

Investigation of a Sulfur-Utilizing Perchlorate-Reducing Bacterial Consortium

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INVESTIGATION OF A SULFUR-UTILIZING PERCHLORATE-REDUCING BACTERIAL CONSORTIUM

A Dissertation Presented

by

TERESA ANNE CONNEELY

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Department of Microbiology

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TERESA ANNE CONNEELY

Approved as to style and content by:

Klaus Nüsslein, Chair

Jeffery Blanchard, Member

James F. Holden, Member

Sarina Ergas, Member

John Lopes, Department Head Department of Microbiology

DEDICATION

Lé grá dó mo chlann

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ABSTRACT

INVESTIGATION OF A SULFUR-UTILIZING PERCHLORATE-REDUCING BACTERIAL CONSORTIUM

MAY 2011

TERESA ANNE CONNEELY, B.A., SUFFOLK UNIVERSITY Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Klaus Nüsslein

We present research investigating how, with in depth knowledge of the community, microbial communities may be harnessed for bioremediation of hazardous water contaminants. We focused on the bacterial reduction of perchlorate, a common water contaminant. For this we studied the structure and capabilities of a novel sulfurutilizing, perchlorate-reducing bacterial (SUPeRB) consortium. Initially, we characterized the minimal consortium that retained functional capabilities, using 16S rRNA and functional gene analysis. A diverse functional consortium dominated by Beta-Proteobacteria of the family Rhodocyclaceae and sulfur-oxidizing Epsilon-Proteobacteria was found. We also examined the optimal growth conditions under which perchlorate degradation occurred and uncovered the upper limits of this function. Bacterial isolates were screened for function and the presence of functional genes.

We expanded to bioreactor studies at bench- and pilot-scale, and first used a perchlorate-reducing, bench-scale bioreactor to probe the stability of the microbial ecosystem. During stable reactor function, a core consortium of Beta- and Epsilon-Proteobacteria reduced perchlorate and the co-contaminant nitrate. A disturbance of the

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consortium led to a failure in function and to higher system diversity. This suggests that the SUPeRB consortium was not metabolically flexible and high population diversity was necessary for a return to stable function. In a pilot-scale bioreactor we determined that the SUPeRB consortium could stably degrade low levels of perchlorate to below the EPA maximum recommended limit. Field conditions, such as temperature extremes and intermittent perchlorate feed, did not negatively impact overall function. When all reactor consortia were compared we observed that the volume of the reactor and the initial inoculum were not as important to stable reactor function as the acclimatization of the consortium to the system and maintenance of favorable conditions within the reactor.

In summary we found that the SUPeRB consortium successfully degraded perchlorate in multiple systems. The study of this novel consortium expands our knowledge of the metabolic capabilities of perchlorate-reducing bacteria and suggests potential evolutionary pathways for perchlorate-reduction by microorganisms. The SUPeRB consortium may be used to establish bioremediation systems for perchlorate and other environmental contaminants.

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CHAPTER 1

INTRODUCTION

Perchlorate as an Environmental Contaminant

Perchlorate is currently under regulatory determination by the United States Environmental Protection Agency (USEPA) due to health risks associated with ingestion of this inorganic water contaminant. Perchlorate has a similar ionic radius and charge to iodine and can block the sodium-iodide symporter. Iodine uptake is thus inhibited by perchlorate leading to potential hazardous effects to human health. Iodine is an essential component of thyroid hormones (Siglin *et al.*, 2000) and impairment of thyroid function in expectant mothers may impact the fetus and newborn, resulting in adverse changes in behavior, delayed development, and decreased learning capability (Coates and Achenbach, 2004). To address these health concerns the USEPA (2008) has adopted an interim drinking water health advisory level of <15 μ g/L. The Commonwealth of Massachusetts became the first state in the U.S. to promulgate drinking water standards for perchlorate, setting the maximum detection limit at 2 μ g/L (CDPH, 2007).

Water or soil contamination results from many natural or man-made sources of perchlorate. Natural sources include Chilean nitrate fertilizer, and atmospheric deposition from rain, snow and lightning (Dasgupta *et al.*, 2005). Man-made sources include disposal of unused and outdated perchlorate propellants, road flares, fireworks, electroplating, and natural rubber manufacture (Cunniff *et al.*, 2006) and perchlorate can be a by-product of water disinfectants (Greiner *et al.*, 2008).

Following the development and implementation of a sensitive analytical method (USEPA, 1999), perchlorate was detected in the groundwater of 37 states and US territories (MADEP, 2008). Currently, perchlorate can be detected at concentrations as low as 0.11 µg/L (Metrohm, Riverview, Fl, 2010). Perchlorate can accumulate in many food sources and consumable liquids (Dasgupta *et al.*, 2006; Smith, 2006; Seyfferth and Parker, 2007) and has been found in consumables as diverse as milk (Dyke *et al.*, 2007), vitamins and mineral supplements (Snyder *et al.*, 2006), irrigation water for fruit (Sanchez *et al.*, 2006), seaweed, tap water, bottled water, wine, beer, and produce from many countries including Brazil, Chile, Columbia, Guatemala, Italy, Mexico, Poland, Spain, and Turkey (El Aribi *et al.*, 2006).

Perchlorate Bioremediation

Perchlorate ions consist of a stable structure of one chlorine atom in the center of a tetrahedral grouping of four oxygen atoms. The even distribution of negative charge over the four oxygen atoms makes the ion nonreactive with positively charged metallic centers. Perchlorate ions do not accept electrons directly from reductants and generally do not form complexes with minerals or organics; therefore, conventional water treatment methods such as precipitation do not remove perchlorate (Urbansky, 1998; 2000).

Established remediation methods involve physical removal of perchlorate by ion exchange or chemical reduction. However, physical methods produce a perchlorate contaminated matrix which must be further treated and chemical reduction is expensive (Tripp and Clifford, 2000; Urbansky, 2000; Gu and Brown, 2006). Bioremediation using perchlorate-reducing bacteria (PRB) has been successfully implemented as a method to reduce perchlorate to the innocuous by-products chloride and oxygen and is considered a cost-effective method of perchlorate reduction (Xu *et al.*, 2003; Coates and Achenbach, 2004).

Perchlorate-Reducing Bacteria (PRB)

PRB occur naturally in the environment and are found in perchlorate contaminated sites as well as pristine areas, possibly due to their ability to use alternate electron acceptors such as oxygen and nitrate (Coates and Achenbach, 2004; Waller *et al.*, 2004; Rikken *et al.*, 1996; Wallace *et al.*, 1996; Michaelidou *et al.*, 2000). Approximately 70 dissimilatory PRB are now in pure culture (i.e., Bruce *et al.*, 1999; Coates *et al.*, 1999; Wolterink *et al.*, 2005; Thrash *et al.*, 2010a; b) (Table 1.1). Known PRB are phylogenetically diverse with the most common PRB found in the Proteobacteria (Achenbach *et al.*, 2001; Zhang *et al.*, 2002; Coates and Achenbach, 2004). Ongoing research reveals PRB are also present in other phyla (see Table 1.1) (Balk *et al.*, 2008; 2010).

Current PRB isolates are generally characterized as denitrifying, facultative anaerobes that can either degrade or cometabolize perchlorate (Xu *et al.*, 2003; Coates and Achenbach, 2004). Perchlorate is highly oxidized and is an energetically favorable electron acceptor in microaerophilic or anaerobic environments (Herman and Frankenberger, 1998; Coates and Achenbach, 2004). PRB, in both mixed and/or pure cultures, can be heterotrophic (Cox *et al.*, 1999) or autotrophic, and have a large range of electron donors and acceptors. Organisms capable of autotrophic perchlorate reduction use a variety of inorganic electron donors including hydrogen (Nerenberg *et al.*, 2002;

2006; Zhang *et al.*, 2002; Logan and LaPoint, 2002; Adham *et al.*, 2006), reduced iron (Son *et al.*, 2006, Bardiya and Bae, 2005; Yu *et al.*, 2007), or sulfur compounds (Ju *et al.*, 2007; Sahu *et al.*, 2009). Artificial electron donors like graphite cathodes have also been described (Butler *et al.*, 2010). Sulfur as an electron donor has an energy yield with perchlorate reduction comparable to that of the use of hydrogen as and electron donor (Sahu, 2008; Table 1.2). In general, PRB grow optimally at neutral pH and in a pH-range of 5 to 9 (Coates and Achenbach, 2004; Wang *et al.*, 2008). Attaway and Smith (1993) found a redox potential of -110 mV for perchlorate reduction based on the redox indicator resazurin. To date, no isolated PRB are confirmed to grow by perchlorate respiration in salinities greater than 6%. The morphology of PRB is generally a rod shape; however, the PRB of the Alpha-Proteobacteria are commonly spirilli. Organisms with a high affinity for perchlorate have the ability to grow on low concentrations of perchlorate; therefore, the concentration of perchlorate in contaminated areas may select for different PRB (Waller *et al.*, 2004).

The perchlorate reduction pathway consists of two genes (Figure 1.1), chlorite dismutase (*cld*) (van Ginkel *et al.*, 1996) and perchlorate reductase (*pcrA*) (Kengen *et al.*, 1999). The *pcrA* gene reduces perchlorate and chlorate to chlorite, which is toxic to the bacterial cell. For complete degradation the *cld* gene is necessary to disproportionate chlorite to chloride and oxygen (Rikken *et al.*, 1996). Generally, neither of the intermediates, chlorate or chlorite, accumulate in solution under perchlorate-reducing conditions as the degradation of perchlorate to chlorate is the limiting step (Attaway and Smith, 1993). For each reaction, to convert perchlorate to chlorate, and chlorate to chlorate to chlorate, two electrons must be added to the chlorine center and one oxide ion removed

(Urbansky, 2000). A total of eight electrons are required for complete reduction of perchlorate (Shrout and Perkins, 2006).

The *cld* gene is expressed under both aerobic and anaerobic conditions but transcription is increased under perchlorate-reducing conditions. The *pcrA* gene is only transcribed under perchlorate-reducing conditions (microaerophilic or anaerobic) (Kengen *et al.*, 1999; Achenbach *et al.*, 2006). Previous analyses of PRB genomes indicate that in each genome *cld* and *pcrA* differ in gene organization and transcriptional orientation regardless of phylogenetic similarity. This suggests that horizontal gene transfer was involved in the evolution of the ability of multiple strains to reduce perchlorate (Achenbach *et al.*, 2006). That the ability to degrade perchlorate is transferred by horizontal gene transfer is also supported by differences in phylogenetic comparisons of the *cld* gene and the 16S rRNA gene (Bender *et al.*, 2004).

Sulfur as an Electron Donor

Many known PRB are capable of denitrification and some denitrifiers also are known to have the ability to cometabolize perchlorate (Coates *et al.*, 1999). Because elemental sulfur has been used as an electron donor by sulfur-utilizing bacteria capable of denitrification (Oh *et al.*, 2000; Kimura *et al.*, 2002; Tian *et al.*, 2003; Sengupta *et al.*, 2006; 2007) there is the potential that a perchlorate-reducing system could also use sulfur compounds as autotrophic electron donors. However, with the exception of a few recent studies (Ju *et al.*, 2007; 2008; Sahu *et al.*, 2009), there is no known literature report of successful sulfur-oxidation coupled with perchlorate reduction. Other researchers have unsuccessfully combined perchlorate reduction with elemental sulfur or thiosulfate as

electron donors using a perchlorate-reducing consortium enriched from sewage treatment samples, and also a pure culture of *Dechlorosoma* sp. (Bardiya and Bae, 2005).

The stochiometry for perchlorate reduction using elemental sulfur as an electron donor was derived by Sahu (2008) using the method of McCarty (1972). Assuming a yield factor of 60% for autotrophic growth, the following biochemical reaction was derived:

2.87 S° + 3.32 H₂O + ClO₄ +1.85 CO₂ + 0.462 HCO₃ + 0.462 NH₄⁺
$$\rightarrow$$

5.69 H⁺ + 2.87 SO₄²⁻ + Cl⁻ + 0.462 C₅H₇O₂N

In this reaction, 5.69 moles of H^+ are generated per mole of perchlorate utilized. Two moles of H^+ are produced for every eight moles of nitrate reduced leading to an acidic system (Oh *et al.*, 2000).

Perchlorate Remediation using Bioreactors

General issues for the bioremediation of water contaminants include acceptance by the public, sustainability, the control of microbial, nutrient and electron donor release, operational simplicity, and cost effectiveness to build and operate (Speth and Schock, 2007; Rittmann *et al.*, 2006). Bioreactors using heterotrophic substrates such as acetate or ethanol have been fully implemented for perchlorate reduction (Xu *et al.*, 2003). However, reactors using autotrophic substrates are desirable as these substrates, hydrogen, iron, and sulfur, are highly selective to bacterial growth. Furthermore, elemental sulfur is an excellent substrate as it is used by few microorganisms as an electron donor, thus reducing overgrowth of biofilms, also known as biofouling, and limits the range of byproduct formation. Sulfur pellets are cheap, non-toxic, and plentiful by-products of oil production that can be immobilized in a packed bed reactor and, as sulfur is water insoluble, do not enter the effluent stream.

Elemental sulfur as a packing medium in denitrifying packed bed reactors has been successfully implemented (Koenig and Liu, 1996; 2001; Sengupta *et al.*, 2007). Perchlorate-reduction in a packed bed rector with elemental sulfur was investigated by Sahu (2008) and Ju *et al.* (2007; 2008). However, the microbiology of these bioreactors was only briefly discussed leaving much to be discovered about these unique systems (Sahu *et al.*, 2009).

Microbial Ecology within Bioreactors

The goal of microbial ecology is to understand microbial communities and their interactions with and within their environment. In particular, microbial ecology determines which microorganisms are present, their community structure, the functional capabilities of the community, the relationships among the community members, and the ability of the community to respond to perturbations, i.e., community stability and resilience. To understand the connections between community structure and function microorganisms must be measured both spatially and temporally. For a bacterial community to function in a bioreactor it must be stable and resilient (Rittmann *et al.*, 2006). Bioreactors are manageable systems for studying these microbial ecology connections. However, few studies have examined the microbiology of consortia in perchlorate-reducing reactors (Zhang *et al.*, 2005; Chung *et al.*, 2009; Xiao *et al.*, 2010).

Function may remain stable regardless of changes in the bacterial community structure and interactions between all populations in the bioreactor, whether minor or dominant, may be important for maintaining the ecosystem stability (Briones and Raskin, 2003). The ecological principles underlying microbial community dynamics are poorly understood but elucidation of these principles and application to reactor design and operation could potentially improve system function stability (Wang *et al.*, 2010).

Hypotheses and Research Objectives

Preliminary studies show that a PRB enrichment culture, given the acronym SUPeRB for Sulfur-Utilizing, Perchlorate-Reducing Bacteria, reduced perchlorate at low concentrations (5 mg/L) (Sahu *et al.*, 2009).

The goal of this research project was to investigate the novel microbiological process of perchlorate reduction utilizing elemental sulfur. We hypothesized that a unique SUPeRB consortium is responsible for this process. To test this hypothesis the following research projects were conducted and are described in detail in this dissertation. Outlined below are goals and research objectives for each research project.

Goal 1: Characterization of the Microbial Consortium Coupling Perchlorate-Reduction to Sulfur-Utilization

In Chapter 2, the SUPeRB consortium carrying out the novel function of perchlorate-reduction using sulfur as an electron donor was investigated. To accomplish this, the minimal consortium capable of this process was identified and the characteristics of this consortium were examined. The objectives of this study were to (1) determine the minimal SUPeRB consortium that retained function, (2) characterize the growth parameters of the consortium, and (3) identify whether direct bacterial attachment to the

sulfur is necessary for growth. By addressing the objectives of this study we are the first to report on this consortium.

Goal 2: Phylogenetic Structure and Functional Relationships in a Bench-Scale

Bioreactor

In Chapter 3, the ecology and stable function of the SUPeRB consortium in a bench-scale bioreactor was investigated. To accomplish this, the consortium was inoculated into the reactor and the microbial consortium structure was examined spatially and temporally within the reactor. The objectives of this study were to (1) determine the consortium that stably reduced perchlorate over time, (2) examine spatial and temporal changes in the stably functioning consortium, and (3) determine the effect of a disturbance, i.e., the addition of nitrate as a competing electron accepting contaminant. By addressing the objectives of this study, this chapter presents answers to core microbial ecology questions of the role of microbial diversity in function and long-term stability of this function.

Goal 3: Microbiological Investigation of the SUPeRB Consortium from the Pilot-Scale Bioreactor

In Chapter 4, the effect of scale-up on the microbial ecology of the SUPeRB consortium was investigated in a pilot-scale bioreactor. To accomplish this, the microbiology of the pilot-scale reactor was examined when stable degradation of perchlorate was established. The objectives of this study were to (1) successfully scale-up and inoculate a large culture of SUPeRB into a pilot-scale reactor, (2) examine the

microbial structure of the pilot-scale reactor, and (3) determine whether microbial processes at this scale are inhibitory to the consortium. By addressing the objectives of this study, this chapter answers whether the consortium is robust in field conditions and whether perchlorate degradation occurs regardless of shifts in temperature, perchlorate concentration, nutrient availability, oxygenation of feed water or build up of by-products.

Goal 4: Comparing SUPeRB Consortia to Elucidate Core Structure

In Chapter 5 the similarities among SUPeRB consortia from several starting inocula and in different growth vessels were determined. To accomplish this, the nucleic acid based community composition of enrichment cultures and the 0.2 L, 1 L, and 200 L bioreactors were compared using the software program mothur (Schloss *et al.*, 2009) and principle component analysis. The objectives of this study were (1) to determine the similarities in the consortia from different starting inocula and (2) to determine the core SUPeRB consortium. By addressing the objectives of this study, this chapter answers whether stable function is due to metacommunities independently stabilizing to form a core community of SUPeRB.

Significance

This research adds to the understanding of autotrophic perchlorate reduction, the microbial community involved and the environment in which the microbes function. Insights into microbial ecology and biogeochemical cycles are obtained by studying microorganisms in their niches where many transformations are catalyzed by consortia

and not by single species of microorganisms. SUPeRB may be used as a cost-effective biological treatment for perchlorate contaminated water supplies.

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Phylogeny	E- donor	E- acceptor	Source	Temperature /pH/Salinity	Reference
Alpha-Proteobacteria				· · · ·	
Azospirillum sp. TT1	Acetate	ClO_4^-, ClO_3^-	Contaminated soil		Coates et al., 1999
Magnetospirillum (Dechlorospirilium) anomalous sp. WD	Includes: Acetate, Ethanol, FeCl ₂	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Swine waste lagoon, contaminated and uncontaminated soils and sediments	25-37°C (35) /6.5-7.5 (7.2) /<1.0% NaCl	Coates <i>et al.</i> , 1999; Michaelidou <i>et al.</i> , 2000; Trash et al., 2010a
Azospirillium AJ2, ABL1, PMS1, PMS2, SN1A, SN1B, SN2	Acetate, Oleate, Molasses, Canola oil	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻	Contaminated soil		Waller <i>et al.</i> , 2004
(Dechlorospirillum) VDY	H ₂ , Acetate, AH2DS, Ethanol, Glucose, Yeast extract, Lactate, Casamino acids	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Cathode chamber of bioelectrical reactor with creek water enrichment		Trash <i>et al.</i> , 2007
Dechlorospirilium anomalous strain JB116	Acetate	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻	Primary settling tank of sewage treatment plant, South Korea	25–35°C /7–7.8 /<0.5% NaCl	Bardiya and Bae, 2008
Magnetospirillum bellicus sp. nov. VDY ^T	Includes: H ₂ , Acetate, AHDS, Ethanol, FeCl ₂	$\begin{array}{c} ClO_4, \\ (Transient \\ ClO_3) ClO_3, \\ O_2, NO_3, \\ NO_2, NO_2, N_2O \end{array}$	Cathode chamber of bioelectrical reactor with creek water enrichment	10-42°C (42) /6.8 /<1.5% NaCl	Trash <i>et al.</i> , 2010a
<i>Dechlorospirillum</i> sp. SN1					Achenbach and Coates, unpublished AY171615

Table 1.1 Review of perchlorate-reducing bacteria.

