure E-4. AQUASIM model of co-culture test 2 with a 7:1 ratio of HCAP-C to PDA. The predicted K value was 42.6 mg/L and the predicted $q_{\text{max}}$ was 3.3 mgS/mgX-d.
Figure E-5. AQUASIM model of co-culture test 2 with a 1:1 ratio of HCAP-C to PDA. The predicted K value was 16.4 mg/L and the predicted $q_{\text{max}}$ was 2.2 mgS/mgX-d.
Figure E-6. AQUASIM model of co-culture test 2 with a 1:3 ratio of HCAP-C to PDA. The predicted $K$ value was 22.72 mg/L and the predicted $q_{\text{max}}$ was 3.01 mgS/mgX-d.
Figure E-7. Model of a batch test of HCAP-C and PDA in co-culture grown on perchlorate with a ratio of 7:1 HCAP-C to PDA. Maximum chlorate accumulation was approximately 6.9 mg/L.
Figure E-8. Model of a batch test of HCAP-C and PDA in co-culture grown on perchlorate with a ratio of 1:1 HCAP-C to PDA. Maximum chlorate accumulation was approximately 0.9 mg/L.
Figure E-9. Model of a batch test of HCAP-C and PDA in co-culture grown on perchlorate with a ratio of 1:3 HCAP-C to PDA. Maximum chlorate accumulation was approximately 0.4 mg/L.
Figure E-10. Model of a Batch test of HCAP-C grown on perchlorate. Maximum chlorate accumulation was approximately 39 mg/L.
APPENDIX F

AQUASIM MODELING OF CO-CULTURES WITH HCAP IN A CSTR

Results of modeling co-cultures with AQUASIM are presented in figures F-1 through F-6.

Figure F-1. Model of a CSTR of HCAP and PCRB in co-culture grown on perchlorate. Steady state chlorate accumulation was approximately 8.6E-5 mg/L.
Figure F-2. Model of a CSTR of HCAP and CRB in co-culture grown on perchlorate. Steady state chlorate accumulation was approximately 0.05 mg/L.
Figure F-3. Model of a CSTR of PCRB and CRB in co-culture grown on perchlorate. Steady state chlorate accumulation was approximately 8.4E-5 mg/L.
Perchlorate Degradation in a CSTR (Co-Culture of HCAP, PCRB, and CRB)

Figure F-4. Model of a CSTR of HCAP, PCRB, and CRB in co-culture grown on perchlorate. Steady state chlorate accumulation was approximately 8.4E-5 mg/L.

Perchlorate Degradation in a CSTR (HCAP)

Figure F-5. Model of a CSTR of HCAP grown on perchlorate. Steady state chlorate accumulation was approximately 2.4 mg/L.
Figure F-6. Model of a CSTR of PCRB grown on perchlorate. Steady state chlorate accumulation was approximately $8.4 \times 10^{-5}$ mg/L.
APPENDIX G

NUCLEOTIDE SEQUENCES OF _DECLOROMONAS AGITATA_ (AY180108) AND _DECHLOROMONAS AROMATICA_ (CP000089)

The following are nucleotide sequences of the _pcrA_ gene of _Decloromonas agitata_ (AY180108) and _Dechloromonas aromatica_ (CP000089).

