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Perchlorate Reduction by Sulfur Oxidizing Bacteria

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A Masters Project Presented

by

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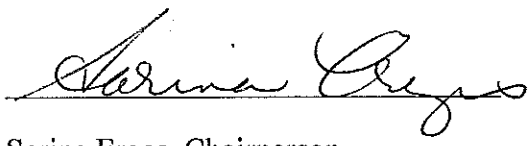

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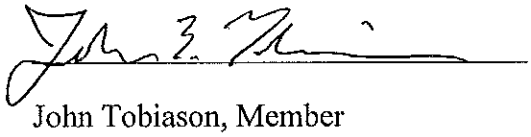

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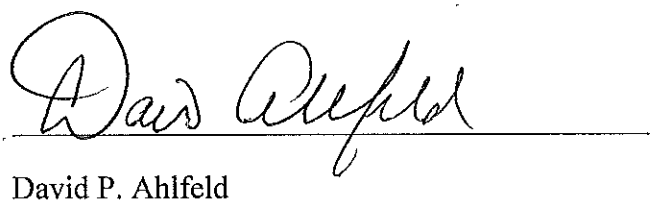

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The material presented in Chapters 2 and 3 are two articles to be submitted to the journals *Biotechnology and Bioengineering* and *Bioresource Technology*. The full articles are presented to maintain completeness. Note that contributions to the writing were made by other students and proper credit is due. Robert McKeever designed and constructed the 267 L bioreactor as a part of his honor's project and produced the bioreactor schematic (Figure 2.2). Teresa Conneely developed methodology and performed laboratory procedures and data analyses on molecular work including PCR and protein

and sulfide measurements for Chapter 2. She also provided the data for the attachment study presented in Chapter 3.

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ABSTRACT

Perchlorate Reduction by Sulfur Oxidizing Bacteria

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Perchlorate (ClO_4-) contaminated water is becoming a wide-spread problem as more sites are being identified worldwide. Biological perchlorate reduction is a promising alternative to conventional physical/chemical treatment processes and has the advantage of reducing perchlorate to the benign products, chloride and oxygen. A number of bacteria are capable of reducing perchlorate using a variety of electron donors including organic carbon compounds, hydrogen, iron, and reduced sulfur compounds. Previous studies in our laboratory successfully used a novel, sulfur oxidizing bacterial consortium (SUPeRB) to reduce perchlorate in both batch culture and in packed bed reactors (PBR).

There were two main objectives of this research. The first objective was to construct and operate an ex-situ pilot scale PBR using SUPeRB cultures, with elemental sulfur pellets and crushed oyster shells as a packing material. The second objective was to investigate the role of the oyster shell as a buffer, organic carbon source, adsorbent, and/or attachment site to gain a better understanding of the SUPeRB process.

The first study examined the scale up of a PBR for treatment of water from a perchlorate and RDX contaminated aquifer in Massachusetts with low-level background nitrate levels. The pilot-scale PBR (~250-L) was constructed with elemental sulfur and crushed oyster shell packing media and was inoculated with SUPeRB cultures enriched from a wastewater seed. Sodium sulfite provided a good method of dissolved oxygen

removal in batch cultures, but was found to promote the growth of sulfate reducing bacteria, which inhibited perchlorate reduction in the pilot system. After terminating sulfite addition, the PBR successfully removed 96% of the influent perchlorate in the groundwater at an empty bed contact time (EBCT) of 12 hours (effluent perchlorate of 4.2 μg L^{-1}). Simultaneous perchlorate and nitrate degradation was observed in the lower half of the reactor before reactions shifted to sulfur disproportionation. Analyses of water quality profiles were supported by molecular analysis showing distinct groupings of perchlorate and nitrate degrading organisms in the bottom of the PBR, while sulfur disproportionation was the primary biological process occurring in the top of the reactor.

The use of crushed oyster shells as an alkalinity source in the SUPeRB process was found to enhance perchlorate degradation. The second study examined the role of oyster shells as a buffer, organic carbon source, attachment site, and adsorbent in the SUPeRB process. Perchlorate degradation was monitored in microcosms comparing the base case (sulfur and oyster shells) to systematic variations. The necessity for direct microbial attachment was examined by isolating sulfur pellets, oyster shells, or bacteria from the culture using membranes. The oyster shell maintained a favorable pH for perchlorate reduction ($k=23.7$ day^{-1} g $protein^{-1}$), but this could not completely explain the enhanced perchlorate reduction rates. SUPeRB cultures were found to be capable of mixotrophic metabolism, which increased rates of perchlorate reduction fivefold. Heating oyster shells impaired perchlorate degradation due to the diminished availability of organic carbon for cellular synthesis. Oyster shells reduced bacterial toxicity, possibly by hydrogen sulfide adsorption. The necessity for direct microbial attachment to the solid

oyster shell matrix was unclear, though proximity to oyster shells was more important than to sulfur.

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CHAPTER 1: INTRODUCTION

1.1 Overview

Perchlorate (ClO_4^-) is a highly stable, soluble anion that is of growing concern due to evidence of widespread surface and ground water contamination (Parker et al. 2008). Most perchlorate is manufactured in the form of ammonium, sodium, or potassium salts, though they also occur naturally. Perchlorate salts are oxidants that are widely used as a component in fireworks, roadside flares, rocket fuel, propellants and munitions. A naturally occurring perchlorate source is Chilean saltpeter, which when used as fertilizer can lead to soil and groundwater contamination. Low level perchlorate concentrations have also been detected in rainwater and snow as a result of natural atmospheric reactions.

Advances in analytical instrumentation have enabled low level perchlorate detection (<4.0 µg/L), which has increased the identification of perchlorate contaminated waters. Chronic exposure to low perchlorate concentrations in drinking water and food may be harmful to human health. The US EPA has set an Interim Health Advisory Level of 15 µg L^{-1} and plans to regulate perchlorate under the Safe Drinking Water Act at a specified maximum contaminant level (MCL) (USEPA, 2008; 2011). A number of US states have set their own MCLs for perchlorate, including Massachusetts (2 µg L^{-1}; MassDEP, 2006) and California (6 µg L^{-1}; CDPH, 2007).

A number of studies have found evidence that perchlorate interrupts thyroid hormone synthesis by interfering with iodine uptake (Capen 1994; Braverman et al. 2004; Von Burg 1995; Urbansky 1998). The most sensitive populations to perchlorate toxicity

are developing fetuses, premature infants and newborns, where adequate iodine nutrition is necessary for thyroid hormone synthesis (Cao et al. 1994; Leung et al. 2010). Insufficient thyroid hormone production during fetal development or after birth has been linked to problems with visual attention and processing, visuospatial skills, fine and gross motor skills, delays in responding, language and memory impairment (Zoeller and Rovett 2004). The most common route of human perchlorate exposure is through ingestion of contaminated water, food, or breast milk. Drinking water sources may be contaminated or perchlorate can be unintentionally generated at low levels during disinfection with hypochlorite. Perchlorate is not known to accumulate in the body and its effects can sometimes be reversed once exposure ceases.

Current physical and chemical treatment technologies for perchlorate contaminated water include ion exchange (IX) and reverse osmosis (RO; Motzer 2001; Urbansky 2000; 2002). Advantages of IX and RO include the ability to treat high water flow rates with low level perchlorate concentrations and produce low effluent levels. Additionally, co-contaminants such as nitrate and other explosive residuals, RDX and HMX, may be removed concurrently. The Massachusetts Military Reservation (MMR) in Bourne, MA employs an onsite pump, treat, and reinjection system using IX resin for perchlorate remediation and granular activated carbon (GAC) for RDX and HMX remediation. However, a major drawback of these technologies is the generation of contaminated resins or brine solutions that require further treatment or disposal.

Biological perchlorate reduction offers a promising alternative to physical/chemical treatment processes (Urbansky 1998). In biological treatment, acclimated bacteria use two central enzymes, perchlorate reductase and chlorite

dismutase, to completely degrade perchlorate to the benign products chloride and oxygen (Coates and Achenbach, 2004). The reaction occurs under anaerobic conditions, though the organisms are known to be facultative anaerobes as well as denitrifiers. The ubiquity of organisms capable of perchlorate reduction is possibly due to perchlorate's presence in rainwater and snow. Perchlorate reducing bacteria are metabolically diverse and are capable of using a variety of organic and inorganic compounds as electron donors. Inorganic electron donors include hydrogen gas and reduced iron and sulfur compounds (Coates et al. 1999; Okeke and Frankenberger 2005; Wu et al. 2001; Kim and Logan 2001; Ju et al., 2007; Sahu et al. 2009a; Son et al. 2006).

Biofilm reactors, such as fluidized bed reactors (FBR) and up and down-flow packed bed reactors (PBR), are good approaches for biodegradation of low level contaminants because of their ability to achieve high mass transfer rates, which frequently limits biodegradation at low concentrations (Rittman and McCarty, 2001). Both types of reactors rely on bacterial attachment and growth on some type of media within the reactor. A PBR is filled with structured or random dump media usually composed of plastic, ceramic, or metal. An FBR operates with media in suspension, requiring small solid materials resistant to abrasion, such as sand grains, diatomaceous earth, or GAC (Rittman and McCarty, 2001).

A number of studies (Table 1.1) have investigated using PBR and/or FBR biofilm reactors to biologically reduce perchlorate. The influent and operating parameters varied greatly from one another from study to study. Reactor sizes ranged from very small (0.14-1.0 L) to pilot-(35-657 L), and full scale. Influent perchlorate concentrations have been investigated over several orders of magnitude from 0.01 mg L^{-1} to 120 mg L^{-1}.

Table 1.1: Summary of published studies of perchlorate reduction in biofilm reactors

Reactor Type	**Packing Media**	**Electron Donor**	**Influent Perchlorate (mg L^{-1})**	**Removal Efficiency**	**Other Compounds (mg L^{-1})**	**Reactor Volume (L)**	**Retention Time (hr)**	**Reference**
PBR	S^o/ Oyster shells 3:1	S^o pellets	0.10	96%	RDX (0.008) NO_3^-	267	EBCT = 11.9 HRT = 5.5	This study
PBR	S^o/ Oyster shells 3:1	S^o pellets	4-8 0.06-0.12	>99% >95%	NO_3-N	1.0	EBCT = 13 EBCT = 7.5	Sahu et al. 2009b
PBR	S^o/ Oyster shells 3:1	S^o pellets	---	>99%	NO_3-N (20)	190	EBCT = 8	Sengupta et al. 2007
PBR	S^o/ Limestone 1:1	S^o pellets	---	96%	NO_3-N (9-103)	0.4	HRT = 1.8– 31	Sierra-Alvarez et al. 2007
PBR	Gravel & Anthracite	Acetate	0.05	96%	---	657	EBCT = 1.2	Dugan et al. 2009

Reactor Type	Packing Media	Electron Donor	Influent Perchlorate (mg L^{-1})	Removal Efficiency	Other Compounds (mg L^{-1})	Reactor Volume (L)	Retention Time (hr)	Reference
PBR	Sand or Plastic	Acetate	0.077	> 95%	NO_3^- (4)	384	EBCT = 1.7	Min et al. 2004
PBR	GAC	Acetate	0.05	>99%	---	35	EBCT = 0.42	Brown et al. 2005
PBR	Plastic	Acetate	0.01-0.40 1.0	>95% >98%	NO_3-N (0, 10, 16)	12.5	EBCT = 8	Choi & Silverstein 2007; 2008
PBR	Diatom. Earth	Acetate	0.80	>99%	NO_3-N (20)	3.1	HRT = 0.3	Losi et al. 2002
PBR	Diatom. Earth	Acetate	0.73-0.55	>99%	NO_3 (0, 26)	0.38	EBCT = 5	Giblin et al. 200

Reactor Type	Packing Media	Electron Donor	Influent Perchlorate (mg L^{-1})	Removal Efficiency	Other Compounds (mg L^{-1})	Reactor Volume (L)	Retention Time (hr)	Reference
PBR	Sand	Acetate	20	>99%	---	0.14	EBCT = 0.04	Kim et al. 2001
PBR	Glass beads/ Fe^o 19:1	Fe^o granules	65	>99%	---	0.15	HRT = 48	Son et al. 2006
FBR	Silica sand	Ethanol	120	>99%	RDX (20)	7.9	---	Atikovic et al. 2009
FBR	GAC	Acetate or Molasses	0.10	>96% >96%	RDX (0.19)	4.0	---	Fuller et al. 2007

Acetate was commonly used as an electron donor and organic carbon source, though elemental sulfur (S^o) and zero-valent iron (Fe^o) were also used as both inorganic electron donors and packing media. A number of studies examined the impact of competing electron donors, such as nitrate and oxygen, and the presence of RDX, as a common co-contaminant.

Several bench-scale FBR studies examined the impact of the presence of RDX as a co-contaminant (Atikovic et al 2008; Fuller et al. 2007). In a study with high containment levels, perchlorate and RDX removal was dependant on the added organic substrate concentration and better removal was seen when reactors were fed only one contaminant (Atikovic et al. 2008). Using GAC as media in an FBR was a successful approach for simultaneously removing low-level concentrations of both perchlorate and RDX (Fuller et al. 2007). Although GAC was used as an adsorptive surface for RDX removal, there was evidence of microbial mediated RDX degradation. Since FBR configurations require careful control of bed fluidization, they were less commonly employed for biological perchlorate reduction compared to PBRs (Rittman and McCarty, 2001).

A number of PBR studies have demonstrated that acetate or acetic acid can be successfully used as an added electron donor for perchlorate reduction (Brown et al. 2005; Choi and Silverstein 2007, 2008; Dugan et al. 2009; Kim and Logan 2001; Losi et al. 2002; Min et al. 2004). The equation for biological perchlorate reduction with acetate was presented by Losi et al. (2002) as:

$$CH_3CHOO^- + ClO_4^- \rightarrow Cl^- + H^+ + 2HCO_3^- \quad (1)$$

Adding acetate in excess of the stoichiometric requirements improved perchlorate degradation because competing electron acceptors, such as dissolved oxygen and nitrate, were also consumed (Choi and Silverstein 2007, 2008; Dugan et al. 2009; Losi et al. 2002; Min et al. 2004). However, operating PBRs this way has some problems including increased requirements for maintenance and backwashing, vulnerability to pump failures, and costly post-treatment requirements to remove excessive biomass and unused substrate (Min et al. 2004).

Elemental sulfur, zero-valent iron, and other solid inorganic electron donors can be used as packing material in PBRs. Several authors have investigated sulfur oxidizing perchlorate reduction in batch cultures (Sahu et al.2008; 2009b, Ju et al. 2007; 2008).The stoichiometric equation for the sulfur utilizing perchlorate reducing bacterial (SUPeRB) process was presented by Sahu et al. (2009b) as:

$$S^0 + 3.32\ H_2O + ClO_4^- + 1.85\ CO_2 + 0.462\ HCO_3^- + 0.462\ NH_4^+$$

$$\rightarrow 5.69\ H^+ + 2.87\ SO_4^{-2} + Cl^- + 0.462\ C_5H_7O_2N \qquad (2)$$

The reaction also produces acidity (H^+), requiring an alkalinity source to maintain a near neutral pH (7 to 8), which we have recently found to be optimal for sulfur oxidizing perchlorate reducing bacteria (Conneely, 2011). Elemental sulfur and a solid phase buffer such as crushed oyster shells (OS) provided a good medium in PBRs for perchlorate reduction or denitrification, as the provision of a continuous supply of electron donor or alkalinity source were unnecessary. Sengupta et al. (2007) investigated a variety of solid-phase buffers for sulfur oxidizing denitrification and found that crushed oyster shell was a superior buffer over limestone or marble chips. Specifically, oyster shells were found to have a better dissolution rate, produce lower effluent turbidity, enhance denitrification

rates, and overall be more economically viable than limestone or marble chips. Sulfur oxidizing bacteria are slow growing autotrophs, producing little excess biomass, thus minimizing the need for extensive backwashing and maintenance for onsite wastewater treatment (Sahu et al., 2009b; Sengupta et al., 2007).

In a prior study in our laboratory, Sahu et al. (2009b) carried out bench scale up-flow PBR experiments with elemental sulfur and crushed oyster shells as a packing media. The effects of perchlorate concentration, empty bed contact time (EBCT) and the presence of nitrate on reactor performance were investigated. At the high perchlorate concentrations associated with source areas (4 to 8 mg L^{-1}), perchlorate was consistently reduced to < 0.5 mg L^{-1} at an EBCT of 13 h. At the low perchlorate concentrations (60-120 µg L^{-1}) found more typically at contaminated groundwater sites, perchlorate was reduced to < 4 µg L^{-1} at an EBCT of 7.5 h. The use of internal recirculation to increase mass transfer within the reactor was investigated; however, the authors found that recirculation had a negative impact on perchlorate degradation, possibly due to the loss of microbial biomass caused by shearing. The presence of nitrate in the feedwater was found to inhibit perchlorate reduction kinetics; however, the reactor performance was not diminished, as reduction occurred deeper in the PBR.

Numerous studies have investigated biological perchlorate reduction using biofilm reactors. While there have been several pilot scale bioreactor studies using organic electron donors and inert packing media, no pilot scale studies using inorganic electron donors have been investigated. The results of bench scale studies performed by Sahu et al. (2009b) using SUPeRB cultures were promising, warranting further research into the feasibility of scaling up such a system.

1.2 Research Objectives

The objective of this project was to investigate factors that control perchlorate removal in engineered systems that use elemental sulfur as an electron donor. An environmental engineering approach was used for the work presented in this thesis. However, the larger goal of the project was to carry out molecular biology and remediation studies in tandem in order to see if molecular biology work could be used to gain insight into reasons for fluctuations in treated water quality.

The specific objectives of this report were:

1. Investigation of operating conditions and performance of a packed bed sulfur oxidizing bioreactor for direct treatment of perchlorate contaminated water.

2. Investigation of the role of oyster shells in the SUPeRB process as a buffer, organic carbon source, adsorbent, and/or microbial attachment site.

Chapter 1 References

Atikovic E, Suidan MT, Maloney SW. 2008. Anaerobic treatment of army ammunition production wastewater containing perchlorate and RDX. Chemosphere. 72:1643-1648.

Braverman LE, He X, Pino S, Cross M, Magnani B, Lamm SH, Kruse MB, Engel A, Crump KS, Gibbs JP. 2005. The effect of perchlorate, thiocyanate, and nitrate on thyroid function in workers exposed to perchlorate long-term. J Clin Endoc Metab 90(2):700-706.

Brown JC, Anderson RD, Min JH, Boulous L. 2005. Fixed-bed biological treatment of perchlorate contaminated drinking water. J Am Water Works Ass 7(9)70-81.

Cao XY, Jiang XM, Dou ZH, Rakeman MA, Zhang ML, O'Donnell K, Ma T, Amette K, DeLong N, DeLong GR. 1994. Timing of vulnerability of the brain to iodine deficiency in endemic cretinism. N Engl J Med 331(26):1739-1744.

Capen CC. 1994. Mechanisms of chemical injury of thyroid gland. Prog Clin Biol Res 387:173-191.

CDPH. 2007. Perchlorate in drinking water. California Department of Public Health. Publication CDPH R-16-04. Sacramento, CA.

Choi H, Silverstein J. 2007. Effluent recirculation to improve perchlorate reduction in a fixed biofilm reactor. Biotechnol Bioeng 98(1):132-140.

Choi H, Silverstein J. 2008. Inhibition of perchlorate reduction by nitrate in a fixed biofilm reactor. J Hazard Mater. 159:440-445.

Coates JD, Achenbach LA. 2004. Microbial perchlorate reduction: rocket-fueled metabolism. Nat Rev Microbiol 2(7):569-580.

Coates JD, Michaelidou U, Bruce RA, O'Connor SM, Crespi JN, Achenbach LA. 1999. Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Appl Environ Microb 65(12):5234-5241.

Conneely T, 2011, Investigation of a sulfur-utilizing perchlorate-reducing bacterial consortium [Ph.D.], Amherst: University of Amherst, p 188.

Dugan NR, Williams DJ, Meyer, M, Schneider RR, Speth TF, Metz DH. 2009. The impact of temperature on the performance of anaerobic biological treatment of perchlorate in drinking water. Water Res 43:1867-1878.

Fuller ME, Hatzinger PB, Condee CW, Tonga AP. 2007. Combined treatment of perchlorate and RDX in ground water using a fluidized bed reactor. Ground Water Monit R 27(3):59-64.

Giblin R, Herman D, Deshusses MA, Frankenberger WT Jr. 2000. Removal of perchlorate in ground water with a flow-through bioreactor. J Environ Qual 29:578-583.

Ju X, Field JA, Sierra-Alvarez R, Salazar M, Bentley H, Bentley R. 2007. Chemolithotrophic perchlorate reduction linked to the oxidation of elemental sulfur. Biotechnol Bioeng 96(6):1073-1082.

Ju X, Sierra-Alvarez R, Field JA, Byrnes DJ, Bentley H, Bentley R. 2008. Microbial perchlorate reduction with elemental sulfur and other inorganic electron donors. Chemosphere 71(1):114-122.

Kim K, Logan BE. 2001. Microbial reduction of perchlorate in pure and mixed culture packed-bed bioreactors. Water Res 35(13):3071-3076.

Leung AM, Pearce EN, Braverman LE. 2010. Perchlorate, iodine and the thyroid. Best Pract Res Clin Endocrinol Metab 24(1):133-141.

Losi ME, Giblin T, Hosangadi V, Frankenberger Jr. WT. 2002. Bioremediation of perchlorate contaminated groundwater using a packed bed reactor. Bioremediation Journal 6(2):97-103.

MassDEP. 2006. Maximum contaminant level for inorganic chemicals. Massachusetts Department of Environmental Protection. Office of Research and Standards. Code of Massachusetts Regulations 310 CMR 22.06. Boston, MA.

Min B, Evans PJ, Chu AK, Logan BE. 2004. Perchlorate removal in sand and plastic media bioreactors. Water Res 38(1):47-60.

Motzer WE. 2001. Perchlorate: problems, detection, and solutions. Environmental Forensics 2:301-311.

Okeke BC, Frankenberger Jr. WT. 2004. Use of starch and potato peel waste for perchlorate bioreduction in water. Sci Total Environ 347(1-3):35-45.

Parker DR, Seyfferth AL, Reese BK. 2008. Perchlorate in groundwater: a synoptic survey of "pristine" sites in the coterminous United States. Environ Sci Technol 42(5):1465-1471.

Rittman BE, McCarty PL. 2001. Environmental Biotechnology: Principles and Applications. New York, NY: McGraw-Hill Companies.