Beta-Proteobacteria					
Dechlorosoma sp. GR-1	Acetate, Succinate,	ClO_4 , ClO_3 ,	Activated sludge from a	30°C	Rikken et al., 1996
	Malate, Propionate,	NO_3 , O_2 , Mn	domestic waste water	/1	
	Caprionate	(IV)	treatment plant		
Dechloromonas agitata CKB	Acetate	ClO_4^-, ClO_3^-, O_2^-	Paper mill waste sludge		Bruce <i>et al.</i> , 1999 Achenbach <i>et al.</i> , 2001
Dechloromonas sp. NM,	Includes Acetate,	$ClO_4^-, ClO_3^-,$	Contaminated and		Coates et al., 1999
CL	Propionate, Lactate	O ₂	uncontaminated soils and sediments		
Dechloromonas sp.	Includes Acetate,	ClO_4 , ClO_3 ,	Contaminated and		Coates et al., 1999
MissR, SIUL	Ethanol, Lactate	NO_3 , O_2	uncontaminated soils		
			and sediments		
Azospira oryzae	Includes Acetate,	ClO_4 , ClO_3 ,	Contaminated and	37°C	Coates et al., 1999;
(Dechlorosoma suillum)	Ethanol, Lactate	NO_3 , O_2	uncontaminated soils	/6.5	Achenbach et al.,
sp. PS, Iso1, Iso2,			and sediments, primary	/0% NaCl	2001
SDGM			treatment lagoon of		
D 11	T 1 1 4 4 4		swine waste	20,4000 (25	
Dechlorosoma sp.	Includes Acetate,	CIO_4 , CIO_3 ,	Biosolids enrichment	20-40°C (25-	Herman and
Perclace	Y east extract	NO ₃		(50)	Frankenberger,
				/6.5-8.5 (/.0-	1999
Dechlenemen as an IM	II with agatata	C10 - C10 -	A stiveted sludge	1.2)	Millor and Logan
Dechioromonas sp. Jivi	n ₂ with acetate	$NO_2^- O_2$	aeration basin of a		2000
		1003, 02	wastewater treatment		2000
			nlant		
Dechloromonas sp					Achenbach <i>et al</i>
CCO CL24 CL24+					2001
FL2, FL8, FL9					
Dechloromonas	4-chlorobenzoate	ClO_4 , ClO_3 ,	Aquatic sediment		Coates et al., 2001

aromatica sp. RCB		NO_3 , O_2			
Dechlorosoma sp. PDC,	Lactate, Acetate	ClO_4 , ClO_3 ,	Primary digester sludge		Logan et al., 2001
PDD, PDE		O ₂	enrichment with lactate		
Dechlorosoma sp. PDX,	Lactate, Acetate	ClO_4 , ClO_3 ,	Primary digester sludge		Logan <i>et al.</i> , 2001
PDY		NO_3 , O_2	enrichment with lactate		
Dechlorosoma sp. KJ,	Lactate, Acetate	ClO_4 , ClO_3 ,	Perchlorate-degrading		Logan et al., 2001
KJ3, KJ4		NO_3^-, O_2	bioreactor with acetate		
			feed		
Dechloromonas sp. HZ	H ₂ , Acetate	ClO_4 , ClO_3 ,	Perchlorate-reducing		Zhang et al., 2002
		NO_3^-, O_2	bioreactor		
Dechloromonas sp.,	Acetate, Molasses,	ClO_4 , ClO_3 ,	Groundwater		Waller et al., 2004
EAB1, EAB2, EAB3,	Oleate, Canola oil	NO ₃ ⁻			
ABL2, PMC,					
RC1, RC2, PR, INS					
Dechloromonas sp.	H ₂ , Butyrate, Lactate,	ClO_4 , ClO_3 ,	H ₂ -fed microcosm with	30°C	Shrout <i>et al.</i> , 2005
JDS5, JDS6	Acetate, Propionate,	NO_3^- ,	contaminated		
		Fumarate	groundwater and soil		
			Grows in flocs/clumps		
Dechloromonas	Acetate, Propionate	ClO_4 , ClO_3 ,	Garden soil	30°C	Wolterink et al.,
<i>hortensis</i> sp. nov. $MA-I^{T}$		NO_{3}^{-}, O_{2}		/7.2	2005
Dechloromonas sp. PC1	H ₂ , Acetate	ClO_4 , ClO_3 ,	H ₂ ,-based, autotrophic		Nerenberg et al.,
		NO_3^-, O_2	hollow-fiber membrane		2006
			biofilm reactor		
Dechlorosoma sp.	Acetate, H ₂ ,	ClO_4 , ClO_3 ,	Municipal activated		Dudley et al., 2008
HCAP-C (PCC)	Accumulates	O_2 , NO_3^- ,	sludge		
	chlorate, cannot	NO_2^-			
	reduce ClO_4 - < 200				
	mg/L				
Propionivibrio militaris	Includes Acetate,	ClO_4 , ClO_3 ,	Cathode chamber of	10-37°C (30)	Thrash <i>et al.</i> , 2010b
sp. nov. MP^{T}	Lactate, Fe(II), H ₂ ,	$NO_{3}^{-}, O_{2},$	bioelectrical reactor	/ 6.0-7.5 (6.8)	

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	AHDS	NO ₂	with creek water enrichment	/1% NaCl	
Propionivibrio militaris sp. nov. CR	Includes Acetate, Lactate, Yeast extract, AHDS, Ethanol	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂	Cathode chamber of bioelectrical reactor with creek water enrichment	30°C /7 /<1% NaCl	Thrash <i>et al.</i> , 2010b
Dechlorobacter hydrogenophilus LT-1 ^T	Includes Acetate, Yeast extract, Ethanol, H ₂ , AHDS	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , O ₂ Mn (IV)	Perchlorate contaminated soil	4–37°C (37) /6.0–7.2 (6.5) /1% NaCl	Thrash <i>et al.</i> , 2010b
Epsilon-Proteobacteria <i>Wolinella succinogenes</i> Hap1	H ₂ , Formate	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻ , Fumarate, Asparatate, Malate	Anaerobic sewage enrichment culture	20-45°C (40) /6.5-8 (7.1)	Wallace <i>et al.</i> , 1996
Gamma-Proteobacteria					
Vibrio dechloraticans Cuznesove B-1168	Acetate, Ethanol	ClO ₄ , ClO ₃ , NO ₃			Romanenko <i>et al.</i> , 1976
Citrobacter sp. IsoCock1	Yeast extract, Acetate	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻	High salt and/or high density hydrocarbon oxidizing enrichments	20-35°C (30) /6.0-9.0 (7.5) /5% NaCl	Okeke <i>et al.</i> , 2002
Citrobacter amalonaticus strain JB101 Citrobacter farmeri strain JB109	Acetate	ClO ₄ ⁻ , ClO ₃ ⁻ , NO ₃ ⁻	Primary settling tank of sewage treatment plant, South Korea		Bardiya and Bae, 2004
Pseudomonas stutzeri PseudoaeroA1	Nutrient broth	ClO ₄	Soil from a perchlorate- manufacturing factory	25-50°C /5-9	Shete <i>et al.</i> , 2008
Actinobacteria					
Sinomonas sp.	Nutrient broth	ClO ₄	Soil from a perchlorate-	25-50°C	Shete <i>et al.</i> , 2008

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ArthroaeroA2,			manufacturing factory	/5.0-9.0	
ArthroaeroA3					
Firmicutes (Clostridia)					
Moorella	Includes Methanol,	ClO_4 , ClO_3 ,	Underground gas	40-70°C (55-	Balk et al., 2008
perchloratireducens sp.	CO, Glucose,	NO ₃ ⁻ , AQDS,	storage tank	60)	
nov. An10		thiosulfate,	_	/7	
		Fe(III)		/1% NaCl	
		complexes			
Sporomusa sp. An4	Includes H ₂ /CO ₂ ,	ClO_4 , ClO_3 ,	Underground gas	20-40°C (37)	Balk et al., 2010
	Methanol, Ethanol,	NO ₃ ⁻	storage reservoir	/5.5-8.0 (7.0)	
	CO, Lactate				
Unknown					
D-8	Lactate, Acetate	ClO_4 , ClO_3 ,	Activated-sludge		Logan et al., 2001
		NO_3 , O_2	aeration basin with		
			lactate		

AQDS is the humic substances analog: anthrohydroquinone-2,6-disulfonate. Temperature /pH/Salinity: Optimum values presented in parentheses.
Table 1.2. Comparison of energy yields from elemental sulfur and hydrogen.

Autotrophic electron donors for perchlorate and nitrate reduction, values for hydrogen are adapted from Nerenberg *et al.*, 2002 and those for elemental sulfur from Sahu, 2008.

Acceptor	∆Go [kJ/e- with hydrogen]	∆Go [kJ/e- with sulfur]
Perchlorate	-112.1	-113.0
Nitrate	-112.2	-91.0



Figure 1.1. Per(chlorate) reduction pathway.

CHAPTER 2

CHARACTERIZATION OF THE MICROBIAL CONSORTIUM COUPLING PERCHLORATE-REDUCTION TO SULFUR-UTILIZATION

<u>Abstract</u>

The unique consortium capable of using sulfur as an electron donor and perchlorate as an electron acceptor (SUPeRB) was characterized. Members of the consortium were isolated on solid medium but in pure culture were not capable of perchlorate reduction in this system. A perchlorate-reducing strain with this unique metabolic ability did not grow in pure culture but was identified by functional gene analysis and potentially by 16S rRNA to be a Beta-Proteobacterium within the family Rhodocyclaceae with a distant similarity to *Azospira* sp. Within the consortium perchlorate was reduced optimally at low concentrations, anaerobically, at 20°C, and at near neutral pH of 7 to 8. The consortium also reduced nitrate, chlorate, selenate, thiosulfate, and nitrite using sulfur as an electron donor. Attachment to the solid electron donor was not necessary for perchlorate reduction. The study of this novel consortium may be used to establish bioremediation systems for perchlorate and other environmental contaminants.

Introduction

Novel species and functions of perchlorate-reducing bacteria (PRB) continue to be discovered (Balk *et al.*, 2010; Thrash *et al.*, 2010). Known perchlorate-reducing isolates and consortia have been isolated from an array of environments and are physiologically diverse. PRB reduce perchlorate at a wide range of perchlorate concentrations, temperatures and salinities, use diverse electron donors and acceptors, and can be autotrophic or heterotrophic (Xu *et al.*, 2003; Coates and Achenbach, 2004).

Autotrophic perchlorate-reduction has been described for organisms that use inorganic compounds as electron donors such as hydrogen (Giblin *et al.*, 2000; Nerenberg *et al.*, 2002; 2006; Zhang *et al.*, 2002; Logan and LaPoint, 2002; Adham *et al.*, 2006), reduced iron (Bardiya and Bae, 2005; Son *et al.*, 2006; Yu *et al.*, 2006), sulfur compounds (Ju *et al.*, 2007; Sahu *et al.*, 2009), or graphite cathodes (Butler *et al.*, 2010), and inorganic carbon is used as a carbon source.

The sulfur-utilizing autotrophic denitrification (SLAD) process is reported to be robust and cost-effective for treating water contaminated with nitrate, a common cocontaminant to perchlorate (Koenig and Liu, 2002). However, few species of autotrophic bacteria can carry out sulfur dependent denitrification, and knowledge of these bacteria is limited (Soares, 2002; Wang and Qu, 2003). Even less is known about the bacteria that carry out the recently discovered sulfur-utilizing, perchlorate-reducing bacterial (SUPeRB) process (Sahu *et al.*, 2009).

In this chapter the microbial ability of SUPeRB is described. It was hypothesized that two or more bacterial species worked in a consortium to oxidize sulfur and reduce perchlorate. The presence of other bacterial species may also be necessary to remove waste products created by the SUPeRB process or to create habitable conditions for the SUPeRB. The community structure of the consortium was characterized by phylogenetic analysis of the universal structural gene, 16S rRNA, and perchlorate-specific functional genes, *pcrA* and *cld*. Optimal growth parameters were investigated, including perchlorate

and sulfur concentration, pH, temperature, oxygen level, and requirement of the trace element molybdenum. The ability to grow with increased salinity, increased surface area of powdered sulfur, and alternate electron acceptors and donors was also examined. The necessity of bacterial attachment for perchlorate reduction to occur was determined in batch cultures containing the solid electron donor (sulfur pellets) and medium buffer (oyster shells).

Materials and Methods

Consortium Enrichment

The SUPeRB culture was enriched in minimal medium from an inoculum of mixed liquor suspended solids taken from the denitrification zone of a wastewater treatment facility using methanol as an electron donor (Lanesboro, MA, June 2008). This enrichment was referred to as E1. The minimal medium contained the following components per liter of ground water: 6.5 mg of NaClO₄, 0.5 mg of NaHCO₃, 8.5 mg of KH₂PO₄, 21.75 mg of K₂HPO₄, 33.4 mg of Na₂HPO₄·7H₂O, 22.5 mg of MgSO₄·7H₂O, 0.25 mg of FeCl₃·6H₂O, 27.5 mg of CaCl₂, 10 mg of (NH₄)₂SO₄, 1 ml/L of 0.05% resazurin, 30 g sulfur pellets (Georgia Gulf Sulfur Corporation, Valdosta, GA), and 10 g oyster shell (Myco Supply, Pittsburgh, PA). The medium was sparged with a mixture of 80% N₂ and 20% CO₂ for 30 minutes and incubated at 20°C while shaking at 120 RPM. A parallel culture was also inoculated with a frozen SUPeRB culture consisting of a perchlorate-reducing consortium from the active zone of a perchlorate-degrading bioreactor (Sahu *et al.*, 2009). This latter enrichment was referred to as E2.

Consortium Dilution

When perchlorate was reduced by E1 and E2, a 1:10 dilution series was performed (Figures 2.1 and 2.2). Balge tubes with 10 ml volumes of minimal medium were gassed for 12 minutes with a mixture of 80% N₂ and 20% CO₂, then stoppered, crimp capped and autoclaved. Two inocula were prepared by filtering 20 ml of the enrichments through a 0.22 µm filter. The filters were placed in 2 ml of phosphate buffer and agitated at 150 rpm for 15 min. This inoculum was equally divided over three balge tubes. Each series was then diluted a further seven times. Uninoculated tubes served as a control. The cultures were incubated at 20°C in the dark. The medium contained particulate matter making visualization of cell growth by turbidity impossible. Biological activity was indirectly measured by perchlorate, sulfate, and chloride measurement. The most dilute culture where perchlorate-reduction was observed was used to inoculate a second dilution series. Two further dilution series were performed for E1 for a total of four dilution series. The fourth dilution series was in 125 ml serum bottles containing 50 ml of minimal medium. One further dilution series was performed for E2 for a total of three dilution series.

Analytic Measurements

Perchlorate, sulfate, chloride, nitrate and nitrite concentrations were measured by ion chromatography (IC) using a Metrohm 850 Professional IC AnCat MCS system equipped with an 858 Professional Sample Processor, a Metrosep A Supp 7 – 250 column and a Metrosep RP Guard column (Metrohm-Peak, LLC, Houston, TX). The final eluent consisted of 20% acetronitrile and 10 mM sodium carbonate and a final flow rate of 0.6

mL/min (USEPA, 1999). The detection limit for perchlorate was 5 μ g/L. Thiosulfate, chlorate, and selenate were measured with a Metrosep A Supp 7 – 250/4.0 column and a Metrosep RP Guard column (both from Metrohm-Peak, LLC, Houston, TX) with an eluent of 3.2 mM sodium bicarbonate and 1.0 mM sodium carbonate and a final flow rate of 0.6 mL/min.

Phylogenetic Analysis of the Minimal Consortium

Clone libraries based on the 16S rRNA gene were constructed from the second dilution series of enrichments E1 and E2, namely, of the dilution steps immediately before and after the dilution step that still indicated perchlorate reduction. The fourth dilution series of E1 was sampled on days 0, 11, 19, 25, and 30. The consortium composition in a culture grown with 50 mg/L perchlorate was also examined. For each sample, perchlorate, sulfate, and chloride were measured (Figures 2.3 and 2.4) and 5 ml of the culture was filtered through a 0.2 μ m filter. The filters were stored at -30° C until DNA was extracted using the RapidWater® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene was amplified from total genomic DNA in triplicate PCRs. A 30 µl reaction volume had the following final concentrations: 0.5 ng/µl DNA, 0.5 µM of each primer 8F and 1492R (Weisburg et al., 1991), 2 mM MgCl₂, 10x PCR buffer, 0.25 mM of each dNTP, 0.08 U/µl Taq DNA polymerase, and 400 ng/µl BSA. The following PCR program was run: 95°C for 3 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; one cycle of 72°C for 5 min, on an MJ Research Peltier Thermal Cycler PTC-200 (GMI, Inc., Ramsey, MN). Triplicate PCR products were pooled to reduce amplification bias, and cleaned using a QIAquick® PCR purification kit (Qiagen Inc., Valencia, CA). The expected fragment was visualized on a 1% agarose gel stained with ethidium bromide. The 16S rRNA gene was cloned into the pGEM-T Easy Vector Systems kit (Promega, Madison, WI), and E. coli JM109 high-efficiency competent cells (Promega, Madison, WI) were transformed in accordance with manufacturer's instructions. Clones were grown into colonies and positive colonies were randomly picked. Amplification of the 16S rRNA gene from each clone was carried out in a 30 μ l reaction volume with the following final concentrations: 0.5 ng/ μ l DNA, 0.33 µM of each pGEMf (5'-GCA AGG CGA TTA AGT TGG G-3') and pGEMr (5'-ATG ACC ATG ATT ACG CCA AG-3') primers; 1.75 mM MgCl₂; 10x PCR buffer; 0.17 mM of each dNTP; 0.1 U/µl of *Taq* DNA polymerase. The following PCR program was used: 95° C for 3 min; 30 cycles of 94° C for 30 s, 65° C for 30 s, 72° C for 30 s; one cycle 72° C for 5 min. Selected clones from each sample were submitted for 16S rRNA gene sequencing. PCR amplified products were pooled, cleaned, amplified with BigDye®Terminator V.3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA). Sequences were manually edited, checked for possible chimeric structures using the software package Mallard (http://www.cf.ac.uk/biosi/research/biosoft/Mallard/index .html, School of Biosciences, Cardiff University), compared to the NCBI database (Altschul et al., 1997), and classified using Ribosomal Database Project (Release 9.57, Wang et al., 2007) for nearest matches.

<u>Functional Gene Detection</u>: A forward and reverse primer pair, pcrAF and pcrAR, was created and tested using BLAST. This primer pair specifically selects for all available conserved regions of protein and DNA PRB sequences of the *pcrA* functional gene. The *cld* gene was amplified as outlined by Bender *et al.* (2004) with the exception

that the PCR reactions were carried out in 30 μ l reactions. The *pcrA* and *cld* genes were amplified from the minimal consortium total DNA in triplicate PCR reactions. The pcrA gene was amplified in a 30 μ l reaction volume with the following final concentrations: 0.5 ng/µl DNA, 0.4 µM of each primer pcrAF 5'-ACTACATGTATGGNCCGCATCG-3' and pcrAR 5'-CGTGRTCRCYGTACCAGTCRAA-3', 1.5 mM MgCl₂, 1x PCR buffer, 0.20 mM of each dNTP and 0.05 U/ μ l Taq DNA polymerase, and 250 ng/ μ l BSA. The following PCR program was used: 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; one cycle of 72°C for 10 min. The appropriate PCR product size, cld (365 bp) and pcrA (278 bp), was confirmed on a 1% agarose gel. Triplicate PCR products were pooled to reduce amplification bias, and the mixture was cleaned using a QIAquick[®] PCR purification kit (Qiagen Inc., Valencia, CA). The two functional genes were sequenced and closest relatives identified as previously outlined. Multiple sequence alignments were created using the program ClustalX, V.1.83 (Thompson et al., 1997), and phylogenetic analyses were conducted using the software package MEGA V.4 minimum evolution analysis, using the Tamura-Nei model, with bootstrap values of 1,000 replicates (Tamura et al., 2007).

Characterization of the Minimal Consortium

The most dilute minimal consortium of E1 that showed perchlorate reduction in the fourth serial dilution was characterized in liquid minimal medium to determine growth parameters. A total of eleven parameters were tested to characterize the minimal consortium (Table 2.1). Each parameter was measured in triplicate in balge tubes containing 15 ml minimal medium. Perchlorate reduction was used as an indicator of metabolic activity of the SUPeRB consortium. Perchlorate was added at 5 mg/L unless otherwise indicated. All chemicals were from Fisher Scientific, Fair Lawn, New Jersey, except the electron donors and acceptors (Sigma, St. Louis, MO).

The polysulfide stock solution was prepared as follows: 12 g of crystalline sodium sulfide and 1.6 g powdered sulfur were added to 30 ml anoxic water, shaken for 1 hr at room temperature, and the solution was brought to a volume of 100 ml for a final concentration of 50 mM sodium polysulfate.

Growth of Isolates from the Minimal Consortium

Aliquots from the tests of perchlorate concentration as a growth parameter at 5 mg/L and 50 mg/L were diluted 1:10 for four dilution steps. The five dilutions were plated anoxically on solid R2A medium containing 5 mg/L perchlorate, 1 ml/L of a 0.5 mg/ml resazurin stock solution, and 0.25 mM of L-cysteine hydrochloride, and incubated at 20°C. The plates were placed in gas-tight bags with an atmosphere of 80% N₂ and 20% CO_2 . Isolates were selected, tested for the presence of the functional gene and identified by the 16S rRNA gene.

Quantitative PCR (qPCR)

Standard curves were created from a *pcrA* gene amplified from the control strain *Dechlorosoma* (renamed *Azospira*) *suillum* PS and cloned into a plasmid. The copy number of the plasmid was calculated by measuring absorbance at 260 nm. A dilution series from 10^6 to one gene copies/ml of the DNA was performed and the cycle threshold (C_T) values were plotted against gene copy number per volume. The copy numbers of

samples were calculated after real-time amplification from the linear regression of the standard curve.

DNA extracted from the fourth dilution of E1 was tested for the relative quantity of the functional gene, *pcrA*, at each dilution. PCR amplification was performed in 20 µl final volumes containing 1 µl of DNA, 0.16 µM each of pcrAF and pcrAR, and 10 µl of GoTaq® qPCR Master Mix (Promega, Madison WI). All amplifications were carried out in Thermo-Fast® white 96-well PCR plates (Thermo Scientific, Epsom, UK) on a DNA Engine Opticon® 2 System (Bio-Rad, Hercules, CA) with an initial step of 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by an elongation step at 72°C for 5 min. All samples were performed in triplicate.

Attachment of SUPeRB to Solid Electron Donor or Buffer Material

Each attachment possibility, sulfur pellets, oyster shells and SUPeRB consortium, was constrained to determine if attachment to a solid surface was necessary for successful perchlorate reduction. In 50 ml conical tubes containing 35 ml of perchlorate minimal medium each constraint was measured in triplicate with the exception of the negative and positive controls, which were measured in duplicate due to the number of constraint devices. The dialysis device was made of ultra-pure biotech cellulose ester membrane with an 8-10 Kda cutoff with a 1 ml volume fitted to a resealable container (Spectra/Por® Float-A-Lyzer®, Spectrum Laboratories, Inc., Rancho Dominguez, CA).

The positive and negative controls had sulfur pellets and oyster shell added directly to the tube along with an empty resealable dialysis device. The negative control had no bacteria added. To test whether direct contact with the sulfur pellets was necessary, samples (termed SP) had sulfur pellets constrained by the dialysis device, while oyster shell and bacteria were added directly to the tube. In parallel samples the need for direct contact with oyster shells was tested. In these samples (termed OS) oyster shell was added into the dialysis device and sulfur pellets and bacteria were added directly to the tube. In a third series (termed B) the bacteria were constrained by adding them to the dialysis device, while oyster shell and sulfur pellets were added directly to the tube. The bacterial inoculum was from the fourth dilution of the minimal consortium E1. The media was sparged with 80% N₂ and 20% CO₂. The conical tubes were sealed with tape and incubated at 20°C in a gas-tight jar with a BBLTM GasPakTM plus anaerobic system envelopes with palladium catalyst (BD, Sparks, MD), which created a H_2/CO_2 atmosphere.

Results

Perchlorate Reduction in Consortium Dilutions

<u>Dilution series 1</u>: For E1, perchlorate was reduced from approximately 6.0 mg/L to below the detection limit in the fifth dilution within 45 days. For E2, perchlorate was 53% reduced from 6.0 mg/L to 2.8 mg/L in the third dilution within 45 days. The fifth dilution of E1 and the third dilution of E2 were used for the second dilution series (Figures 2.1 and 2.2).

<u>Dilution series 2:</u> For E1, perchlorate was reduced to below the detection limit in the first five dilutions as measured on day 67 of incubation. In dilutions six and greater no perchlorate reduction was measured compared to the control. For E2, perchlorate was reduced to under the detection limit in dilution two in 72 days, while no perchlorate

reduction was seen in further dilutions. The fifth dilution of E1 and the second dilution of E2 were used for the third dilution series (Figures 2.1 and 2.2).

<u>Dilution series 3:</u> For E1, perchlorate reduction varied within the three replicates with perchlorate reduction to below the detection limit occurring after 46 days in dilutions 3, 4, and 5 in only one replicate. For E2, perchlorate reduction to below the detection limit only occurred in the first dilution of one of three replicates after 46 days. Only E1 dilution five, where perchlorate was reduced to below the detection limit, was used to inoculate a fourth dilution series (Figure 2.1).

