PERCHLORATE REDUCTION USING SALT-TOLERANT CULTURES

by

Yeyuan Xiao

M.A.Sc., Tongji University, 2002
B.A.Sc., Tongji University, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The College Of Graduate Studies

(Civil Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA

(Okanagan)

February 2012

ABSTRACT

The wide use of ion-exchange processes to remove perchlorate from drinking water creates an urgency for the regeneration or treatment of perchlorate-laden ion-exchange resins and/or regenerant brines. The use of biological processes with a salt-tolerant culture NP30 has been demonstrated as a promising cost-effective approach. In this study, the kinetics and ecology of NP30 were studied. A pure culture was isolated from the mixed culture, identified and characterized.

Perchlorate–laden ion-exchange resins were effectively regenerated by the mixed culture in laboratory batch reactors. A numerical model was developed to describe the regeneration process and for design predictions. A unique "resin phase" regeneration, in which the culture degraded perchlorate on the resin instead of only what desorbed into the bulk medium, was proposed in the model. The model generated an acceptable correlation to experimental data and the degradation from the "resin phase" accounted for the majority of the perchlorate removal.

The microbial composition of NP30 and the changes during a pilot plant experiment treating perchlorate- and nitrate-laden ion-exchange brine were analyzed using DGGE (denaturing gradient gel electrophoresis) and FISH (fluorescence *in situ* hybridization). *Halomonas* was the dominant (>18%) nitrate-reducing organism and *Azoarcus/Denitromonas* was the dominant (>22%) perchlorate-reducing organism. A shift towards nitrate-reducing organisms with time in the reactors was observed and attributed to the non-obvious perchlorate reduction seen in operation data.

A pure salt-tolerant, perchlorate-reducing strain P4B1 (*Marinobacter multirespiro* sp. nov. proposed name) was successfully isolated from the mixed culture. P4B1 could grow in the presence of 1.8%-10.2% NaCl. A molar Mg^{2+}/Na^{+} ratio of ~0.11 optimized the perchlorate degradation and cell growth when perchlorate was the sole electron acceptor. It could use perchlorate, nitrate and oxygen as electron acceptors. P4B1 preferred perchlorate to nitrate as the electron acceptor. A perchlorate reductase, which is only induced by perchlorate, is active in both perchlorate and nitrate reduction. When nitrate was used as the sole electron acceptor, the strain eventually lost the ability to reduce nitrate. The maximum specific substrate utilization rate (V_m) and the half saturation coefficient (K_S) for P4B1 were determined to be 0.050 ± 0.007 mg ClO_4^-/mg VSS-hr and 22 ± 12 mg ClO_4^-/L respectively.

PREFACE

A version of chapter 3 has been published as: Xiao, Y., Basu, A., Kashyap, V. & Roberts, D. (2010) Experimental and Numerical Analysis of Biological Regeneration of Perchlorate Laden Ion-Exchange Resins in Batch Reactors. *Environmental Engineering Science.* 27: 75-84. The experiments were done in the University of Houston by Kashyap, under the supervision of Roberts. The section of "Introduction" and "Materials & Methods" (experimental part) were originally drafted by Basu. I developed the model and wrote the draft of remaining part of the manuscript. Roberts finalized the manuscript.

A version of chapter 4 has been published as: Xiao, Y., Zuo, G., Roberts, D., Badruzzaman, M. & Lehman, G. (2010) Characterization of Microbial Populations in Pilot-Scale Fluidized Bed Reactors Treating Perchlorate- and Nitrate-Laden Brine. *Water Research.* 44: 4029-4036. The pilot-plant experiments were conducted by Badruzzaman and Lehman. The experiment of denaturing gradient gel electrophoresis (DGGE) was performed in University of Houston by Zuo. I did the experiment of fluorescence *in situ* hybridization (FISH) and drafted the manuscript. Roberts finalized the manuscript.

A version of chapter 5 has been submitted for publication to *Applied and Environmental Microbiology.* as: Xiao, Y., Hooker, L. & Roberts, D.. *Marinobacter multirespiro* sp. nov., a New Salt-Tolerant Perchlorate-Reducing Bacteria. I did the experiments and drafted the manuscript. Roberts finalized the manuscript. Hooker helped on the DGGE experiments.