Sahu, AK. 2008. Biological reduction of nitrate and perchlorate by autotrophic bacteria. [Ph.D.], Amherst: University of Amherst, p 265.

Sahu AK, Conneely T, Nusslein K, Ergas SJ. 2009a. Hydrogenotrophic denitrification and perchlorate reduction in ion exchange brines using membrane biofilm reactors. Biotechnol Bioeng 104(3):483-491.

Sahu AK, Conneely T, Nusslein KR, Ergas SJ. 2009b. Biological perchlorate reduction in packed bed reactors using elemental sulfur. Environ Sci Technol 43(12):4466-4471.

Sengupta S, Ergas SJ, Lopez-Luna E. 2007. Investigation of solid-phase buffers for sulfur-oxidizing autotrophic denitrification. Water Environ Res 79(13):2519-2526.

Sierra-Alvarez R, Beristain-Cardoso R, Salazar M, Gomez J, Razo-Flores E, Field JA. 2007. Chemolithotrophic denitrification with elemental sulfur for groundwater treatment. Water Res 41(6):1253-1262.

Son A, Lee J, Chiu PC, Kim BJ, Cha DK. 2006. Microbial reduction of perchlorate with zero-valent iron. Water Res 40(10):2027-2032.

USEPA. 2008. Interim drinking water health advisory for perchlorate. Publication EPA 822-R-08-025. Washington, D.C.: Office of Water, U.S. Environmental Protection Agency.

USEPA. 2011. Fact sheet: final regulatory determination for perchlorate. Publication EPA 815-F-11-003. Washington, D.C.: Office of Water, U.S. Environmental Protection Agency.

Urbansky ET. 1998. Perchlorate chemistry: implications for analysis and remediation. Bioremediation Journal 2(22):81-95.

Urbansky ET. 2000. American Chemical Society. Division of Environmental Chemistry., American Chemical Society. Meeting. Perchlorate in the environment. New York: Kluwer Academic/Plenum. xv, 299 p. p.

Urbansky ET. 2002. Perchlorate as an environmental contaminant. Environ Sci Pollut Res Int 9(3):187-192.

Von Burg R. 1995. Toxicology update: perchlorates. Journal of Applied Toxicology 15(3):237-241.

Wu J, Unz RF, Zhang H, Logan BE. 2001. Persistence of perchlorate and the relative numbers of perchlorate- and chlorate-respiring microorganisms in natural waters, soils, and wastewater. Bioremediation Journal 5(2):119-130.

Zoeller RT, Rovett J. 2004. Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. Journal of Neuroendocrinology 16:809-818.

CHAPTER 2: Pilot Scale Packed Bed Bioreactor Studies Using a Novel Sulfur Oxidizing Perchlorate Reducing Bacterial Consortium

This paper has been formatted for submission to the journal *Biotechnology and Bioengineering* as:

Pilot Scale Bioreactor Studies Using a Novel Sulfur Oxidizing Perchlorate Reducing Bacterial Consortium

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Abstract

A novel sulfur-utilizing perchlorate reducing bacterial (SUPeRB) consortium was successfully used in prior batch and bench-scale packed bed reactor (PBR) studies for biological perchlorate (ClO_4^-) reduction. This study examines the scale up of the SUPeRB process for treatment of water from a ClO_4^- and RDX contaminated aquifer in Massachusetts, with low-level background nitrate (NO_3^-) levels. A pilot-scale upflow PBR (~250-L) was constructed with elemental sulfur and crushed oyster shell packing media. The PBR was inoculated with SUPeRB cultures enriched from a wastewater seed. Sodium sulfite provided a good method of dissolved oxygen removal in batch cultures, but was found to promote the growth of sulfate reducing bacteria, which inhibited ClO_4^- reduction in the pilot system. After terminating sulfite addition, the PBR successfully removed 96% of the influent ClO_4^- in the groundwater at an empty bed contact time (EBCT) of 12 hours (effluent ClO_4^- of 4.2 µg L^{-1}). Simultaneous ClO_4^- and NO_3^- degradation was observed in the lower half of the reactor before reactions shifted to sulfur disproportionation. Analyses of water quality profiles were supported by molecular analysis showing distinct groupings of ClO_4^- and NO_3^- degrading organisms at the inlet of the PBR, while sulfur disproportionation was the primary biological process occurring in the top of the reactor.

2.1 Introduction

Perchlorate (ClO_4^-) has been used in a number of applications including as a solid rocket fuel, in flares, fireworks, fertilizers and some munitions (MassDEP, 2006a; USEPA, 2011). Advances in analytical instrumentation have assisted in the detection of ClO_4^- at over 150 sites in more than 38 states, including a large number of military bases (Gu and Coates, 2006). Perchlorate is known to disrupt thyroid function by interfering with iodine uptake (Braverman et al., 2005; Capen, 1994; 2004; Urbansky, 1998; Von Burg, 1995). The most sensitive populations are developing fetuses, premature infants, and newborns, where adequate iodine is necessary for thyroid hormone synthesis (Cao et al., 1994; Leung et al., 2010). Insufficient thyroid hormone production in developing fetuses and infants has been linked to problems with visual attention and processing, visuospatial skills, fine and gross motor skills, language delays and memory impairment (Zoeller and Rovett, 2004). ClO_4^- is regulated under the US Safe Drinking Water Act; although no maximum contaminant limit (MCL) is currently specified, an Interim Health Advisory Level of 15 μg L^{-1} has been established (USEPA, 2008; 2011). Several states have set their own MCLs for ClO_4^-, including Massachusetts (2 μg L^{-1}; MassDEP, 2006b) and California (6 μg L^{-1}; CDPH, 2007). Current water treatment technologies for ClO_4^- include ion exchange (IX), reverse osmosis (RO), and biological treatment (Motzer, 2001; Urbansky, 2000; 2002). Although IX and RO can treat high volumes of water with low ClO_4^- concentrations, a major drawback is the high disposal cost (4-6% of total treatment costs) associated with the generation of contaminated resins or brine solutions (Stotter, 2008).

Biological ClO_4^- reduction offers a promising alternative to physical/chemical

treatment processes (Urbansky, 1998). In biological treatment, acclimated bacteria use two central enzymes, perchlorate reductase and chlorite dismutase, to degrade ClO_4^- to the benign products, chloride and oxygen (Coates and Achenbach, 2004). The reaction occurs under anaerobic conditions and the organisms are known to be facultative anaerobes and denitrifiers. Ubiquitous and metabolically diverse, ClO_4^- reducing bacteria are capable of using a variety of organic and inorganic electron donors, including hydrogen, and reduced iron and sulfur compounds (Coates et al., 1999; Ju et al., 2007; Kim and Logan, 2001; Okeke and Frankenberger, 2005; Sahu et al., 2009a; Son et al., 2006; Wu et al., 2001). The presence of nitrate (NO_3^-), a common groundwater co-contaminant (Parker et al., 2008), has been shown to decrease ClO_4^- reduction rates, although simultaneous NO_3^- and ClO_4^- metabolism has also been shown to occur (Bardiya and Bae, 2005; Ju et al., 2007; Min et al., 2004; Sahu et al., 2009b; Tipton et al., 2003).

A number of researchers have investigated microbial communities capable of using elemental sulfur (S^0) as an electron donor for biological NO_3^- reduction (Batchelor and Lawrence, 1978; Darbi et al., 2003; Sengupta et al., 2007; Sierra-Alvarez et al., 2007). A few studies have expanded on this work to investigate S^0-oxidizing ClO_4^- reduction in batch cultures and bench scale packed bed reactors (PBR; Ju et al., 2007; 2008; Sahu et al., 2009b). The stoichiometric equation for biological S^0-oxidizing ClO_4^- reduction was presented by Sahu et al. (2009b) as:

$$S^0 + 3.32\ H_2O + ClO_4^- + 1.85\ CO_2 + 0.462\ HCO_3^- + 0.462\ NH_4^+ \rightarrow$$

$$5.69\ H^+ + 2.87\ SO_4^{-2} + Cl^- + 0.462\ C_5H_7O_2N \qquad (1)$$

The reaction consumes alkalinity, requiring a pH buffer to maintain a pH between 7 and

8, which we have recently found to be optimal for S^0-oxidizing ClO_4^- reducing bacteria (Conneely, 2011). Sengupta et al. (2007) found that crushed oyster shell (OS) was a superior buffer over limestone or marble chips for S^0-oxidizing denitrification.

Biofilm reactors, such as fluidized bed reactors (FBRs) and PBRs, are good approaches for biodegradation of low level contaminants because of their ability to achieve high rates of substrate mass transfer to the biofilm (Rittmann and McCarty, 2001), which frequently limits biodegradation of groundwater contaminants at low concentrations. Several researchers (Choi et al., 2007; Losi et al., 2002; Min et al., 2004) have successfully employed PBRs with various packing materials for treatment of low concentration (<1 mg L^{-1}) ClO_4^- contaminated groundwaters using acetate as an electron donor. A disadvantage of this type of system includes the need for frequent backwashing and post-treatment to remove excessive biomass and unused organic substrate from the product water to minimize disinfection byproduct formation and biofilm growth in drinking water distribution systems. Autotrophic S^0-oxidizing ClO_4^- reducing bacteria are slow growing autotrophs, which produce little excess biomass, thus minimizing the need for backwashing.

Few studies of ClO_4^- reduction in bioreactors have examined consortium microbiology. Molecular techniques were used by Zhang et al. (2004) and Chung et al. (2009) to analyze community composition using the 16S rRNA gene from biofilms growing on plastic and granular activated carbon (GAC) media in acetate-fed reactors. After six-months of operation, Zhang et al. (2004) used fluorescent in situ hybridization (FISH) and found that although the ClO_4^- reducer *Dechlorosoma sp.* was added to the reactor, indigenous groundwater organisms such as *Dechloromonas sp.* became

dominant. In high salinity, denitrifying, and ClO_4^- reducing reactors, Chung et al. (2009) used 16S rRNA gene analysis and found that *Clostridium* sp. and *Rhodocyclaceae* were the dominant species growing on plastic supports. Xiao et al. (2010) used denaturing gradient gel electrophoresis (DGGE) and FISH to determine the dominant species on GAC. Using terminal restriction fragment length polymorphism (t-RFLP), Park et al. (2008) found that microbial community diversity decreased with ClO_4^- addition in an acetate fed biofilm reactor established with a wastewater inoculum. An increase in salinity to 3% also changed the community structure but did not affect its diversity. In a hydrogen-fed community, Park et al. (2008) found Alpha- and Gamma-Proteobacteria were dominant. However, 16S rRNA gene analysis revealed no sequences with similarity to previously known ClO_4^- reducers.

In a prior study in our laboratory, Sahu et al. (2009b) carried out bench scale upflow PBR experiments with S^0 and OS packing media. At high ClO_4^- concentrations (4 to 8 mg L^{-1}), ClO_4^- was consistently reduced to < 0.5 mg L^{-1} at an empty bed contact time (EBCT) of 13 h. At low ClO_4^- concentrations (60-120 µg L^{-1}) found more typically at contaminated groundwater sites, ClO_4^- was reduced to < 4 µg L^{-1} at an EBCT of 7.5 h. This study builds on our previous work by investigating the scale up of a S^0-oxidizing PBR to treat groundwater from a ClO_4^- contaminated aquifer at the Massachusetts Military Reservation (MMR) in Bourne, MA. The effects of feedwater deoxygenation method, EBCT, and internal recirculation on ClO_4^- removal and byproduct formation were examined over a ten-month period. The microbial community structure in the PBR was investigated using DGGE community fingerprint analysis, and functional gene amplification, identification and quantification.

2.2 Materials and Methods

2.2.1 Batch Experiments

Cultures capable of using S^0 as an electron donor for ClO_4^- reduction were enriched from a denitrifying wastewater seed using the method described by Sahu et al. (2009b). Briefly, cultures were set up in 1-L glass flasks containing 4 mm diameter lentil-shaped S^0 pellets (30 g; Martin Midstream Partners, Seneca, IL), OS (10 g; Myco Supply, Pittsburgh, PA; >97% $CaCO_3$; sieve mesh size analysis results: 80% #4, 52% #6, 18% #10), and N_2 sparged local groundwater (Amherst, MA). Reagent grade chemicals (Fisher Scientific, Pittsburgh PA; Sigma-Aldrich, St. Louis, MO) were used for all stock solutions and standards. Sodium perchlorate and sodium nitrate and potassium phosphate nutrients were added to achieve a ClO_4^- concentration of 5 mg L^{-1}, nitrogen at two times the stoichiometric requirements (Equation 1) and phosphorous at 20% of the nitrogen. The flasks were inoculated with 250-mL of mixed liquor suspended solids (MLSS) supernatant collected from a denitrification reactor at the Berkshire Mall WWTP in Lanesboro, MA. Mercuric chloride (20 mg L^{-1}) was added to killed control cultures, which were run in parallel. To grow the large volume of inoculum needed for the pilot-scale PBR, the enrichments were transferred to 2-L flasks after 20 days and then divided into three 20-L containers after an additional 54 days. Fresh nutrient medium, S^0 and OS were added in the same ratios with each transfer. As N_2 sparging would be impractical and costly in the pilot-scale PBR, sodium sulfite deoxygenation (Na_2SO_3; 100 mg L^{-1}) was compared with N_2 sparging in 1-L batch cultures inoculated from the 62-day old enrichment cultures (Reising and Schroeder, 1996). An additional uninoculated flask containing ClO_4^-, nutrients, S^0 pellets, OS and SO_3^{-2} was used to control for abiotic

reactions.

2.2.2 Bioreactor Studies

Pilot-scale upflow PBR experiments were carried out down gradient of Demolition Area 1 (3.0 ha), at MMR in Bourne, MA. Demolition Area 1 is a naturally formed topographical depression with permeable sandy soils, which allow rapid groundwater movement (0.3-0.6 meters per day). From the mid-1970s through 1997, the site was used as a disposal area for munitions, fireworks, explosives, and other items. The US Army performed extensive surveys of explosives and propellants in the soil and groundwater and detected ClO_4^- contaminated groundwater at this site (Clausen et al., 2004). The Army's Impact Area Groundwater Study Program (IAGWSP) identified a 2,700 by 305 meter plume containing ClO_4^- concentrations ranging from 2 to 500 μg L^{-1} down gradient of Demolition Area 1. (Fig. 2.1). RDX (cyclotrimethylenetrinitramine), HMX (cyclotetramethylene-tetranitramine), and NO_3^- were also identified in the plume. A four-inch diameter monitoring well, designated as MW-211, provided access to ClO_4^- contaminated groundwater at a depth of 55 m. Concentrations of ClO_4^-, RDX, and HMX detected at MW-211 on December 23, 2008 were 116, 8.22, and 0.16 μg L^{-1} respectively. Background levels of NO_3^- were also detected at 152 μg L^{-1}, which is well below the MCL (44 mg L^{-1}). MMR is currently using a pump-and-treat system, which uses IX and GAC to remove ClO_4^-, RDX and HMX from the groundwater.

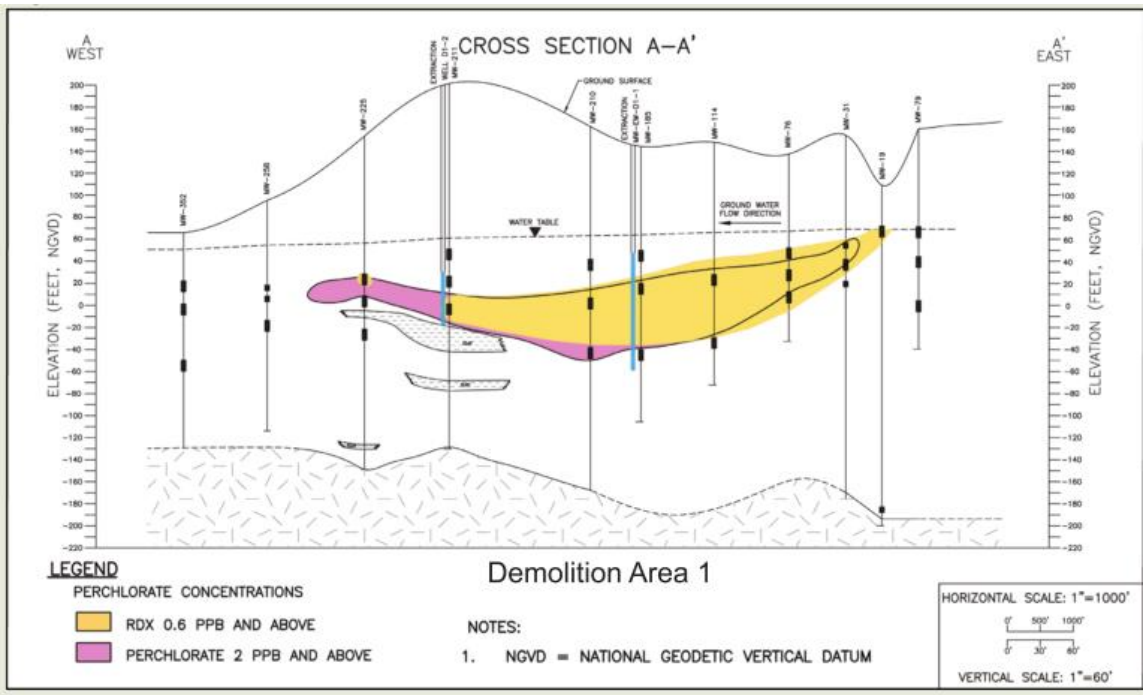

Image obtained from: http://www.mmr.org/Community/teams/pct2/meetings.htm

Figure 2.1: Cross section of Demolition Area 1 ClO_4^- and RDX plume.

The pilot-scale upflow PBR (Fig. 2.2) was constructed from three plastic injection molded risers (Polylok Inc. Wallingford, CT; overall reactor size: 91.4 cm height, 61.0 cm diameter). Sample ports were placed at depths of 9.5, 16.5, 22.9, 33.7, 44.4, 55.2, 65.4, 76.2, and 86.4 centimeters from the bottom inlet. The closer grouping towards the base allowed for finer data collection in the area of maximum ClO_4^- degradation. A check valve and stainless steel mesh screen (80/80) upstream of the inlet prevented liquid and particles from flowing back into the well. The tank was filled (from the bottom up) with 12.7-cm of washed coarse gravel (Amherst Farmers Supply, Amherst MA) and 81.3 cm of mixed S^0 and OS media (3:1 v:v), composed of 4-mm lentil shaped S^0 pellets and sieved OS. The media porosity and bulk density were determined to be 30% and 1.22 g

mL^{-1}, respectively using the method presented by Blake and Hartge (1986). The overall reactor volume was 267-L and the media volume including voids was 200-L.

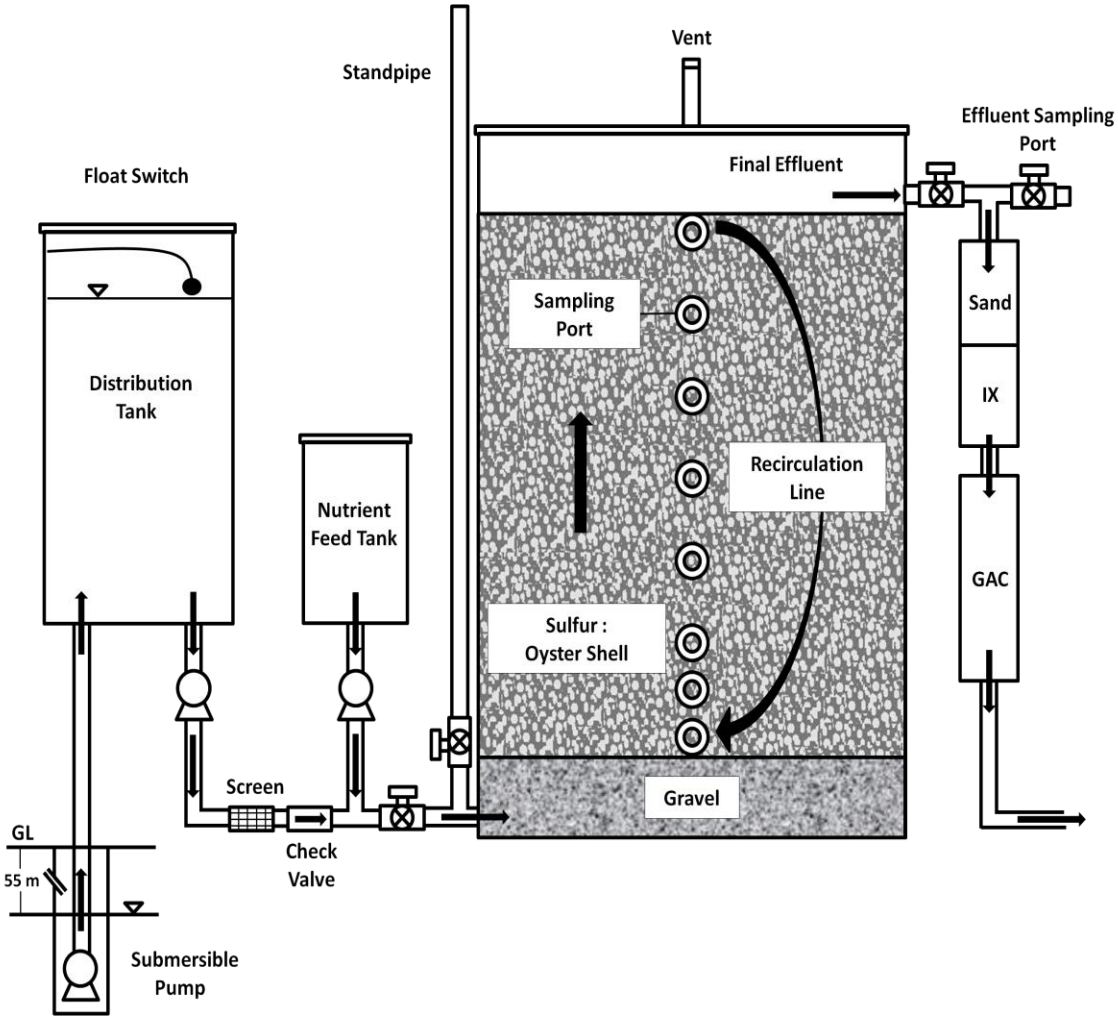

Figure 2.2: Upflow PBR schmatic

The ClO_4^- contaminated groundwater was initially pumped to a distribution tank and subsequently pumped to the bioreactor with added nutrients. The location of the internal recirculation line is shown with a black arrow. A post-effluent treatment train consisting of sand, IX resin, and GAC removed residual contaminants prior to discharge.