<u>Dilution series 4:</u> Perchlorate was reduced to below the detection limit within 19 days in two of the triplicates of dilution 4. Perchlorate in the third triplicate was reduced to below the detection limit within 37 days (Figure 2.3). Sulfate remained constant from 27 to 55 ± 2 to 7 mg/L over days 0 to 43. Chloride increased from 10 to 63 mg/L from days 0 to 4 and remained constant from days 4 to 43 at 36 to 63 ± 2 to 5 mg/L (Figure 2.4).

Phylogenetic Analysis of the Minimal SUPeRB Consortium

<u>Dilution series 2:</u> The minimal consortium was analyzed in E1 dilutions four, five, and six, and in E2 dilutions 1 and 2 (Table 2.2). In the actively degrading consortia, Alpha- and Beta-Proteobacteria were found in similar quantities and Epsilon-Proteobacteria were a minor population of the consortium. The Alpha-Proteobacteria were of the orders Sphinomondales and Rhizobiales, family Rhizobiaceae genus *Agrobacterium* sp. and species *Rhizobium selenireducens*. The Beta-Proteobacteria were generally of the family Rhodocyclaceae, distantly related to the genus *Azospira* and the family Hydrogenophilales, distantly related to the genus *Thiobacillus sp.* The Epsilon-Proteobacteria were most similar to the genus *Sulfuricurvum* of the family Helicobacteraceae. In dilutions where perchlorate was no longer reduced the numbers of Alpha-Proteobacteria tended to increase as the Beta-Proteobacteria decreased.

<u>Dilution series 4:</u> To analyze the E1 consortium, samples were taken from the combination of filters from two replicates of dilution four on days 0, 11, 19, 25, and 30 (Table 2.3). Samples for time 0 did not yield any positive clones. When clones for other time-points were checked for an insert of the correct size by gel electrophoresis, few clones were positive for the correct insert.

<u>High perchlorate enrichment:</u> The consortium from the 50 mg/L perchlorate concentration tube parameter was also analyzed (Table 2.3).

Functional Gene Detection and Identification

<u>Dilution two:</u> The *cld* gene from E1 appeared in two clusters. One cluster was most closely related to *D. agitata* AY124796, while the other cluster was most closely related to *Azospira oryzae* AY540964 (Figure 2.5). The *pcrA* gene was most closely related to *D. agitata* AY180108 (Figure 2.6). The *cld* gene from E2 was most closely related to *A. oryzae* AY540960 (Figure 2.7), and the *pcrA* gene was most closely related to *Azospira* sp. cl-6 GU320252 and *Dechloromonas* sp. MissR EU273890 (Figure 2.8).

<u>Dilution four:</u> On days 19, 25 and 30 *pcrA* was detected by PCR, while *cld* was not detected on any day by nested PCR. The *cld* positive control gave a PCR product of the correct size.

<u>High perchlorate enrichment:</u> The *pcrA* gene was detected but not the *cld* gene. The *cld* positive control gave a PCR product of the correct size.

Characterization of Minimal Consortium Growth Parameters

Perchlorate was reduced to below the detection limit at 0.5 and 5 mg/L, and reduction was slowed at concentrations of 50 mg/L (Table 2.4). At 50 mg/L perchlorate was reduced by 38% in the first 44 days and a further 35% in the next 44 days for a total of 73%. Perchlorate reduction was inhibited at 100 mg/L or higher (Table 2.4). The temperature range for perchlorate reduction was from 16°C to 30°C with an optimum of 20°C. Perchlorate was reduced completely at pH 7.5 and 8.0 with a minimum pH for perchlorate reduction at 6.5. The upper pH maximum for perchlorate reduction was not determined. At atmospheric oxygen levels perchlorate was reduced 40% (n = 3, \pm 20%). At dissolved oxygen concentrations of 1 mg/L and 0.1 mg/L perchlorate was not reduced compared to the control.

The electron donor elemental iron precipitated after filtering through a 0.22 μ m filter and was not measured on the IC. With ferrous iron as the electron donor perchlorate was not reduced. With acetate as the electron donor perchlorate was reduced to below the detection limit in one of the triplicate tubes tested. With hydrogen gas as the electron donor perchlorate was reduced to below the detection limit in two of the triplicate tubes tested. The alternate electron acceptors nitrate and chlorate were fully reduced by the SUPeRB consortium. Using sulfur as an electron donor selenate was reduced by 99%, thiosulfate was reduced 71%, and nitrite was reduced 67% compared to an uninoculated control. Sulfate was not reduced by the consortium that used sulfur as an electron donor.

Under saline conditions, in 88 days, perchlorate was not reduced at 2% or 3% NaCl. At 1% NaCl perchlorate was reduced 20% on average compared to the control (n = $3, \pm 20\%$). At 0.5% NaCl perchlorate was reduced 67% compared to the control with a standard deviation of 30% (n = $3, \pm 30\%$). When excess molybdenum was added as sodium molybdate to the media the perchlorate-reducing capability of the consortium was inhibited and perchlorate was reduced by 19% compared to the control with a standard deviation of 18% (n = 3). When elemental sulfur was provided in powder form perchlorate was reduced 70% compared to the control with a standard deviation of 43% (n = 2). The concentration of elemental sulfur from 30 g/L to 1 g/L did not affect perchlorate reduction. The morphology of all members of the mixed community was short rods.

Growth of Isolates from the Minimal Consortium

Three colony types grew on the minimal medium plates. The morphology of the colonies was (1) large beige, (2) small beige, and (3) large white. The presence of the *pcrA* and *cld* genes were not detected by PCR. A large-sized band of greater than 1500 bp was seen with the *pcrA* primer set for the large white colony. The *pcrA* PCR product was sequenced twice but neither gave a readable sequence. By 16S rRNA gene sequencing this isolate was identified as an Epsilon-Proteobacterium of the genus *Sulfurospirillum*, a sulfur-oxidizing bacterium. When inoculated alone into the SUPeRB minimal medium no reduction of perchlorate occurred within 40 days. The large beige colony was also identified as *Sulfurospirillum* sp. The cells of the small beige colony were identified as

99% similar to the Alpha-Proteobacteria *Rhizobium selenireducens* EF440185 and *Agrobacterium* sp. HQ222282.

Functional gene copy quantitation

There was no *pcrA* gene detection by qPCR in the fourth dilution. The standard curve had an R^2 value of 0.999 (Figure 2.9). The detection limit was 10 gene copies/ml.

Attachment of SUPeRB to Solid Electron Donor or Buffer Material

Perchlorate was reduced to below the detection limit in the positive control as well as when the sulfur pellets were separated from the oyster shell and bacteria (Table 2.5). When the bacteria were separated from the oyster shell and the sulfur pellets two of the three replicates reduced perchlorate completely, while the third reduced perchlorate 54% compared to the control. When the oyster shell was separated from the sulfur pellets and bacteria, two of the three replicates showed no perchlorate reduction while the third reduced perchlorate completely.

Discussion

The goal of this research was to characterize a novel consortium that uses elemental sulfur as an electron donor while reducing perchlorate. Two enrichments were compared: a well established SUPeRB consortium revived from frozen stock and a freshly enriched SUPeRB consortium further enriched by serial dilution. Members of each enrichment were phylogenetically identified. Growth parameters and attachment abilities were also investigated. The reconstituted SUPeRB consortium did not fully recover function. The time to degrade perchlorate remained at approximately 45 days and was only reduced in the first dilution after three serial dilutions suggesting that only approximately 10 cells/ml of a key member of the SUPeRB consortium were present in the initial culture. Less than 50 ml of this consortium was available for dilution potentially excluding sufficient quantities of significant members of the consortium for the entire consortium to retain perchlorate-reduction function.

A fresh SUPeRB consortium (E1) was readily enriched from an inoculum collected from the denitrification zone of the wastewater treatment plant where the original SUPeRB consortium was obtained (Sahu *et al.*, 2009). This consortium was enriched for function during four serial dilutions. The time needed to reduce perchlorate to below the detection limit fell from approximately 45 to 19 days. If only one perchlorate-reducing cell was necessary for growth into a fully functioning consortium, and function was generally lost after the fifth dilution, it may be extrapolated that the number of perchlorate-reducing cells in the starting culture was at least 1 x 10^5 cells/ml. This is within the range reported in the literature of 3 x 10^3 to 4.01×10^5 CFU/ml and 2.3×10^3 to 2.40×10^6 cells/g sample with varying electron donors and varying concentrations of perchlorate in varied environments (Gal *et al.*, 2008; Wu *et al.*, 2001; Coates *et al.*, 1999). The limiting factor is considered to be biotic rather than abiotic as, although cells are visible in subsequent dilutions, there is no measurable function. The limiting species may be a PRB or another essential member of the SUPeRB consortium.

The composition of the SUPeRB consortium remained diverse particularly within the Proteobacteria. Based on current knowledge (see Chapter 1, Table 1.1) the majority of known PRB are found in the Alpha- and Beta-Proteobacteria. However, even after several dilutions series the phyla Actinobacteria and Acidobacteria were still identified in the cultures. As the presence of these phyla was not always detected by clone libraries they did not appear to have a dominant function in the SUPeRB process. Members of the Acidobacteria have the ability to reduce nitrate and Actinobacteria may be involved in oxygen removal, thus indirectly supporting the SUPeRB process. The perchloratereducing strain appeared to be a member of the Beta-Proteobacteria. Clones with similarity to uncultured Beta-Proteobacteria and to *Thiobacillus denitrificans* were found. T. denitrificans is a predominant sulfur-utilizing denitrifier and may reside in a biofilm on the sulfur pellets (Soares, 2002; Wang and Qu, 2003; Liu, 2005). It is a facultative anaerobe, with the ability to grow optimally at neutral pH and to oxidize sulfite to sulfate. A denitrifying Beta-Proteobacterium was also identified as a dominant species in a perchlorate-reducing biocathode community (Butler et al., 2010). This community was more diverse than, and shared little overlap with, a nitrate-reducing biocathode community. In a hydrogen-fed membrane biofilm reactor Proteobacteria again dominated the biofilm communities. As no known PRB were detected in the membrane reactor it was suggested that denitrifiers reduced perchlorate, probably by secondary-utilization (Van Ginkle et al., 2010). An enrichment of biosolids from a water treatment plant resulted in a four-strain consortium for the reduction of perchlorate with hydrogen. Although one of the strains was identified as similar to D. agitata, a known perchloratereducer, no perchlorate reduction was seen without the presence of all four strains (Giblin et al., 2000). Miller and Logan (2000) also found that a consortium of microorganisms might be necessary for perchlorate-reduction in an autotrophic reactor utilizing hydrogen

as an electron donor. Another enrichment from a denitrifying wastewater treatment plant reduced perchlorate and nitrate under high salt conditions. *Clostridium* sp. and a bacterium belonging to the Rhodocyclaceae were identified as the dominant clones (Chung *et al.*, 2009). As *Azospira* species include known PRB and were distantly related to the Beta-Proteobacteria clones it is likely that the PRB in the SUPeRB consortium is a novel species within the family Rhodocyclaceae (Borole *et al.*, 2009).

Because most 16S rRNA gene clone identities were distant, and even if the identity had greater than 99% similarity, sequences can exhibit 30–70% dissimilarity across complete genomes (Zhang *et al.*, 2002), also, closely related species may or may not have the ability to reduce perchlorate; therefore, functional genes are also used to indicate the presence of perchlorate-reducers (Bender *et al.*, 2004). The *cld* gene from the fresh SUPeRB consortium (E1) clustered in two groups identified as most closely related to *D. agitata* and *Azospira oryzae*; both species are Beta-Proteobacteria of the order Rhodocyclales and family Rhodocyclaceae. The *pcrA* gene also clustered with *D. agitata*. The *cld* gene from the frozen SUPeRB consortium also clustered with the same *A. oryzae* clone as E1. The *pcrA* gene clustered most closely with a different *Azospira* sp. clone cl-6 and was also closely related to a *Dechloromonas* sp. strain MissR. The *cld* gene phylogeny distinctly separates into two clades with Alpha-Proteobacteria in one and the Beta- and Gamma-Proteobacteria in the other (Achenbach *et al.*, 2006).

It was also seen by Gal *et al.* (2008) that clones identified by the *cld* gene from perchlorate-contaminated soil had a low similarity (80 to 84%) to Azospira oryzae, Dechloromonas aromatica RCB, Dechlorospirillum sp. DB, Dechloromarinus chlorophilus and Pseudomonas sp. PK in the Alpha-, Beta- and Gamma-Proteobacteria.

Primer sets created for *pcrA* and *cld* contain mismatches with known PRB, which may underestimate the numbers detected (deLong *et al.*, 2010; O'Connor and Coates, 2002). The *cld* gene of a Beta-Proteobacterium with the alternate metabolism of growing on benzene with chlorate as the electron acceptor was too divergent from known *cld* genes to detect with a standard primer set (Weelink *et al.*, 2008). There is less known about the *pcrA* gene. Although by standard PCR *pcrA* was detected on day 25 of the fourth incubation no *pcrA* product was seen by qPCR. As standards gave the expected results it may be that the qPCR method has a greater sensitivity to mismatches within the primer sets.

Although the SUPeRB consortium has some similarities to characteristics of pure cultures of *Azospira* sp. and *Dechloromonas* sp. there were differences suggesting that the PRB within the SUPeRB consortium were novel members of the Rhodocyclaceae (Coates *et al.*, 1999; Achenbach *et al.*, 2001; Table 1.1). The morphology of our cultures was short rods while *Dechloromonas* sp. are rod shaped, *Azospira* sp. are curved rods.

Levels of less than 50 mg/L perchlorate were reduced by the SUPeRB culture. In general, isolates are obtained from heterotrophic cultures grown at concentrations of perchlorate higher than 50 mg/L. However, bioreactors are generally run with levels of perchlorate at 5 mg/L and less. The inhibition of perchlorate-reduction at higher concentrations of perchlorate was also seen by Simon and Weber (2006) where at 10 mg/L perchlorate it took more than 20 days to reduce perchlorate to below the detection limit and at 100 mg/L there was no perchlorate reduction seen within 40 days.

Isolates generally grew optimally from 25 to 37°C (Coates and Achenbach 2004). However, perchlorate was completely reduced by *A. oryzae* within 12 days at 22°C and

26 days at 10°C with acetate as the electron donor (Sturichio, 2007). The SUPeRB consortium appeared to be acclimatized to temperatures around 20°C. Perchlorate was degraded to below the detection limit at a pH of 7.0 to 8.0. This pH range appears to be the common optimum for perchlorate-reduction, with the exception of Azospira orzyae, which has an optimum pH of 6.5 (Coates *et al.*, 1999). The reduction of perchlorate or nitrate contributes protons that eventually leads to an environment that is too acidic for the bacteria to function. Our system may be adapted to a more alkaline pH due to the buffering capacity of the oyster shell which was found to have high dissolution rates in water when used as an alkalinity source for denitrification using elemental sulfur (Moon et al., 2006; Sengupta et al., 2006). Microaerophilic conditions inhibited perchlorate degradation. Cyanide inhibited Azospira sp. KJ growth on perchlorate or chlorate due to the accumulation of dissolved oxygen (Sturichio, 2007). Perchlorate reduction was inhibited at salt concentrations above 0.5% NaCl, while Azospira sp. was inhibited by NaCl addition (Table 1.1). Excess molybdate partially inhibited perchlorate degradation. Pure culture studies using *Dechloromonas sp.* and *Azospira sp.* required molybdenum as a trace element for perchlorate reduction (Chaudhuri et al., 2002).

A concentration of sulfur pellets as low as 1 g/L did not slow perchlorate degradation, but powdered sulfur pellets at 30 g/L partially inhibited perchlorate reduction. Powdered sulfur was seen by Ju *et al.* (2007) to reduce perchlorate; however, this reduction may have been stimulated by the addition of yeast extract. Yeast extract was not added in our study to maintain autotrophic conditions. The amount of sulfur pellets per unit volume was also observed by Ju *et al.* (2007) to affect perchlorate reduction.

In our study the degradation was measured at one time point and it is possible that lower concentrations of sulfur reduced the perchlorate at a slower rate than higher concentrations, but this was not captured by our measurement timeframe.

Reduced molecules with redox properties may play a role in the biological reduction of inorganic electron acceptors such as nitrate and perchlorate (Van der Zee and Cervantes, 2009). Elemental sulfur can be reduced to water soluble linear polysulfide, polysulfide is reduced to sulfite, and sulfite to sulfate (Takahashi *et al.*, 2010). Elemental sulfur can also be dissimilated to sulfide (Ju *et al.*, 2007) and in alkaline solution can disproportationate to polysulfide and thiosulfate (Yamamoto *et al.*, 2010). However, in our tests, both sulfate and polysulfide with or without a buffer of potassium phosphate at pH 7 failed to reduce perchlorate abiotically or with the SUPeRB consortium.

As a control, *Azospira* (*Dechlorosoma*) sp. PS was tested with the same electron donors as the SUPeRB consortium. *Azospira* sp. PS did not reduce perchlorate using elemental sulfur, hydrogen or ferrous iron as electron donors but reduced perchlorate to below the detection limit with elemental iron and acetate. The acetate and hydrogen results support previous results, whereas sulfur and elemental iron were not previously tested and ferrous iron was previously found to reduce perchlorate (Coates *et al.*, 1999). Sahu (2008) tested the initial SUPeRB batch culture enrichment with sodium acetate, hydrogen, elemental iron, and ferrous iron. The amount of ferrous iron used as an electron donor was adjusted for electron availability. Perchlorate reduction was observed with acetate and ferrous iron (2.5 mg/L to below the detection limit). No perchlorate reduction was observed using hydrogen or elemental iron as electron donors. In the Sahu (2008) study the pH of the batch culture was 9.5. Other researchers have also shown limited or no perchlorate reduction with elemental iron at a higher pH (>9.8) (Shrout *et al.*, 2005; Yu *et al.*, 2006). PRB use hydrogen formed by the oxidation of elemental iron in water under anoxic conditions (Sanchez *et al.*, 2004); however, at higher pH, hydrogen production rates are reduced (Reardon, 1995).

In our study perchlorate was not reduced with ferrous iron as an electron donor. It is possible that the bacteria that carried out this function were not present in the second SUPeRB enrichment or that this function was lost in subsequent dilutions with sulfur as the sole electron donor. As the control strain *Azospira* sp. strain PS also failed to grow with ferrous iron as the donor in our study there is the possibility that a sufficient concentration was not used. Perchlorate was reduced with the electron donors acetate (one of three triplicates) and hydrogen (two of three triplicates). Hydrogen was not used by the consortium in the Sahu (2008) study but it is possible that there were no hydrogenotrophic PRB present. In our study oyster shell was included in the media with the elemental iron and hydrogen cultures and the pH remained neutral, possibly negating the effects of the high pH seen by Sahu (2008). It is also possible that a different bacterial strain present at low levels in the current SUPeRB consortium was capable of hydrogenotrophic growth. If the hydrogenotrophic strain was not present in all inoculums this may also explain the lack of reduction in one of the three replicates.

The alternate electron acceptors nitrate and chlorate were fully reduced to below the detection limit and selenate was also reduced by the SUPeRB consortium. The amino acid sequence of the subunits encoded by the perchlorate reductase showed similarities with subunits of chlorate reductase, nitrate reductase, and selenate reductase all of which were members of the type II DMSO reductase family (Achenbach *et al.*, 2006; Thorell *et al.*, 2003). The chlorate reductase could also partially reduce selenate (Thorell *et al.*, 2003) suggesting that the perchlorate reductase could also have this ability. A bacterial community established in a perchlorate-reducing reactor with hydrogen as an electron donor was also found to reduce selenate (Chung *et al.*, 2007). A perchlorate-enriched, facultative anaerobic consortium that reduced perchlorate with acetate as an electron donor also used oxygen, chlorate, chromium, and selenate as alternate electron acceptors (Bardiya and Bae, 2005). Thiosulfate and nitrite were partially reduced but sulfate was not reduced with the SUPeRB culture and sulfur pellets as an electron donor. *T. denitrificans* was found to grow with thiosulfate as an electron donor and nitrate as the electron acceptor (Claus and Kutzner, 1985).

Isolates from the SUPeRB consortium grown on solid medium either did not have perchlorate functional genes or they were undetectable with the available primer sets. The isolate identified as *Sulfuricurvum* sp., although found in all communities where perchlorate was degraded by the SUPeRB consortium, did not reduce perchlorate when inoculated into the SUPeRB media by itself. Other perchlorate-reducing isolates, e.g., *Dechoromonas* sp. strain HZ, were reported to be isolated in liquid medium but could not be grown on solid medium containing the same electron donors and acceptors (Zhang *et al.*, 2002). Attaway and Smith (1993) also, could not successfully obtain an isolate from a consortium. Isolates are required for a comprehensive understanding of the physiology of an organism. However, only a fraction of microorganisms present in an environment can be easily cultivated. This is often due to lack of knowledge of the conditions necessary for cultivation. In our study, although we are selecting for certain growth conditions, the interdependency among species is unknown and could be difficult to mimic on solid medium.

Oyster shell has been investigated for effects on perchlorate reduction. Sahu (2008) found no perchlorate reduction with organic-free oyster shell in the absence of an electron donor. Oyster shell that had not been treated to remove organics might initially support perchlorate reduction; however, reduction could not be sustained without the addition of electron donor. It was proposed that for initial growth microorganisms could use the trace concentrations of organic carbon from the oyster shells as an electron donor. Oyster shells consist of a hard tissue of calcium carbonate and organic matrices. The organics were identified as protein and carbohydrate in the oyster species *Crassostrea virginica* (Simkiss, 1965). The oyster shell used in the assays discussed in this chapter were treated at high temperatures to remove organics. This may have slowed the initial biomass growth and resulted in a longer lag time than untreated oyster shell.

It was expected that the SUPeRB consortium would be established in a biofilm on the solid electron donor or alkalinity source. In denitrifying bioreactors a biofilm was found to be readily visible on sulfur pellets (Koenig, 2004). In our study multiple tests suggested that the highest concentration of biomass and function was associated with the pore water, then the oyster shell, and lastly the sulfur pellets. These test included protein (Sahu, 2008), DNA extraction and functional gene detection (see Chapter 4). The location of the functional bacteria and the necessity of attachment were tested by physical separation of the components from the bacteria. The results show that attachment of the bacteria to the solid electron donor or alkalinity source was not necessary. However, it was noted that when the oyster shell was constrained perchlorate reduction was reduced. Potentially this was due to the inability of the oyster shell to act as a pH buffer rather than an attachment substrate.

In summary, the unique SUPeRB consortium capable of using sulfur as an electron donor and perchlorate as an electron acceptor was characterized. The perchlorate-reducing strain was identified by functional gene analysis and potentially by 16S rRNA to be a Beta-Proteobacterium within the family Rhodocyclaceae with a distant similarity to *Azospira* sp. Within the consortium perchlorate was reduced optimally at low concentrations of perchlorate, anaerobically, at 20°C, and at near neutral pH of 7 to 8. The consortium also reduced nitrate, chlorate, selenate, thiosulfate, and nitrite using sulfur as an electron donor. Attachment to the solid electron donor was not necessary for perchlorate reduction.