>`Decloromonas agitata`

1 atggcaegac tgtgctcag agatattttg aaggcatccg cgcagcactct gtcggttaat  
61 taactcact tcacacattt cagaggccaccc tcggccctt cagatattg ccgaagcaat cgtggctt  
121 ggctgggaaa attttcatcg caaccagtgg tcggtggcaca agaaaactcg cggagcgcac  
181 ctgatcaact gtactggggc ttgcccgcac ttcgtttata cgaaagatgg tgtggtcatt  
241 cgcgaggagc actcaagga cttccgcgcac tccggaatt aaccagcagc ggcggagcgc  
301 gcggagcgac ccgcacaccg ctgctgctac tcggccacat ttcctttctc cggctgctt  
361 cgctgctgct gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
421 gtcccatttg gccgagtcag gctcctccag cggagggttg gcggggtcag ccgcacac  
481 cgagtcggtg acggggttcc gccggtcctc ggcggagcgc cccagcgcct cgggggctt  
541 gtctccgctc ggcggagcgc gctcctccag cggagggttg gcggggtcag ccgcacac  
601 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
661 ctgctgctgct gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
721 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
781 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
841 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
901 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
961 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1021 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1081 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1141 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1201 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1261 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1321 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1381 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1441 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1501 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1561 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1621 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1681 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1741 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  
1801 gcgtcctcgc gcgcgtcgtc gcggagcgc acgcgaaccc gcggggagtc gtcggtctct  

111
1801 ctatattctg atgttgttc gccttcggct cactgtatg aaaaagtctga tctcaatgtg
1861 accgaagagc acacatttat caatatgacc gagccggcga tcaagcggcag tgggaatcg
1921 aagggctatc agaaatttaa tgaagcaagag ttaaatggg cagcataatcg
1981 ctggacaatg ccccggctg gccgaggacg ctggccagc ccaatcatt
2041 tggaaaccaga tgcagatgga cggaaaactc ggcaggagcg ctgaggcagc ccaatacatt
2101 cgcgattaac gcgagttg ttcgtaaaat ggcgcaaćg cccctggtgga aaccgagcag
2161 cgccttcgag cgacatcctc tcaagtctgaa aagaggggtg tgccttcgag
2221 aatatttgtt gtataaaaaa ggcgcaaacg cccctggtgga aaccgagcag
2281 gatcagcaga cgttcttcgat tatggaggtt gaattgecg gcgtataacg gccttcgag
2341 gcagacaat atcaccatcgc ctttacta cccctogcg acgcaacagc gccttcgag
tcacttcgac
2401 ttaaagacga tgtgtttgtg gtcctttttg cagcgccggg gaccatcctg tgccttcg
2461 tcaatcggac caaagactct cgtatcagac gccaggtt gccgtgcagc tggatgtgaac
2521 catggaagaag tcatggtgcg gtccttcpeg gcgcagcgacg cggcaatcatt
2581 atgtggagc ccccggggatgt ccttctgggc aagggggttg cgcgtgggctc
2641 ccccttcgac gccgcaac ccaacctcgc gcgcagcgacg cggcaacatccttggtttt
cgccttcgag
2701 aattactacg gccgcaacgc cgcagtcgagc gacgtcgccg tgaacatcagacgccta
tagaeta
APPENDIX H

ALIGNMENTS OF THE PCRA GENE

The following is the nucleotide alignment of the pcrA gene of *Dechloromonas aromatica*, *Dechloromonas agitata*, *Dechlorosoma* sp. HCAP-C, *Dechloromonas* sp. PC1, and *Dechlorosoma* sp. KJ.
The following is an amino acid alignment of the pcrA gene of *Dechloromonas aromatica*, *Dechloromonas agitata*, *Dechlorosoma* sp. HCAP-C, *Dechloromonas* sp. PC1, and *Dechlorosoma* sp. KJ.
APPENDIX I

CONSERVED DOMAINS OF THE PCRA GENE

The following are regions of conserved domains of the amino acid sequences of
the pcra gene of Dechloromonas aromatica, Dechloromonas agitata, Dechlorosoma sp.
HCAP-C, Dechloromonas sp. PC1, and Dechlorosoma sp. KJ.
Figure I-1 Conserved domains of the *pcrA* gene of *D. aromatica*.

Figure I-2 Conserved domains of the *pcrA* gene of *D. agitata*.

Figure I-3 Conserved domains of the *pcrA* gene of HCAP-C.

Figure I-4 Conserved domains of the *pcrA* gene of PC1.

Figure I-5 Conserved domains of the *pcrA* gene of KJ.