For field implementation, a Grundfos (Olathe, KS) Redi Flo-2 variable speed sampling pump was used to pump groundwater from MW-211 into a 1,600-L covered polyethylene distribution tank equipped with a float valve. The distribution tank was normally filled twice weekly during the continuous flow phase of the experiment. A peristaltic pump (Masterflex L/S Economy Pump (EW-77910-20) Cole Palmer, Loveland CO) delivered groundwater from the distribution tank to the bioreactor. A second peristaltic pump (Masterflex C/L Dual Channel Variable-Speed Compact Pump (EW-77120-62)) was connected to the influent line via a T-fitting to supply nutrients from a 20-L carboy. Nitrogen (NH_4Cl) and phosphorous (K_2HPO_4) were added to maintain concentrations of 45 µg L^{-1} and 17 µg L^{-1} respectively at the reactor inlet. The nutrient feed pump speed was adjusted periodically to maintain target nitrogen and phosphorous concentrations. During the initial stages of the experiments, Na_2SO_3 was added at 0.79 mg L^{-1} (groundwater DO of 10 mg L^{-1}), but was discontinued on day 232 of reactor operation as discussed below. A post-effluent treatment train, consisting of a sand pre-filter, two IX resin cartridges (Purolite A-520E Nitrate Selective Resin) and GAC (Carbon Activated Corp), was used to maintain compliance with Massachusetts DEP drinking water standards (2 µg L^{-1} for both ClO_4^- and RDX).

A tracer test was performed on day 292 by injecting sodium bromide (20 mg L^{-1}; tracer volume 28-L) into the influent. Samples were collected every 10 minutes over an eight-hour period. The reactor exhibited dispersed plug flow behavior with initial breakthrough occurring at 2.75 hours and the peak concentration exiting at 5.5 hours.

2.2.3 Experimental Program

Phase I—Acclimation: The pilot scale reactor was inoculated with the enrichment cultures on December 23, 2008 (Day 0). The bioreactor was filled with approximately 60-L of ClO_4^- contaminated groundwater and 60-L of the enrichment culture and sparged with N_2. $NaClO_4$ (1.6 g) was added to bring the reactor ClO_4^-concentration to 13 mg L^{-1}, along NH_4Cl (795-mg), and K_2HPO_4 (182-mg). A peristaltic pump (L/S Variable Speed Modular Drive (EW-07553-70) Cole Palmer, Loveland, CO) was used to recirculate water from the top sample port to the inlet at a flow rate of 400 mL min^{-1}. Samples were collected every three days during this period.

Phase II—Batch Operation: Between days 17-176 of reactor operation, groundwater pumping was intermittent due to pump malfunction. The bioreactor was operated in batch mode with weekly ClO_4^- and nutrient spikes (640-mg $NaClO_4$, 318-mg NH_4Cl, 73-mg K_2HPO_4). The mass of ClO_4^- added was similar to the expected mass loading rate with the system operating in continuous flow mode resulting in a completely mixed reactor ClO_4^- concentration of 5 mg L^{-1} after each spike.

Phase III—Continuous Flow: The bioreactor was operated continuously for 134 days. Modifications were made several times during Phase III based on the analytical results (Table 2.1). Na_2SO_3 was removed from the feed on day 232. A recirculation loop (400 mL min^{-1}) was added for a period of 37 days. The influent flow rate was increased from 250 mL min^{-1} to 375 mL min^{-1} in stages over the course of the experiment to investigate bioreactor performance at varying EBCT.

Table 2.1: Experimental conditions for each phase and days of operational changes and intensive sampling campaigns.

Day	Phase		Flow Rate (mL min $^{-1}$)	EBCT (hr)
0	I	Inoculation (December 23, 2008)	--	--
0-16	I	Acclimation		
1-175	II	Batch operation with weekly spikes		
176-310	III	Continuous flow	250	17.8
204	III	Flow rate increased	360	12.4
210	III	Recirculation added, flow rate decreased	250	17.8
232	III	Sodium sulfite addition terminated	250	17.8
247	III	Internal recirculation terminated	250	17.8
250	III	Profile sampling	250	17.8
288	III	Flow rate increased	360	12.4
292	III	Tracer study	360	12.4
296	III	Flow rate increased	375	11.9
310	III	Bioreactor decommissioned, profile sampling	375	11.9

2.2.4 Analytical Methods

Samples were normally collected weekly for anion analysis. Samples were collected for RDX/HMX analysis five times (days 195, 210, 215, 271, and 281). Samples were collected from ports along the length of the reactor on days 250 and 310. Anion (ClO_4^-, Cl^-, NO_3^-, SO_4^{2-}) concentrations were measured following EPA method 314.0 using a Metrohm (Riverview, FL) 850 Professional Ion Chromatograph AnCat MCS, equipped with the 858 Professional Sample Processor and Metrosep A Supp 7-250 column. *Standard Methods* (Eaton et al. 2005) were used to measure alkalinity (2320B), pH (4500-H^+-B), and S^{-2} (4500-S^{2-}). RDX and HMX analysis were performed using the aqueous low-level salt extraction method following USEPA method 8330, using a Waters 2690 (Milford, MA) HPLC equipped with Restek Pinnacle II Cyano 5 mm column (Bellefonte, PA) bridged to a Waters 996 Photodiode Array detector. Method detection limits (MDLs) for ClO_4^-, NO_3^-, S^{-2}, RDX, and HMX were 0.5, 1.0, 0.0016, 0.05, and 0.05 µg L^{-1} respectively.

2.2.5 Microbiological Analysis

On days 281 and 310, 1-L pore water samples were taken from each port, the influent and the effluent and, filtered through a 0.22 µm filter (Millipore, Billerica, MA). The filters were stored at –30°C. The 16S rRNA gene was amplified from total DNA extracted from the filters (RapidWater® DNA Isolation Kit; MO BIO Laboratories, Inc., Carlsbad, CA). DGGE community fingerprint analysis was performed as described in Conneely (2011). 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. DNA was PCR amplified

from excised bands of interest and sequenced as described in Conneely (2011). A partial nucleotide sequence was submitted to GenBank with the accession number JF325874. Pore water samples (days 281 and 310) were tested for the presence of the functional genes, *pcrA* and *cld*, using PCR amplification, and PCR products were sequenced as outlined in Conneely (2011). Partial nucleotide sequences were submitted to GenBank with the accession numbers JF304786-JF304791. The relative quantity of the gene *pcrA* was determined by quantative PCR (qPCR) analysis. Standard curves for qPCR were created from cloned DNA extracted from the control strain *Dechlorosoma* (*Azospira*) *suillum* PS. A DNA dilution series from 10^6 to one gene copies mL^{-1} was performed and cycle threshold (C*T*) values were plotted against gene copy number mL^{-1}. Plasmid copy numbers were determined by measuring absorbance at 260 nm and adjusting for reactor porosity.

Matrix S^0 and OS samples were collected in triplicate at sampling ports 1, 2, 4, and 7 and pore water was collected from each port, the influent and the effluent during decommissioning (day 310). All samples were transported to the laboratory on ice and stored at 4°C. Protein concentrations in pore water samples (day 310) were measured within 24 hours, while matrix samples were analyzed 48 days after collection. To dislodge biomass from the matrix samples, approximately 10 g of S^0 and OS medium were mixed with 10 mL phosphate buffered saline (PBS) and vortexed for one minute. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL). Protein data are reported as mg biomass L^{-1} bioreactor by correcting for media porosity (0.30) and bulk density (1.22 g mL^{-1}).

2.3 Results and Discussion

2.3.1 Batch Cultures

In 1-L batch cultures, ClO_4^- was reduced from 3.9 mg L^{-1} to below detection limit (BDL) in less than 17 days. After subsequent transfer to the 20-L carboys, ClO_4^- concentrations were reduced from 3.9 mg L^{-1} to 1.4 mg L^{-1} in 45 days (data not shown). Initial first order decay coefficients were 0.10 d^{-1} in the 1-L and 2-L cultures, while the 20-L cultures had a slower decay rate of 0.069 d^{-1}, likely due to a lower biomass density after dilution. Insignificant ClO_4^- removal was observed in killed controls. Sulfite was a suitable substitute for N_2 sparging for DO removal in batch cultures. Perchlorate reduction rates were identical in both the SO_3^{2-} and N_2 deoxygenated cultures. Perchlorate losses in abiotic controls were negligible, indicating that SO_3^{-2} did not reduce ClO_4^-.

2.3.2 Packed Bed Bioreactor Studies

A plot of effluent ClO_4^- concentration versus. time during reactor acclimation (Phase I) is shown in Fig. 2.3. Perchlorate was reduced from 12.6 mg/L to BDL in 17 days with no lag period. A first order decay constant of 0.17 d^{-1} was calculated from the data, which was an order of magnitude higher than observed in the batch cultures used for inoculation. The improved ClO_4^- degradation in the PBR compared to the batch cultures may have been due to internal recirculation applied during this period, which may have increased ClO_4^- mass transfer to the biomass. Alternatively, the presence of the large volume of S^0 pellets and OS media in the reactor may have increased the rate of ClO_4^- reduction. Ju et al. (2007) found ClO_4^- reduction was dependent on S^0 surface area and suggested that S^0 should be added in excess. Once ClO_4^- degradation was established,

ClO_4^- spikes were degraded from 5 mg L^{-1} to BDL within seven days throughout Phase II (data not shown). The pH in the reactor remained stable during this period at 7.3 ± 0.2.

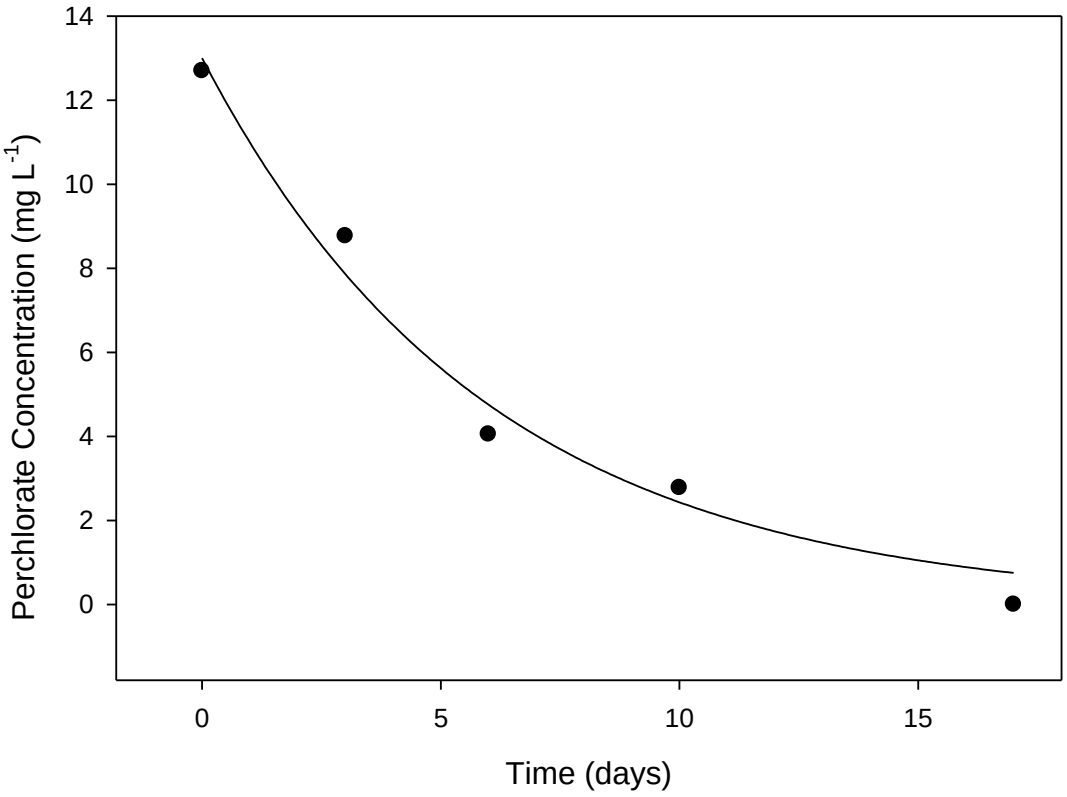

Figure 2.3: Initial acclimation effluent ClO_4^- concentration versus time fitted with first-order degradation curve (k_1 = 0.17 d^{-1}) during the initial pilot-reactor acclimation period (days 0-17).

Bioreactor influent and effluent ClO_4^- concentrations during Phase III are shown in Fig. 2.4. A summary of the average reactor performance under each set of operating conditions is shown in Table 2.2. The influent ClO_4^- concentration decreased slightly over time (average 96.4 µg L^{-1}; range 84.5-113 µg L^{-1}). The influent pH (6.7 ± 0.2) and alkalinity (4.5 ± 1.8 mg L^{-1} as $CaCO_3$) were consistent throughout Phase III. The initial

bioreactor performance was promising. At a flow rate of 250 mL min^{-1} (EBCT = 17.8 h), effluent ClO_4^- concentrations decreased from 48.2 µg L^{-1} (50% removal) to 28.3 µg L^{-1} (72% removal) over eight days. The pH in the reactor (average pH from ports 2, 4, and 7) was 8.0 (±0.6) on day 176. However, ClO_4^- removal diminished as the reactor pH increased to 8.6 (±0.5) on day 195, 8.8 (±0.14) on day 210, and to 9.0 (±0.15) on day 226. At the beginning of Phase III, Na_2SO_3 was added to the reactor for DO removal. Although SO_3^{-2} did not inhibit ClO_4^- reduction in batch cultures, in the pilot system, SO_3^{-2} addition appeared to promote the growth of SO_4^{-2} reducing bacteria, most likely due to decreased redox conditions in the reactor. The activity of SO_4^{-2} reducing bacteria raised the pH and produced high concentrations of S^{-2}, which inhibited ClO_4^- reduction. On day 195, H_2S gas production became evident (due to the strong odor) and the reactor was uncapped and sparged with N_2 and the influent flow rate was increased to 360 mL min^{-1} (EBCT = 12.4 h) to flush toxic by-products from the system. Reactor performance temporarily improved when the flow rate was returned to 250 mL min^{-1} and internal recirculation was added (arrow A in Fig. 2.4). On day 226, SO_3^{-2} addition was terminated (arrow B) and on day 250, recirculation was discontinued (arrow C). After day 250, no further alterations were made to the reactor other than increasing the flow rate. The average pH in the reactor remained >8.5 until day 250, when the effluent pH fell to 7.5 (±0.03).

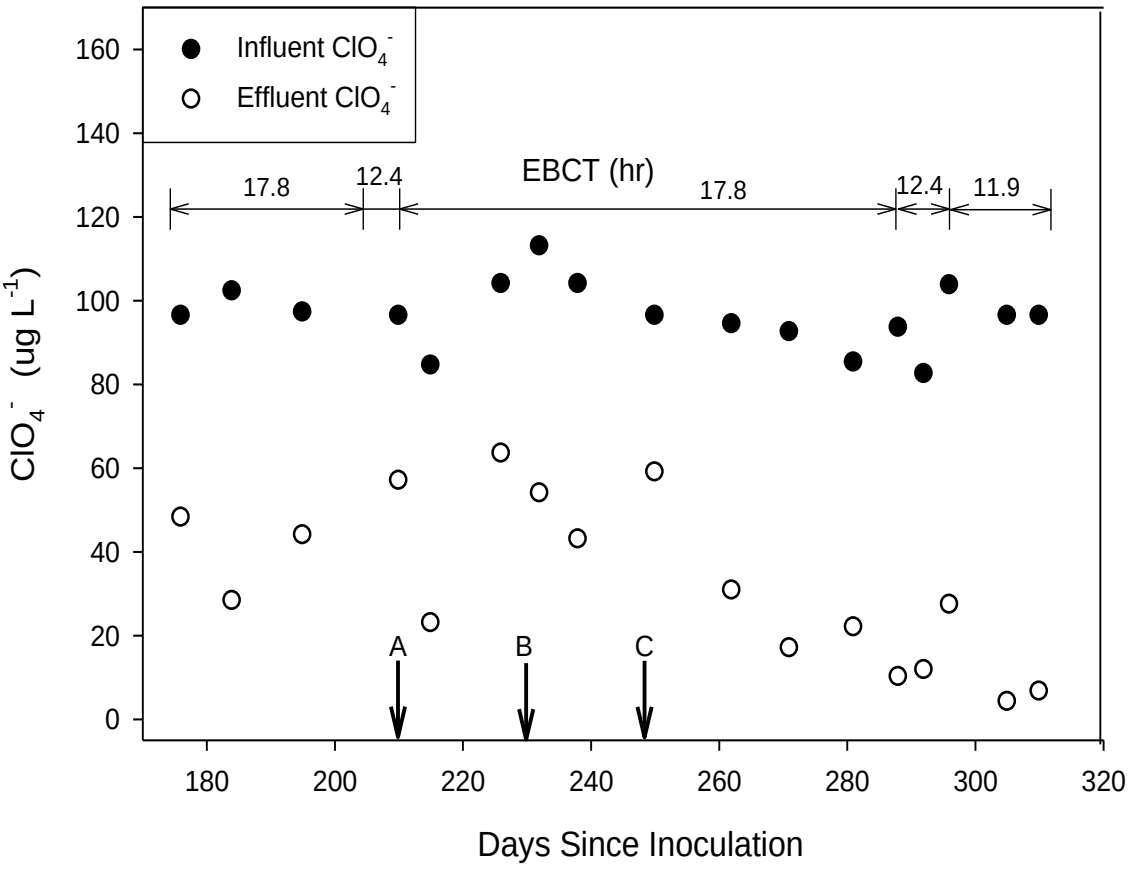

Figure 2.4: Influent and effluent ClO_4^- concentrations and EBCTs throughout the continuous flow period (Phase III). Arrows indicate addition of internal recirculation (A), termination of sulfite addition (B) and discontinuation of internal recirculation (C).

Table 2.2: Average bioreactor performance under each set of operating conditions during Phase III.

Day	Flow rate (mL min^{-1})	EBCT (hr)	Removal Efficiency (%)	Mass Removal Rate (mg $m^{-3}d^{-1}$)	Effluent ClO_4^- (µg L^{-1})
184	250	17.8	73	112	23.0
288	250	17.8	88	133	10.1*
292	360	12.4	87	205	11.8*
296	375	11.9	70	173	27.4
305	375	11.9	96	249	4.2*
310	375	11.9	93	240	6.7*

*Meets EPA interim level 15 µg L^{-1}

From day 250 to 288, effluent ClO_4^- concentrations decreased with the flow rate at 250 mL min^{-1}. On Day 288, the flow rate was increased to 360 mL min^{-1}, when the effluent concentration was 10.1 µg L^{-1}. Approximately one week later (day 296), the flow rate was increased to the highest rate in the study of 375 mL min^{-1} (EBCT = 11.9 h). Overall, the reactor performance improved even though the EBCT was decreased as was seen in our prior column studies (Sahu et al., 2009b), most likely due to biomass growth and acclimation. Perchlorate removal may also have improved due to increased mass transfer or flushing of inhibitory waste products at higher flow rates. The best removal efficiency (96%) occurred on the five days prior to the final day of operation, with an effluent ClO_4^- concentration of 4.2 µg L^{-1} and ClO_4^- degradation rate of 249 mg m^{-3} d^{-1}. During this period, the bioreactor effluent ClO_4^- concentration was able to meet California's MCL of 6 µg L^{-1}, but not the more stringent Massachusetts MCL of 2 µg L^{-1}.

RDX concentrations were measured periodically to examine the effect of S^0-oxidizing ClO_4^- reduction on RDX degradation. The average influent RDX concentration was 9.2 µg L^{-1} (±1.3), while the average effluent concentration was 7.1 µg L^{-1} (±2.0). RDX removal efficiency ranged from 43% to 8%, with an average of 25%. While RDX was not the focus of this study and no statistical analyses of the data were performed, consistent RDX removal was observed. Previous studies have documented microbial RDX utilization as either a nitrogen or carbon source in anaerobic systems (Boopathy et al., 1998; Adrian and Arnett, 2006). Boopathy et al. (1998) used a SO_4^{-2} reducing bacterial consortium to oxidize RDX in soil slurries. In the current study, SO_4^{-2} reducing conditions were present in the bioreactor. Additionally, no external carbon source was added and microorganisms may have used RDX for cellular synthesis. Mixotrophic metabolism by sulfur oxidizing ClO_4^- reducing bacteria (use of S^0 as an electron donor and an organic substrate for cell synthesis) was observed by both Ju et al. (2007) and Conneely (2011).

On October 29, 2009 (day 310) the reactor was decommissioned due to nighttime freezing. Samples were collected from each sampling port along the length of the bioreactor. Profiles of ClO_4^- concentration and pH with depth and retention time are plotted in Fig. 2.5. Most of the ClO_4^- reduction occurred close to the inlet of the reactor (bottom 22.9 cm), where the influent ClO_4^- (95.0 µg L^{-1}) was reduced by 82% by port 3. Between the influent and port 3, S^{-2} and pore water protein concentrations (Fig. 2.6 and 2.7) remained low (< 160 µg L^{-1} S^{-2}; 10 to 19 mg protein L^{-1}), while SO_4^{-2} concentrations and pH increased steadily. The matrix protein concentration was highest in the area of highest ClO_4^- degradation and decreased along the reactor length. An additional 9% of

the ClO_4^- was removed between ports 3 and 4 and little degradation occurred for the remaining media depth. A decrease in ClO_4^- reduction was observed with a rise in pH as the influent pH of 6.5 (±0.12) rose to 7.9 (±0.04) by port 3, and to 8.8 (±0.02) at port 4 where ClO_4^- reduction slowed. Between ports 3 and 5, the primary metabolic reactions shifted from ClO_4^- degradation to sulfur disproportionation, as discussed below, as rapid increases in S^{-2} (from 160 to 2,405 µg L^{-1}) and pore water protein concentrations were observed. Protein concentrations increased from 19 to 67 mg L^{-1} and remained high (67 to 99 mg L^{-1}) in the remaining ports of the reactor. Both SO_4^{-2} and S^{-2} decreased gradually from port 5 to port 8 as the pH steadied and reached a maximum of 9.0 (±0.006) at port 7. The steady decrease in matrix associate biomass coupled with the increase in pore water associated biomass further support the transition in metabolic reactions from solid phase S^0-oxidation to aqueous phase SO_4^{-2} reduction.