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Growth	Range	Comment		
parameter	-			
Perchlorate concentration	0.5 – 500 mg/L	0.5, 5, 50, 100, 250, and 500 mg/L		
Temperature	6 - 30°C	30, 20, 16, 12, and 6°C.		
рН	4.5 – 8 in 0.5 steps	NaH ₂ PO ₄ .H ₂ O and Na ₂ HPO ₄ .7H ₂ O replaced oyster shell		
Oxygen concentration	0.1 mg/L, 1 mg/L, and atmospheric air	O ₂ was calculated using Henry's constant		
Electron donor	sodium acetate, elemental iron, ferrous iron (30 g/L), hydrogen gas and polysulfide (0.5, 5, and 50 mM).			
Electron	sodium perchlorate, sodium			
acceptor	sodium sulfate, sodium			
	thiosulfate, sodium nitrite, and			
	sodium selenate. All at 5 mg/L			
Salinity	0.5, 1, 2, and 3% NaCl			
Excess sodium molybdate	0.25 μg/ml			
Elemental sulfur powder	450 mg/L	Crushed sulfur pellets		
Sulfur pellets	16 – 1 g/L, 240, 120, 60, 30, and 15 mg sulfur pellets /15 ml tube	The average weight of a single sulfur pellet was 15 mg (with a range from 13 mg to 17 mg		
Morphology	100X magnification	Nikon Eclipse 6400 microscope (Nikon, Inc., Melville, NY), equipped with a Nikon 100X 1.30 NA oil objective and a Hamamatsu digital camera (Hamamatsu, Bridgewater, NJ)		

Table 2.1. Parameters measured for the SUPeRB consortium.

Dilution series 2	E1:4	E1:5	E1:6	E2:1	E2:2
(# of clone)	(15)	(10)	(20)	(13)	(23)
Phylogenetic group	Total of Community (%)				
Phylum: Proteobacteria					
Class					
Family					
Alpha-					
Sphinomondales	7	30	30	30	13
Rhizobiales	40	40	55	8	48
Beta-					
Burkholderiales	7		5	54	31
Hydrogenophilales	7				4
Rhodocyclaceae	26	10			
Epsilon-			-		
Campylobacterales	13	20		8	4
Gamma-	-	-		-	-
Pseudomonadales			5		
Xanthomonadales			5		

Table 2.2. Phylogenetic analysis of the minimal SUPeRB consortium.

Dilution	Number	Phylum	Description	Accession	%
series 4	of clones			number	Identity
Day					
11	2	Beta-	Uncultured	AB425068	96-97
		Proteobacteria	Thiobacillus sp.		
19	1	Actinobacteria	Propionicimonas	EF440185	97
			sp.		
	1	Alpha-	Agrobacterium sp.	HQ222282	99
		Proteobacterium	Rhizobium	EF440185	99
			selenireducens		
25	2	Acidobacteria	Uncultured	HM146712	97-99
			Geothrix	HM146770	
	1	Epsilon-	Sulfurospirillum	DQ234237	99
		Proteobacterium	sp.		
	1	Beta-	Thiobacillus sp.	AB425068	98
		Proteobacterium			
	2	Alpha-	Rhizobium	EF440185	99
		Proteobacteria	selenireducens		
			Agrobacterium sp.	HQ222282	99
30	1	Acidobacteria	Geothrix sp.	HM141900	98
	1	Epsilon-	Sulfurospirillum	DQ234237	95
		Proteobacteria	sp.		
	5	Beta-	Uncultured	AF407390	84
		Proteobacteria	<i>Thiobacillus</i> sp.	AB425068	81-98
			<i>Azospira</i> sp.	FJ823940	81
	1	Alpha-	Rhizobium	EF440185	99
		Proteobacterium	selenireducens		
			<i>Agrobacterium</i> sp.	HQ222282	99
50 mg/L	4	Epsilon-	Sulfurospirillum	DQ234237	96-98
		Proteobacteria			
	3	Beta-	<i>Thiobacillus</i> sp.	AB425068	95-97
		Proteobacteria		AB161272	79
			<i>Rhodoferax</i> sp	HQ222266	98
	8	Alpha-	Rhizobium	EF440185	97-99
		Proteobacteria	selenireducens		
			<i>Agrobacterium</i> sp.	HQ222282	82-99

 Table 2.3. Phylogenetic analysis of the SUPeRB consortium dilution series.

Table 2.4. Perchlorate reduction with increasing starting concentrations.

Perchlorate (mg/L)	0.5	5	50	100	200	400
Day 44	0	0	31	94	193	385
Day 88	0 ± 0	0 ± 0	13 ± 2	93 ± 18	201 ± 11	417 ± 9

Day $88 \pm$ standard deviation from the mean, n=3.

Mg/L	Positive (n=2)	Negative (n=2)	SP (n=3)	OS (n=3)	B (n=3)
Day 0					
Perchlorate	7.3	ND	ND	ND	ND
Sulfate	68.9	ND	ND	ND	ND
Chloride	39.8	ND	ND	ND	ND
Day 40					
Perchlorate	0	6.4 ± 4.6	0	6.6 ± 5.7	1.0 ± 1.7
Sulfate	217.7 ± 89.5	143.5 ± 8.6	297.7 ± 41.7	221.9±113.7	173.2±137.3
Chloride	58.7 ± 3.3	66.4 ± 14.7	61.4 ± 4.6	57.1 ± 3.4	59.5 ± 4.1

Table 2.5. Attachment study anion measurements.

SP = sulfur pellet sequestered, OS = oyster shell sequestered, B = bacteria sequestered. \pm = the standard deviation from the mean. ND = not determined.





Serial dilutions were as indicated by the arrows. Dilutions indicated in the dark gray were used for phylogenetic analysis.



Figure 2.2. Dilution scheme for the frozen SUPeRB enrichment (E2).

Serial dilutions were as indicated by the arrows. Dilutions indicated in the dark gray were used for phylogenetic analysis.



Figure 2.3. Perchlorate reduction by the SUPeRB consortium.

Perchlorate reduction over time in the fourth dilution series of the E1 enrichment, fourth dilution (closed square: \blacksquare), fifth dilution (closed triangle: \blacktriangle). Error bars show the standard deviation within the mean of triplicate samples.



Figure 2.4. Perchlorate reduction and chloride production by the SUPeRB consortium.

Chloride (\blacklozenge), sulfate (\blacksquare), and perchlorate (\checkmark) measurements for the fourth dilution of the fourth dilution series of the SUPeRB consortium over time. Error bars indicate a standard deviation from the mean (n= 3).


Figure 2.5. Phylogenetic relationship of the *cld* gene from the fresh SUPeRB consortium (E1).

Phylogenetic relationship of the *cld* gene from the fresh (E1) SUPeRB consortium as identified by class and phylum. The comparative analysis was inferred by Minimum Evolution analysis of the *cld* gene from clones in concert with public nucleotide databases. Clones were designated by the prefix C24. The scale bar represents 5% estimated sequence divergence. Bootstrap values are shown for all nodes in an analysis of 1,000 replicates.



Figure 2.6. Phylogenetic relationship of the *pcrA* gene from the fresh SUPeRB consortium (E1).

Phylogenetic relationship of the *pcrA* gene from the fresh SUPeRB consortium (E1) as identified by class and phylum. The comparative analysis was inferred by Minimum Evolution analysis of the *pcrA* gene from clones in concert with public nucleotide databases. Clones were designated by the prefix p26. The scale bar represents 10% estimated sequence divergence. Bootstrap values are shown for all nodes in an analysis of 1,000 replicates.



Figure 2.7. Phylogenetic relationship of the *cld* gene from the frozen SUPeRB consortium (E2).

Phylogenetic relationship of the *cld* gene from the frozen SUPeRB consortium as identified by class and phylum. The comparative analysis was inferred by Minimum Evolution analysis of the *cld* gene from clones in concert with public nucleotide databases. Clones were designated by the prefix S21. The scale bar represents 5% estimated sequence divergence. Bootstrap values are shown for all nodes in an analysis of 1,000 replicates.



Figure 2.8. Phylogenetic relationship of the *pcrA* gene from the frozen SUPeRB consortium (E2).

Phylogenetic relationship of the *pcrA* gene from the frozen SUPeRB consortium (E2) as identified by class and phylum. The comparative analysis was inferred by Minimum Evolution analysis of the *pcrA* gene from clones in concert with public nucleotide databases. Clones were designated by the prefix S22. The scale bar represents 10% estimated sequence divergence. Bootstrap values are shown for all nodes in an analysis of 1,000 replicates.



Figure 2.9. qPCR standard curve for the *pcrA* gene.

Obtained from serially diluted *Dechlorosoma suillum* (*Azospira oryzae*) PS genomic DNA. C_T values are the average of three replicates. Error bars represent standard deviations.

CHAPTER 3

PHYLOGENETIC STRUCTURE AND FUNCTIONAL RELATIONSHIPS IN A BENCH-SCALE BIOREACTOR

Abstract

Ecosystem stability usually correlates positively with diversity. However, in this perchlorate- and nitrate-reducing bioreactor system, disturbance to function led to higher system diversity while the stably functioning reactor had lower diversity. The unintentional disruption of feed and potential of simultaneous oxygen influx had a more distinct disturbance effect than the intentional disturbance of nitrate addition. With the community disturbance there was a reduction in function suggesting that the optimized sulfur-utilizing, perchlorate-reducing bacterial (SUPeRB) consortium was not metabolically flexible and a higher population diversity was necessary to return to stable function. Under stable conditions the structure of the reactor SUPeRB consortium was similar to that found in the minimal consortium experiments. Perchlorate and nitrate were both reduced to below the detection limit with presence of function correlated with perchlorate-reducing bacteria (PRB) quantities. Novel Beta-Proteobacteria, distantly related to the *Azospira/Dechloromonas* group of PRB, were thought to be responsible for perchlorate-reduction. Members of the Beta-Proteobacteria and Epsilon-Proteobacteria known to have the capability to reduce nitrate using sulfur as an electron donor were found.

Introduction

It has been fully recognized that an understanding of the microbial ecology of bioreactors involved in the treatment and bioremediation of various biological processes is important for the successful long-term function of these processes (Rittmann, 2002; Briones and Raskin, 2003). However, the benefit of the inverse, the use of bioreactors to understand complex ecology questions, has not been fully explored (van der Gast *et al.*, 2006). In ecosystems the distribution and interaction of species and species function are largely governed by chance (Botton *et al.*, 2006). Bioreactors are managed systems and therefore ideal for studies in microbial ecology and, in particular, the effects of system disturbance and the recovery of the system (Briones and Raskin, 2003). A bioreactor provides the capability of having a single measurable function, a reduced complexity of microbial interactions, sampling variability may be controlled, and system disturbances and recoveries can be measured in a reasonable timeframe.

However, studies of microbial ecology concepts in bioreactors have given variable answers to studies of ecosystem stability and disturbance. In reactors, microbial communities may or may not have stable community membership even with stable function (Gentile *et al.*, 2007b; Wang *et al.*, 2010). This contradiction seems to be specific to reactor function. For example, in denitrifying reactors stable community dynamics correlated with functional stability while the highly variable community structure of methanogenic reactors had higher functionally stable (Hashsham *et al.*, 2000; Gentile *et al.*, 2006; 2007a). If a community is functionally highly flexible, such as reported for denitrifying communities, lower diversity can still provide resiliency to perturbation (Botton *et al.*, 2006; Gentile *et al.*, 2006). Yet, in methanogenic reactors,

function is supported by a greater richness in which a mixture of species performs more successfully than individual species in isolation (Konopka, 2009; Wittebolle *et al.*, 2009). A minimum number of species is necessary in an ecosystem to maintain function; however, availability of a greater diversity of species grants a better suited response to diverse disturbances or changes in the environment (Botton *et al.*, 2006). In bioreactors there is the potential to have both keystone species and rare species, with the rare species becoming keystone species upon a disturbance (Botton *et al.*, 2006; Gentile *et al.*, 2006). The identification of the species that appear when function is stable or disrupted is important in linking microbial ecology to functional stability (Gentile *et al.*, 2007a).

Nitrate addition to a reactor containing the SUPeRB consortia was previously seen to inhibit perchlorate reduction (Sahu *et al.*, 2009) and therefore is a disturbance to the function of the system. Nitrate is often found as a co-contaminant of perchlorate and many known PRB are also capable of denitrification (Coates *et al.*, 1999; Stetson *et al.*, 2006). The presence of nitrate may have either an inhibitory or positive effect on perchlorate reduction (Herman and Frankenberger, 1999; Chaudhuri *et al.*, 2002; Xu *et al.*, 2004; Coates and Achenbach, 2006). A positive effect may be due to cometabolism of the perchlorate. The inhibitory effect may be due to preferential use of nitrate by the consortium or the accumulation of the toxic intermediate nitrite (Attaway and Smith, 1993, Gentile *et al.*, 2007a).

The goal of this research project was to investigate the microbial ecology and stable function of the SUPeRB consortium in a bench-scale bioreactor. We hypothesized that the SUPeRB consortium would remain in the bioreactor due to association with the solid electron donor, that the community would reduce both perchlorate and nitrate, and that the consortium would change in structure in response to a perchlorate and nitrate gradient. Also, even with the reduced complexity of the SUPeRB consortium, microbial diversity in the bioreactor was necessary for function and long-term function stability. Denitrifying and perchlorate-reducing communities are diverse metabolically and physiologically, using a range of electron donors and acceptors making these excellent functions with which to measure stability (Coates and Achenbach, 2004; Wittebolle *et al.*, 2009).

The use of analytical techniques was combined with molecular methods for community analysis of intermittent samples to make predictions about this particular system and to evaluate community dynamics. The consortium was inoculated into the reactor and the microbial consortium structure was examined spatially and temporally within the reactor. We determined the effect of a disturbance by adding nitrate as a competing contaminant. The investigation of this novel and complex system outlined in this chapter answers broader core microbial ecology questions of the role of microbial diversity in function and long-term functional stability.

Materials and Methods

Bench-Scale Bioreactor Set-up

The bioreactor had a working volume of 195 ml (Figure 3.1). It was constructed from glass with an inner diameter of 2.5 cm and height of 40 cm, with sample ports distributed along the height of the reactor and sealed with septa for sampling. The ports were distributed to ensure maximum coverage of spatial perchlorate-reduction. The bioreactor packing material was a 3:1 ratio of 99.9% pure S° pellets (Georgia Gulf Sulfur Corporation, Valdosta, GA) and washed crushed oyster shell (Myco Supply, Pittsburgh, PA). The feed consisted of deionized water that was sparged with 80% N_2 and 20% CO₂ for 30 minutes, 2.5 mg/L ammonium chloride, 0.5 mg/L potassium phosphate and approximately 5 mg/L perchlorate were added, or 2.5 mg/L perchlorate and 2.5 mg/L nitrate were added (Table 3.1). The bioreactor was operated in an up-flow mode at an empty bed contact time (EBCT) of approximately 6.5 hours. The bioreactor was inoculated with the E1 fresh SUPeRB consortium also used for the Chapter 2 experiments (Berkshire, MA, June 2008) and incubated at 20°C with an attached tedlar bag filled with 80% N_2 and 20% CO₂.

Reactor Sampling

The influent and effluent were monitored until the effluent perchlorate measured below the detection limit in three consecutive samples. This acclimatization period took 170 days which was then considered day 0. A sample from each port was taken on days 0, 10, and 30. Nitrate was then added and the amount of perchlorate was halved to supply an equal electron acceptor equivalent. All reactor ports were sampled on days 0, 1, 2, 7, 14, and 28 of nitrate addition. Perchlorate and/or nitrate was measured and a 5 ml sample was filtered onto a 0.2 μ M pore size membrane filter and stored at –30°C.

Microscopic Observation

A Nikon Eclipse 6400 microscope (Nikon, Inc., Melville, NY), equipped with a Nikon 100X 1.30 NA oil objective, and a digital camera (Hamamatsu, Bridgewater, NJ), was used to examine the morphology of cells residing in the pore water.

Analytical Measurements

<u>Anion Measurement:</u> Perchlorate, sulfate, chloride, nitrate and nitrite concentrations were measured by ion chromatography (IC), using a Metrohm 850 Professional IC AnCat MCS system equipped with an 858 Professional Sample Processor, a Metrosep A Supp 7 – 250 column, and a Metrosep RP Guard column all from Metrohm-Peak, LLC (Houston, TX). The final eluent consisted of 20% acetronitrile and 10 mM sodium carbonate and a final flow rate of 0.6 mL/min (USEPA, 1999). The detection limit was 5 μ g/L. Triplicate samples were originally taken; however, due to the small pore volume size for liquid media in the reactor, this proved detrimental to the consortium perhaps due to the suction of oxygen from the effluent outlet.

<u>pH:</u> The pH was measured using an Orion 720A meter (Cole-Parmer Instrument Co., Vernon Hills, IL).

<u>Total organic carbon (TOC) and total nitrogen (TN):</u> TOC and TN were measured at one time point after sampling was completed using a Shimadzu TOC-VCPN analyzer with TN unit and ASI-V autosampler (Shimadzu Corporation, Kyoto, Japan).

<u>Alkalinity:</u> Alkalinity was measured at one time point after sampling was completed by adding 0.1 N HCl to the solution until the pH reached 4.5 using a Metrohm titrator (Metrohm USA Inc., Riverview, FL).

<u>Heterotrophic plate counts</u>: Unfiltered pore water samples were measured at one time point after sampling was completed. Samples were diluted in phosphate buffered saline (PBS) to 10⁷ and each dilution was plated onto R2A agar (Difco, BD, Sparks, MD), in triplicate, within 24 hours of collection. The plates were incubated for seven days at 20°C. On day seven the colony forming units (CFU) on the agar plates were counted.

<u>Oxidation/Reduction potential (ORP):</u> The ORP was measured once after sampling was completed using an Orion 720A meter (Cole-Parmer Instrument Co., Vernon Hills, IL).

Phylogenetic Analysis by 16S rRNA Gene Sequencing

DNA Extraction and Amplification: DNA was extracted from 5 ml of pore water filtered onto 0.22 μ M filters and stored at -30°C using the RapidWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene was amplified from the DNA in triplicate PCR amplifications. A 30 μ l reaction volume had the following final concentrations: 0.5 ng/µl DNA, 0.5 µM of each primer 8F and 1492R (Weisburg et al., 1991), 2 mM MgCl₂, 10x PCR buffer, 0.25 mM of each dNTP and 0.08 U/µl Taq DNA polymerase, 400 ng/µl BSA. The following PCR program was used: 95°C for 3 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; one cycle of 72°C for 5 min on an MJ Research Peltier Thermal Cycler PTC-200 (GMI, Inc., Ramsey, MN). Triplicate PCR products were pooled to reduce amplification bias and cleaned using a QIAquick® PCR purification kit (Qiagen Inc., Valencia, CA). The expected fragment was visualized on a 1% agarose gel stained with ethidium bromide. The 16S rRNA gene was cloned into the pGEM-T Easy Vector Systems kit (Promega, Madison, WI), and E. *coli* JM109 high efficiency competent cells (Promega, Madison, WI) were transformed in accordance with manufacturer's instructions. Clones were grown into colonies and positive clones were then randomly picked. Amplification of the 16S rRNA gene from each clone was carried out in a 30 µl reaction volume with the following final concentrations: 0.5 ng/µl DNA, 0.33 µM of each pGEMf and pGEMr primers; 1.75 mM MgCl2; 10x PCR buffer; 0.17 mM of each dNTP; 0.1 U/µl of *Taq* DNA polymerase. The following PCR program was used: 95°C for 3 min; 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 30 s; one cycle 72°C for 5 min. Selected clones from each sample were submitted for 16S rRNA gene sequencing. PCR amplified products were pooled, cleaned, amplified with BigDye®Terminator V.3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) and submitted for sequencing. RNA was unsuccessfully extracted from the same filters.

Sequence Analysis: Sequences were edited, checked for chimeras using Mallard (Ashelford *et al.*, 2006), compared to the NCBI database (Altschul *et al.*, 1997), and classified using the program mothur V.1.14.0 (Schloss *et al.*, 2009) and checked with RDP V.9.57, with an 80% confidence threshold (Wang *et al.*, 2007). The community tree was newick-formatted according to the Yue & Clayton theta structural diversity measure (Schloss *et al.*, 2009).

<u>Functional Gene Detection</u>: Samples of pore water from each port were tested for the presence of the functional genes *pcrA* and *cld* using PCR amplification. The *pcrA* and *cld* genes were amplified from total DNA in triplicate PCR reactions. The *cld* gene was amplified as outlined by Bender *et al.* (2004) with the exception that the PCR reactions were carried out in 30 μ l reactions. The *pcrA* gene was amplified in a 30 μ l reaction volume with the following final concentrations: 0.5 ng/ μ l DNA, 0.4 μ M of each primer pcrAF (5'-ACTACATGTATGGNCCGCATCG-3') and pcrAR (5'-CGTGRTCRCYGTACCAGTCRAA-3'), 1.5 mM MgCl2, 1x PCR buffer, 0.20 mM of each dNTP and 0.05 U/ μ l *Taq* DNA polymerase, 250 ng/ μ l BSA. The following PCR program was run: 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; one cycle of 72°C for 10 min. Triplicate PCR products were pooled to reduce amplification bias and cleaned using a QIAquick® PCR purification kit (Qiagen Inc., Valencia, CA). The two functional genes were sequenced and closest relatives identified as previously outlined. Multiple sequence alignments were created using the program ClustalX, V.1.83 (Thompson *et al.*, 1997) and phylogenetic analyses were conducted using the software package MEGA V.4, minimum evolution analysis with the Tamura–Nei model, and bootstrap values of 1,000 replicates (Tamura *et al.*, 2007).

Quantitative PCR (qPCR)

Standard curves were created from DNA extracted from the control strain *Dechlorosoma suillum (Azospira oryzae)* PS and cloned into a plasmid. A dilution series from 10^6 to one *pcrA* gene copies/ml of the DNA was performed and the cycle threshold (C_T) values were plotted against gene copy/ml. The copy number of the plasmid was calculated by measuring absorbance at 260 nm. DNA was extracted from the pore water samples of the bioreactor fed perchlorate and nitrate on days 1, 2, 6, 14 and 28 from the ports where perchlorate was reduced below the detection limit. PCR amplification was performed in 20 µl final volumes containing 1 µl of DNA, 0.16 µM each of the primers pcrAF and pcrAR and 10 µl of GoTaq® qPCR Master Mix (Promega, Madison WI). All the amplifications were carried out in Thermo-Fast® white 96-well PCR plates (Thermo Scientific, Epsom, UK) on a DNA Engine Opticon® 2 System (Bio-Rad, Hercules, CA) with an initial step of 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30

s, 72°C for 30 s, followed by an elongation step at 72°C for 5 min. All samples were performed in triplicate. Gene copy numbers were adjusted for a reactor porosity of 30%.

Results

Microscopic Observation

The morphology of the cells in the pore water from throughout the reactor, and over time, was short rods.

Analytical Measurements

As the sole electron acceptor perchlorate was reduced gradually from approximately 7 to 2.5 mg/L by port 10 (18.8 cm) and to below the detection limit by port 11 (24 cm) (Table 3.2), as measured on day 178 of the bioreactor run, referred to hereafter as day 0 (Figure 3.2a). Ten days later the reactor reduced perchlorate gradually from 5 to 2.5 mg/L from the influent to port 9 (17.3 cm) and to below detection by port 10 (18.8 cm). Between days 10 and 30 a disturbance occurred as a malfunctioning pump interrupted perchlorate feed flow. Perchlorate was reduced 60% from the influent to the feed (Figure 3.2a).

On day 30 perchlorate and nitrate were added to the reactor feed. Perchlorate was gradually reduced from 2.5 to 1.2 mg/L by port 8 (15.8 cm) and then to below the detection limit by port 9 (17.3 cm) on day 0 (Table 3.2, Figure 3.3a). Within 24 hours, on day 1, perchlorate was reduced from 2.5 to 1.2 mg/L by port 7 (13.6 cm) and to below the detection limit by port 8 (15.8 cm). On day 2 perchlorate was gradually reduced from 2.5 to 1.2 mg/L by port 7 (13.6 cm). On day 6 perchlorate was gradually reduced from 2.5 to 1.0 mg/L by port 4 (8.4 cm) and to

below the detection limit by port 5 (10.6 cm). On day 14 perchlorate was gradually reduced from 2.5 to 1.5 mg/L by port 3 (6.9 cm) and then to below the detection limit by port 4 (8.4 cm) (Figures 3.3a and 3.4). On day 28 perchlorate was gradually reduced from 3.0 to 1.2 mg/L by port 5 (10.6 cm) and then to below the detection limit by port 6 (12.1 cm). On days 0 and 28 perchlorate was detected in the effluent at approximately 1.6 mg/L. Nitrate was reduced from approximately 2.5 mg/L to less than 1.0 mg/L by port 1 (3.2 cm) on each day and to below the detection limit by port 2 (5.4 cm) on days 1, 2, 6, and 14. On day 0 a low level of nitrate was detected in all ports but was reduced to below detection in the effluent. On day 28 a low level of nitrate remained throughout the reactor and in the effluent (Figure 3.5a). Nitrite was undetectable at any time point in any port. Chloride was variable throughout the ports and time points (Figure 3.5b). Sulfate levels remained constant throughout the time points from days 0 to 28 (Figure 3.5c). Sulfate increased gradually from approximately 10 mg/L to 40 to 90 mg/L at port 12 (29.2 cm) and then spiked in the effluent for each time point. The highest levels of sulfate in the effluent were on days 0 and 14. The pH measured in the influent was approximately 6.5 and measured 7.5 to 8.0 in the effluent.

On day 19, after the addition of perchlorate and nitrate, the following parameters were measured:

<u>Total organic carbon and total nitrogen:</u> TOC was 2.59 ± 0.90 mg/L and TN was 1.34 ± 0.13 mg/L (n=3).

<u>Alkalinity:</u> 1.15 ml HCl was added to 20 ml of sample to attain a pH of 4.5 from pH 7.5. Alkalinity (mg CaCO₃/L) was determined by multiplying the volume of acid

added by the normality of the acid by 50,000 per mL sample. Alkalinity was 287.5 mg CaCO₃/L (Standard Methods, 1997).

<u>Heterotrophic plate counts:</u> Counts were measured in CFU/ml (Table 3.3).

Oxidation/Reduction potential (ORP): ORP was -0.1 mV.

Phylogenetic Analysis

<u>16S rRNA Gene:</u> More than 1000 clones that indicated positive for the correct insert were picked for sequencing. However, when visualized on an agarose gel, approximately 200 clones were observed to have an insert of the desired size.

With perchlorate at 5 mg/L the composition of the consortium was identified from ports 9 and 10, spatially located before the area of perchlorate reduction to below detection, and from ports 11 and 12, spatially located after perchlorate was reduced to below detection (Figures 3.2b and 3.6; Table 3.4). On day 30 the composition of the consortium was identified where perchlorate was reduced by 60% (Figure 3.2b and Table 3.5). The phylogenetic tree showed a distant clustering of the unclassified Beta-Proteobacteria found in the reactor fed only perchlorate with the *Azospira/Dechlorosoma* group of PRB (Figure 3.6).

With perchlorate and nitrate at 2.5 mg/L the composition of the consortium in the reactor was identified from before and after perchlorate was degraded to below the detection limit (Figure 3.3b and Table 3.6). The phylogenetic tree showed a distant clustering of the unclassified Beta-Proteobacteria found in the reactor fed with both perchlorate and nitrate with the *Azospira/Dechlorosoma* group of PRB (Figure 3.7).

<u>Functional genes *pcrA* and *cld*</u>: The functional genes were not amplifiable from the reactor fed only perchlorate. In the reactor fed nitrate and perchlorate the *cld* gene was amplified from day 0 ports 7 and 10 (13.6 and 18.8 cm), from day 1 ports 6 and 9 (12.1 and 17.3 cm), from day 2 ports 5, 6, 7, and 8 (10.6, 12.1, 13.6 and 15.8 cm), from day 6 ports 3, 4, 5, and 6 (6.9, 8.4, 10.6 and 12.1 cm), and day 28 ports 4, 5, 6, and 7 (8.4, 10.6, 12.1 and 13.6 cm). The *cld* gene was not detected from the day 14 samples. The *pcrA* gene was only detected in samples from day 14 in ports 2 and 3 (5.4 and 6.9 cm). The *cld* gene from day 1 port 9 (17.3 cm) and the *pcrA* gene from day 14 port 3 (6.9 cm) were identified thorough sequencing. Both functional genes were most closely related to the functional genes from PRB of the Beta-Proteobacteria. The *cld* gene was distantly related to the uncultured bacterium clone ASH-4 chlorite dismutase gene (DQ151571) at 81% similarity and *Dechloromonas agitata* (AY124796) at 79% similarity. The *pcrA* gene was distantly related to the uncultured bacterium clone PNA3 perchlorate reductase alpha subunit (FJ602710) at 81% similarity, *Dechloromonas* sp. MissR (EU273890) at 80% similarity and *Azospira* sp. clone cl-6-Sarno river (GU320252) at 79% similarity.

Functional gene copy quantitation

The assay was linear over six orders of magnitude and the detection limit was approximately 10 gene copies/ml (Figure 3.8). The *pcrA* gene copy number was calculated based on the standard curve and with the assumption that full DNA extraction was attained and there was one copy of the *pcrA* gene per cell. Gene copy numbers for reactor two day 0 port 9 (17.3 cm), day 1 port 8 (15.8 cm), day 2 port 7 (13.6 cm), day 6 port 5 (10.6 cm), and day 28 port 6 (12.1 cm) were below the detection limit, day 14 port 4 (8.4 cm) had $3.8 \times 10^5 \pm 2.5 \times 10^4$ gene copies/L.

Discussion

The goal of this study was to determine whether function remains stable with disturbance, and whether the community was flexible and remains stable, or if rare species dominated during a disturbance. A return to stable function after a disturbance is thought to be coupled with high population diversity combined with functional redundancy (Briones and Raskin, 2003; Botton *et al.*, 2006; Konopka, 2009; Wittebolle *et al.*, 2009). Exploring the microbial ecology of the bioreactor over time we found that the SUPeRB consortium remained similar to the minimal consortium persisting after numerous serial dilutions as discussed in Chapter 2 of this dissertation. The function of the SUPeRB consortium was resilient and returned over time despite disturbances such as competing electron acceptors.

The reactor acclimatized in approximately 170 days. This long period of adaptation may be necessary since this is a slow-growing, autotrophic consortium. Even with an organic electron donor an 85-day acclimatization period was needed for perchlorate reduction (Dugan *et al.*, 2009). Perchlorate was consistently reduced to below the detection limit over the 58 days of bioreactor port measurements with the exception of the day 30 sampling of run one. Before day 30 the reactor feed was interrupted, possibly allowing the introduction of oxygen into the system. A feed flow interruption also occurred between days 14 and 28 of run two when perchlorate and nitrate were added to the reactor. This resulted in a reduction of perchlorate further up in the reactor again possibly due to an influx of oxygen and use as a competitive electron acceptor. Dissolved oxygen was not measured in this reactor but concentrations less than 2 mg/L were enough to inhibit perchlorate reduction by *A. suillum* (Coates and Achenbach,

2004). In another bioreactor study it was considered that bacterial species other than PRB aided in perchlorate removal efficiency by removing oxygen (Li *et al.*, 2010).

When only perchlorate was added as an electron acceptor, perchlorate was reduced to below the detection limit in the upper part of the bioreactor. Perchlorate was seen to decrease gradually until approximately 60% was reduced, at which point the perchlorate concentration was reduced to below the detection limit from one port to the next. Previous studies of up-flow bioreactors also show perchlorate-reduction occurring closer to the feed inlet in successive measurements (Kim and Logan, 2000). There was no lag time for nitrate reduction by the SUPeRB consortium when nitrate was added as an electron acceptor for the SUPeRB consortium for the first time. Nitrate and perchlorate reduction occurred separately in different areas of the reactor. Nitrate reduction occurred within the first port of the reactor while perchlorate reduction started in the upper part of the reactor at port 10 (18.8 cm). Rather than an inhibitory effect nitrate addition appeared to stimulate perchlorate reduction. Perchlorate reduction to below the detection limit occurred closer to the feed inlet of the reactor in successive measurements. This faster reduction within the reactor may be due to adaptation of the consortium within the reactor. It may also be due to the removal of oxygen by nitrate-reducers in the lower parts of the reactor to create anaerobic conditions that were more conducive to the function of the SUPeRB consortium lower in the reactor. Stimulation of perchlorate-reduction by removal of oxygen rather than adaptation of the consortium is probable as the faster reduction occurred quickly whereas the consortium was normally seen to adapt to perchlorate degradation after a long lag phase. It has also been seen that nitrate presence helped reduce low levels of perchlorate in a membrane bioreactor (Adham et al., 2004).

There is also the potential within this oligotrophic, autotrophic environment that some members of the consortium may provide organic carbon to the functioning mixotrophic PRB (Adham *et al.*, 2004). It was also seen by Ju *et al.* (2007) and Boles *et al.* (2010) that low levels of yeast extract as an organic carbon source enhance the reduction of perchlorate by SUPeRB consortia.

Nitrite levels were below detection in all ports on all days measured. Degradation of nitrate occurred quickly before the first sampling port; therefore, nitrite may also have been degraded before the first sampling port and there was no inhibitory effect of nitrite on perchlorate reduction. The nitrate reduction gradient occurred too quickly to detect changes in community structure between the nitrate and perchlorate degraders. There was a gradual community change observed over time from the reactor fed perchlorate only to the reactor fed nitrate and perchlorate. However, the consortium had a greater change along the perchlorate gradient. The gradient had an effect on community structure in terms of evenness with the numbers of certain consortium members increasing with perchlorate degradation. Even with the reduced complexity of the SUPeRB consortium the microbial diversity in the reactor was still diverse with members from several phyla represented. This diversity appeared necessary for recovery of function with rare species from phyla other than the Proteobacteria appearing when there was a disturbance to the stable reduction of perchlorate within the reactor.

Once the consortium was established in the reactor it appeared to recover quickly from interruptions to electron acceptor access. Factors found to contribute to disturbance of bioreactor function include: (1) flow rate, an increased rate decreases perchlorate reduction, (2) an uneven biomass distribution in the reactor, (3) unstable pH levels, and (4) limited delivery of electron donor to the bacteria (Giblin *et al.*, 2000). In our reactor, a stable degradation was found when the flow rate of the feed remained constant at a residence time of 330 minutes. PRB are most active within a pH range of 6-8 (Adham et al., 2004; Raye-Hoponick, 2006) and in our study the pH remained constant due to the buffering capacity of the oyster shell. It was not examined whether the biomass was evenly distributed in the reactor but species composition of the consortium remained constant within areas where perchlorate was reduced. As electron donor was constantly available there was the potential that the consortium could also use alternative electron acceptors such as thiosulfate or oxygen in the absence of perchlorate while briefly available. It was previously discussed in Chapter 2 that sulfate was not reduced by the SUPeRB consortium with sulfur as the electron donor. Other studies have found that when feed was interrupted perchlorate reduction recovered quickly due to cells protected by biofilms (Wallace et al., 1998). Another reactor study showed that a 24-hour long disruption took 24 hours to recover and a three-day organic feed failure resulted in a nine-day recovery period (Brown et al., 2003). Oxygen and nitrate have been shown to reduce or inhibit perchlorate utilization in some strains as perchlorate is used after oxygen and nitrate. Dechlorosoma suillum did not reduce perchlorate until nitrate was completely removed in a medium containing equal moles of the two electron acceptors (Chaudhuri et al., 2002). D. agitata could not use nitrate as a sole electron acceptor (Bruce et al., 1999), but could simultaneously conduct complete perchlorate reduction and partial denitrification from nitrate to nitrite (Chaudhuri et al., 2002), presumably because nitrate can be co-reduced by (per)chlorate reductase (Coates and Achenbach,

2006). *Dechlorosoma* strain GR-1 grown on perchlorate could not reduce nitrate (Rikken *et al.*, 1996).

The same short rod morphology that dominated the enrichment cultures and pilotplant reactor was again observed in this bench-scale reactor. The short rod morphology of the cells found in the bioreactor was similar to that seen for *Dechloromonas* species (Coates and Achenbach, 2004) while the *Azospira* have a slight curved morphology (Reinhold-Hurek and Hurek, 2000).

Although no direct attachment of the consortium appeared necessary for the consortium to remain in the reactor we observed that the influent and effluent communities were dominated by microorganisms other than those identified in the reactor body where the electron donor resides. Gamma-Proteobacteria found in the effluent are related to the genus Acidithiobacillus that can utilize sulfur as an electron donor and grow aerobically on the effluent tubing (Kelly and Wood, 2000). Species identified in the influent were not found in further ports in the reactor or in the effluent suggesting that these organisms remain in the reactor below the sampling port or are quickly washed out of the reactor. It is possible that the SUPeRB were attached, either to the solid electron donor or buffer source, at numbers below the detection limit of DNA extraction or visualization of DAPI stained cells. An alternative explanation is that SUPeRB require contact with, but not permanent attachment to, the solid electron donor or buffer source. From the experiments outlined in Chapter 2 of this dissertation it appeared that no contact was required, leading to the possibility that a soluble product leaching from elemental sulfur was responsible for perchlorate reduction and the consortium remained within the release area of this product (Nealson et al., 2002). The

low water solubility of elemental sulfur, 5 mg/L at 20°C, may also play a role in maintaining the bacteria close to the pellet (Yamamoto *et al.*, 2010). Recovery of community structure stability was slower than functional stability in a denitrifying reactor study leading to speculation that rare species that were dominant during a disturbance remained in the biofilm of the reactor (Gentile *et al.*, 2006). As this slow recovery was not observed in our reactor, biofilm may not play an important role in the establishment of the SUPeRB consortium.

The *cld* gene was detected in areas where the *pcrA* gene was not detected suggesting that chlorate-reducing bacteria were also present in the system. However, the cld gene was not detected in areas where the pcrA gene was detected perhaps due to unspecificity of the primers used to the PRB present in the consortium. The pcrA gene could only be detected in the same region of the reactor where perchlorate was degraded and on day 14 when the gene was detected by both PCR and qPCR. The *pcrA* gene copy number was in the same range as those for the perchlorate-degrading section of the pilotscale reactor reported in Chapter 4. A low quantity of PRB within the reactor as a fraction of the total was to be expected. Low concentrations of perchlorate result in low quantities of PRB, thus rendering their detection more difficult (Adham et al., 2004; Li et al., 2010). Also, with mixed inoculum, other studies have seen perchlorate reduction with levels of PRB ranging from 28% to 47% even with the addition of yeast (Wallace et al., 1998). In a reactor supplied with organic substrate PRB of the *Dechloromonas* species represented only approximately 12% of the community, even with Beta-Proteobacteria as the dominant species. This low abundance of PRB was thought to be due to the low concentrations of perchlorate fed to this reactor. With the addition of dissolved oxygen,

the numbers of Beta-Proteobacteria and PRB decreased and phyla such as Bacteroidetes increased (Young *et al.*, 2008). In an autotrophic, hydrogen-fed reactor *Dechloromonas* sp. were found at up to 49% of the total community by FISH (Nerenberg *et al.*, 2008). In a biofilm, *Dechlorosoma* sp. was found in the deepest part of the biofilm at 3–5% of the community while *Dechloromonas* sp. remained at the biofilm surface with 23% of the total community (Zhang *et al.*, 2005). This may also suggest that *Dechlorosoma* sp. is more sensitive to oxygen than *Dechloromonas* sp.

In run one, with perchlorate as the only electron acceptor, it was observed that Beta-Proteobacteria dominated in areas where perchlorate was reduced, while Epsilon-Proteobacteria remained consistent throughout the body of the reactor. Thiobacillus denitrificans, a sulfur-oxidizing, nitrate-reducing bacterium, was the dominant Beta-Proteobacterium species (Oh et al., 2000). Sulfurovum sp. was the dominant Epsilon-Proteobacteria and can also use elemental sulfur as an electron donor and nitrate as an electron acceptor (Yamamoto et al., 2010). Gamma-Proteobacteria are dominant in the effluent and influent though few are seen in the body of the reactor. Although Alpha-Proteobacteria are a dominant member of the enrichment culture, as discussed in Chapter 2 of this dissertation, few are seen in the body of the bioreactor. In reactor run two, with perchlorate and nitrate provided, Beta-Proteobacteria and Epsilon-Proteobacteria were again the dominant phyla while Alpha-Proteobacteria again were present in low quantities and Gamma-Proteobacteria were observed after perchlorate reduction. From the community tree it was observed that only those samples that showed reduction of perchlorate clustered together and upon addition of nitrate, the communities became less similar over time (Figure 3.9). The exception is day 30 of the perchlorate only run where

perchlorate was not fully reduced and which clustered with day 6 of run two. This may be due to the more diverse nature of the community from day 30; however, the number of sequences used to create this tree was too few to make adequate inferences.

Several water quality parameters were measured in the bioreactor effluent to determine whether the effluent from this bioreactor could be released to the environment without further detriment to water quality. As filtered deionized water was used for the reactor feed heterotrophic plate counts were higher than expected in the influent. The plate counts increased by two-log at port 1 (3.2 cm) likely due to a higher dissolved oxygen level from the influent. The counts dropped two logs by the next measured port, port 6 (12.1 cm), and remained at this level to port 12 (29.2 cm) likely due to the absence of oxygen and aerobic microorganism growth. In the effluent, high levels of aerobic cells were observed. This may be due to the presence of sulfur and oxygen creating favorable growth conditions on the effluent tubing.

The total organic carbon in the effluent could be released to the environment without further detriment to water quality. The total organic carbon in the effluent was higher than that found for groundwater (Leenheer and Croué, 2003) but lower than that found for freshwater sources (Reckhow *et al.*, 2007). The release of carbon corresponds to approximately 3 x 10^7 cells/L. This is similar to the numbers of heterotrophic cell counts from port 12 of the reactor but less than the heterotrophic counts found in the effluent. The aerobic Gamma-Proteobacteria found in the effluent tubing could contribute to this difference.

As no nitrate remained in the effluent the total nitrogen is thought to come from the cell respiration. Total nitrogen in the effluent was of similar concentration to the nitrate found in the influent samples. Alkalinity measurements show that there was enough buffering capacity available to maintain a stable pH in the reactor. Levels of 20 to 200 mg/L alkalinity are normal for freshwater and the alkalinity found in our reactor exceeds the upper level of this range. Denitrification also adds base adding to the buffering capacity of the system (Adham *et al.*, 2004).

The oxidation reduction potential was in the high end of the range for perchlorate reduction of 0 to -100 mV as reported by Raye-Hoponick (2006) but considerably higher than the redox potential suggested by Attaway and Smith (1993) and Shrout and Parkin (2006). Attaway and Smith (1993) based their reported redox potential on the color change of resazurin whereas Raye-Hoponick (2006) measured ORP values with a probe perhaps giving a more accurate measurement. Shrout and Parkin (2006) saw some perchlorate degradation at an ORP higher than 0 mV and suggested that excess electron donor may compensate for an oxygen presence. The high ORP may be due to a lag time between taking the sample and measuring ORP, or it could indicate that the upper part of the reactor supported aerobic conditions while the areas where perchlorate reduction occurred did not. Perchlorate degradation can occur effectively under slightly reducing conditions, whereas nitrate occurs effectively from 50 to -50 mV (Raye-Hoponick, 2006) and sulfate reduction takes place at -200 to -240 mV a redox level that may not occur in our reactor.

Based on our ORP measurements the ORP may not be low enough in our reactor for sulfate reduction to occur. Sulfate reduction is undesirable because it produces hydrogen sulfide. Sulfate was below the EPA recommended limit of 250 mg/L at all ports and in the effluent (Raye-Hoponick, 2006). Sulfate peaks occurred in the effluent

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possibly due to the action of the microbes in the upper part of the reactor. Chloride generally increased while perchlorate was reduced and remained constant after perchlorate was reduced to below the detection limit. This was observed in run two day 14 where perchlorate reduction correlated with chloride and sulfate production (Figure 3.5).

In summary this bioreactor system proved a useful tool to test the resilience of the community structure, the stable functioning and response to perturbations of this unique consortium. The microbiology of this system is novel, yet complex, and this research will add to the knowledge of autotrophic perchlorate-reduction, an understanding of the microbial community involved and the environment in which the microbes function. Furthermore, SUPeRB may be used as a cost-effective biological treatment for perchlorate contaminated drinking water supplies with effluent that can be readily treated for downstream applications.

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Table 3.1. Parameters for bench-scale bioreactor.

Experimental run 1 and run 2 with empty bed contact time (EBCT), and influent perchlorate and nitrate concentrations, ND = not determined.

Run	Time point (day) (Days of	EBCT	Influent perchlorate	Influent nitrate
#	reactor operation)	(h)	(mg/L)	(mg/L)
1	0 (178)	ND	7.1	-
	10 (188)	ND	5.2	-
	30 (208)	6.8	5.4	-
2	0 (208)	6.66	2.5	7.7
	1 (209)	ND	2.7	2.1
	2 (210)	6.66	2.5	1.9
	6 (214)	6.86	2.5	2.3
	14 (222)	6.90	2.8	1.6
	28 (236)	ND	3.1	3.0

 Table 3.2. Bench-scale bioreactor perchlorate measurements.