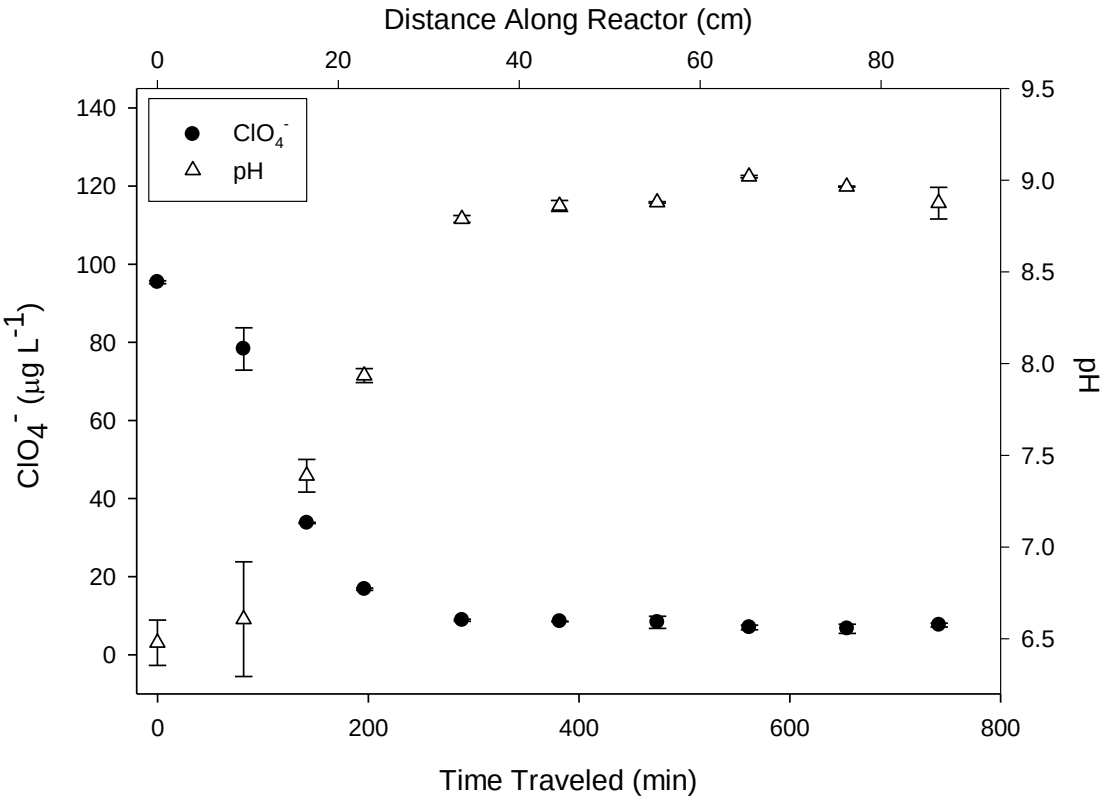

Figure 2.5: ClO_4^- concentration and pH versus depth and residence time on day 310 of bioreactor operation (at a flow rate of 375 mL min^{-1}). Error bars show standard deviations of duplicate samples.

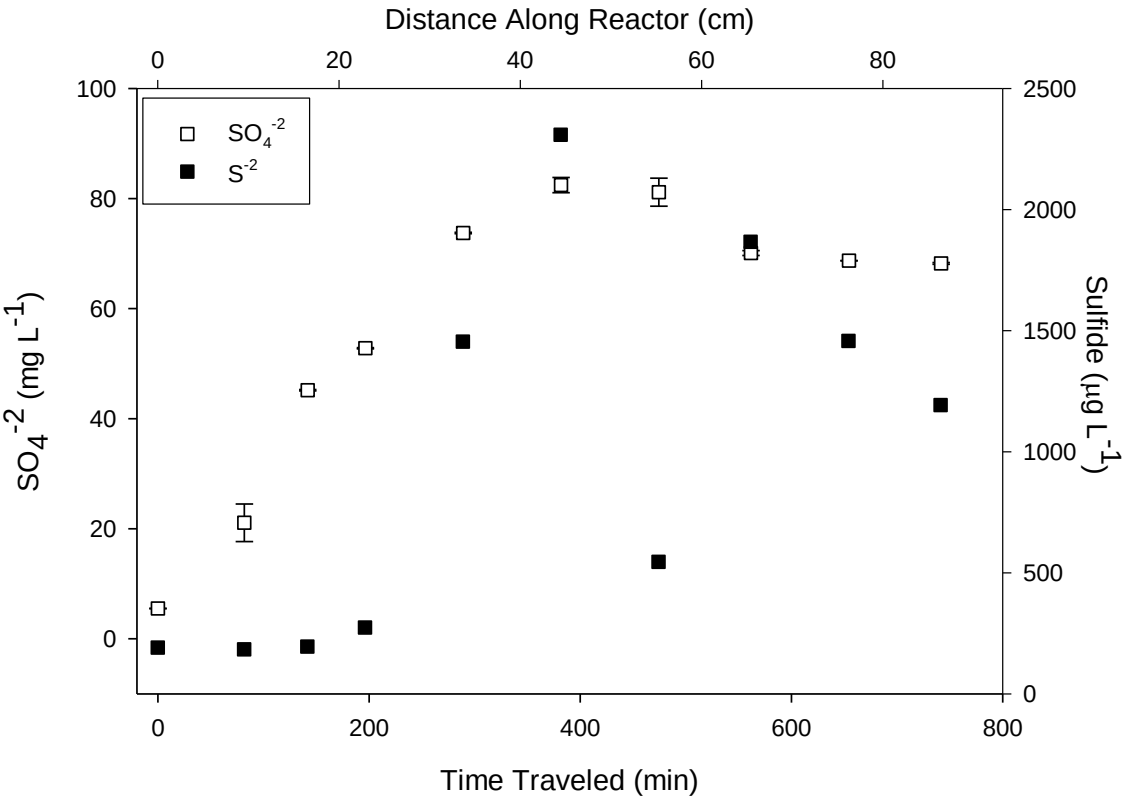

Figure 2.6: SO_4^{-2} and S^{-2} concentrations versus depth and residence time on day 310 of bioreactor operation (at a flow rate of 375 mL min^{-1}). Error bars on SO_4^{2-} show standard deviations of duplicate samples. S^{-2} samples were measured only once.

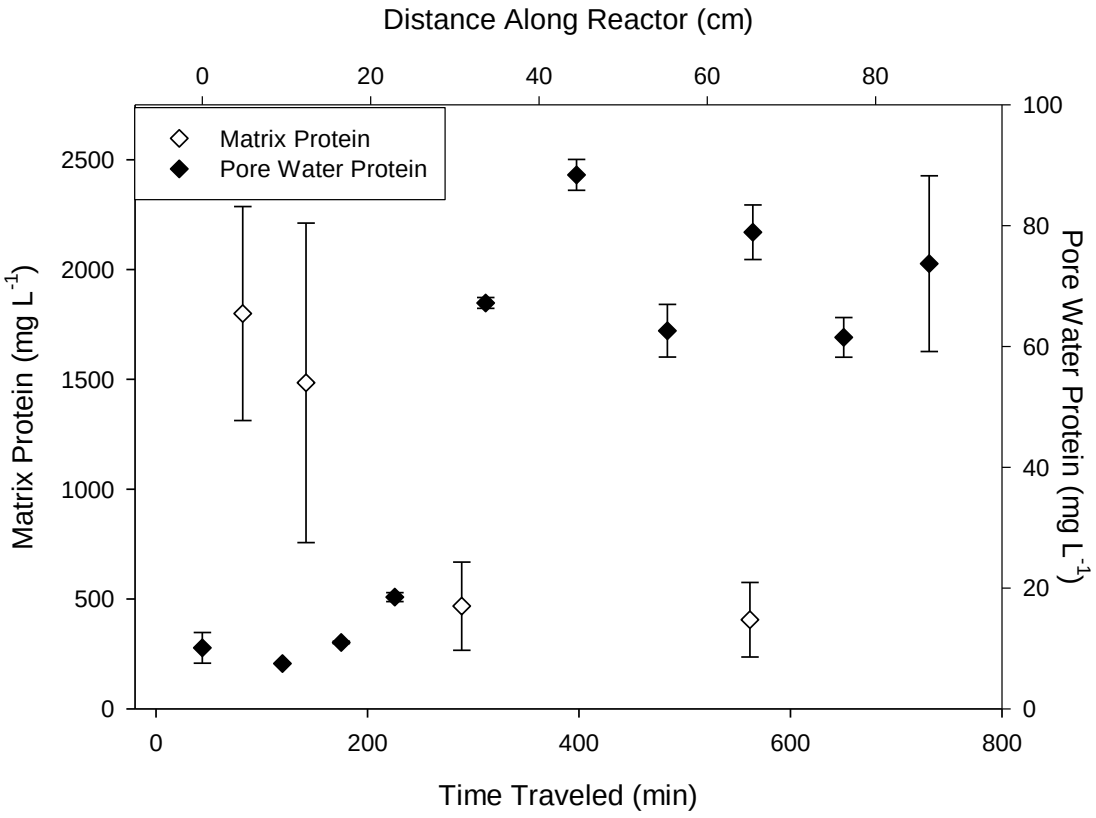

Figure 2.7: Matrix and pore water protein concentrations versus depth on day 310 of bioreactor operation (at a flow rate of 375 mL min^{-1}). Error bars on pore water protein show standard deviations of duplicate samples. For the matrix, averages and standard deviations are shown for media extractions from three locations at each depth. Concentrations are presented as protein per unit volume bioreactor.

Simultaneous low-level NO_3^- and ClO_4^- removal was observed (Fig. 2.8), with the majority of degradation occurring close to the inlet (within 33.4 cm) of the PBR. The microorganisms appeared to preferentially use the more energetically favorable NO_3^-, as influent NO_3^- (137.6 µg L^{-1}) concentrations decreased by nearly 30% at port 1, 80% at port 2, and completely degraded to BDL by port 4. Perchlorate was used more slowly than NO_3^-, with nearly all degradation completed by port 4. In the acclimated bioreactor, ClO_4^- and NO_3^- reduction occurred concurrently, which is similar to results observed in prior studies (Choi et al., 2007; Herman and Frankenberger, 1999; Losi et al, 2002, Min et al., 2004). The presence of NO_3^- may have inhibited sulfur disproportionation, as Ju et al. (2007) found in a previous study, since evidence of sulfur disproportionation was not present until after all NO_3^- was consumed.

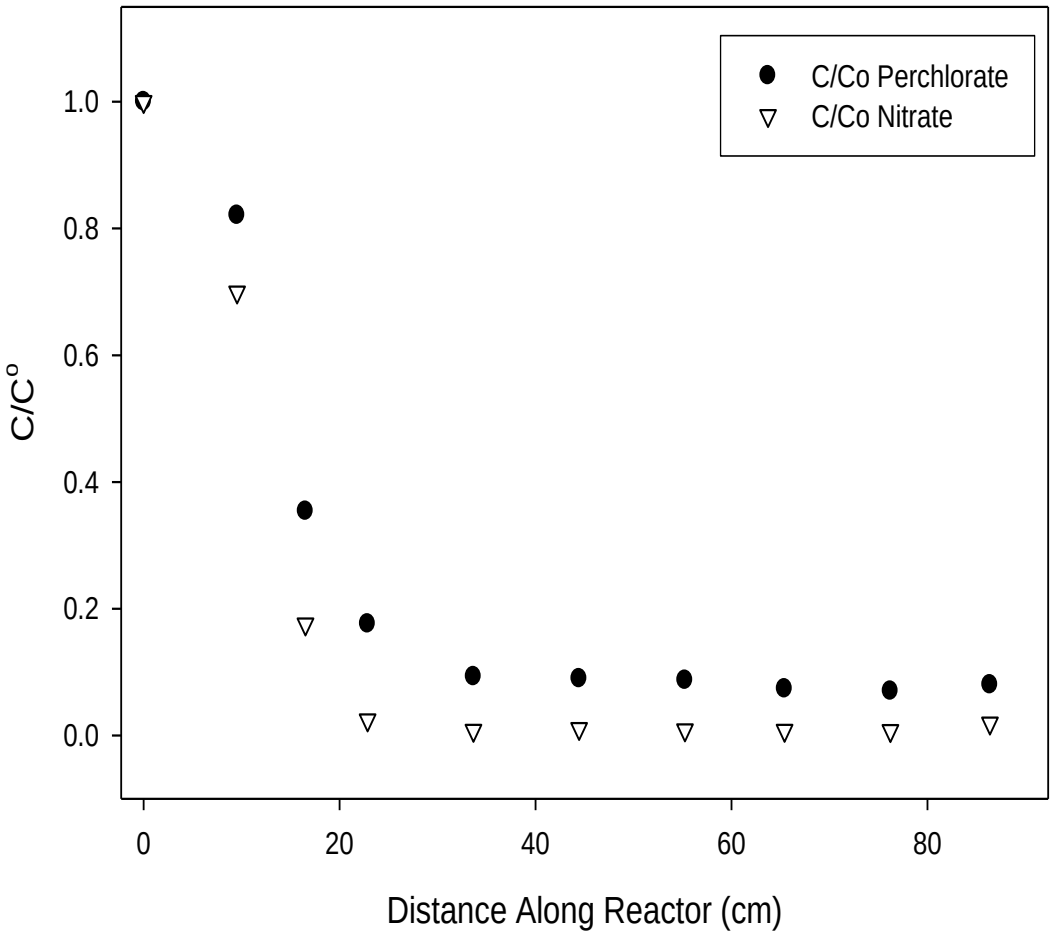

Figure 2.8: Normalized ClO_4^- and NO_3^- concentrations versus depth on day 310 of bioreactor operation (at a flow rate of 375 mL min^{-1}). Initial ClO_4^- and NO_3^- concentrations were 95.0 µg L^{-1} and 137.6 µg L^{-1} respectively.

The results of the analysis of microbial community structure support the chemical and protein analyses, with the structure changing from the inlet (bottom ports 1 to 4) to the outlet (top ports 5 to 8) of the reactor (Fig. 2.9). This result was expected since ClO_4^- and NO_3^- degradation were the dominant reactions in ports 1 to 4, while sulfur disproportionation was the primary biological process occurring in ports 5 to 8. The effluent sample (86.4 cm) grouped most closely with ports 5 to 8, while 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 had 98-99% similarity to an uncultured bacterium clone from a sulfur spring and 96% related to an uncultured Epsilon-Proteobacterium clone from iron-rich, deep-sea, microbial mats.

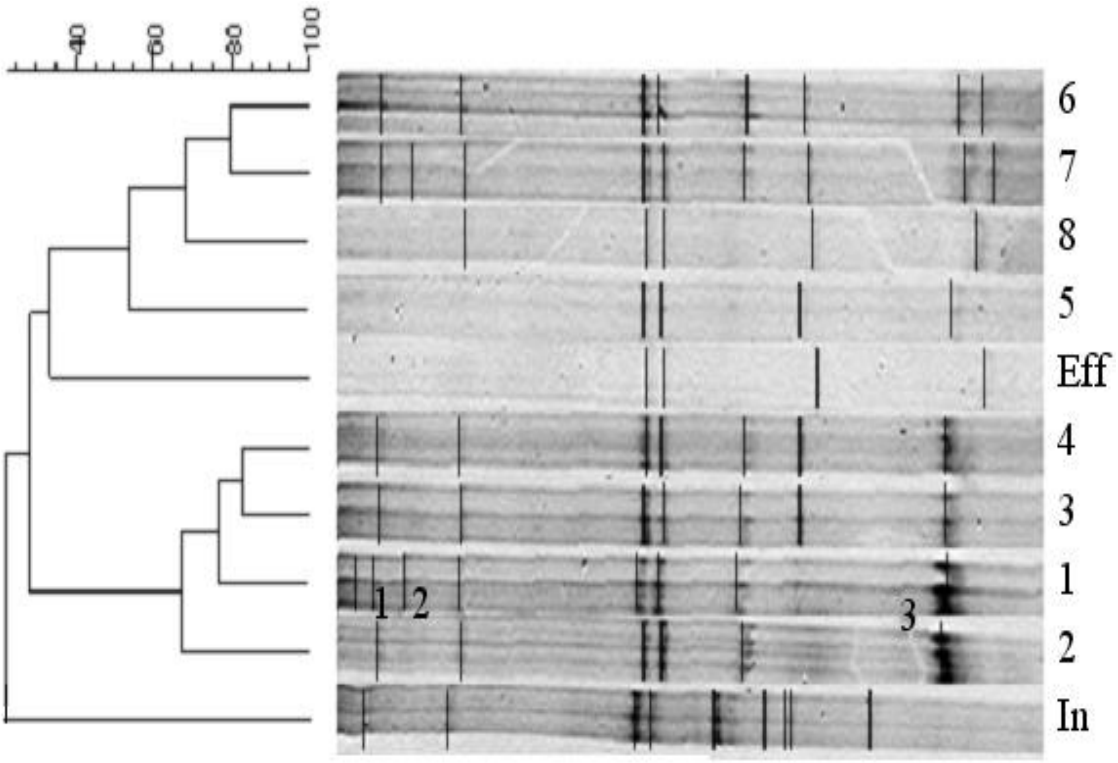

Figure 2.9: Community structure analysis from the bioreactor pore water on day 310 of bioreactor operation from influent (IN) and ports 1 (9.5 cm), 2 (16.5 cm), 3 (22.9 cm), 4 (33.7 cm), 5 (44.5 cm), 6 (55.3 cm), 7 (65.4 cm), 8 (76.2 cm), and effluent (EFF). Bands 1, 2 and 3 had 98-99% similarity to clone DQ145977 isolated from a sulfur spring and 96% to Epsilon-Proteobacterium clone FJ497346.

PCR and qPCR analyses were used to measure the presence of the functional genes *pcrA* and *cld.* Both genes were most closely related to functional genes from ClO_4^- reducing bacteria of the Beta-Proteobacteria. The *cld* gene was detected by PCR in ports 1 and 2 on day 281, but only in port 1 on day 310. 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 present in the pore liquid at port 1 on day 281 at $4.2 \times 10^5 \pm 9.8 \times 10^4$ gene copies/L and $6.3 \times 10^4 \pm 1.7 \times 10^4$ gene copies/L on day 310. The *pcrA* gene was distantly related to the uncultured bacterium clone PNA3 ClO_4^- reductase alpha subunit at 82% similarity *Dechloromonas* sp. MissR at 81% similarity (Fig. 2.10 and 2.11).

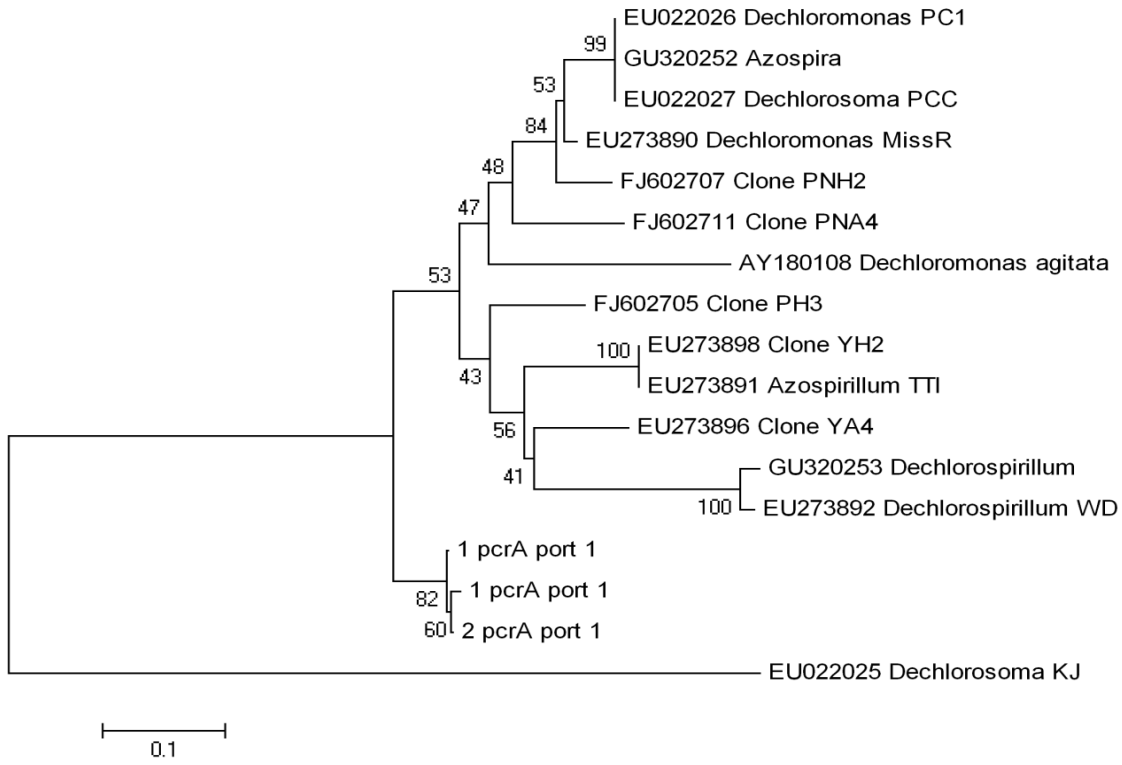

Figure 2.10: Phylogenetic relationship of the *pcrA* gene to known *pcrA* gene sequences deposited in the Genbank database as of September 2010. The comparative analysis was inferred by Minimum Evolution analysis of 3 aligned port 1 *pcrA* clones designated by 1 for day 281 and 2 for day 310 collection. The scale bars represent 10% estimated sequence divergence. Bootstrap values are shown for 1,000 replicates.