Experimental run 1 and run 2, $n = n$ mucht and ETT = cindent.														
Run:	Perchlorate concentration along the bioreactor (mg/L)													
Day														
		Port												
Run 1	IN	1	2	3	4	5	6	7	8	9	10	11	12	EFF
1:0	7.1	5.1	4.5	4.4	3.8	3.5	3.9	3.1	2.8	2.5	2.4	0	0	0
1:10	5.2	3.4	2.6	3.3	2.6	2.5	2.8	2.1	2.1	2.6	0	0	0	0
1:30	5.4	4.0	3.7	3.8	3.5	3.4	3.3	3.0	2.9	2.7	2.7	2.6	2.2	2.1
Run 2														
2:0	2.5	2.1	1.9	1.5	1.4	1.4	0	1.2	1.2	0	0	0	0	1.4
2:1	2.7	2.0	1.7	1.7	0	0	1.4	1.2	0	0	0	0	0	0
2:2	2.5	2.0	1.7	1.6	1.4	0	1.2	0	0	0	0	0	0	0
2:6	2.5	1.6	1.3	1.3	1.0	0	0	0	0	0	0	0	0	0
2:14	2.8	1.5	1.3	1.5	0	0	0	0	0	0	0	0	0	0
2:28	3.1	5.7	1.5	1.3	1.3	1.3	0	0	0	0	0	0	0	1.7

Experimental run 1 and run 2, IN = influent and EFF = effluent.

 Table 3.3. Heterotrophic plate counts from the bench-scale bioreactor.

Reactor Area	Cell Counts (CFU/ml)
influent	$7.17 \ge 10^4 \pm 1.25 \ge 10^4$
port 1	$1.17 \ge 10^6 \pm 5.77 \ge 10^5$
port 6	$4.27 \ge 10^4 \pm 5.03 \ge 10^3$
port 12	$4.03 \times 10^4 \pm 4.04 \times 10^3$
effluent	$2.00 \times 10^7 \pm 2.50 \times 10^6$

Table 3.4. Phylogenetic analysis of the perchlorate fed bench-scale bioreactor on
days 0 and 10.

Phylogenetic classification for community members found in the bench-scale reactor in areas before and after perchlorate reduction. Clone libraries are based on 16S rRNA gene sequences. Sequences are designated unclassified if there were no close matches in the NCBI database or matches were closely related to uncultured organisms only.

Area of Reactor	eactor Phylum Order		% of Total
(# of clones)			(# of clones)
Influent (7)	Alpha-Proteobacteria	Caulobacterales	14(1)
		Rhizobiales	14(1)
	Beta-Proteobacteria	Burkholderiales	29(2)
	Bacteroidetes	Sphingobacteria	43(3)
ports 1 to 4:	Alpha-Proteobacteria	Rhodobacterales	14(1)
3.2 to 8.4 cm (7)	Beta-Proteobacteria	Hydrogenophilales	29(2)
		Unclassified	14(1)
	Epsilon-Proteobacteria	Campylobacterales	14(1)
	Gamma-Proteobacteria	Unclassified	29(2)
ports 5 to 8:	Beta-Proteobacteria	Hydrogenophilales	50(4)
10.6 to 15.8 cm		Rhodocyclales	13(1)
(8)		Unclassified	37(3)
ports 9 to 10	Beta- Proteobacteria	Hydrogenophilales	23(3)
17.3 to 18.8 cm		Burkholderiales	15(2)
(13)		Unclassified	31(4)
	Epsilon-Proteobacteria	Campylobacterales	23(3)
	Delta-Proteobacteria	Unclassified	8(1)
ports 11 to 12:	Alpha-Proteobacteria	Unclassified	5(1)
24.0 to 29.2 cm	Beta-Proteobacteria	Hydrogenophilales	24(5)
(21)		Burkholderiales	5(1)
		Unclassified	32(7)
	Epsilon-Proteobacteria	Campylobacterales	24(5)
	Delta-Proteobacteria	Desulfobulbus	5(1)
	Bacteroidetes	Unclassified	5(1)
Effluent (8)	Beta-Proteobacteria	Hydrogenophilales	12(1)
		Burkholderiales	12(1)
	Epsilon-Proteobacteria	Campylobacterales	12(1)
	Gamma-Proteobacteria	Acidithiobacillus	64(5)

Table 3.5. Phylogenetic analysis of the perchlorate fed bench-scale bioreactor on day30.

Phylogenetic classification for community members found in the bench-scale reactor in areas before and after perchlorate reduction. Clone libraries are based on 16S rRNA gene sequences. Sequences are designated unclassified if there were no close matches in the NCBI database or matches were closely related to uncultured organisms only.

Area of Reactor	Phylum	Order	% of Total
(# of clones)			(# of clones)
Influent (2)	Alpha-Proteobacteria	Caulobacterales	50(1)
		Rhizobiales	50(1)
ports 1 to 4:	Epsilon-Proteobacteria	Campylobacterales	75(3)
3.2 to 8.4 cm (4)	Actinobacteria	Actinomycetales	25(1)
ports 5 to 8:	Alpha-Proteobacteria	Rhizobiales	25(1)
10.6 to 15.8 cm (4)	Beta-Proteobacteria	Hydrogenophilales	50(2)
		unclassified	25(1)
ports 9 to 12:	Beta-Proteobacteria	Burkholderiales	38(3)
17.3 to 29.2 cm (8)		Unclassified	12(1)
	Epsilon-Proteobacteria	Campylobacterales	12(1)
	Actinobacteria	Holophagae	12(1)
	Chloroflexi	Unclassified	12(1)
	Firmicutes	Clostridia	12(1)

Table 3.6. Phylogenetic analysis of the perchlorate and nitrate fed bench-scale bioreactor.

Phylogenetic classification for community members found in the bench-scale reactor in areas before and after perchlorate reduction. Clone libraries are based on 16S rRNA gene sequences. Sequences are designated unclassified if there were no close matches in the NCBI database or matches were closely related to uncultured organisms only.

Area of Reactor (# of clones)	Phylum	Order	% of Total (# of clones)
Before	Alpha-Proteobacteria	Rhizobiales	11 (2)
perchlorate	Beta-Proteobacteria	Burkholderiales	44 (8)
reduction (18)		Hydrogenophilales	
		Rhodocyclales	
	Epsilon-Proteobacteria	Campylobacterales	33 (6)
	Chloroflexi	Anaerolineales	6 (1)
	Firmicutes	Unclassified	6 (1)
After	Alpha-Proteobacteria	Rhodobacterales	7 (2)
perchlorate		Rhizobiales	
reduction (29)	Beta-Proteobacteria	Burkholderiales	34(10)
		Hydrogenophilales	
		Rhodocyclales	
	Epsilon-Proteobacteria	Campylobacterales	31 (9)
	Gamma-Proteobacteria	Thiothrix	21 (6)
	Chlorofexi	Pseudomonas	7 (2)
		Anaerolineales	




Positioning of ports from influent (cm): Port 1: 3.2, 2: 5.4, 3: 6.9, 4: 8.4, 5: 10.6, 6: 12.1, 7: 13.6, 8: 15.8, 9: 17.3, 10: 18.8, 11: 24.0, and 12: 29.2. The influent was kept anoxic by replacing feed medium volume with a mixture of N_2 :CO₂ in the headspace.





A) Perchlorate concentrations as a percentage of the influent in Run 1 days 0 (\blacklozenge), 10 (\blacksquare), and 30 (\blacktriangle). B) Community analysis, as a percentage of the total community, corresponding to perchlorate concentration. Alpha- (\Diamond), Beta- (\blacksquare), Gamma- (\times), Delta-(Δ), Epsilon- (\Box) Proteobacteria, and Bacteroidetes (\ast) in areas of the reactor community analysis of days 0 and 10 only. Influent (0 cm), ports 1 to 4, ports 5 to 8, ports 9 and 10, and ports 11 and 12 were combined, Effluent (40 cm). Error bars indicated the standard deviation of the combined ports from the average.





A) Perchlorate concentrations as a percentage of the influent in Run 2, days 0 (\blacklozenge), 1 (\blacksquare), 2 (\blacktriangle), 6 (\square), 14 (\times), and 28 (\diamondsuit). B) Community analysis, as a percentage of the total community, corresponding to perchlorate concentration before and after reduction to below the detection limit. Alpha- (\diamondsuit), Beta- (\blacksquare), Gamma- (\times), Epsilon-(\square) Proteobacteria, Chloroflexi (Δ), and Firmicutes (\blacklozenge) in areas of the reactor community analysis of days 0 and 10 only. Influent (0 cm), ports 1 to 3, ports 4 to 6, ports 7 and 9, and ports 10 and 12 were combined, Effluent (40 cm). Error bars indicated the standard deviation of the combined ports from the average.



Figure 3.4. Perchlorate and nitrate fed bioreactor anion profile.

Perchlorate (\blacksquare), nitrate (x), nitrite (\blacktriangle), and chloride (\blacklozenge), on the left Y-axis with solid lines and sulfate (*) on the right Y-axis for day 14, run 2.



Figure 3.5a. Nitrate reduction in the bench-scale bioreactor. Figure 3.5b. Chloride production in the bench-scale bioreactor. Figure 3.5c. Sulfate production in the bench-scale bioreactor.

A) Nitrate as a percentage of the influent concentration **B**) Chloride increase within the reactor in mg/L **C**) Sulfate increase within in the reactor in mg/L. Day 0 (\blacklozenge), 1 (\blacksquare), 2 (\blacklozenge), 6 (\Box), 14 (\times), and 28 (\diamondsuit). Influent (0 cm), ports 1 to 3, ports 4 to 6, ports 7 and 9, and ports 10 and 12 were combined, Effluent (40 cm). Error bars indicated the standard deviation of the combined ports from the average.



Figure 3.6. Phylogenetic relationship of the 16S rRNA gene of the perchloratedegrading bench-scale bioreactor.

Phylogenetic relationship and distribution of bacterial isolates as identified by phyla from the reactor fed perchlorate only. The comparative analysis was inferred by Minimum Evolution analysis of aligned 16S rRNA sequences from clones in concert with public nucleotide databases. The scale bar represents 2% estimated sequence divergence. Bootstrap values are shown for nodes that had 50% support in an analysis of 1,000 replicates. Known PRB (\diamond), clones from run one, days 1 (\bullet), 10 (\blacksquare), and 30 (\blacktriangle).



Figure 3.7. Phylogenetic relationship of the 16S rRNA gene of the perchlorate- and nitrate-degrading bench-scale bioreactor.

Phylogenetic relationship and distribution of bacterial isolates as identified by phyla from the reactor fed perchlorate and nitrate. The comparative analysis was inferred by Minimum Evolution analysis of aligned 16S rRNA sequences from clones in concert with public nucleotide databases. The scale bar represents 5% estimated sequence divergence. Bootstrap values are shown for nodes that had 50% support in an analysis of 1,000 replicates. Known PRB (O), clones from run two days 1 (•), 6 (\bigstar), 14 (\checkmark), and 28 (\bigstar).



Figure 3.8. qPCR standard curve for the *pcrA* gene

Standard curve to quantify the *pcrA* gene obtained from serially diluted *Dechlorosoma* (renamed *Azospira*) *suillum* PS genomic DNA. C_T values are the average of three replicates. Error bars represent standard deviations.



Figure 3.9. Community cluster dendrogram to compare sequential samples of the bench-scale bioreactor.

Run one (R1): days 0 (0), 10 (10), and 30 (30) and run two (R2): days 1 (1), 2 (2), 6 (6), 14 (14), and 28 (28). The scale bar represents 5% estimated sequence divergence.

CHAPTER 4

MICROBIOLOGICAL INVESTIGATION OF THE SUPERB CONSORTIUM FROM THE PILOT-SCALE BIOREACTOR

<u>Abstract</u>

The sulfur-utilizing, perchlorate-reducing bacterial (SUPeRB) consortium successfully degraded low levels of perchlorate (100 μ g/L) to below the EPA recommended contamination limit of 15 μ g/L under field conditions in a 200 L pilot-scale reactor. The presence of the common co-contaminants nitrate and RDX had no effect on perchlorate degradation. The community structure composition in the lower half (influent) of the reactor differed from the upper half (effluent). The presence of perchlorate-reducers was located by functional gene analysis in the influent end of the reactor, and sulfur-oxidizers were found to be dominant in this bioreactor system. Our study shows that the function of the SUPeRB consortium is stable under field conditions, including temperature extremes, intermittent perchlorate feed, and oxygenation of feed water. Furthermore, by-products such as sulfide and sulfate did not prevent successful bioremediation of perchlorate and did not reach hazardous levels.

Introduction

This chapter describes the microbial community structure within a 200 L, pilotscale, perchlorate-reducing bioreactor with elemental sulfur as the electron donor. The reactor set-up and perchlorate degradation kinetics were reported by McKeever (2009) and Boles *et al.* (2009).

Many reactors use microbes to reduce perchlorate (Xu et al., 2003); however, few studies have examined the microbiology of the reactors. Molecular techniques were used to analyze community composition of the microbial biofilm on plastic and granular activated carbon (GAC) supports in acetate-fed reactors. Zhang et al. (2005), using fluorescent *in situ* hybridization (FISH), found that after a six-month groundwater feed Dechloromonas sp., became dominant in an up-flow reactor even though the perchloratereducer *Dechlorosoma* sp. was originally inoculated into the reactor. In high salt, denitrifying, and perchlorate-reducing reactors, Chung et al. (2009) used 16S rRNA gene analysis and found that Clostridium sp. and Rhodocyclaceae were the dominant species on plastic supports. Xiao et al. (2010) used denaturing gradient gel electrophoresis (DGGE) and FISH to determine the dominant species over time on a GAC support. Using terminal restriction fragment length polymorphism (t-RFLP), Park et al. (2008) found that the diversity of the microbial community, established with a wastewater sludge inoculum and acetate as an organic substrate, decreased with the addition of perchlorate. Addition of salinity, to 3%, also changed the structure of the community but did not affect diversity. In a hydrogen-fed community, Alpha- and Gamma-Proteobacteria were dominant after 90 days. However, using 16S rRNA gene analysis no previously known perchlorate reducers were identified in the Park et al. study.

In our study, the microbial community structure was examined by microscopic visualization, DGGE community fingerprint analysis, and functional gene amplification, identification and quantification. Heterotrophic plate counts were used to determine if numbers of microorganisms could lead to excess contamination from the effluent of the reactor. Protein measurements were used as a proxy for microbial biomass.

Measurements were performed to determine potential conversion of sulfur by microbial processes to sulfide, an undesirable by-product.

Materials and Methods

Scale-Up of Enrichment Culture and Bioreactor Inoculation

One liter of perchlorate minimal medium was inoculated with an actively degrading perchlorate enrichment culture. When this culture was degrading perchlorate at a constant rate it was used to inoculate a 20-liter carboy containing 20 L of perchlorate minimal medium. The minimal medium contained the following components per liter of filtered ground water: 5 mg of ClO₄, 0.5 mg of NaHCO₃, 8.5 mg of KH₂PO₄, 21.75 mg of K₂HPO₄, 33.4 mg of Na₂HPO₄·7H₂O, 22.5 mg of MgSO₄·7H₂O, 0.25 mg of FeCl₃·6H₂O, 27.5 mg of CaCl₂, 10 mg of (NH₄)2SO₄, 30 g sulfur pellets (Georgia Gulf Sulfur Corp., Valdosta, GA), and 10 g oyster shell (Core Calcium & Shell Products, Mobile, AL). The media was sparged with nitrogen for one hour and incubated at room temperature with internal stirring and a gas-tight tedlar bag (SKC, Eighty Four, PA) filled with nitrogen. The contents of the carboy were divided into two carboys and the media refilled with fresh ground water, sulfur pellets, oyster shell and 2.5 mg/L NH₄Cl, and 0.5 $mg/L K_2HPO_4$. One of the two carboys was deoxygenated with sulfite instead of nitrogen to ensure that this mode of deoxygenation did not negatively affect perchlorate reduction. An additional 20 L carboy containing sulfur pellets, oyster shell and ground water was inoculated with 1 L of enriched SUPeRB, 2.5 mg/L NH₄Cl, 0.5 mg/L K₂HPO₄ and then made oxygen-free by adding nitrogen gas. Samples for perchlorate measurements and pH were taken regularly. Perchlorate was slowly reduced over the 90-day incubation and pH

remained near neutral at 7.5. The pilot-scale bioreactor was inoculated with the total 60liters of enrichment cultures on day 0 of the pilot study.

Sample Collection for Microbiological Analysis

On day 103 of bioreactor operation, while the reactor was alternating between recirculation and flow-through mode, 100 ml of pore liquid from ports 1, 4, and 7 were filtered through a 0.2 μ m pore size membrane filter and transported to the lab on ice. The filters were then stored at -30°C.

On days 281 and 310 of bioreactor operation, during continuous flow-through mode, two complete sets of pore water samples were taken. Approximately one liter of pore water was taken from each of the eight ports and from the influent and effluent water. The pore water samples were kept on ice, transported to the laboratory and stored at 4°C. Approximately 50 ml of each sample was frozen at -30°C and the remainder was filtered through a 0.22 μ m filter (Millipore, Billerica, MA) within 24 hours of collection. The filters were then stored at -30°C.

The bioreactor was disassembled on day 310. Sulfur pellet and oyster shell matrix samples were collected at depths within the reactor equal to the location of each sampling port. Matrix samples were taken next to the sample port (A), in the center of the reactor (C), and approximately halfway along the radius between the center and the port (B) for a total of three samples for each port level. The matrix samples were kept on ice, and then transported to the laboratory where they were stored at 4°C.

Microscopic Observation

A Nikon Eclipse 6400 microscope (Nikon, Inc., Melville, NY), equipped with a Nikon 100X 1.30 NA oil objective, and a digital camera (Hamamatsu, Bridgewater, NJ), was used to examine the morphology of cells residing in the pore water from day 103 samples.

Biomass Measurements

Protein content: The unfiltered pore water and matrix samples taken on day 310 were analyzed for protein content, as a proxy for biomass, using a BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. The protein concentration in the pore water samples was measured within 24 hours of collection directly from the liquid samples and was adjusted for a reactor media porosity of 30%. The protein concentration in the matrix samples was measured 48 days after sample collection. For the matrix samples approximately 10 g of sulfur pellet and oyster shell medium from each of the three regions sampled, A, B and C, were collected at ports 1, 2, 4 and 7, at 9.5, 16.5, 33.7, and 65.4 cm distance from the reactor influent, respectively. The samples were vortexed for one minute to dislodge biomass and the protein concentrations in the PBS were measured and adjusted for a bulk density of 1.22 g solid medium/ml volume of medium.

<u>Heterotrophic plate counts</u>: Unfiltered pore water samples from days 210 and 310 were diluted in PBS to 10^{-7} and each dilution was plated onto R2A agar (Difco, BD,

Sparks, MD), in triplicate, within 24 hours of collection. The plates were incubated at 20°C and, after seven days, the colony forming units (CFU) were counted.

Sulfide Analysis

Sulfide concentrations in the unfiltered pore water from each port, and in both the influent and effluent, were measured in the day 310 samples which were stored frozen at -30° C. The sulfide concentration was measured using the methylene blue method with a detection limit of 0.05 nM (Chen and Mortenson, 1977).

Phylogenetic Analysis

DNA Extraction and Amplification: Approximately 10 ml of PBS supernatant from the matrix samples were filtered through a 0.22 μm pore membrane filter (Millipore, Billerica, MA) with a GF/F glass microfiber filter (Whatman International, Ltd., Maidstone, England) placed on top. The microfiber filter was used to trap the larger oyster shell and sulfur pellet particles while the 0.22 μm filter trapped the microbial cells. DNA was extracted from the pore water and matrix sample filters using the RapidWater® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene was amplified from the DNA in triplicate PCRs.

Amplifications for samples taken on day 103 were run with a standard protocol in which a 30 μ l reaction volume had the following final concentrations: 0.5 ng/ μ l DNA, 0.5 μ M of each primer 8F and 1492R (Weisburg *et al.*, 1991), 2 mM MgCl₂, 10x PCR buffer, 0.25 mM of each dNTP and 0.08 U/ μ l *Taq* DNA polymerase, 400 ng/ μ l BSA. The following PCR program was run: 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s and finally one cycle of 72°C for 5 min. All PCRs were run

in triplicate on an MJ Research Peltier Thermal Cycler PTC-200 (MJ Research Inc., Waltham, MA), pooled to reduce amplification bias, cleaned using a QIAquick® PCR purification kit (Qiagen Inc., Valencia, CA) and the expected fragment was visualized on a 1% agarose gel stained with ethidium bromide. The 16S rRNA gene was cloned into the pGEM-T Easy Vector Systems kit (Promega, Madison, WI), and E. coli JM109 high efficiency competent cells (Promega, Madison, WI) were transformed in accordance with manufacturer's instructions. Clones were grown into colonies and positive clones were then randomly picked. Amplification of the 16S rRNA gene from each clone was carried out in a 30 μ l reaction volume with the following final concentrations: 0.5 ng/ μ l DNA, 0.33 µM of each pGEMf and pGEMr primers, 1.75 mM MgCl₂, 10x PCR buffer, 0.17 mM of each dNTP, 0.1 U/µl of *Taq* DNA polymerase. The following PCR program was used: 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 30 s and finally one cycle of 72°C for 5 min. Selected clones from each sample were submitted for 16S rRNA gene sequencing. For all sequence submissions, PCR amplified products were pooled, cleaned, and amplified with BigDye®Terminator V.3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, CA).

For days 281 and 310 samples the following PCR conditions were used in 30 μ l reaction volumes: 0.5 ng/ μ l DNA, 0.5 μ M of each primer 341F (5'-CC TAC GGG AGG CAG CAG-3' containing a 40-bp GC clamp at the 5' end) and 786R (5'-CTA CCA GGG TAT CTA ATC-3') (Baker *et al.* 2003), 2 mM MgCl₂, 1x PCR buffer, 0.25 mM of each dNTP and 0.08 U/ μ l *Taq* DNA polymerase, 400 ng/ μ l BSA. The following PCR program was run: 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s and finally one cycle of 72°C for 5 min.

Sequence Analysis: For day 103, 59 sequences were edited, checked for chimeras using Mallard (Ashelford *et al.*, 2006), compared to the NCBI database (Altschul *et al.*, 1997), and classified using RDP V.9.57, with an 80% confidence threshold (Wang *et al.*, 2007). Distance based OTU, richness determination and diversity index of each port was calculated using DOTUR (Schloss and Handelsman, 2005).