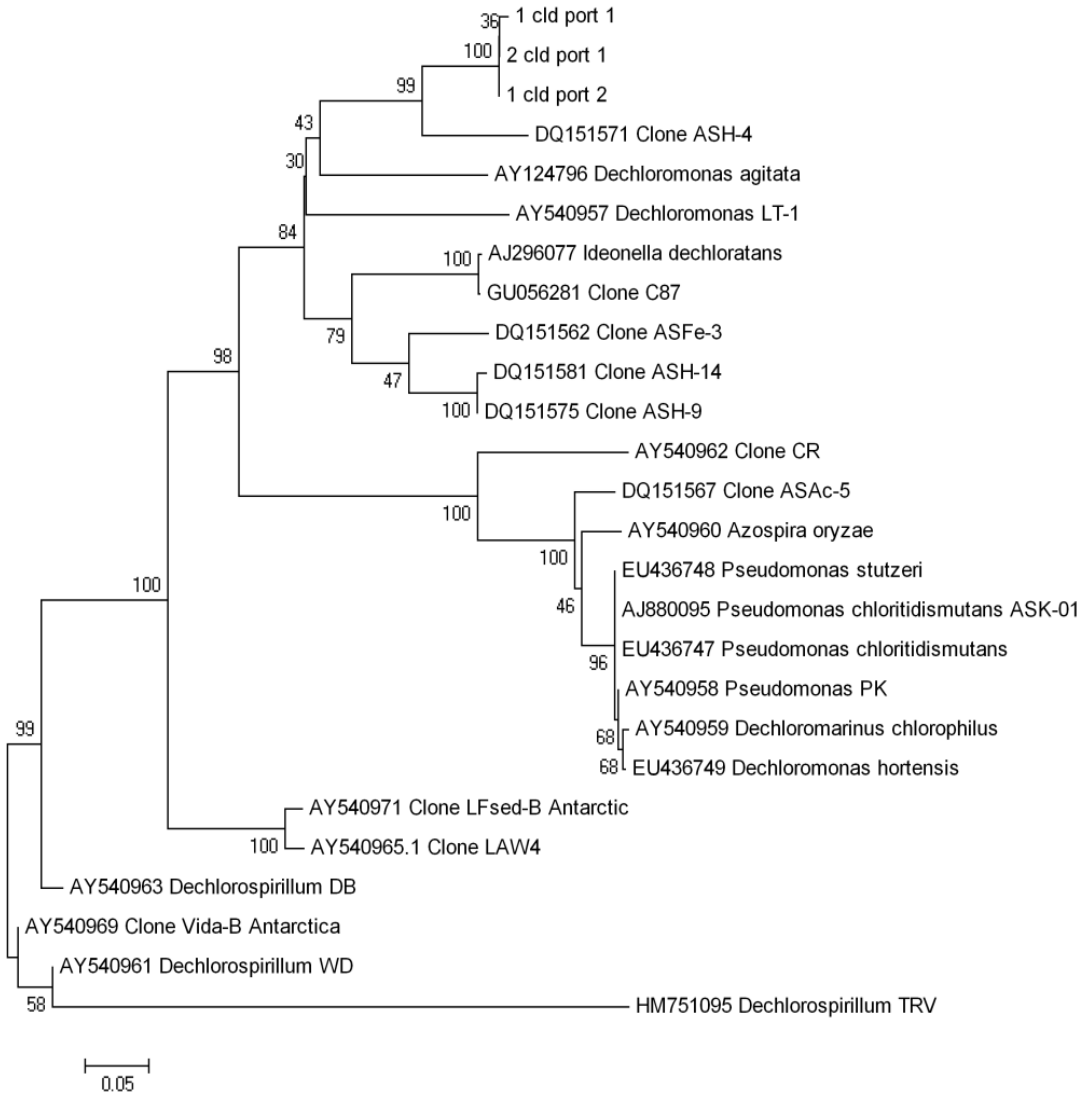

Figure 2.11: Phylogenetic relationship of the *cld* gene to known *cld* gene sequences deposited in the Genbank database as of September 2010. The comparative analysis was inferred by Minimum Evolution analysis of 3 aligned port 1 or port 2 *cld* clones designated by 1 for day 281 and 2 for day 310 collection. The scale bars represent 5% estimated sequence divergence. Bootstrap values are shown for 1,000 replicates.

2.4 Conclusions

This study examined scale-up and operation of a sulfur oxidizing ClO_4^- reducing upflow PBR for treatment of groundwater from a ClO_4^- contaminated aquifer at MMR. Batch ClO_4^- degradation kinetics were slower than observed in prior studies; however, degradation rates increased in the PBR compared to the batch cultures, possibly due to better mass transfer of the ClO_4^- to the biofilm. Sodium sulfite provided a good method of DO removal in batch cultures, but was found to promote sulfate reduction and inhibit ClO_4^- reduction in the pilot system. Once SO_3^{2-} was removed from the feed, the PBR successfully removed 96% of the influent perchlorate in the groundwater at an EBCT of 12 hours. Simultaneous ClO_4^- and NO_3^- degradation was observed near the inlet of the reactor. The presence of the genes *cld* and *pcrA*, from the perchlorate-reduction pathway near the reactor influent and the distinct clustering patterns of the microbial community support the water quality profiles.

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Chapter 2 References

Adrian NR, Arnett CM. 2006. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) serves as a carbon and energy source for a mixed culture under anaerobic conditions. Curr Microbiol 53(2):129-134.

Bardiya N, Bae JH. 2005. Bioremediation potential of a perchlorate-enriched sewage sludge consortium. Chemosphere 58(1):83-90.

Batchelor B, Lawrence AW. 1978. A kinetic model for autotrophic denitrification using elemental sulfur. Water Resources 12:1075-1084.

Bender KS, Rice MR, Fugate WH, Coates JD, Achenbach LA. 2004. Metabolic primers for detection of (per)chlorate-reducing bacteria in the environment and phylogenetic analysis of cld gene sequences. Appl Environ Microb 70(9): 5651-5658.

Blake, GR, Hartge KH. 1986. Bulk density. In: A. Klute, editor. Methods of soil analysis, part I. physical and mineralogical methods. Madison, WI: American Society of Agronomy, Soil Science Society of America. pp. 363-375.

Boopathy R, Gurgas M, Ullian J, Manning JF. 1998. Metabolism of explosive compounds by sulfate-reducing bacteria. Curr Microbiol 37(2):127-131.

Braverman LE, He X, Pino S, Cross M, Magnani B, Lamm SH, Kruse MB, Engel A, Crump KS, Gibbs JP. 2005. The effect of perchlorate, thiocyanate, and nitrate on thyroid function in workers exposed to perchlorate long-term. J Clin Endos Metab 90(2):700-706.

Cao XY, Jiang XM, Dou ZH, Rakeman MA, Zhang ML, O'Donnell K, Ma T, Amette K, DeLong N, DeLong GR. 1994. Timing of vulnerability of the brain to iodine deficiency in endemic cretinism. N Engl J Med 331(26):1739-1744.

Capen CC. 1994. Mechanisms of chemical injury of thyroid gland. Prog Clin Biol Res 387:173-191.

CDPH. 2007. Perchlorate in drinking water. California Department of Public Health. Publication CDPH R-16-04. Sacramento, CA.

Chen J-S, Mortenson LE. 1977. Inhibition of methylene blue formation during determination of the acid-labile sulfide of iron-sulfur protein samples containing dithionite. Anal Biochem 79(1-2): 157-165.

Choi H, Silverstein J. 2007. Effluent recirculation to improve perchlorate reduction in a fixed biofilm reactor. Biotechnol Bioeng 98(1):132-140.

Chung JS, Shin S, Oh J. 2009, Characterization of a microbial community capable of reducing perchlorate and nitrate in high salinity. Biotechnol Lett 31(7):959-966.

Clausen J, Robb J, Curry D, Korte N. 2004. A case study of contaminants on military ranges: Camp Edwards, Massachusetts, USA. Environ Pollut 129:13-21.

Coates JD, Achenbach LA. 2004. Microbial perchlorate reduction: rocket-fueled metabolism. Nat Rev Microbiol 2(7):569-580.

Coates JD, Michaelidou U, Bruce RA, O'Connor SM, Crespi JN, Achenbach LA. 1999. Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. Applied and Environmental Microbiology 65(12):5234-5241.

Conneely T, 2011, Investigation of a sulfur-utilizing perchlorate-reducing bacterial consortium [Ph.D.], Amherst: University of Amherst, p 188.

Darbi A, Viraraghavan T, Butler R, Corkal D. 2003. Pilot-scale evaluation of select nitrate removal technologies. J Environ Sci Health A Tox Hazard Subst Environ Eng 38(9):1703-1715.

Eaton AD, Clesceri LS, Rice, EW, Greenberg AE, Franson, MAH. 2005. Standard Methods for the Examination of Water and Wastewater. Washington DC. American Public Health Association.

Gu B, Coates JD. 2006. Perchlorate : environmental occurrence, interactions and treatment. New York: Springer. xii, 411 p.

Herman DC, Frankenberger Jr. WT. 1999. Bacterial reduction of perchlorate and nitrate in water. Journal of Environmental Quality 28(3):1018-1024.

Jang A, Bum M, Kim S, Ahn Y, Kim IS, Bishop PL. 2005. Assessment of characteristics of biofilm formed on autotrophic denitrification. JMicrobiol Biotechn 15(3): 455-460.

Ju X, Field JA, Sierra-Alvarez R, Salazar M, Bentley H, Bentley R. 2007. Chemolithotrophic perchlorate reduction linked to the oxidation of elemental sulfur. Biotechnol Bioeng 96(6):1073-1082.

Ju X, Sierra-Alvarez R, Field JA, Byrnes DJ, Bentley H, Bentley R. 2008. Microbial perchlorate reduction with elemental sulfur and other inorganic electron donors. Chemosphere 71(1):114-122.

Kim K, Logan BE. 2001. Microbial reduction of perchlorate in pure and mixed culture packed-bed bioreactors. Water Res 35(13):3071-3076.

Kodama Y, Watanabe K. 2004. Sulfuricurvum kujiense gen. nov., sp. nov., a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium isolated from an underground crude-oil storage cavity. Int J Syst Evol Micr 54: 2297-2300.

Lawler, D, Benjamin, M. 2006. Draft of Chapter 2, Textbook in Preparation by Professor Lawler and Professor Benjamin, The McGraw-Hill Companies.

Leung AM, Pearce EN, Braverman LE. 2010. Perchlorate, iodine and the thyroid. Best Pract Res Clin Endocrinol Metab 24(1):133-141.

Losi ME, Giblin T, Hosangadi V, Frankenberger Jr. WT. 2002. Bioremediation of perchlorate contaminated groundwater using a packed bed reactor. Bioremediation Journal 6(2):97-103.

MassDEP. 2006a. Update to "perchlorate toxicological profile and health assessment." Massachusetts Department of Environmental Protection. Office of Research and Standards. Boston, MA.

MassDEP. 2006b. Maximum contaminant level for inorganic chemicals. Massachusetts Department of Environmental Protection. Office of Research and Standards. Code of Massachusetts Regulations 310 CMR 22.06. Boston, MA.

Min B, Evans PJ, Chu AK, Logan BE. 2004. Perchlorate removal in sand and plastic media bioreactors. Water Res 38(1):47-60.

Motzer WE. 2001. Perchlorate: problems, detection, and solutions. Environmental Forensics 2:301-311.

Nozawa-Inoue M, Jien M, Hamilton NS, Stewart V, Scow KM, Hristova KR. 2008. Quantitative detection of perchlorate-reducing bacteria by real-time pcr targeting the perchlorate reductase gene. Appl Environ Microb 74(6):1941–1944.

Okeke BC, Frankenberger Jr. WT. 2004. Use of starch and potato peel waste for perchlorate bioreduction in water. Sci Tot Environ 347(1-3):35-45.

Park, J., Rhee, J., Oh, H., Ahn, C.-H., Oh, J., and Rittmann, B. 2008. Microbial ecology of emerging contaminant bioremediation. International Symposium on Microbiology, Daejeon, Korea.

Parker DR, Seyfferth AL, Reese BK. 2008. Perchlorate in groundwater: a synoptic survey of "pristine" sites in the coterminous United States. Environ Sci Technol 42(5):1465-1471.

Reising AR, Schroeder ED. 1996. Denitrification incorporating microporous membranes. Journal of Environmental Engineering 122(7):599-604.

Rittman BE, McCarty PL. 2001. Environmental Biotechnology: Principles and Applications. New York, NY: McGraw-Hill Companies.

Sahu AK, Conneely T, Nusslein K, Ergas SJ. 2009a. Hydrogenotrophic denitrification and perchlorate reduction in ion exchange brines using membrane biofilm reactors. Biotechnol Bioeng 104(3):483-491.

Sahu AK, Conneely T, Nusslein KR, Ergas SJ. 2009b. Biological perchlorate reduction in packed bed reactors using elemental sulfur. Environ Sci Technol 43(12):4466-4471.

Sengupta S, Ergas SJ, Lopez-Luna E. 2007. Investigation of solid-phase buffers for sulfur-oxidizing autotrophic denitrification. Water Environ Res 79(13):2519-2526.

Sierra-Alvarez R, Beristain-Cardoso R, Salazar M, Gomez J, Razo-Flores E, Field JA. 2007. Chemolithotrophic denitrification with elemental sulfur for groundwater treatment. Water Res 41(6):1253-1262.

Son A, Lee J, Chiu PC, Kim BJ, Cha DK. 2006. Microbial reduction of perchlorate with zero-valent iron. Water Res 40(10):2027-2032.

Stotter JM. 2008. Electrochemical treatment and recycling of spent perchlorate contaminated ion-exchange regneration brine. Eltron Research Inc.; Boulder, CO: Environmental Protection Agency.

Tamura K, Dudley J, Nei M, Kumar S. (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. (1997) The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882.

Tipton DK, Rolston DE, Scow KM. 2003. Transport and biodegradation of perchlorate in soils. J Environ Qual 32(1):40-46.

USEPA. 2005. Method 314.1. Determination of perchlorate in drinking water using inline column concentration/matrix elimination ion chromatography with suppressed conductivity detection. Cincinnati, OH: USEPA.

USEPA. 2008. Interim drinking water health advisory for perchlorate. Publication EPA 822-R-08-025. Washington, D.C.: Office of Water, U.S. Environmental Protection Agency.

USEPA. 2011. Fact sheet: final regulatory determination for perchlorate. Publication EPA 815-F-11-003. Washington, D.C.: Office of Water, U.S. Environmental Protection Agency.

Urbansky ET. 1998. Perchlorate chemistry: implications for analysis and remediation. Bioremediation Journal 2(22):81-95.

Urbansky ET. 2000. American Chemical Society. Division of Environmental Chemistry., American Chemical Society. Meeting. Perchlorate in the environment. New York: Kluwer Academic/Plenum. xv, 299 p. p.

Urbansky ET. 2002. Perchlorate as an environmental contaminant. Environ Sci Pollut Res Int 9(3):187-192.

Von Burg R. 1995. Toxicology update: perchlorates. Journal of Applied Toxicology 15(3):237-241.

Wu J, Unz RF, Zhang H, Logan BE. 2001. Persistence of perchlorate and the relative numbers of perchlorate- and chlorate-respiring microorganisms in natural waters, soils, and wastewater. Bioremediation Journal 5(2):119-130.

Xiao Y, Roberts DJ, Zuo G, Badruzzaman M, Lehman GS. 2010. Characterization of microbial populations in pilot-scale fluidized-bed reactors treating perchlorate- and nitrate-laden brine Water Res 44(14): 4029-4036.

Xu J, Song Y, Min B, Steinberg L, Logan BE. 2003. Microbial degradation of perchlorate: principles and applications. Environ Eng Sci 20(5): 405-422.

Zhang H, Logan BE, Regan JM, Achenbach LA, Bruns MA. 2005. Molecular assessment of inoculated and indigenous bacteria in biofilms from a pilot-scale perchlorate-reducing bioreactor. Microb Ecol 49(3): 388-398.

Zoeller RT, Rovett J. 2004. Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. Journal of Neuroendocrinology 16:809-818.

CHAPTER 3: Oyster Shell Waste Product Enhances Perchlorate Degradation in Sulfur Oxidizing Bacterial Consortium

This paper has been formatted for submission to the journal *Bioresource Technology* as:

Oyster shell waste product enhances perchlorate degradation in sulfur oxidizing bacterial consortium

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Abstract

The use of crushed oyster shells as an alkalinity source for autotrophic sulfur oxidizing perchlorate (ClO_4^-) reduction was found to enhance perchlorate degradation in previous studies. This study examines the role of oyster shells as a pH buffer, source of organic carbon for mixotrophic metabolism, biofilm attachment site and/or adsorbent in the sulfur oxidizing perchlorate reduction process. Perchlorate reduction kinetics were compared in microcosm studies carried out with different pH buffers and organic carbon sources. Microbial attachment was also examined by isolating sulfur pellets, oyster shells, or bacteria from the perchlorate-nutrient media using dialysis bags. The addition of oyster shells to the media maintained a favorable pH for perchlorate reduction (7.9); however, this mechanism alone could not explain the enhanced perchlorate reduction rates observed. Small amounts of organic carbon in the oyster shell matrix may have enhanced degradation, as the cultures were capable of mixotrophic metabolism. Addition of oyster shells that were heated to 550 °C impaired perchlorate degradation, possibly due to loss of oyster shell surface area or liberation of inhibitory compounds. The attachment studies indicated that oyster shells reduced the toxicity of the media, possibly by adsorption of hydrogen sulfide, a byproduct of sulfur disproportionation.

3.1 Introduction

Perchlorate (ClO_4^-) has been used in a number of applications including as a solid rocket fuel, in flares, fireworks, fertilizers and some munitions. Perchlorate contamination of surface and groundwater has been detected at over 150 sites in more than 38 states (Gu and Coates, 2006; MA DEP, 2006). Perchlorate may inhibit thyroid hormone production by interfering with iodine uptake (Capen, 1994; Urbansky, 1998). Although no maximum contaminant limit (MCL) has been specified for perchlorate by the US EPA, an Interim Health Advisory Level of 15 μg L^{-1} has been established (US EPA, 2008). Several US states have set their own MCLs for perchlorate, including Massachusetts at 2 μg L^{-1} and California at 6 μg L^{-1} (MA DEP, 2006; CDPH, 2007).

Current treatment technologies for perchlorate contaminated water include ion exchange (IX), reverse osmosis (RO), and biological treatment (Motzer, 2001; Urbansky, 2002). Biological perchlorate reduction offers a promising alternative to physical/chemical treatment processes because acclimated bacteria are able to completely degrade perchlorate under anaerobic conditions to the benign products chloride and oxygen (Coates and Achenbach, 2004; Urbansky, 1998). Perchlorate reducing bacteria are metabolically diverse and can use both organic and inorganic compounds as electron donors. Inorganic electron donors that have been used for biological perchlorate reduction include hydrogen gas and reduced iron and sulfur compounds (Ju et al., 2007; Kim and Logan, 2001; Sahu, 2008; Sahu et al., 2009; 2009; Son et al., 2006).

Several authors have enriched microbial communities capable of using elemental sulfur (S^o) as an electron donor for biological perchlorate reduction (Ju et al., 2007; 2008; Sahu, 2008; Sahu et al., 2009). The stoichiometric equation for the Sulfur Utilizing

Perchlorate Reducing Bacterial (SUPeRB) process was presented by Sahu et al. (2009) as:

$$2.87\ S^0 + 3.32\ H_2O + ClO_4^- + 1.85\ CO_2 + 0.462\ HCO_3^- + 0.462\ NH_4^+ \rightarrow$$
$$5.69\ H^+ + 2.87\ SO_4^{-2} + Cl^- + 0.462\ C_5H_7O_2N \qquad (1)$$

The reaction consumes alkalinity, requiring an alkalinity source to maintain a near neutral pH (7 to 8), which has been shown to be optimal for sulfur oxidizing perchlorate reduction (Conneely, 2011). Elemental sulfur and a solid phase buffer such as crushed oyster shells (OS) or limestone provide an excellent medium in packed bed reactors (PBR) for perchlorate reduction or denitrification, continuously supplying both an electron donor and an alkalinity source. Sengupta et al. (2007) compared solid-phase buffers for sulfur oxidizing denitrification and found that crushed oyster shell was a superior buffer over limestone or marble chips. Specifically, oyster shells had a higher dissolution rate, produced lower effluent turbidity, enhanced denitrification rates, and overall were found to be more cost effective than limestone or marble chips. In a similar study, Moon et al. (2006) found that oyster shell buffer achieved the highest denitrification rate compared with calcite or dolomite. The authors proposed that this was due to the high initial calcium carbonate ($CaCO_3$) dissolution rate of oyster shell. In addition, in sulfur oxidizing denitrification reactors used for onsite wastewater treatment less backwashing was required to maintain high denitrification rates in PBRs supplied with oyster shells compared to limestone (Sahu et al., 2009; Sengupta et al., 2007).

Oyster shells are byproducts of the shellfish industry that have been used in a number of applications including as a catalyst (Nakatani et al., 2009), construction material (Yoon et al., 2003), soil conditioner (Lee et al., 2008), and poultry feed

supplement (Scheideler, 1998). Several studies have investigated using oyster shells as a filter and adsorptive material for nutrient removal in wastewater treatment and for *in situ* remediation of nitrogen and phosphorous (Kwon et al., 2003; Park and Polprasert, 2009). Enhanced denitrification was observed in a closed, recirculating seawater system for culturing black tiger shrimp broodstock with addition of oyster shells and a supplemental organic carbon source (Menasveta et al., 2000). Asaoka et al. (2009) used oyster shells to adsorb hydrogen sulfide, which can be toxic to benthic organisms and can deplete dissolved oxygen.

Raw oyster shells are chemically composed of 93 to 97% $CaCO_3$, trace minerals, and 2% (w/w) organic carbon (Asaoka et al., 2009; Moon et al., 2006; Yoon et al., 2003). $CaCO_3$ mineral has a number of naturally occurring crystalline structures or polymorphs (Patnaik, 2003). The most common polymorphs are calcite, which is the most stable, and aragonite, which is highly crystallized and created by both physical and biological mechanisms (Patnaik, 2003). Oyster shells grow in two primary microstructures: hard, laminated layers horizontally oriented to the plane (sheet phase) and brittle, porous layers (bulky) between the sheet layers (Asaoka et al., 2009; Yoon et al., 2003). The nacre layer is the location of an organic matrix of $CaCO_3$ crystallized as aragonite (Balmain et al., 1999). An organic scleroprotein called *conchiloin* binds the layers together (Sengupta et al., 2007). Sengupta et al. (2007) also identified an abundance of nanoflakes in the oyster shell structure, which increases the total surface area.

In prior research in our laboratory (Sahu, 2008), oyster shells were shown to enhance sulfur oxidizing perchlorate degradation over limestone or phosphate buffered media in batch microcosm experiments. The possible benefits of using oyster shell in the

SUPeRB process include: 1) the oyster shells slowly dissolve to contribute an alkalinity source, maintaining the media at a favorable pH for perchlorate reduction, 2) trace amounts of organic matter in the untreated oyster shells are used for biosynthesis in mixotrophic metabolism, 3) the oyster shells reduce the toxicity of sulfur byproducts to the bacteria by adsorbing hydrogen sulfide produced by sulfur disproportionation, or 4) the solid oyster shell matrix is used as an attachment surface for SUPeRB. This research confirms that using oyster shells as an alkalinity source enhances the rate of sulfur oxidizing perchlorate reduction kinetics and also provides insight into mechanisms that enhance sulfur oxidizing perchlorate reduction.

3.2 Materials and Methods

3.2.1 Enrichment cultures

Cultures capable of using elemental sulfur as an electron donor for perchlorate reduction were enriched from a denitrifying wastewater seed using the method described by Sahu et al. (2009). Briefly, cultures were set up in 1-L glass flasks containing 4 mm sulfur pellets (30 g; Martin Midstream Partners, Seneca, IL), crushed oyster shells (10 g; Myco Supply, Pittsburgh, PA; >97% $CaCO_3$; sieve mesh size analysis results: 80% #4, 52% #6, 18% #10), and N_2 sparged local groundwater (Amherst, MA). Perchlorate and nutrients were added (mg L^{-1}: $NaClO_4$ (6.2), $(NH_4)_2SO_4$ (10), K_2HPO_4 (8.5), KH_2PO_4 (21.75)) to achieve a perchlorate concentration of 5 mg L^{-1} and nitrogen and phosphorous in excess of the stoichiometric requirements for biosynthesis (Equation 1). The flasks were inoculated with 250-mL of mixed liquor suspended solids (MLSS) supernatant from the denitrification zone of the Berkshire Mall wastewater treatment plant in Lanesboro,

MA. The cultures were maintained for a period of one year with fresh perchlorate and nutrients added each time the perchlorate concentration fell below 0.5 mg L^{-1}.

3.2.2 Oyster shell and buffer studies

A summary of the microcosm experiments performed in this study is shown in Table 3.1. An orthogonal matrix was used for the experimental design to ensure that all results were statistically independent. The base case consisted of elemental sulfur pellets, oyster shell buffer, and no organic carbon amendment. Buffer conditions and organic amendments within the microcosms were systematically altered from the base case to gain insight into how the oyster shell affected perchlorate biodegradation. To investigate the impact of oyster shells as a buffer, the base case was compared with limestone and no added buffer. The base case was compared to cultures with heat treated oyster shells to evaluate the role of the oyster shell associated organic matter as an organic carbon source. Organic matter was stripped from the heat treated oyster shells by combusting them for 15 minutes at 550^{o}C in a muffle furnace (following Standard Methods 2540 B for volatile solids). Yeast extract (Difco, Lawrence, KS) was added as a supplemental organic carbon source to both the base case and the heat treated oyster shell treatment at a concentration of 0.42 mg-YE L^{-1} (0.125 mg-C L^{-1}) which was approximately 10% of the stoichiometric requirements as shown in the following equations for perchlorate reduction using a protein (2) and a carbohydrate (3) as electron donors.

$$0.44C_4H_8O_2N + ClO_4^- + 0.44H^+ \rightarrow 1.78CO_2 + 0.44NH_4^+ + 1.33H_20 + Cl^- \quad (2)$$

$$2CH_2O + ClO_4^- + H^+ \rightarrow 2CO_2 + 2H_2O + Cl^- + H^+ \quad (3)$$

Killed (20 mg L^{-1} sodium azide) and uninoculated microcosms were run in parallel as controls for abiotic losses of perchlorate and to assess changes in water chemistry after addition of sulfur, oyster shells, and heat treated oyster shells. Analyses of anions, pH, protein, and other parameters were performed on the uninoculated controls after one week of incubation at 20^{o}C.

Microcosms were set up in triplicate in sterilized 125 mL glass serum bottles with rubber butyl stoppers and crimp tops. A minimal perchlorate media, free of sulfate and chloride, was developed to track accumulation of these metabolic byproducts. The media consisted of local groundwater (Amherst, MA), perchlorate (5 mg L^{-1}), and nutrients (10 mg L^{-1} $(NH_4)_2HPO_4$). Approximately 100 mL of minimal media was added to each of the bottles, which were sparged for 45 minutes with N_2 prior to adding 20 mL of the enrichment culture. Microcosms were set on a shaker table at 100 rpm in the dark in a 20^{o}C incubator. Samples were collected after a two week acclimation period and microcosms exhibiting active degradation (ClO_4^- <2.5 mg L^{-1}) were spiked to a perchlorate concentration of 10 mg L^{-1}. Data collection (day 0) began at this point. Because little change was observed in perchlorate concentrations in the no sulfur and killed control, perchlorate concentrations remained at 5 mg L^{-1} throughout the study in these microcosms.

Table 3.1: Microcosm treatments for buffer comparison, organic carbon supplementation, and uninoculated control experiments.

Treatment	S^0	Buffer	Additional Amendments	First-order Decay Rate (day^{-1} g $protein^{-1}$)
Base case	3.0 g	1.0 g OS[a]	None	23.7 (±0.005)
(-) Buffer	3.0 g	None	None	12.6 (±0.017)
Limestone Buffer	3.0 g	1.0 g Limestone	None	4.62 (±0.015)
(-) Organics	3.0 g	1.0 g HOS	None	4.94 (±0.039)
(+) YE	3.0 g	1.0 g OS	YE	35.5 (±0.001)
(-) Organics (+) YE	3.0 g	1.0 g HOS	YE	20.6 (±0.006)
(-) Sulfur[b]	None	1.0 g OS	None	0
Killed[b]	3.0 g	1.0 g OS	SA	0
(-) Bacteria[c]	3.0 g	1.0 g OS	None	---
(-) Bacteria	3.0 g	1.0 g HOS	None	---

[a]OS = oyster shells, HOS = heat treated oyster shells, YE = yeast extract, SA = sodium azide

[b]Note: No perchlorate removal was observed in these microcosms during the initial acclimation period so the perchlorate concentration remained at 5 mg L^{-1}.

[c]Uninoculated control microcosms were run in quadruplicate and analyzed after one week.

3.2.3 Attachment Studies

Cultures were set up in 50 mL conical tubes with 35 mL of perchlorate minimal media. Resazurin was added as a visual indicator of anaerobic conditions. Sulfur pellets (1.5 g), oyster shells (0.5 g) and enrichment culture inoculums were added to the tubes along with a membrane dialysis bag (Spectra/Por® Float-A-Lyzer® #G235031; Spectrum Laboratories, Inc., Rancho Dominguez, CA) in the configurations outlined in Figure 3.1. Each experimental setup was carried out in triplicate. Negative and positive controls were carried out in duplicate. To assess the importance of bacterial attachment to the sulfur and/or oyster shell on perchlorate reduction, each experimental setup varied which substrates were placed into the dialysis bag as follows: sulfur pellets (SP), oyster shells (OS), and bacteria (B). The positive control had sulfur pellets, oyster shells, and bacteria added directly to the tube with an empty dialysis bag. The negative control had the same configuration with no added bacteria. The conical tubes were sealed with tape and placed in a gas jar with BBL™ GasPak™ plus anaerobic system envelopes with palladium catalyst (BD, Sparks, MD). The jar was incubated at 20ºC. Perchlorate, chloride, and sulfate concentrations were measured at the beginning of the experiment (day 0) and on day 40.

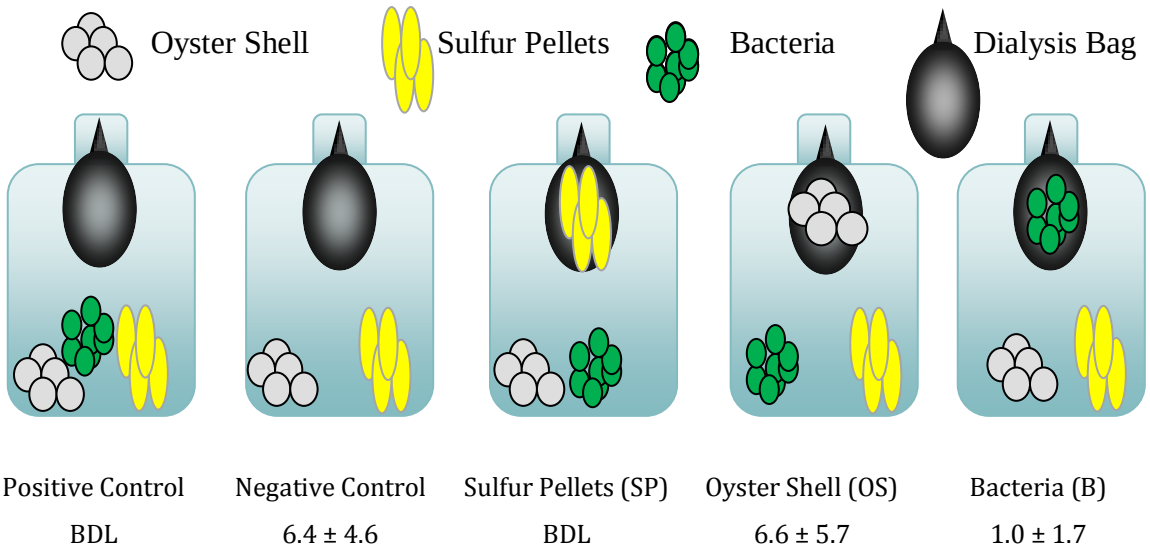

Figure 3.1: Attachment study experimental setup. Initial perchlorate concentration was 7.3 mg L^{-1}. Day 40 average perchlorate concentrations (mg L^{-1}) and standard deviations are shown below each figure.

3.2.4 Analytical Methods

Reagent grade chemicals (Fisher Scientific, Pittsburgh PA; Sigma-Aldrich, St. Louis MO) were used for all stock solutions and standards. Anion analyses were performed using a Metrohm (Riverview, FL) 850 Professional Ion Chromatograph AnCat MCS, equipped with an 858 Professional Sample Processor. A Metrosep A Supp 7-250 column was used to determine anion concentrations (ClO_4^-, Cl^-, NO_3^-, and SO_4^{2-}) following US EPA method 314.0. Other anions (F^-, Br^-, NO_2^-, and PO_4^{3-}) were analyzed using a Metrosep A Supp 5-250 column following Standard Methods 4110. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Due to the small sample volume, colorpHast pH indicator strips (#9578; pH 2-9) were used to measure pH (EM Science, Gibbstown, NJ) in microcosms. *Standard Methods* (Eaton et al., 2005) protocols were used to measure pH (4500-H+-B), DOC/TN (dissolved organic carbon; total nitrogen) (5310B), and conductivity (2510) for the oyster shell media composition analyses. DOC and TN were measured using a Shimadzu TOC-VCPN analyzer with TN unit and ASI-V auto sampler (Shimadzu Corporation, Kyoto, Japan).

3.2.5 Statistical Analyses

For each individual microcosm, perchlorate concentrations over time were plotted and fit with an exponential regression curve. Data points were checked for outliers, defined as falling more than two standard deviations away from the regression curve, and none were identified. The statistical significance of each set was determined by performing a paired Student *t* test ($\alpha = 0.05$) on the slopes of the regression lines at each time step versus the base case data set. This test determined, with a 95% confidence interval, whether the degradation seen in the experimental treatment was significantly different than the base case set (i.e. sulfur and oyster shell). Two-sample two-tailed *t* tests ($\alpha = 0.05$) assuming equal variances were performed to compare the results of the inoculated control microcosms.

3.3 Results

3.3.1 Oyster shell and buffer studies

In the base case treatment (sulfur and oyster shell), perchlorate was degraded from 10.6 mg L^{-1} to below detection limit (BDL = 1.0 μg L^{-1}) in 21 days (Figure 3.2). Based on the stoichiometry (Equation 1), it was expected that the chloride concentration

would increase as perchlorate was degraded in a 1:1 molar ratio, resulting in a final chloride concentration of 3.8 mg L^{-1}. However, the accumulated chloride over 21 days in the base case was 10.5 mg L^{-1}, which is 2.8 times greater than expected. The excess chloride may have been contributed by the oyster shells themselves, as it is known that oyster shells contain sodium chloride (Lee et al., 2007). In a study using oyster shells as a soil conditioner, the soluble NaCl concentration was determined to be 2.7 mg NaCl/1.0 g oyster shell (Lee et al., 2007). In the present study, this would equate to 13.1 mg L^{-1} using 1.0 gram of oyster shell, which could account for the excess chloride concentration. The uninoculated control (Table 3.2) accumulated chloride over seven days to a comparable concentration of 10.1 mg L^{-1}. The sulfate concentration was found to be 2.5 times higher than expected based on Equation 1, possibly due to sulfur disproportionation (discussed below), which has been seen in previous sulfur oxidizing perchlorate reduction studies (Ju et al., 2007; 2008). Note that Table 3.2 also shows a small measurable increase in nitrate in the uninoculated microcosms containing oyster shells. The SUPeRB cultures are known denitrifiers (Batchelor and Lawrence, 1978) and previous research on this consortium has shown that they are capable of simultaneous perchlorate reduction and denitrification (Sahu et al., 2009). Thus, the crushed oyster shells may have provided an additional source of electron acceptor to the anoxic consortium.

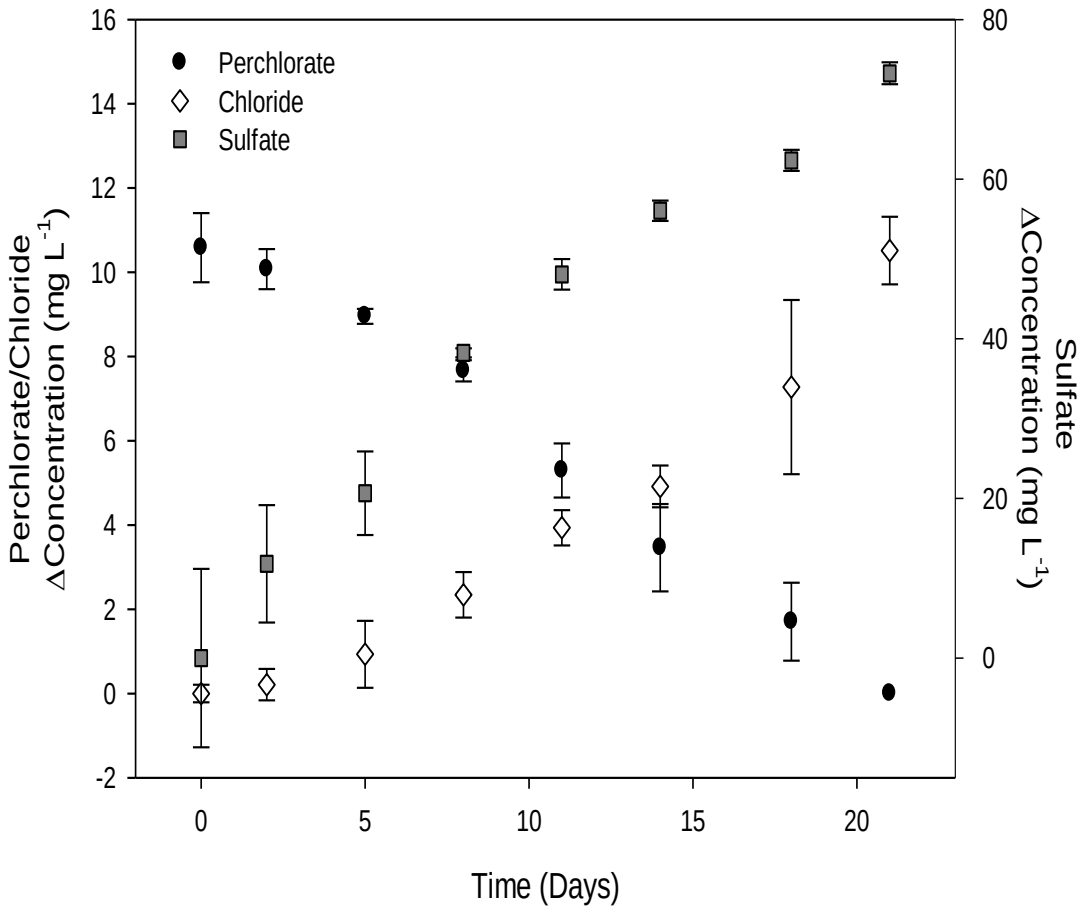

Figure 3.2: Base case perchlorate, chloride, and sulfate concentrations versus time. Chloride and sulfate concentrations show accumulation over time.

Table 3.2: Results of uninoculated control microcosms: base case and heat treated oyster shell. Each microcosm treatment was carried out in quadruplicate.

		μS	mg L^{-1}								
	pH	Conductivity	Protein	DOC	TN	Phosphate	Sulfate	Fluoride	Chloride	Nitrite	Nitrate
Media No Buffer	7.25	---	BDL	---	---	5.89	BDL	--	10.11	BDL	BDL
OS Buffer	7.63 ±0.05	160 ±8.57	3.13 ±2.46	1.48 ±0.35	2.26 ±0.12	6.81 ±0.15	21.1 ±0.52	0.43 ±0.08	10.11 ±0.11	BDL	0.42 ±0.03
HOS Buffer	8.50 ±0.27**	154 ±2.38	1.59 ±1.70	1.52 ±0.61	2.19 ±0.05	5.65 ±0.40**	23.7 ±1.69**	0.71 ±0.19**	10.12 ±0.20	BDL	0.49 ±0.07

HOS = heated oyster shell

**Indicates a statistical difference from the oyster shell buffer at $P<0.05$.

The pH values in the microcosms over a period of 28 days are shown in Figure 3.3. All microcosms were set up in media with an initial pH of 7.25. The pH changed in all microcosms within three days, with the exception of the oyster shell only treatment, where no perchlorate degradation occurred and the pH remained close to 7.0. The sulfur only treatment exhibited the lowest buffering capacity, with pH values decreasing steadily over the first 13 days of the incubation period to stabilize at 6.4. The pH in the microcosms with limestone buffer initially decreased to below 7.0 over five days, but increased to a final pH of 7.25. The rate of alkalinity dissolution has been found to be slower with limestone than oyster shells, which may explain the delay in buffering (Moon et al., 2006). The pH increased in both the oyster shell and heat treated oyster shell microcosms to the highest pH in the study of 7.9. The organic carbon amended treatments followed the same pattern as the unamended treatments (data not shown).

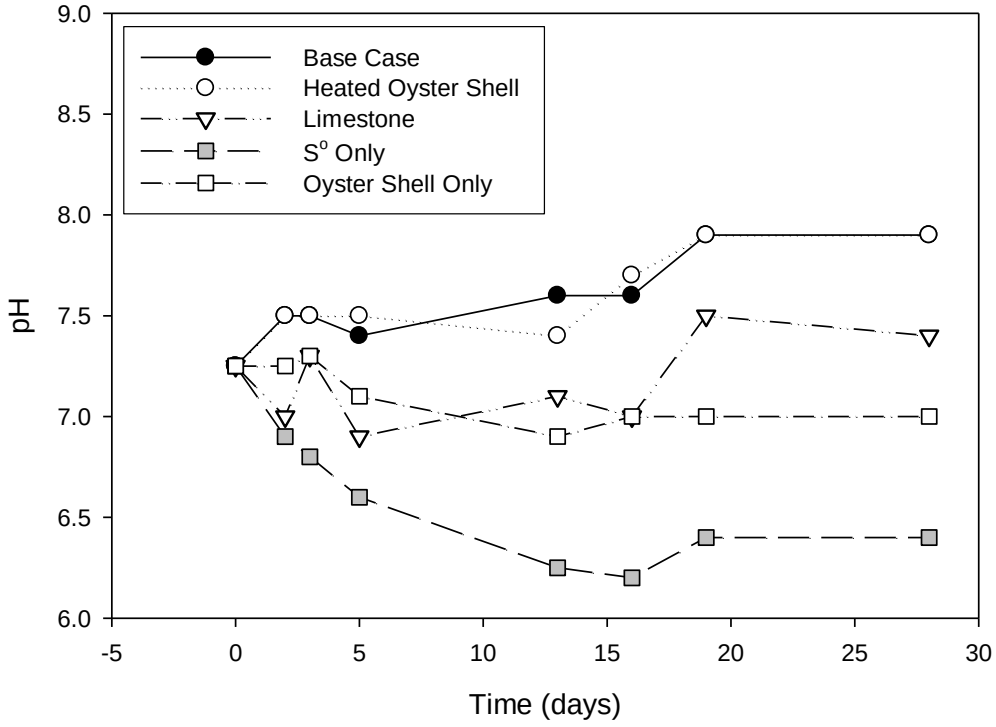

Figure 3.3: Microcosm pH versus time. Initial pH for all microcosms was 7.25 and final pH ranged from 6.4 to 7.9.

Plots of normalized perchlorate concentration versus time in the microcosms are shown in Figures 3.4, 3.5, and 3.6. Pseudo-first-order perchlorate decay coefficients were calculated from the data (summarized in Table 3.1**)** and were tested for significant differences (P=0.05). Note that care was taken to maintain the same initial biomass concentration in all microcosms so that differences in degradation rates were not due to differences in biomass density. The first-order perchlorate decay coefficient in the base case ($k=23.7\ day^{-1}\ g\ protein^{-1}$) was significantly higher than with the limestone buffer ($k=4.62\ day^{-1}\ g\ protein^{-1}$) treatment (Figure 3.4). No lag period was observed in either treatment, indicating that the organisms were acclimated to each condition prior to the start of the experiment. The observed enhanced degradation in the treatment with oyster shell buffer was similar to the results of previous sulfur oxidizing perchlorate reduction and denitrification studies that compared oyster shells with limestone and other $CaCO_3$ buffers (Moon et al., 2006; Sahu, 2008; Sengupta et al., 2007).

Perchlorate reduction occurred at a higher rate under the base case conditions (elemental sulfur and oyster shell), than in the sulfur only, oyster shell only, and heat treated oyster shell with sulfur treatments (Figure 3.5). The presence of oyster shell was found to improve the rate of perchlorate reduction compared with sulfur oxidizing microcosms without buffer ($k=12.6\ day^{-1}\ g\ protein^{-1}$), though the differences were not found to be significant. Little degradation was observed in the microcosms with oyster shell and no sulfur, showing that sulfur is an essential component in the SUPeRB process and that organic matters in the oyster shell were not present in adequate amounts for significant heterotrophic perchlorate degradation. Heating the oyster shells had a significant negative impact on the perchlorate reduction. A 14 day lag period was

observed and the rate decreased by a factor of 4.7. The effect of heat treatment on oyster shell chemistry is discussed in detail below.

Yeast extract was added to provide a supplemental carbon source to microcosms containing sulfur and oyster shells and sulfur and heat treated oyster shells (Figure 3.6). The yeast extract was added at a concentration below the stoichiometric requirement to serve as an electron donor for perchlorate reduction (Equations 2 and 3). The addition of sources of organic carbon improved perchlorate degradation in both treatments. In the base case supplemented with yeast extract, the degradation rate ($k=35.5\ day^{-1}\ g\ protein^{-1}$) was nearly four times that of the unamended base case. The degradation rate in the heat treated oyster shells with yeast extract increased fivefold ($k=20.6\ day^{-1}\ g\ protein\text{-}^{1}$), which was approximately the same as in the base case rate.