DGGE Analysis: For days 281 and 310 total community fingerprint analysis was performed with the DCode Universal Mutation Detection System (BIO-RAD, Hercules, CA). DGGE gels were created with a 30 to 50% denaturing gradient. The PCR product was mixed with 2X loading dye and 20 µl loaded onto the gel. The samples were run for 16 hours at 80V in 1X TAE buffer preheated to 60°C. The gels were stained for one hour with ethidium bromide and visualized using Epi Chemi II darkroom (UVP, LLC, Upland, CA). Dendrograms were created with Gelcompar II (Applied Maths, Inc., Austin, TX) using the Pearson correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm. Bands of interest were excised from the gel and DNA was eluted in PCR-grade water overnight. DNA was PCR amplified in triplicate with the same amplification reaction as described above, with the exception that the forward primer did not have the GC clamp and the DNA submitted for sequencing.

<u>Functional Gene Detection</u>: Samples of pore water and matrix from days 281 and 310 were tested for the presence of the functional genes *pcrA* and *cld*, using PCR amplification. The *pcrA* and *cld* genes were amplified from total DNA in triplicate PCR reactions. The *cld* gene was amplified as outlined by Bender *et al.* (2004) with the exception that the PCR reactions were carried out in 30 µl reactions. The *pcrA* gene was amplified in a 30 µl reaction volume with the following final concentrations: 0.5 ng/µl

DNA, 0.4 μ M of each primer pcrAF 5'-ACTACATGTATGGNCCGCATCG-3' and pcrAR 5'-CGTGRTCRCYGTACCAGTCRAA-3', 1.5 mM MgCl2, 1x PCR buffer, 0.20 mM of each dNTP and 0.05 U/ μ l *Taq* DNA polymerase, 250 ng/ μ l BSA. The following PCR program was run: 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and finally one cycle of 72°C for 10 min. The two functional genes were sequenced and their closest relatives identified as previously outlined. Multiple sequence alignments were created using the program ClustalX, V.1.83 (Thompson *et al.*, 1997) and phylogenetic analyses were conducted using the software package MEGA V.4 minimum evolution analysis, using the Tamura–Nei model, with bootstrap values of 1,000 replicates (Tamura *et al.*, 2007).

Quantitative PCR (qPCR)

Standard curves were created from DNA extracted from the control strain *Dechlorosoma* (renamed *Azospira*) *suillum* PS and cloned into a plasmid. The copy number of the plasmid was calculated by measuring absorbance at 260 nm. A DNA dilution series in seven steps from 10^6 to one gene copies/ml was performed and the cycle threshold (C_T) values were plotted against gene copy number/ml. DNA extracted from the bioreactor pore water samples of days 281 and 310 were tested for the relative quantity of functional gene, *pcrA*, at each port and the influent and effluent. PCR amplification was performed in a 20 µl final volume containing 1 µl of DNA, 0.16 µM each of the primers pcrAF and pcrAR and 10 µl of GoTaq® qPCR Master Mix (Promega, Madison, WI). All amplifications were carried out in Thermo-Fast® white 96-well PCR plates (Thermo Scientific, Epsom, UK) on a DNA Engine Opticon® 2 System (Bio-Rad, Hercules, CA)

with an initial step of 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by an elongation step at 72°C for 5 min. All samples were analyzed in triplicate. Gene copy numbers were adjusted for a reactor porosity of 30%.

Results

Microscopic Observation

On day 103 of the bioreactor run two distinct morphologies were observed, short rods and spirilli (Figure 4.1). The short rod was the dominant morphology.

Biomass Measurements

Protein content: In the pore water, biomass increased from approximately 10 to 19 mg of protein/L between the influent and port 3 (0.0 cm to 22.9 cm), with the greatest increase between ports 3 (22.9 cm) and 4 (33.7 cm) from 19 to 67 mg/L (Figure 4.2). The protein levels remained high (62 to 88 mg/L) in the remaining ports of the bioreactor. The protein level in the matrix was measured in three areas of the reactor: A, B, and C, at the level of ports 1, 2, 4, and 7. Biomass concentrations decreased in the matrix between ports 2 and 4 (Figure 4.3). For areas A, B, and C the protein levels decreased between ports 1 and 7, from 2,342 to 555 μ g/L, 1,787 to 387 μ g/L, and 1,269 to 275 μ g/L, respectively. However, area C also had a biomass increase between the first and second port from 1,269 to 2,342 μ g/L protein. In the port 1 matrix sample protein concentration was greatest in the outer area A, followed by area B, and then the central area C. In port 2 the reverse was found with the greatest protein concentrations of protein were measured. The

lower matrix protein concentration, measured in the upper part of the bioreactor, was approximately equal to the highest concentration found in pore liquid, also in the upper part of the reactor.

<u>Heterotrophic plate counts</u>: On day 210 of bioreactor flow-through, heterotrophic plate counts in the influent were $1.61 \times 10^6 \pm 2.47 \times 10^5$ CFU/ml and $2.00 \times 10^5 \pm 0$ CFU/ml in the effluent of the reactor. On day 310 the counts in the influent were 4.26 x $10^4 \pm 1.92 \times 10^4$ CFU/ml and 2.00 x $10^4 \pm 4.62 \times 10^3$ CFU/ml in the effluent of the reactor.

Sulfide Analysis

Sulfide concentrations remained low, at less than 5 μ M sulfide, until port 3 (22.9 cm), increased from 5 to 75 μ M by port 5 (44.5 cm), and decreased gradually from port 5 (44.5 cm) to port 8 (76.2 cm) from 75 to 35 μ M with one point at port 6 (55.3 cm) decreasing to approximately 18 μ M. Sulfide spanned concentrations from 0.18 to 2.25 μ g/L (Figure 4.4).

Phylogenetic Analysis

Sequence Analysis: For ports 1, 4, and 7, the number of clones sequenced were 24, 24 and 10, respectively. Diversity estimates were calculated and Chao values of 34, 45, and 23 for ports 1, 4, and 7, respectively, indicated that further sequencing of the communities was necessary to achieve complete coverage. Although the Simpson diversity indexes (all values < 0.1) indicated that each community had high levels of species diversity further sequence analysis would be necessary for a more accurate measurement. The bacterial community structure was distinct among the three zones

(Table 4.1). Members of the phylum Epsilon-Proteobacteria increased from bottom to top, from 25% to 70% of clones sequenced. These clones were identified as the genus *Sulfuricurvum*, a sulfur-oxidizing bacterium (Kodama and Watanabe, 2004). Members of the phylum Beta-Proteobacteria decreased from bottom to top, from 50% to 20% of the clones sequenced, and Alpha-Proteobacteria only appeared in the middle port at 21% of the clones sequenced.

DGGE Analysis: The cluster analysis dendrogram created based on the DGGE patterns (Figure 4.5) showed that the community structure was most similar among ports 1 to 4 (9.5 to 33.7 cm) and ports 5 to 8 (44.5 to 76.2 cm). The effluent sample (86.4 cm) grouped most closely with ports 5 to 8. The influent aquifer water did not group with any other sample. The bacterial species represented by certain gel bands that appeared in the first port and disappeared in subsequent ports were identified. Bands 1, 2 and 3 extracted from the DGGE gel (Figure 4.5) had 98-99% similarity to an uncultured bacterium clone from a sulfur spring and 96% related to an uncultured Epsilon-Proteobacterium clone found in iron-rich, deep-sea, microbial mats.

<u>Functional Gene Detection</u>: In the day 281 sample the *pcrA* gene was detected by PCR in the pore liquid from port 1 (9.5 cm) and the *cld* gene was detected by PCR in the pore liquid from ports 1 and 2 (9.5 to 16.5 cm). From the day 310 sample the *pcrA* and *cld* genes were detected in the pore liquid from port 1 (9.5 cm). Both functional genes were most closely related to the functional genes from perchlorate-reducing bacteria (PRB) of the Beta-Proteobacteria. The *cld* gene was distantly related to the uncultured bacterium clone ASH-4 chlorite dismutase gene at 87% similarity and *Dechloromonas agitata* at 78% similarity. The *pcrA* gene was distantly related to the uncultured

bacterium clone PNA3 perchlorate reductase alpha subunit at 82% similarity *Dechloromonas* sp. MissR at 81% similarity (Figures 4.6 and 4.7).

Functional gene copy quantitation

The assay was linear over six orders of magnitude and the detection limit was approximately 10 gene copies/ml. The *pcrA* gene copy number was calculated based on the standard curve, with the assumption that full DNA extraction was attained and that there was one copy of the *pcrA* gene per cell (Figure 4.8). The highest gene copy numbers were found in port 1 (9.5 cm) of the pore water samples collected on days 281 and 310 at 4.2 x $10^5 \pm 9.8 \times 10^4$ and 6.3 x $10^4 \pm 1.7 \times 10^4$ gene copies/L, respectively (Figure 4.9). For all other ports (0.0 cm and 16.5 to 86.4 cm) on day 281 there were between 5.9 x 10^3 and 1.8×10^4 gene copies/L. For all other ports (0.0 cm and 16.5 to 86.4 cm) on day 310 gene copies ranged from below the detection limit to 1.7×10^4 gene copies/L. In the influent on day 281 the copy number was below the detection limit of the assay, while the day 310 sample measured 9.4 x 10^3 gene copies/L. For both days the effluent measured below the detection limit of the assay. The matrix samples measured from 2.8 x 10^3 to 9.3 x 10^3 gene copies/L with the highest copy numbers in port 2 (16.5 cm).

Discussion

The protein, heterotrophic plate counts, sulfide and molecular assays indicated that there was a community change in the reactor over the eight ports from the inlet to the outlet. With a similar sulfur/limestone process for denitrification there was an increase in the number of bacteria, concentrations of assimilable organic carbon and the byproduct sulfide in the bioreactor effluent (Kimura *et al.*, 2002). Therefore, it is important to examine microbially mediated effects on the effluent of the reactor.

The biomass density, as indicated by protein measurements from matrix and pore water samples, showed opposing trends with reference to location. For the matrix, biomass concentration was highest in the lower ports, in at least ports 1 and 2 at 9.5 and 16.5 cm from the influent, respectively, and decreased with flow through the bioreactor. In the lower ports there was no clear trend regarding the location of the highest biomass concentration in the width of the reactor, whereas biomass was homogenous throughout the width of the reactor toward the middle and upper parts of the reactor. For the pore liquid, the majority of the biomass was in the upper part of the bioreactor, from ports 4 to 8 at 33.7 to 76.2 cm from the influent. This suggested that most biofilm formed in the lower part of the bioreactor closest to the inlet. The biofilm may detach and washout of the reactor resulting in higher protein concentrations in the upper port pore water. Also, in the upper regions of the bioreactor, perchlorate depletion may lead to lower biomass, either in the pore water or the biofilm. This was also seen by Xiao *et al.* (2010) where biomass was not detected at the top of their up-flow reactor.

The heterotrophic plate counts taken in the summer were higher compared to those taken in the autumn which may be expected due to the higher temperatures in the summer when compared to the autumn. The counts in the influent were higher than those in the effluent giving an overall trend of a decrease in aerobic heterotrophs within the bioreactor. This decrease was contrary to the protein concentrations found in the pore liquid indicating that the majority of cells contributing to the biomass were not cultivatable or could not grow under aerobic conditions. There was also the possibility that protozoa in the system assimilate the bacteria as a food source. The high CFU/ml found in the summer sample were perhaps due to storage of the feed water in a tank coupled with growth within the sampling tubing. However, groundwater was found to support only up to 3 x 10^4 CFU/ml within seven days when stored at 25° C (Payment *et al.*, 1997). Samples for heterotrophic plate counts were only taken twice over the course of the pilot run and further data would determine whether the high counts in the summer sample were representative of the entire season.

A strong odor of hydrogen sulfide from the reactor pore water indicated production in the reactor. As the reactor was anaerobic, this production was thought to be microbially produced by sulfate-reducing bacteria rather than by abiotic oxidation of the sulfur pellets. Sulfate-reducers may produce detectable levels of sulfide in strictly anaerobic niches within the reactor when reducing sulfate released by sulfur-oxidizing bacteria. Sulfide concentrations were found to increase in the upper part of the reactor from ports 4 to 8, at 33.7 to 76.2 cm from the influent, coinciding with the increase in biomass in pore liquid and reduction of perchlorate. Ju *et al.* (2007) also found that elemental sulfur disproportionated into sulfate and sulfide by abiotic disproportionation and microbial fermentation. This reaction also started after the perchlorate concentration decreased to a low concentration. One anomalous point found at port 6, at 55.2 cm from the influent, coincided with a decrease in biomass in the pore liquid indicating there may be a decrease in the microbial population responsible for the disproportionation of the elemental sulfur.

While sulfur particles have previously been noted to support an autotrophic denitrifying biofilm (Jang et al., 2005), the biomass dislodged from the matrix in our study did not appear to contain PRB. In further support of the absence of PRB in the biofilm, in our study, results indicated that it was not necessary for the PRB to maintain constant direct contact with the matrix of sulfur pellets or oyster shell particles. Also, the functional genes for perchlorate-reduction were only present at concentrations detectable by PCR amplification in the pore water from the lower ports. It was assumed that PRB were only present in detectable levels at the ports where the majority of perchlorate was actively degraded (Boles et al., 2009). A PRB level of 4.2 x 10⁵ cells/L and 6.3 x 10⁴ cells/L appeared to be adequate for perchlorate reduction to occur as shown by the qPCR results (Figure 4.9). The approximately seven-fold difference in cell numbers between the two sample days suggests that the PRB were becoming more efficient in degrading the concentration of perchlorate available. Nozowa-Inoue et al. (2008) also saw a minimum detection of approximately 10 copies of the *pcrA* gene and detected 3.4×10^4 to 4.5×10^5 pcrA gene copies/g dry soil. De Long et al. (2010) found that even when they prepared qPCR reactions containing known copy numbers of pcrA the measured copy number was approximately two orders of magnitude smaller than the theoretical copy number. Populations of PRB were reported to range from 2.31 x 10^3 to 2.4 x 10^6 cells/g sample in perchlorate-contaminated groundwater sediment as measured by most probable number (MPN) with acetate as an electron donor (Coates *et al.*, 1999). Given the difference in cell numbers found in soil and sediment compared to free water, numbers of potential PRB were comparable between our study and those reported in the literature (Coates et al., 1999; Nozowa-Inoue et al., 2008).

The *pcrA* and *cld* genes were identified as Beta-Proteobacteria but had only 81% similarity to the functional gene of PRB submitted to Genbank. The closest match to the *cld* gene was a clone whose sequence was submitted to Genbank by a group working on perchlorate and nitrate reduction in soil with the addition of acetate and hydrogen (Son *et al.*, 2006). When the *cld* gene sequence was translated to amino acid sequence there was 83% similarity to *D. agitata*. The closest match to the *pcrA* gene was a clone whose sequence was submitted to Genbank by a group working on perchlorate and nitrate reduction using acetate and hydrogen (Nozawa-Inoue *et al.*, 2008). When the *pcrA* gene sequence was translated to amino acid sequence and nitrate reduction using acetate and hydrogen (Nozawa-Inoue *et al.*, 2008). When the *pcrA* gene sequence was translated to amino acid sequence there was 88% similarity to these clones and also *Dechloromonas* sp. MissR. This distant similarity indicated that the PRB in the SUPeRB system is a novel species within the Beta-Proteobacteria.

The community structure analysis also showed a divide in the reactor between the lower and upper ports. The effluent sample clustered less closely with the upper ports perhaps due to a potential exposure to air in the effluent tubing or due to the absence of perchlorate. The influent sample had a diverse community and was most different in structure from the bioreactor ports communities, perhaps due to indigenous aquifer microorganisms entering the system. When ports 1, 4 and 7 were sequenced while the reactor was in recirculation mode there were single species representatives of phyla other than Proteobacteria in the bottom and middle ports (Table 4.1). No phylum other than Proteobacteria was seen in port 7. This limitation in detected diversity may be due to the small number of clones sequenced for port 7 as on recirculation there should be a more even species diversity assuming there is a greater transport of perchlorate throughout the reactor.

DNA fragments extracted from the port 1 sample DGGE bands were identified as Epsilon-Proteobacteria. Previous studies on this sulfur-utilizing, perchlorate-reducing system (Sahu et al., 2009) and previous sequence analysis of this bioreactor (Conneely et al., unpublished results 2009) showed that sulfur-oxidizers of the Epsilon-Proteobacteria, namely Sulfuricurvum kujiense, were a dominant species throughout the SUPeRB reactors. S. kujiense is a facultative anaerobe that can utilize elemental sulfur and sulfide as electron donors and nitrate and oxygen as electron acceptors (Kodama and Watanabe, 2004). Xiao et al. (2010) also used DGGE and FISH to examine nitrate and perchloratereducing reactors and found that the dominant species that were present at approximately 50% of the bacterial community in the biofilm as detected by FISH could not be detected by DGGE. Therefore, although we did not sequence every DGGE band, it was possible that a band corresponding to the bacterial species responsible for perchlorate reduction would not be present. Although no known PRB were found in the 16S rRNA clone libraries for ports 1, 4, and 7, closely related members were found. In the Alpha-Proteobacteria, members similar to the genus Magnetospirillum, which are closely related to the perchlorate-reducing *Dechlorospirillum* species, were found. In the Beta-Proteobacteria, members of the family Rhodocyclaceae, which also contains the perchlorate-reducing *Dechloromonas* species, were found (Coates *et al.*, 1999). Unlike our study, Xiao et al. (2010) found that all cells were attached to the biofilm support and did not detect a PCR product in the pore water.

Our study shows that although the SUPeRB culture was transferred many times from the original inoculum to the final pilot-scale test and subjected to flow-through conditions the function of the SUPeRB consortium remained stable under field conditions. It was also seen that even with the multiple transfers to fresh enrichments and given the flow-through conditions the SUPeRB consortium remained diverse but with a core consortium community of Epsilon-, Beta- and Alpha-Proteobacteria. Although the *pcrA* and *cld* genes were most closely related to a Beta-Proteobacteria this similarity was very distant to *Dechloromonas* species suggesting that the SUPeRB strain is a novel species within the Beta-Proteobacteria. The high gene copy numbers found in the lower ports of the bioreactor do not translate into corresponding numbers of PRB in the clone libraries. A different identification method such as FISH could be used to identify key members of the SUPeRB consortium. Comparing the SUPeRB consortium structure from different enrichments and bioreactors may conclusively answer which is the main functional perchlorate-reducer in the consortium. This will be the focus of the next chapter of this dissertation.

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Table 4.1. Community structure within the pilot-scale bioreactor.

Comparison of the community structure of pore water samples from Ports 1, 4 and 7, of the pilot-scale bioreactor while on recirculation as identified by 16S rRNA. Numbers are in percentage of the whole and numbers in parenthesis are the actual number of clones sequenced.

Port	1: Clone % (#)	4: Clone % (#)	7: Clone % (#)
Phylum			
Beta-Proteobacteria	50 (12)	21 (5)	20 (2)
Epsilon-Proteobacteria	25 (6)	38 (9)	70 (7)
Alpha-Proteobacteria		21 (5)	
Bacteroidetes	8 (2)	4 (1)	
Gamma-Proteobacteria	4 (1)	8 (2)	10 (1)
Delta-Proteobacteria	4 (1)		
Actinobacteria	4 (1)		
Planctomycetes	4 (1)		
Verrucomicrobia		4 (1)	
Firmicutes		4(1)	



Figure 4.1. Morphology of cells from pilot-scale bioreactor. Scale bar = $100 \ \mu m$.



Figure 4.2. Pore water protein measurements from the pilot-scale bioreactor. Samples taken on day 310 of bioreactor flow-through from influent (0.0 cm), to effluent (86.4 cm). Concentrations were presented as protein per unit volume bioreactor. Error bars present the standard error from the mean of two measurements for each sample.



Figure 4.3. Matrix protein measurements from the pilot-scale bioreactor. Samples taken on day 310 of bioreactor flow-through from ports 1, 2, 4, and 7 at 9.5, 16.5, 33.7, and 65.4 cm from the influent, respectively. A (\bullet) = samples from by the port, B (o) = samples from between the center and the port and C ($\mathbf{\nabla}$) = samples from the middle of the reactor. Concentrations were presented as mg protein per liter of PBS. Error bars present the standard deviation from the mean of two measurements for each sample. Inset is the open reactor with A, B and C corresponding to sampling areas of reactor matrix.



Figure 4.4. Pore water sulfide measurements from the pilot-scale bioreactor. Samples taken on day 310 of bioreactor flow-through from influent (0.0 cm), to effluent (86.4 cm). Each sample was measured once.



Figure 4.5. Pore water community structure analysis from the pilot-scale bioreactor. Samples were taken on day 310 of bioreactor flow-through from influent (IN) at 0.0 cm, ports 1 (9.5 cm), 2 (16.5 cm), 3 (22.9 cm), 4 (33.7 cm), 5 (44.5 cm), 6 (55.3 cm), 7 (65.4 cm), 8 (76.2 cm), and effluent (EFF) at 86.4 cm from the influent. Band 1, 2 and 3 had 98-99% identity to clone DQ145977 isolated from a sulfur spring and 96% related to Epsilon-Proteobacterium clone FJ497346



Figure 4.6. Phylogenetic analysis of the *pcrA* gene from the pilot-scale bioreactor.

Phylogenetic relationship of the *pcrA* gene to known *pcrA* gene sequences deposited in the Genbank database as of September 2010. The comparative analysis was inferred by Minimum Evolution analysis of 3 aligned port 1 *pcrA* clones designated by 1 for day 281 and 2 for day 310 collection. The scale bar represents 10% estimated sequence divergence. Bootstrap values are shown for 1,000 replicates.


Figure 4.7. Phylogenetic analysis of the *cld* gene from the pilot-scale bioreactor.

Phylogenetic relationship of the *cld* gene to known *cld* gene sequences deposited in the Genbank database as of September 2010. The comparative analysis was inferred by Minimum Evolution analysis of 3 aligned port 1 or port 2 *cld* clones designated by 1 for day 281 and 2 for day 310 collection. The scale bar represents 5% estimated sequence divergence. Bootstrap values are shown for 1,000 replicates.



Figure 4.8. qPCR standard curve for the *pcrA* gene.

The *pcrA* gene was obtained from serially diluted *Dechlorosoma* (renamed *Azospira*) *suillum* PS genomic DNA. C_T values are the average of three replicates with the exception of the 10⁵ point which was an average of two points. Error bars represent standard deviations.





CHAPTER 5

COMPARING SUPERB CONSORTIA TO ELUCIDATE CORE STRUCTURE

Abstract

This study investigates whether functional SUPeRB consortia maintain a core structure by comparing the phylogenetic structure of the SUPeRB consortia from different reactors and enrichments by sequence and cluster analysis. The key and minor species in the consortium for the perchlorate-degrading function were identified. The most stable function was achieved after the optimal perchlorate reduction consortium in these sulfur-utilizing reactor systems had been selected. Acclimatization of the consortium to the system and maintenance of favorable conditions within the reactor were of greater importance to stable reactor function than the volume of the reactor and the initial inoculum.

Introduction

Reproducibility of bacterial communities and reactor function is important when establishing bioreactor systems for the remediation of contaminated water. However, it is still unclear whether identical process set-ups will result in reproducible bacterial communities and community function (Wittebolle *et al.*, 2009). Even less certain is whether identical systems established in multiple countries with varied inocula will function consistently (Curtis and Sloan, 2004). The original inoculum is usually complex and even when one specific electron acceptor and donor are utilized by the bioreactor community there remains a metacommunity of diverse interactive communities each subject to various perturbations (Fernandez *et al.*, 2000). Bacterial inocula for biological water treatment processes are commonly obtained from established full-scale treatment systems, yet these inocula can also differ in composition due to perturbations during transfer to the bench-scales systems, even with parallel-run reactors (Falk *et al.*, 2009).

Although an inoculum source with a consistent microbial community composition capable of stable function would be advantageous for the reliable start-up and scale-up of remediation processes metacommunities are frequently redundant for function (Briones and Raskin, 2003). This concept has been well studied for methanogenesis, where function is easily replicated regardless of time or place of inocula collection despite unstable community diversity (Curtis and Sloan, 2004). However, since perchlorate contamination has only been prevalent in the environment for about 100 years, specific degradation of perchlorate by microbes is unexpected and has only recently been explored in detail (Romanenko *et al.*, 1976; Rikken *et al.*, 1996; Wallace *et al.*, 1996; Bruce *et al.*, 1999; Herman and Frankenberger, 1998; Coates *et al.*, 1999; Coates and Achenbach, 2004; Trash *et al.*, 2010). Therefore, it may not be possible to repeatedly obtain a perchlorate-reducing consortium with the same metabolic potential.

In this chapter we address the question if the inoculum source or the reactor volume will have an effect on the final community structure of a well performing reactor. Here we compared three perchlorate-reducing reactors, with different volumes and inocula of the sulfur-utilizing, perchlorate-reducing bacterial (SUPeRB) consortium, for their significant overlap in community structure due to similarities in inoculum enrichment, reactor treatment, and the functional ability to degrade perchlorate. We also compared the reactor consortia overlap with enrichment and minimal function consortia. This research will elucidate the role of community dynamics in maintaining specific function.

Materials and Methods

Sequence Source

Three reactors of different volumes were used for this comparison. The 0.2 L bench-scale reactor was described in Chapter 3 of this dissertation. The 1 L bench-scale reactor was described in Sahu *et al.* (2009). The 200 L pilot-scale reactor was described in Chapter 4 of this dissertation. Perchlorate was fed to the bench reactors at 5 and 2.5 mg/L, and to the pilot reactor at 0.1 mg/L. The minimal consortium (referred to here as MM) described in Chapter 2 of this dissertation and the original enrichment culture inoculated into the 1 L reactor were also included in the comparison.

Sequence Analyses

Sequences were edited, checked for chimeras using Mallard (Ashelford *et al.*, 2006), compared to the NCBI database (Altschul *et al.*, 1997), classified using the program mothur V.1.14.0 (Schloss *et al.*, 2009) and checked with RDP V.9.57, with an 80% confidence threshold (Wang *et al.*, 2007). Representative nucleotide sequences of the described clone library from the 1 L bench-scale reactor were submitted to GenBank with the accession numbers FJ593134-FJ593170 (Sahu et al., 2009).

Statistical Analyses

Principle components analyses (PCAs) were calculated using variance or covariance matrices with the community composition transformed using the Hellinger equation (Ramette, 2007). The PCAs were plotted using the software package PC-ORD V.4.41 (MjM Software Design, Gleneden Beach, OR). Cluster analysis was used to construct a hierarchical tree based on Ward's group linkage method and Euclidean distances. The hierarchical dendrogram was scaled by Wishart's percent of information remaining at the centroids also using PC-ORD V.4.41.

Results

For a direct comparison of community composition a total of 128, 74, and 58 clones were compared for the 0.2 L, 1 L, and 200 L reactors, respectively (Table 5.1). Beta- and Epsilon-Proteobacteria comprised 70%, 88%, and 54% of the total community of the 0.2 L, 1 L, and 200 L reactors, respectively. In the areas of the reactor where the greatest perchlorate reduction was observed Beta- and Epsilon-Proteobacteria comprised 65%, 89%, and 67% of the total community of the 0.2 L, 1 L, and 200 L reactors, respectively. In the areas of the minimal community and 68% in the enrichment community.

The reactors remained diverse with eight phyla represented (Figure 5.1 and Table 5.2). Only the Proteobacteria overlapped in all three reactors. Within the Proteobacteria, Beta-, Epsilon- and Gamma-Proteobacteria were in all three reactors (Table 5.2). In the areas where perchlorate was fully reduced only Beta- and Epsilon-Proteobacteria overlapped in all three reactors and total diversity decreased with five phyla represented.

In the minimal community only three phyla were represented. In the enrichment community only four phyla were represented, but phyla other than Proteobacteria comprised a larger percentage of the community (Figure 5.2).

Within the Beta- and Epsilon- Proteobacteria there was little overlap among the three reactor communities, with Beta-Proteobacteria of the order Burkholderiales, family Comamonadaceae and unclassified Beta-Proteobacteria present in areas where perchlorate was fully degraded. Beta-Proteobacteria of the order Rhodocyclales and family Rhodocyclaceae also overlapped within all three reactors when examining total community. Only Epsilon-Proteobacteria of the order Campylobacterales, family Helicobacteraceae, genus *Sulfuricurvum* overlapped in all three reactors (Figure 5.3).

The cluster analysis of the different reactor and enrichment communities showed that the areas in the reactors or the dilutions of the minimal community where perchlorate was reduced was closer in identity to the total community than other areas of perchlorate reduction (Figure 5.4). The community that was diluted to obtain the minimal community that retained function (MM) was least similar to the other communities. The original enrichment culture for the 1 L reactor clustered with the 200 L reactor while the 0.2 and 1 L reactor clustered more closely with each other than with the 200 L reactor. The PCA cluster graph clearly showed this separation, with the first axis (principle coordinate 1) explaining 52% of the variation and the second axis explaining 22% of the variation of the data. A similar clustering pattern was seen with the cluster dendrogram (Figure 5.5). The separation of the minimal functioning community appeared to be related to the presence of Alpha-Proteobacteria, Actinobacteria and Acidobacteria. The clustering of the original enrichment culture with the 200 L reactor appeared to be related to the

unique phyla found in these communities. The 0.2 L reactor appeared to be most affected by the presence of the core community, the Beta- and Epsilon-Proteobacteria.

The diversity estimators, Shannon and Simpson indexes, showed that the communities from the reactors had approximately the same high diversity. The Shannon index had values of 4.05 to 4.76 for the total community and the Simpson index had values of 0.0006 to 0.0037 for the total community.

Discussion

It is reported that stable function of the reactor or disturbances that occur within a reactor have more effect on the community diversity than the origin of the inoculum. Wittebolle et al. (2009) found community functionality drove the reproducibility of ammonium-oxidizing communities in stable, parallel-run bioreactors with the same inoculum and acclimatization period. Langenheder et al. (2005) found that in batch culture the growth media had a greater effect on community diversity than the inoculum. In our study, inocula from one site collected at different times also did not appear to be an important factor in the selection of the functioning community. The minimal consortium, the 0.2 L and the 200 L reactors were inoculated from the same wastewater treatment plant inoculum. The 1 L reactor and the enrichment culture were inoculated from the same wastewater treatment plant inoculum. Neither group clustered most closely with communities that were from the same inoculum. The long acclimatization period of each consortium may negate the effect of the initial inoculum as the environment and subsequent bacterial interaction selects for the optimal consortium for the function required. This was also noted by Falk et al. (2009) when studying seed inoculum for

membrane reactors. Community selection during acclimatization could be expected to result in a reduced diversity in each community and a high similarity between communities (Curtis and Sloan, 2004). However, this is not the case as there remains a high diversity of phyla in the reactors and a small overlap among the reactor communities. Potentially the high diversity remains due to additional metabolic interactions within the reactor, perhaps in support of the perchlorate-reducing bacteria (PRB) while the small overlap may be due to the low numbers of PRB needed to degrade the low levels of perchlorate.

Beta- and Epsilon-Proteobacteria contained the overlapping families of bacteria and are thought to comprise the core SUPeRB consortium. The percentage of Beta- and Epsilon-Proteobacteria as part of total community was consistent among reactor communities. A lesser percentage of Beta- and Epsilon-Proteobacteria in the 200 L reactor and the greater diversity of this community may have been contributed by groundwater influent or the lower concentration of perchlorate in the feed. Spatially throughout the three bioreactors, regardless of whether reduction did or did not occur, phylogenetic analysis showed that the perchlorate-reducing community remains diverse within each reactor and each zone, and all reactors and zones exhibited similar richness. Again, the high diversity within each reactor is potentially due to metabolic interactions other than perchlorate-reduction, perhaps associated with the constant presence of the electron donor.

From a process point of view, only function is significant. The active community that will finally be established in the functioning bioreactor will be selected by the reactor conditions regardless of the original metacommunity (Falk *et al.*, 2009). However, the

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microbiology of these bioreactor systems has not been previously studied and is of interest, particularly if function fails. The new metabolic combination of perchlorate reduction with sulfur-utilization appeared to be achieved by a consortium of microorganisms, but only certain members needed to be present when the reactor was functioning stably. The Beta- and Epsilon-Proteobacteria form a core community of the SUPeRB consortium; however, the association between the two groups in the process of perchlorate-reduction remains to be determined.

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Table 5.1. Numbers of sequenced clones from total and perchlorate-reducing communities of each SUPeRB consortium.

	Total community (% of total)	Total community: perchlorate- reducing area (% of total)	Beta- and Epsilon- Proteobacteria (% of total)	Beta- and Epsilon- Proteobacteria: perchlorate- reducing area (% of total)
0.2 L	128 (100)	43 (34)	90 (70)	31 (24)
1 L	74 (100)	18 (24)	65 (88)	16 (22)
200 L	58 (100)	24 (41)	31 (53)	16 (28)
MM	18 (100)	16 (89)	-	-
Enrichment	110 (100)	-	-	-

Numbers of clones sequenced and identified by phylogenetic analysis for creation of graphs. MM= minimal consortium from Chapter 2.

 Table 5.2. Overlap of total community phyla among bench- and pilot-scale bioreactors.

Phylum	0.2 L	1 L	200 L
Beta- Proteobacteria	Х	Х	Х
Epsilon- Proteobacteria	Х	Х	Х
Gamma-Proteobacteria	Х	Х	Х
Alpha- Proteobacteria	Х		Х
Delta-Proteobacteria	Х		Х
Bacteroidetes	Х		Х
Firmicutes	Х		Х
Verrucomicrobia			Х
Plantomycetes			Х
Chloroflexi	Х	Х	
Acidobacteria	Х	Х	
Actinobacteria	Х		



Figure 5.1. Comparative phylogenetic analysis of the SUPeRB consortia. Comparative phylogenetic analysis of the A) Total community, B) Area of perchlorate reduction, and C) Legend for bargraphs. MM= minimal consortium from Chapter 2.



Figure 5.2. Comparative phylogenetic analysis of Beta- and Epsilon-Proteobacteria from the SUPeRB consortium.

Comparative phylogenetic analysis of the A) Total Beta- and Epsilon-Proteobacteria community, B) Beta- and Epsilon-Proteobacteria from the area of perchlorate reduction, and C) Legend for bargraphs.



Figure 5.3. Overlap of the Beta- and Epsilon-Proteobacteria from the SUPeRB consortium.

Overlap of the Beta- and Epsilon-Proteobacteria sequenced clones from each reactor A) Total Reactor Community and B) Area of Perchlorate Reduction. Numbers are a percentage of the total number of sequences and circle size is representative of the numbers of sequences included from each reactor.



Figure 5.4. Principal components analyses determining influence of community structure on the clustering of different bioreactor and enrichment communities.
Axis 1 explains 52% of variance, axis 2 explains 22% of variance. Bacterial phyla (+), SUPeRB consortium (▲), "All" = total community, "Perc" = the community from the area of perchlorate-reduction, 1= 1 L bioreactor, MM= minimal consortium enrichment,

200=200 L bioreactor, 0.2=0.2 L bioreactor, enrichment= initial enrichment community. Superimposed circles indicate clusters of interest.



Figure 5.5. Cluster analysis of the bioreactors and enrichment communities.

Constructed using a hierarchical tree based on Ward's group linkage method and Euclidean distances. The hierarchical dendrogram was scaled by Wishart's percent of information remaining at the centroids. MM= minimal consortium. "All" = total community, "Perc" = the community from the area of perchlorate-reduction, 1=1 L bioreactor, MM= minimal consortium enrichment, 200=200 L bioreactor, 0.2=0.2 L bioreactor, enri= initial enrichment community.

CHAPTER 6

CONCLUSION

As microbial communities are increasingly harnessed for environmental biotechnology processes a deeper understanding of microbial ecology is necessary for appropriate management of these communities (Briones and Raskin, 2003). Bioremediation of environmental contaminants that support bacterial growth by providing energy as electron donors or acceptors is one such process. Perchlorate is an environmental contaminant with negative human and aquatic health effects and, therefore, the degradation of perchlorate by bacteria to innocuous by-products benefits society (Hines *et al.*, 2002; Coates and Achenbach, 2004; Hines, 2004). The use of perchlorate as an electron acceptor by microbes is also a metabolic process of interest. As perchlorate was thought to be primarily a man-made compound, and only introduced into the environment in the last century, it was unexpected that enzymes specific for perchlorate degradation were present in many classes of Proteobacteria (Coates and Achenbach, 2004).

Although the metabolic capabilities of perchlorate were extensively explored and recognized as being diverse, only two research groups were successful in supporting perchlorate reduction utilizing sulfur as an electron donor (Ju *et al.*, 2007; 2008; Sahu *et al* 2009; Sengupta *et al.*, 2009). Only our group explored the microbial communities with this metabolic potential (Sahu *et al.*, 2009).

The goal of this dissertation was to investigate the novel microbiological process of perchlorate-reduction utilizing elemental sulfur. We hypothesized that a unique sulfurutilizing, perchlorate-reducing bacterial (SUPeRB) consortium is responsible for this

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process. The goal of the project was addressed through three distinct but concurrent experiments, namely: enrichment studies, bench-scale and pilot-scale bioreactor studies. A comparison of the community structure among all reactors in which this system was investigated was also conducted.

Summary of Major Findings

The major findings and conclusions from each project, and a summary of all projects, are outlined below.

In Chapter 2, serial dilutions of the enriched SUPeRB consortium were undertaken to obtain the minimal consortium necessary to maintain the function of perchlorate degradation and to examine the growth parameters of this SUPeRB consortium. A perchlorate-reducing strain was identified by 16S rRNA and functional gene analysis as a Beta-Proteobacterium within the family Rhodocyclaceae with similarity to *Azospira* sp. This perchlorate-reducing bacterium (PRB) has an uncommon metabolism among known perchlorate-reducers as it reduced only low concentrations of perchlorate as a member of the consortium. The consortium also reduced multiple other electron acceptors using sulfur as an electron donor.

In Chapter 3, the microbial ecology of a perchlorate- and nitrate-reducing bioreactor was studied with respect to stability of function and disturbances to function. Contrary to some ecosystem studies (Botton *et al.*, 2006) a disturbance in our bioreactor system led to higher system diversity while the stably functioning reactor had a lower diversity. The disruption of nutrient and electron acceptor feed and the potential influx of oxygen as an alternate electron acceptor had a distinct disturbance effect, whereas the

addition of nitrate, rather than being a disturbance, made the reduction of perchlorate more efficient. While the reactor performance was stable, the SUPeRB consortium composition remained unchanged. Upon a disturbance temporary niches were created for multiple phyla and this higher population diversity appeared necessary to return to stable function. As with Chapter 2, a novel Beta-Proteobacterium, distantly related to the *Azospira/Dechloromonas* group of PRB, was thought to be responsible for perchlorate reduction.

In Chapter 4, the scale of function of the SUPeRB consortium was explored. Perchlorate was successfully degraded in a 200 L pilot-scale reactor. This function and the presence of PRB were correlated by functional gene analysis. Our study shows that the function of the SUPeRB consortium is stable under field conditions, namely: temperature extremes, intermittent perchlorate feed, and with oxygenation of feed water, and that there are no inhibitory levels of by-products.

In Chapter 5 the key and minor species in the consortium for the perchloratedegrading function were identified. It was also found that stable reactor function selects for an optimal perchlorate-reducing consortium. The volume of the reactor and the initial inoculum are not as important to stable reactor function as are acclimatization of the consortium and maintenance of favorable conditions within the reactor.

In summary, our research shows that low levels of perchlorate were continuously degraded by a stable, minimal community with elemental sulfur as an electron donor by a consortium. The PRB within the consortium were identified as novel Beta-Proteobacteria within the Rhodocyclaceae family.

Recommendations for Future Work

Further efforts to isolate the perchlorate-reducing strain in pure culture should be undertaken with variations of media as the three isolates that grew on sulfur-powder and perchlorate containing media did not have perchlorate-reducing capabilities. The consortium, using sulfur as an electron donor, also reduced other electron acceptors, such as selenate, that are considered environmental contaminants (Chung *et al.*, 2006). Further projects should focus on using the consortium to degrade these alternate compounds and to examine the structure of the consortium undertaking those specific functions.

Further bench-scale reactor studies should benefit from the use of FISH probes specific to Beta- and Epsilon-Proteobacteria to visualize the location and number of these bacteria and to correlate the numbers of these Proteobacteria with the quantification of functional genes.

The bench-scale reactor in our study focused on distributing the SUPeRB consortium along the length of the reactor. Yet, perchlorate-reduction still occurred in the lower part of the reactor after acclimatization. The pilot-scale reactor was constructed in a manner such that it could easily be reduced to units that were 30 cm in height (McKeever, 2009). This encompasses the first three ports of the reactor investigated in Chapter 4. Further pilot-scale reactor studies could focus on testing whether this reactor height could be as successful as the full-size reactor. This decrease in reactor volume could also save on space and substrates. Successive or stackable units could also be used to increase throughput.

Broader Impacts

A close collaboration between environmental engineers and microbial ecologists is necessary for exploitation of microbial communities to augment our understanding of existing processes, their performance, and to develop new processes for wastewater and drinking water treatment. Such collaborations would benefit a fast-growing world population that is rapidly running out of clean water (Nielsen and Loosdrecht, 2010). Knowledge of microbial community interaction and the understanding of microbial capabilities is needed to reduce environmental complexity and aid in the difficult transitions from laboratory to field remediation (Table 6.1). Together, we can provide new and improved strategies for the development and implementation of bioremediation processes.

The results of this research can be applied to three broad areas: the microbiology of autotrophic perchlorate reduction, microbial ecology, and bioremediation.

<u>Microbiology of autotrophic perchlorate reduction</u>: This research will add to the understanding of the microbes involved in perchlorate-reduction and the environments in which these microbes function. This research shows that the full complement of bacterial species that can reduce perchlorate and the metabolisms these microbes can use to function is still not fully known. The evolution of the ability to degrade perchlorate by microbes is still under investigation (Coates and Achenbach, 2004; Trash *et al.*, 2010). Only low concentrations of perchlorate are naturally produced and this suggests that the investigation of this consortium, that degrades only low concentrations of perchlorate, may supply insights into the evolution of the enzymatic function (Dasgupta *et al.*, 2005). With the recent discovery of perchlorate on Mars, perchlorate-reducing microbes on

Earth are being investigated as possible analogs of near-surface Martian life (Schulze-Makuch and Houtkooper, 2010). This research also underlines the importance of studying consortia rather than individual species. Particularly as most biogeochemical cycle transformations are catalyzed by consortia and not by single species of microorganisms (Amann, 1995).

<u>Bioremediation:</u> Foods and water sources worldwide are contaminated with perchlorate (Dasgupta *et al.*, 2006; El Aribi *et al.*, 2006). SUPeRB may be used as a costeffective biological treatment for perchlorate-contaminated water supplies. Biological treatment of water for return to the environment or for a potable water source is a sustainable technology. There is no concentrated waste stream and this consortium can degrade multiple contaminants. The knowledge gained from field tests are applicable to a broad range of climates and will also be beneficial in setting up other types of bioreactor systems *in situ*. The general public is wary of using methods involving microbes to clean their drinking water, but this research may relieve concerns of biological treatment use, and further knowledge of bioremediation methods may build public acceptance of these processes.

Biological treatment systems, such as bioreactors or wastewater treatments, are usually carried out by a "black box" microbial community. If the community ceases to function it is unknown what failed. Not only does the process need to be restarted, there is the possibility that the problem will continue. The composition of the microbial community influences both the stability and performance of anaerobic reactors, therefore it is important to understand the diversity and function of individuals in the community as well as community interactions, for effective operation and improvement of a bioreactor performance.

<u>Microbial ecology</u>: Microbial ecosystems are complex with interactions that change over time and space. Because microbes continue to evolve and respond to disturbances, it is helpful to study a pared down ecosystem, such as a bioreactor, where function and disturbances can be manipulated to explore concepts such as resilience, disturbance, and stability. Insights into microbial community composition and the factors that determine composition and function may improve understanding of broader topics such as biogeochemical processes, food web dynamics, biodegradation processes and overall ecosystem health.

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Microbial Ecology Method	Rationale for Method	Information Anticipated	Benefit to Engineering
Culture			
Independent:			
Phylogenetic			
analysis:			
16S rRNA gene	Indication of microbial diversity of organisms	Composition of the degrading community	Can indicate dominant organisms for bioreactor optimization, and potential interfering organisms, e.g. organism that will lead to system fouling
<i>pcrA</i> and <i>cld</i>	Detection indicates (per)chlorate reduction	Metabolic capabilities of community	Use of functional genes as performance indicators <i>in situ</i> or in qPCR to quantitate activity
Microscopy and	First indication of	Under what	Can vary bioreactor
Protein analysis	microorganism	conditions are the	conditions based on
	presence and	microorganisms	where
	quantity	are and in what numbers	found
FISH	Dominant isolate	Under what	Can optimize
	can be tracked in	conditions are the	bioreactor
	real time	dominant functional	conditions by
		organisms present, where are they, and	examining effects of varying conditions
		in what numbers	on dominant
			Tunctional organism
Culture Dependent:			
Isolation of	To identify if an	The minimum	Optimization of the
dominant organisms	individual isolate or	consortium involved	process by
by dilution to	a consortium of two	in perchlorate	characterization and
extinction of	or more organisms	reduction	manipulation of the
cultures	desired process: PRB,		involved

Table 6.1. Microbial ecology methods to elucidate complex interactions in engineering environments.

continued on the next page

	PRB in high saline environment, PRB with co- contaminant		
Cultivation of dominant organisms on anaerobic perchlorate/sulfur culture media	Isolation of the organisms that carry out the desired process	Characteristics of the microorganisms involved	Optimization of the process by characterization and manipulation of the microorganisms involved

Adapted from NSF award abstract #0755670: A novel method for biological perchlorate reduction using elemental sulfur as an electron donor.

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