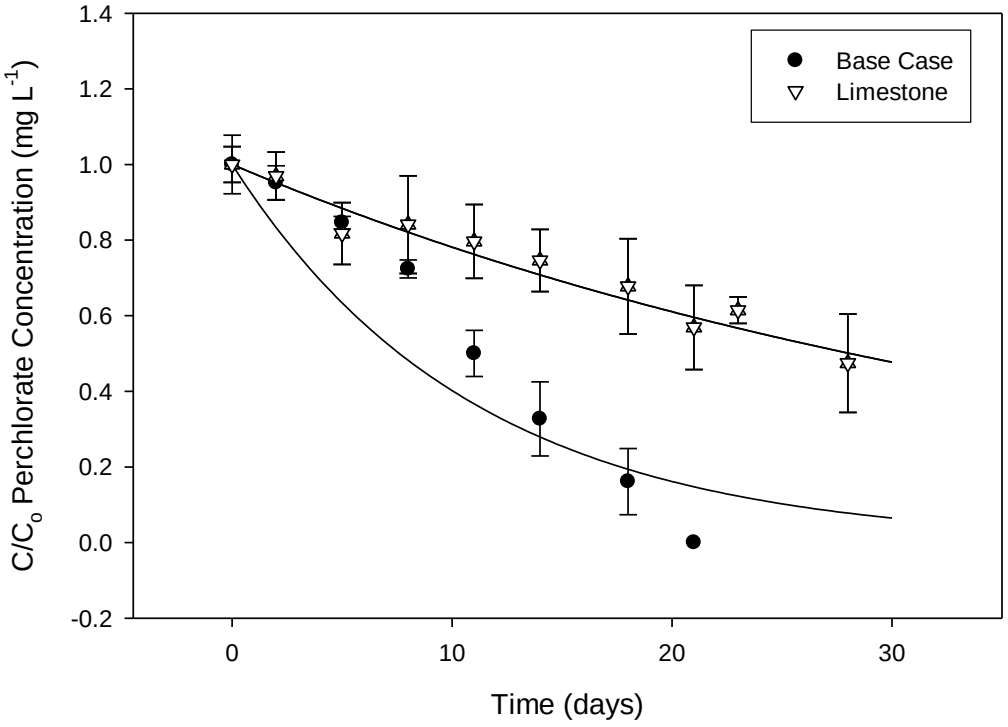

Figure 1: Normalized perchlorate concentrations (initial conc. 11 mg L^{-1}) versus time for microcosms set up with sulfur. The buffer treatments with first order decay coefficients were: oyster shells (23.7 day^{-1} g $protein^{-1}$) and limestone buffer (4.62 day^{-1} g $protein^{-1}$).

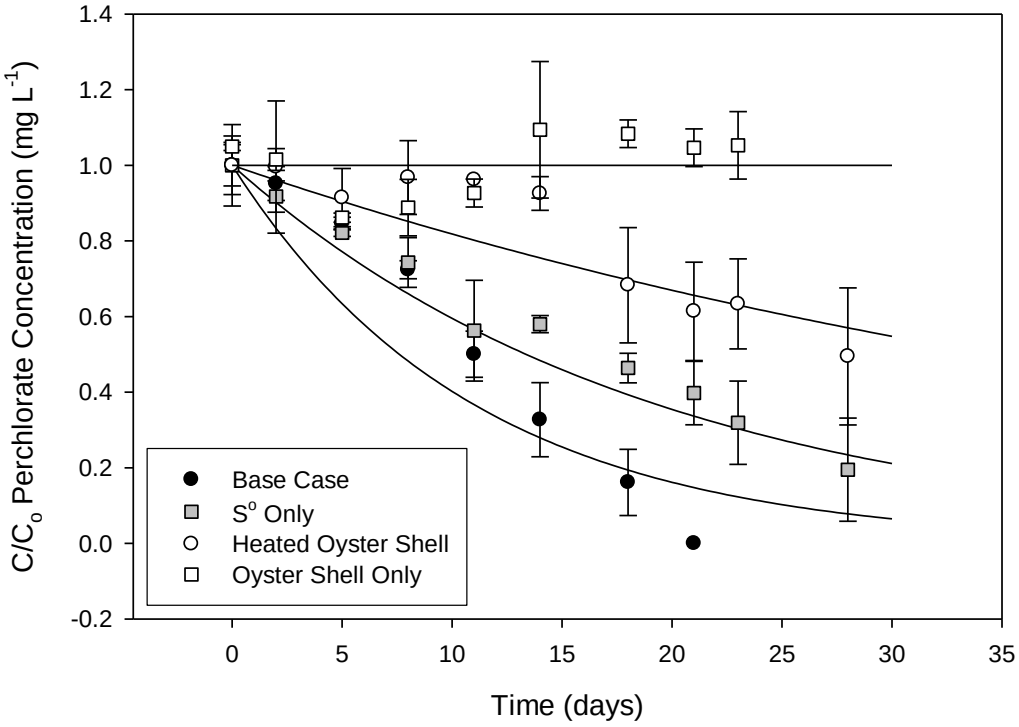

Figure 3.5: Normalized perchlorate concentrations (initial conc. 11 mg L^{-1}) versus time for variations of the base case (sulfur and oyster shells; k = 23.7 day^{-1} g $protein^{-1}$) with heat treated oyster shells (4.94 day^{-1} g $protein^{-1}$) and sulfur without oyster shells (12.6 day^{-1} g $protein^{-1}$). Oyster shells without sulfur showed no perchlorate degradation at 5 mg L^{-1} perchlorate. The base case is plotted again for comparison with other treatments.

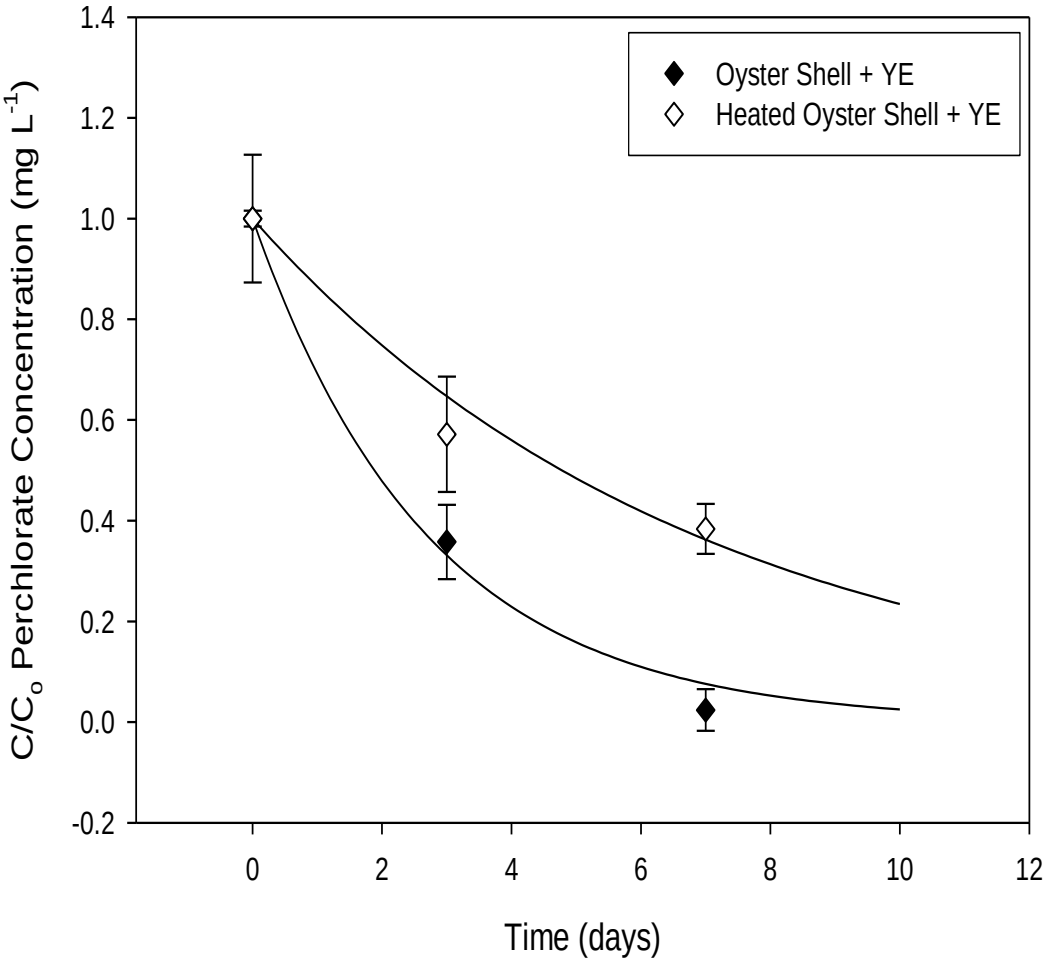

Figure 3.6: Normalized perchlorate concentrations (initial conc. 10 mg L^{-1}) versus time of oyster shells (k = 35.5 day^{-1} g $protein^{-1}$) and heat treated oyster shells (20.6 day^{-1} g $protein^{-1}$) amended with yeast extract (0.125 mg-C L^{-1}).

3.3.2 Attachment studies

Perchlorate was reduced to BDL in the positive control as well as when the sulfur pellets (SP) were separated from the oyster shell and bacteria (Figure 3.1). When the bacteria (B) were separated from the oyster shell and sulfur pellets two of the three replicates reduced perchlorate completely, while the third reduced perchlorate by 54%. The slowed degradation rate in this treatment may have been due to a reduced rate of mass transfer of the substrates to the bacteria imposed by the dialysis bag, as cultures were not shaken. No perchlorate reduction was observed in two of the three replicates when the oyster shell (OS) was separated from the sulfur pellets and bacteria, while the third reduced perchlorate completely.

3.4 Discussion

3.4.1 Oyster shell as pH buffer

Oyster shells have been successfully used to maintain an optimum pH for both biological sulfur oxidizing denitrification and perchlorate reduction in prior studies (Moon et al., 2006; Sahu et al., 2009; Sengupta et al., 2007). Conneely (2011) conducted a series of microcosm studies with the SUPeRB consortium at varying pH values between 4.5 and 8.0 (in pH increments of 0.5) and found the highest perchlorate degradation rates at pH values above 7.5. In this study, however, no clear trend was established between final pH and perchlorate degradation rate. For the microcosms without added yeast extract (Figures 3.4 and 3.5), the highest perchlorate degradation rates were observed in the base case (final pH = 7.9) followed by sulfur only (final pH = 6.4), heat treated oyster shells (final pH = 7.9), and limestone (final pH = 7.4).

Although heat treated oyster shells maintained the pH in the optimal range, heat treatment was generally found to inhibit perchlorate degradation, even in microcosms supplemented with yeast extract (Figure 3.6). The structural and chemical composition of oyster shell is known to change at temperatures between 400^{o}C and 450^{o}C due to the decomposition of the bulky layer between sheets (Asaoka et al., 2009; Balmain et al., 1999; Moon et al., 2006; Yoon et al., 2003; Lee et al. 2008). Heating also drives the conversion of $CaCO_3$ to CaO in carbonaceous minerals at 400^{o}C, with complete destruction of the organic matrix at temperatures between 550 and 600^{o}C (Asaoka et al., 2009; Balmain et al., 1999). The specific surface area for oyster shells heated to 400^{o}C (Asaoka et al 2009) was much lower (0.25 m^2g^{-1}) than studies using unheated oyster shells (1.72-9.59 m^2g^{-1}) (Park and Polprasert 2008; Yoon et al., 2003). A significantly higher pH (8.5) was observed in the uninoculated heat treated oyster shell control compared to the uninoculated base case (7.6), possibly due to the rapid dissolution of $CaCO_3$ nanoflakes (Table 3.2). Both sulfate and fluoride concentrations were also found to be significantly higher for the heat treated oyster shells, though the differences are not great enough to explain inhibition due to toxicity. The phosphate concentration in the heat treated oyster shell was significantly lower than in the base case; however, the concentrations were still substantially higher than the stoichiometric requirements for perchlorate reduction (0.001 mg-P/mg-ClO_4^-).

3.4.2 Oyster shell contributes to mixotrophic metabolism

The highest rate of perchlorate degradation was observed when the base case microcosms were supplemented with yeast extract at 10% of the stoichiometric requirements for heterotrophic perchlorate reduction (Figure 3.6). The increased rate of

perchlorate reduction in the yeast extract amended microcosms may have occurred if the organisms were capable of mixotrophic metabolism. In the presence of organic carbon compounds, some chemolithotrophic bacteria can switch from autotrophic CO_2 fixation to the more thermodynamically favorable mixotrophic metabolism, by using an inorganic electron donor for energy metabolism and an organic carbon source for cellular synthesis (Madigan et al., 2003). Note that the use of heat treated oyster shells in this study was intended to destroy the organic content in the nacre layer, allowing us to test the hypothesis that the oyster shells provided a small amount of organic carbon for mixotrophic metabolism. However, as discussed previously, heat treated oyster shells were found to have an inhibitory effect on sulfur oxidizing perchlorate reduction.

Oh et al. (2001) examined the impact of supplemental organics on sulfur oxidizing denitrification and found that mixotrophic conditions produced the highest denitrification rates, the lowest sulfate production rates, and reduced alkalinity consumption. The authors proposed that some portion of nitrate was reduced by heterotrophic denitrification and that the remaining nitrate was reduced via autotrophic denitrification. In this study, it is unlikely that heterotrophic metabolism occurred in the absence of yeast extract, since no degradation was observed in the oyster shell only treatment. The calculated dissolved organic carbon (DOC) concentration required to reduce 10.6 mg L^{-1} of perchlorate would be 2.27 mg-C L^{-1} (Equation 2). However, the average measured DOC concentration in the uninoculated control microcosms for the base case was 1.48 mg-C L^{-1}, which is insufficient to serve as a sole electron donor for perchlorate reduction, even if all the organic matter present was bioavailable. Organic matter present in the oyster shell matrix (1.44 mg-C L^{-1}), could theoretically consume 6.9

mg L^{-1} perchlorate; however some of the carbon may be recalcitrant and unavailable to the bacteria. Additionally, the cultures had been enriched under sulfur oxidizing conditions for more than one year prior to the experiment.

3.4.3 Oyster shell as adsorptive material

Hydrogen sulfide (H_2S) is known to be toxic to aquatic life and some metabolic processes (Bagarinao, 1992) and is often present in systems using sulfur compounds for energy. Although the bacteria use elemental sulfur as an electron donor, toxic sulfur byproducts may inhibit metabolism when present in sufficient concentrations. Sulfur disproportionation is a metabolic reaction where elemental sulfur is used as both an electron donor and acceptor, generating sulfate and hydrogen sulfide end products (Ju et al., 2007; 2008). Additionally, sulfate may be reduced to generate hydrogen sulfide by sulfate reducing bacteria using dead biomass as an electron donor. Ju et al. (2008) found perchlorate degradation rates were higher in microcosms using lentil shaped sulfur pellets with a lower surface area than powdered sulfur at the same concentration. The powdered sulfur may have produced more toxic sulfur species due to the high surface area available for microbially mediated and chemical reactions. Though sulfide concentrations were not measured in this study, excess sulfate was detected, which could be explained if this type of metabolism were occurring.

Oyster shells have been used to effectively adsorb both phosphate and hydrogen sulfide gas in aquatic environments (Asaoka et al., 2009; Kwon et al., 2004). The oyster shell in the current study appears to reduce the toxicity of elemental sulfur, possibly by acting as an adsorptive surface for hydrogen sulfide. When the oyster shells were isolated in a dialysis bag (Figure 3.1), both the ability to adsorb toxins and the rate of perchlorate

degradation were impaired. However, having the oyster shell near either the bacteria or the sulfur pellets was effective for promoting perchlorate removal.

3.4.4 Oyster shell as microbial attachment surface

No clear conclusions could be drawn from the attachment studies about the necessity of direct bacterial attachment to the sulfur pellets or to the oyster shell. The results suggest that bacterial proximity to oyster shell is more important than proximity to elemental sulfur. When the oyster shell was near either bacteria or sulfur pellets, perchlorate degradation occurred. However, perchlorate reduction did not occur when the oyster shell was separated.

3.5 Conclusions

The purpose of this study was to investigate the role of oyster shell as a buffer, organic carbon source, attachment site, and adsorbent in the SUPeRB process. Major findings of the study were:

- The oyster shell maintained a favorable pH for perchlorate reduction; however, the pH values observed could not completely explain the enhanced perchlorate reduction rates compared with limestone or no buffer.
- The SUPeRB cultures were capable of mixotrophic metabolism, as evidenced by improved degradation when yeast extract was added at 10% of the stoichiometric requirements. Heating oyster shells to remove organics impaired perchlorate degradation; however, issues with toxicity confounded results suggesting that the methodology used in this study was not a good approach for clearly determining the role of organics in oyster shells.

- Evidence was found that oyster shells reduce bacterial toxicity, possibly by adsorption of hydrogen sulfide, which can be produced through sulfur disproportionation. When the oyster shell was near either bacteria or sulfur pellets, perchlorate degradation occurred. Perchlorate degradation was inhibited when the oyster shell was separated from the bacteria.
- The sulfur pellets alone in the attachment study were found to inhibit perchlorate degradation. The necessity for direct microbial attachment to the solid oyster shell matrix was unclear, as degradation still occurred in one of the treatments when the oyster shell was separate.

Acknowledgements

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Chapter 3 References

Asaoka, S., Yamamoto, T., Kondo, S., Hayakawa, S., 2009. Removal of hydrogen sulfide using crushed oyster shell from pore water to remediate organically enriched coastal marine sediments. Bioresource Technolo. 100, 4127-4132.

Bagarinao, T., 1992. Sulfide as an environmental toxicant: tolerance and adaptations in aquatic organisms. Aquat. Toxicol. 24, 21-62.

Balmain, J., Hannoyer, B., Lopez, E., 1999. Fourier transform infrared spectroscopy (FTIR) and x-ray diffraction analyses of mineral and organic matrix during heating of mother of pearl (nacre) from the shell of the mollusc *Pinctada maxima*. J. Biomed. Mater. Res. 48, 749-754.

Capen, C.C., 1994. Mechanisms of chemical injury of thyroid gland. Prog. Clin. Biol. Res. 387, 173-191.

CDPH. 2007. Perchlorate in drinking water. California Department of Public Health. Publication CDPH R-16-04. Sacramento, CA.

Coates, J.D., Achenbach, L.A., 2004. Microbial perchlorate reduction: rocket-fueled metabolism. Nat. Rev. Microbiol. 2, 569-580.

Conneely, T., 2011, Investigation of a sulfur-utilizing perchlorate-reducing bacterial consortium [Ph.D.], Amherst: University of Amherst, p 188.

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg, A.E., Franson, M.A.H., 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington DC.

Gu, B., Coates, J.D., 2006. Perchlorate: environmental occurrence, interactions and treatment. Springer, New York.

Ju, X., Field, J.A., Sierra-Alvarez, R., Salazar, M., Bentley, H., Bentley, R., 2007. Chemolithotrophic perchlorate reduction linked to the oxidation of elemental sulfur. Biotechnol. Bioeng. 96,1073-1082.

Ju, X., Sierra-Alvarez, R., Field, J.A., Byrnes D.J., Bentley, H., Bentley, R., 2008. Microbial perchlorate reduction with elemental sulfur and other inorganic electron donors. Chemosphere. 71, 114-122.

Kim, K., Logan, B.E., 2001. Microbial reduction of perchlorate in pure and mixed culture packed-bed bioreactors. Water Res. 35, 3071-3076.

Kwon, H.B., Lee C.W., Jun, B.S, Yun, J., Weon, S.Y., Koopman, B., 2004. Recycling waste oyster shells for eutrophication control. Resour. Conserv. Recy. 41, 75-82.

Lee, C.H., Lee, D.K., Ali, M.A., Kim, P.J., 2008. Effects of oyster shell on soil chemical and biological properties and cabbage productivity as a liming material. Waste Manage. 28, 2702-2708.

Lee, S.W., Kim, Y.M., Kim, R.H., Choi, C.S., 2008. Nano-structured biogenic calcite: a thermal and chemical approach to folia in oyster shell. Micron. 39, 380-386.

Madigan, M.T., Martinko, J.M., Parker, J. 2003. Brock biology of microorganisms, 10th ed. Prentice Hall/Pearson Education, Upper Saddle River, New Jersey.

MA DEP. 2006. Maximum contaminant level for inorganic chemicals. Massachusetts Department of Environmental Protection. Office of Research and Standards. Code of Massachusetts Regulations 310 CMR 22.06. Boston, MA.

Menasveta, P., Panritdam, T., Sihanonth, P., Powtongsook, S., Chuntapa, B., Lee, P., 2001. Design and function of a closed, recirculating seawater system with denitrification for the culture of black tiger shrimp broodstock. Aquacult. Eng. 25, 35-49.

Moon, H.S., Nam, K., Kim, J.Y., 2006. Initial alkalinity requirement and effect of alkalinity sources in sulfur-based autotrophic denitrification barrier system. J. Environ. Eng.-ASCE. 132, 971-975.

Motzer, W.E.. 2001. Perchlorate: problems, detection, and solutions. Environ. Forensics 2:301-311.

Nakatanki, N., Takamori, H., Tankeda, K., Sakugawa, H., 2009. Transesterifciation of soybean oil using combusted oyster shell waste as a catalyst. Bioresour. Technol. 100, 1510-1513.

Oh, S.E., Yoo, Y.B., Young, J.C., Kim, I.S., 2001. Effects of organics on sulfur-utilizing autotrophic denitrification under mixotrophic conditions. J. Biotechnol. 92, 1-8.

Park, W.H., Polprasert, C., 2008. Roles of oyster shells in an integrated constructed wetland system designed for P removal. Ecol. Eng. 34, 50-56.

Patnaik, P. 2003. Handbook of inorganic chemical. McGraw-Hill Companies, New York.

Rittman, B.E., McCarty, P.L., 2001. Environmental Biotechnology: Principles and Applications. McGraw-Hill Companies, New York.

Sahu, A.K., 2008. Biological reduction of nitrate and perchlorate by autotrophic bacteria. [Ph.D.], Amherst: University of Amherst, p 265.

Sahu, A.K., Conneely, T., Nusslein, K.R, Ergas, S.J., 2009. Biological perchlorate reduction in packed bed reactors using elemental sulfur. Environ. Sci. Technol. 43, 4466-4471.

Scheideler, S.E. 1998. Eggshell calcium effects on egg quality and Ca digestibility in first- or third-cycle laying hens. J. Appl. Poultry Res. 7, 69-74.

Sengupta, S., Ergas, S.J., Lopez-Luna, E., 2007. Investigation of solid-phase buffers for sulfur-oxidizing autotrophic denitrification. Water Environ. Res. 79, 2519-2526.

Son, A., Lee, J., Chiu, P.C., Kim, B.J., Cha, D.K., 2006. Microbial reduction of perchlorate with zero-valent iron. Water Res. 40, 2027-2032.

USEPA. 2008. Interim drinking water health advisory for perchlorate. Publication EPA 822-R-08-025. Washington, D.C.: Office of Water, U.S. Environmental Protection Agency.

Urbansky E.T., 1998. Perchlorate chemistry: implications for analysis and remediation. Bioremediation Journal 2, 81-95.

Urbansky, E.T., 2002. Perchlorate as an environmental contaminant. Environ Sci. Pollut. Res. Int. 9, 187-192.

Yoon, G.L., Kim, B.T., Kim, B.O., Han, S.H. 2003. Chemical-mechanical characteristics of crushed oyster-shell. Waste Manage. 23, 825-834.

CHAPTER 4: Conclusions

This study investigated the operating conditions and performance of a pilot scale upflow PBR packed with elemental sulfur pellets and the use of crushed oyster shells for treatment of low level perchlorate contaminated water. Molecular biology tools were employed in tandem with remediation studies to gain insight into fluctuations in water quality and reactor performance. Additional laboratory studies were conducted to evaluate the role of oyster shells in the SUPeRB process as a buffer, organic carbon source, adsorbent, and attachment site.

- The SUPeRB consortium was successfully used in the operation a pilot scale PBR for biological perchlorate reduction. The PBR removed 96% of the influent perchlorate in the groundwater at an empty bed contact time (EBCT) of 12 hours (effluent ClO_4^- of 4.2 µg L^{-1}).
- Sodium sulfite provided a good method of dissolved oxygen removal in batch cultures, but was found to promote the growth of sulfate reducing bacteria, which inhibited perchlorate reduction in the pilot system.
- Simultaneous perchlorate and nitrate degradation was observed in the lower half of the reactor before reactions shifted to sulfur disproportionation. Additionally, there was evidence of some RDX degradation throughout the study, though future work is needed to optimize this reaction.
- Analyses of water quality profiles were supported by molecular analysis showing distinct groupings of perchlorate and nitrate degrading organisms close to the inlet of the PBR, while sulfur disproportionation was the primary biological process occurring in the top of the reactor.

- Crushed oyster shell buffer enhanced perchlorate degradation in SUPeRB cultures ($k=23.7\ day^{-1}$ g $protein^{-1}$) significantly more than limestone or no buffer. The oyster shells maintained a favorable pH (7 to 8) for perchlorate reduction.
- SUPeRB cultures are capable of mixotrophic metabolism and may use the organic matter in the oyster shells as an organic carbon source for cellular synthesis. Supplementing organic carbon at 10% stoichiometric requirements for heterotrophic perchlorate reduction increased the rate of perchlorate degradation ($k=35.5\ day^{-1}$ g $protein^{-1}$).
- The necessity for direct microbial attachment to the solid oyster shell matrix was unclear, though proximity to oyster shells was found to be important. Oyster shells may reduce the bacterial toxicity of the sulfur, possibly by hydrogen sulfide adsorption.

The results show that the SUPeRB process is a viable option for use in engineered systems treating perchlorate contaminated water. The crushed oyster shell packing material is central to maintaining an optimum pH, and may provide organic carbon for cellular synthesis, and reduce bacterial toxicity in the SUPeRB process. Future work should examine mechanisms behind sulfide production, adsorption, and toxicity in the SUPeRB process, as sulfide was a key inhibitory factor in both studies.

APPENDIX A: Raw Data—Bioreactor Study

DATE	DAY OF OPERATION	NOTES	Sample	Spike
11-Oct-08		Filled Bioreactor with S:OS		
RECIRC				
23-Dec-08	0	Inoculation	Sample	Spike - 13 PPM
26-Dec-08	3		Sample	NO
29-Dec-08	6		Sample	NO
2-Jan-09	10		Sample	NO
9-Jan-09	17	Initial Spike Degraded	Sample	Spike -
16-Jan-09	24		Sample	Spike -
24-Jan-09	32		Sample	Spike -
30-Jan-09	38		Sample	Spike -
8-Feb-09	47		Sample	Spike -
15-Feb-09	54		Sample	Spike -
22-Feb-09	61		Sample	Spike -

FLOW THRU FAILURE				
1-Mar-09	68	RM/KN failed flow thru	Sample	NO
13-Mar-09	80	Rob tries to fix flow thru	Sample	NO
18-Mar-09	85	SE, KN, TC, AB trip to MMR fix up failed flow thru	Sample	NO
5-Apr-09	103	RM TC		Spike-
11-Apr-09	109		Sample	Spike-
17-Apr-09	115	PN only		Spike-
24-Apr-09	122	PN only	NO	Spike-
08-May-09	136		Sample	
18-May-09	*136*	*PN only*	*NO*	*Spike-*
19-May-09	147	AJ help, install GAC filter	Sample	Spike-
03-Jun-09	162	Flushed and run overnight	Sample	
11-Jun-09	170		Sample	

Ident	Chloride Conc	Average Cl Conc	Standard Dev. Chloride	Sulfate Conc	Average Sulfate Conc.	Standard Dev. Sulfate	Perchlorate Conc	Average Perchlorate	Standard Dev. Perchlorate	pH	Average pH	Standard Dev. pH

FLOW THRU	DAY OF OPERATION	EFFLUENT (µg/L)	INFLUENT (µg/L)	Percent Removal	NOTES		Flow Rate (mL/min)	EBCT (min)	EBCT (hr)
17-Jun-09	176	48.2	96.4	50.0%	Flow Through Start; Flow rate 250 mL/min		250	1068	17.8
25-Jun-09	184	28.3	102.3	72.3%			250	1068	17.8
6-Jul-09	195	44.0	97.2	54.7%	Install IX resin	RDX	250	1068	17.8
15-Jul-09	204				Flow rate increased to 360 mL/min, bubbled with N2 gas to flush reactor, skimmed microbial mat		360	742	12.4
21-Jul-09	210	57.0	96.4	40.9%	Internal Recirculation ON; Flow rate decreased to 250 mL/min	RDX	250	1068	17.8
26-Jul-09	215	23.0	84.5	72.8%		RDX	250	1068	17.8
6-Aug-09	226	63.5	104.0	38.9%			250	1068	17.8
12-Aug-09	232	54.0	113.0	52.2%	Sulfite Addition Terminated		250	1068	17.8
18-Aug-09	238	43.0	104.0	58.7%			250	1068	17.8
27-Aug-09	247				Internal Recirculation OFF		250	1068	17.8
30-Aug-09	250	59.0	96.4	38.8%	Profile by Port		250	1068	17.8
11-Sep-09	262	30.8	94.4	67.4%			250	1068	17.8
20-Sep-09	271	17.0	92.5	81.6%		RDX	250	1068	17.8
30-Sep-09	281	22.0	85.2	74.2%		RDX	250	1068	17.8
7-Oct-09	288	10.1	93.5	89.2%	Flow rate increased to 360 mL/min		360	742	12.4
11-Oct-09	292	11.8	82.5	85.7%	Tracer Study		360	742	12.4
15-Oct-09	296	27.4	103.7	73.6%	Flow rate increased to 375 mL/min		375	712	11.9
24-Oct-09	305	4.2	96.4	95.6%			375	712	11.9
29-Oct-09	310	6.7	96.4	93.1%	Profile by Port; Bioreactor Decommission		375	712	11.9

102909	mg/L	mg/L		mg/L			ug/L					
Effluent-2	8.729	8.765	0.051	68.102	68.213	0.157	7.274	7.618	0.486	8.936	8.875	0.086
Effluent-1	8.801			68.324			7.961			8.814		
Port 8-2	8.738	8.725	0.019	68.682	68.711	0.040	5.814	6.655	1.189	8.963	8.965	0.003
Port 8-1	8.711			68.739			7.495			8.967		
Port 7-2	8.727	8.726	0.001	69.781	70.096	0.446	7.428	6.998	0.608	9.015	9.0195	0.006
Port 7-1	8.725			70.411			6.568			9.024		
Port 6-2	8.700	8.714	4.94	79.362	81.155	2.536	9.402	8.305	1.551	8.876	8.879	0.004
Port 6-1	8.728			82.948			7.208			8.882		
Port 5-2	8.713	8.713	0.000	81.473	82.444	1.373	8.564	8.528	0.051	8.839	8.86	0.030
Port 5-1	8.713			83.415			8.492			8.881		
Port 4-2	8.727	8.723	0.005	73.659	73.740	0.115	9.015	8.833	0.257	8.775	8.7885	0.019
Port 4-1	8.719			73.822			8.651			8.802		
Port 3-2	8.679	8.694	0.021	52.868	52.795	0.103	16.984	16.782	0.286	7.962	7.935	0.038
Port 3-1	8.708			52.723			16.580			7.908		
Port 2-2	8.712	8.687	0.034	45.250	45.161	0.126	33.850	33.753	0.138	7.452	7.389	0.089
Port 2-1	8.663			45.071			33.655			7.326		
Port 1-2	8.681	8.679	0.003	23.501	21.086	3.415	74.463	78.305	5.433	6.828	6.607	0.313
Port 1-1	8.677			18.672			82.146			6.386		
Influent-2	8.575	8.602	0.039	5.475	5.499	0.033	95.677	95.398	0.395	6.391	6.4785	0.124
Influent-1	8.630			5.522			95.119			6.566		

sample date	Day of operation	RDX inf	RDX eff	% removal
		µg/L	µg/L	
70709	195	7.2	4.1	43%
72209	210	10	7.65	24%
72709	215	10	7	30%
82109	271	8.8	7	20%
93009	281	10.37	9.57	8%

Average	9.27	7.06
Standard	1.30	1.96

APPENDIX B: Raw Data—Oyster Shell Study

Oyster Shell

Day	OS1	OS2	OS3	Average	STD	C/Co	STD/Co
0	9.93	11.50	10.32	10.58	0.82	1.00	0.08
2	10.62	9.87	9.73	10.07	0.48	0.95	0.04
5	8.78	9.13	8.95	8.95	0.18	0.85	0.02
8	7.49	7.54	7.95	7.66	0.25	0.72	0.02
11	5.08	4.79	6.02	5.30	0.64	0.50	0.06
14	2.93	2.80	4.66	3.46	1.04	0.33	0.10
18	1.13	1.22	2.78	1.71	0.93	0.16	0.09
21	0.00	0.00	0.00	0.00	0.00	0.00	0.00
23							
28							

Sulfur Only

Day	S1	S2	S3	Average	STD	C/Co	STD/Co
0	11.07	11.50	10.32	10.97	0.60	1.00	0.05
2	10.58	9.87	9.73	10.06	0.45	0.92	0.04
5	8.95	9.13	8.95	9.01	0.11	0.82	0.01
8	8.95	7.54	7.95	8.15	0.72	0.74	0.07
11	7.70	4.79	6.02	6.17	1.46	0.56	0.13
14	6.08	6.43	6.56	6.36	0.25	0.58	0.02
18	4.64	5.14	5.49	5.09	0.43	0.46	0.04
21	3.42	4.40	5.26	4.36	0.92	0.40	0.08
23	2.28	3.52	4.70	3.50	1.21	0.32	0.11
28	0.87	1.76	3.79	2.14	1.50	0.19	0.14

Phosphate Buffer

Day	P1	P2	P3	Average	STD	C/Co	STD/Co
0	11.01	11.94	12.32	11.76	0.68	1.00	0.06
2	10.54	11.49	11.51	11.18	0.55	0.95	0.05
5	11.62	10.91	9.06	10.53	1.32	0.90	0.11
8	9.38	9.08	9.64	9.36	0.28	0.80	0.02
11	8.14	8.64	8.97	8.58	0.42	0.73	0.04
14	7.29	8.41	8.79	8.17	0.78	0.69	0.07
18	5.62	7.29	8.32	7.08	1.36	0.60	0.12
21	4.60	6.04	6.93	5.86	1.18	0.50	0.10
23	3.44	5.21	5.77	4.80	1.22	0.41	0.10
28	1.44	3.17	3.68	2.77	1.17	0.24	0.10

Perchlorate Conc. (mg/L)

Limestone Buffer

Day	L1	L2	L3	Average	STD	C/Co	STD/Co
0	10.38	11.39	10.77	10.85	0.51	1.00	0.05
2	11.31	10.13	10.11	10.51	0.69	0.97	0.06
5	9.87	8.53	8.20	8.87	0.89	0.82	0.08
8	10.34	7.59	9.43	9.12	1.40	0.84	0.13
11	9.62	7.51	8.79	8.64	1.06	0.80	0.10
14	8.73	7.07	8.48	8.09	0.89	0.75	0.08
18	8.61	5.90	7.53	7.35	1.37	0.68	0.13
21	7.48	5.10	5.94	6.17	1.21	0.57	0.11
23	6.36	6.55	7.09	6.67	0.38	0.61	0.03
28	6.27	5.61	3.56	5.15	1.41	0.47	0.13

Heat Treated "Baked" Oyster Shell

Day	BOS1	BOS2	BOS3	Average	STD	C/Co	STD/Co
0	11.25	11.13	9.22	10.53	1.14	1.00	0.11
2	11.93	11.12	8.41	10.49	1.84	1.00	0.17
5	10.43	9.66	8.81	9.63	0.81	0.91	0.08
8	11.37	9.48	9.72	10.19	1.03	0.97	0.10
11	10.13	10.13	10.13	10.13	0.00	0.96	0.00
14	10.15	9.23	9.86	9.75	0.47	0.93	0.04
18	9.03	6.49	6.05	7.19	1.61	0.68	0.15
21	8.04	5.81	5.55	6.46	1.37	0.61	0.13
23	7.17	5.24	7.60	6.67	1.26	0.63	0.12
28	3.15	5.55	6.92	5.21	1.91	0.49	0.18

Oyster Shells, No Sulfur

Day	NO S1	NO S2	NO S3	Average	STD	C/Co	STD/Co
0	4.84	4.92	4.88	4.88	0.04	1.00	0.01
2	5.11	5.07	5.18	5.12	0.05	1.05	0.01
5	5.07	5.00	4.80	4.95	0.14	1.02	0.03
8	4.26	4.21	4.14	4.20	0.06	0.86	0.01
11	4.63	4.45	3.92	4.33	0.37	0.89	0.07
14	4.73	4.43	4.40	4.52	0.18	0.93	0.04
18	4.32	5.84	5.85	5.34	0.88	1.09	0.18
21	5.09	5.34	5.43	5.29	0.18	1.08	0.04
23	4.83	5.24	5.25	5.11	0.24	1.05	0.05
28	4.64	5.34	5.43	5.14	0.43	1.05	0.09

Perchlorate Conc. (mg/L)

Oyster Shells w/Yeast Extract

Day	OSYE1	OSYE2	OSYE3	Average	STD	C/Co	STD/Co
0	9.224	9.481	9.48	9.395	0.148091	1	0.015763
3	3.359	4.056	2.67	3.361667	0.693004	0.357814	0.073763
7	0.675	0	0	0.225	0.389711	0.023949	0.041481

Heat Treated "Baked" Oyster Shells w/Yeast Extract

Day	BOSYE1	BOSYE2	BOSYE3	Average	STD	C/Co	STD/Co
0	9.375	9.796	11.823	10.33133	1.308859	1	0.126688
3	4.54	6.623	6.552	5.905	1.182658	0.571562	0.114473
7	3.794	3.559	4.54	3.964333	0.512201	0.383719	0.049577

Days	Oyster Shell	Sulfur Only	Phosphate Buffer	Limestone Buffer	Baked Oyster Shell	OS/YE	BOS/YE	PO4/YE	No Sulfur, OS
0	7.25	7.25	7.25	7.25	7.25	7.25	7.25	7.25	7.25
2	7.5	6.9	6.9	7	7.5	7.5	7.5	6.9	7.25
3	7.5	6.8	7	7.3	7.5	7.5	7.5	6.9	7.3
5	7.4	6.6	6.6	6.9	7.5	7.5	7.5	6.5	7.1
13	7.6	6.25	6.25	7.1	7.4	7.4	7.4	6.2	6.9
16	7.6	6.2	6.2	7	7.7	7.7	7.7	6.2	7
19	7.9	6.4	6.4	7.5	7.9	7.9	7.9	6.4	7
28	7.9	6.4	6.4	7.4	7.9	7.9	7.9	6.4	7

pH

Days	Oyster Shell		Sulfur Only		Phosphate Buffer		Limestone Buffer		Baked Oyster Shell	
	Protein (mg/L)	STD	Protein (mg/L)	STD	Protein (mg/L)	STD	Protein (mg/L)	STD	Protein (mg/L)	STD
0	50.7	3.6	33.0	7.1	31.1	3.7	43.3	2.9	42.4	4.1
2	29.6	1.0	18.8	2.5	24.2	2.8	27.7	1.9	26.7	6.4
3	97.8	11.7	36.0	1.7	32.0	5.0	51.8	5.8	75.5	14.8
12	242.6	25.1	59.2	4.6	57.1	6.4	104.8	21.5	226.3	15.1
15	283.8	30.4	112.1	32.9	111.4	6.9	229.5	55.2	487.6	25.4
28	537.2	34.6	79.9	25.1	66.3	13.4	283.0	35.8	605.5	36.7

Days	OS/YE		BOS/YE		PO4/YE		No Sulfur, OS	
	Protein (mg/L)	STD	Protein (mg/L)	STD	Protein (mg/L)	STD	Protein (mg/L)	STD
0	82.9	1.4	56.1	6.6	41.3	3.4	8.5	2.9
2	37.5	11.5	32.1	10.0	28.1	5.6	10.0	0.5
3	120.7	8.9	84.1	36.6	41.4	11.0	7.2	1.1
12	246.3	19.4	248.4	20.7	55.7	6.1	0.0	0.5
15	407.8	48.7	480.9	33.5	46.6	27.9	0.0	1.1
28	459.7	47.7	582.7	39.2	70.2	3.4	34.4	5.1

		pH	Average	Standard Deviation	Protein ABS	Protein (mg/L)	Average	Standard Deviation	DOC mg/L	Average	Standard Deviation
1A	Raw Oyster Shell	7.673	7.62675	0.05114	0.169	5.15385	3.13462	2.46024	1.652	1.44725	0.34586
1B		7.6			0.156	0.15385			1.822		
1C		7.666			0.161	2.07692			1.092		
1D		7.568			0.169	5.15385			1.223		
2A	Pre-Rinsed Baked Oyster Shell	8.117	8.49975	0.2672569	0.157	0.53846	1.59615	1.70204078	0.9831	1.52303	0.61264726
2B		8.738			0.155	-0.23077			2.345		
2C		8.589			0.164	3.23077			1.138		
2D		8.555			0.163	2.84615			1.626		
3A	Post-Baked Rinsed Oyster Shell	8.555	8.42575	0.22503	0.153	-1	0.44231	2.63445	2.192	1.61148	0.52084
3B		8.474			0.153	-1			1.788		
3C		8.095			0.154	-0.61538			1.515		
3D		8.579			0.167	4.38462			0.9509		
4A	Raw OS + YE	7.652	7.58225	0.08057	0.174	7.07692	7.26923	4.72623	3.349	2.44775	0.62942
4B		7.58			0.176	7.84615			2.054		
4C		7.47			0.189	12.8462			1.979		
4D		7.627			0.159	1.30769			2.409		

		TN (mg/L)	Average	Standard Deviation
Raw Oyster Shell	1A	2.435	2.26325	0.12403
	1B	2.141		
	1C	2.223		
	1D	2.254		
Pre-Rinsed Baked Oyster Shell	2A	2.168	2.18675	12.654443
	2B	2.256		
	2C	2.131		
	2D	2.192		
Post-Baked Rinsed Oyster Shell	3A	2.23	2.30025	0.24549
	3B	2.197		
	3C	2.661		
	3D	2.113		

Protein

STD	Conc (mg/L)	ABS
A	250	0.976
B	125	0.569
D	25	0.22
E	5	0.168
F	0	0.156

	Concentration (mg/L)									
	Fluoride	Average	Standard Deviation	Chloride	Average	Standard Deviation	Nitrite	Average	Bromide	Average
Raw Oyster Shell	0.48694	0.43434	0.07641	9.95948	10.1148	0.11105	-0.03945	**0**	0.38706	0.38706
	0.33424			10.1167			-0.09307		0.38706	
	0.41568			10.2149			-0.09307		0.38706	
	0.50051			10.1682			-0.03562		0.38706	
Pre-Rinsed Baked Oyster Shell	0.53784	0.71004	0.18765336	9.8195	10.1216	0.20212258	-0.09307	**0**	0.44483	0.41594
	0.8093			10.2345			-0.09307		0.44483	
	0.92467			10.2345			-0.09307		0.38706	
	0.56837			10.1977			-0.09307		0.38706	
Post-Baked Rinsed Oyster Shell	0.9111	0.7321	0.16698	9.83423	9.82994	0.28138	-0.09307	**0**	0.38706	0.38706
	0.75161			9.59848			-0.09307		0.38706	
	0.5073			9.66233			-0.09307		0.38706	
	0.7584			10.2247			-0.09307		0.38706	
Raw OS + YE	0.39192	0.44197	0.10979	10.0307	10.2296	0.42431	-0.09307	**0**	0.38706	0.38706
	0.34781			9.79985			-0.02796		0.38706	
	0.42925			10.3008			-0.02796		0.38706	
	0.59891			10.7871			-0.03562		0.38706	

	Bromide STD	Nitrate	Average	Standard Deviation	Phosphate	Average	Standard Deviation	Sulfate	Average	Standard Deviation
1A	Raw Oyster Shell	0.37072	0.41689	0.03172	7.00318	6.8121	0.15204	21.8103	21.13	0.51776
1B		0.42928			6.66083			20.8364		
1C		0.44279			6.85987			20.6362		
1D		0.42477			6.72452			21.2369		
2A	Pre-Rinsed Baked Oyster Shell	0.43378	0.49234	0.06811589	6.23089	5.64769	0.40043761	22.886	23.652	1.68859619
2B		0.43829			5.41083			22.2548		
2C		0.57342			5.36306			26.0892		
2D		0.52387			5.58599			23.378		
3A	Post-Baked Rinsed Oyster Shell	0.57793	0.51637	0.07434	4.46338	5.53822	0.8068	26.9817	24.5665	2.14564
3B		0.53739			5.82484			22.7842		
3C					6.38217			22.7333		
3D		0.43378			5.48248			25.7669		
4A	Raw OS + YE	0.41126	0.49122	0.18505	6.85987	6.74244	0.15539	20.4259	21.0833	0.53337
4B		0.37072			6.62898			21.0909		
4C		0.41577			6.58917			21.7323		
4D		0.76712			6.89172			21.0842		