A version of chapter 6 has been submitted for publication to *Environmental Science & Technology* as: Xiao, Y. & Roberts, D.. Kinetics Analysis of a Perchlorate-Reducing Bacterium: Effects of Sodium, Magnesium and Nitrate. I did the experiments and drafted the manuscript. Roberts finalized the manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2C-75	Dicocoalkyldimethylammonium chloride
AWWA	American Water Works Association
ANOVA	Analysis of variance
APHA	American Public Health Association
BLAST	Basic Local Alignment Search Tool
BV	Bed volume
CDPH	California Department of Public Health
CEPA	California Environmental Protection Agency
cld	Chlorite dismutase
clrA/B/C/D	Chlorate reductase A/B/C/D
CMC	carboxymethyl cellulose
CPC	Cetylpyridinium chloride
CTAB	Cetyltrimethyl ammonium bromide
CTAC	Cetyltrimethylammonium chloride
DAP	Di-ammonium phosphate
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DMSO	Microbial dimethyl sulfoxide
DO	Dissolved oxygen
DTAB	Decyltrimethylammonium bromide
DW	Dry weight
EBCT	Empty bed contact time
ESIX	Electrically switched ion exchange
FAME	Fatty acid methyl esters
FBR	Fluidized-bed reactor
FISH	Fluorescence *in situ* hybridization
GAC	Granular activated carbon
HFTW	horizontal flow treatment well
HRL	health reference level
HRT	Hydraulic retention time

ITRC	Interstate Technology & Regulatory Council
IX	Ion-exchange
IXF	Ion-exchange fiber
MBfR	Membrane biofilm reactor
MFC	Microbial fuel cell
MTAB	Myristyltrimethylammonium bromide
MV	Methyl viologen
NCBI	National Center for Bioinformatics
NF	Nanofiltration
NOM	Natural organic matter
PBR	Packed-bed reactor
PCA	Principal component analysis
PCR	Polymerase chain reaction
pcrA/B/C/D	Perchlorate reductase subunit A/B/C/D
PLEs	Polymeric ligand exchangers
PNRB	Perchlorate- and nitrate-reducing bacteria
PPy	Polypyrrole
PRB	Perchlorate-reducing bacteria
qPCR	Quantitative PCR
RfD	reference dose
RISA	Ribosomal intergenic spacer analysis
RO	Reverse osmosis
SBRs	Sequencing batch reactors
SPBS	Saline phosphate buffer solution
SSm	Scatter search method
T-50	Tallowalkyltrimethylammonium chloride
THAB	Tributylheptylammonium bromide
UF	Ultrafiltration
USEPA	United States Environmental Protection Agency
VSS	Volatile suspended solids
WEF	Water Environment Federation

LIST OF SYMBOLS

$C_{a,t}$	perchlorate concentration in the aqueous phase in control samples at time *t*, eq./L;
$C_{a,b,t}$	perchlorate concentration in the aqueous phase in test samples at time *t*, eq./L;
$C_{e,a,t}$	equilibrium concentrations of perchlorate in samples killed at time *t*, eq./L;
$C_{e,Cl}$	equilibrium concentrations of chloride in the aqueous phase, eq./L;
C_T	total anionic equilibrium concentration in the aqueous phase, eq./L;
EBCT	empty bed contact time during ion-exchange process, min;
f	switching coefficient for the 2-site desorption;
G	amount of air-dried resin used in batch experiments, g;
I.D.	inner diameter of iox-exchange column;
k_{des1}	desorption kinetic constant exhibited during the initial rapid interval, h^{-1};
k_{des2}	desorption kinetic constant exhibited after the initial rapid interval, h^{-1};
K_{Sa}	half saturation constant of perchlorate degradation in the aqueous phase, eq./L;
K_{Sr}	half saturation constant of perchlorate degradation in the liquid film of the resin phase, eq.;
L_a	loading capacity of the air-dried resin, eq./g;
$M_{a,r,t}$	total amount of perchlorate in the resin phase at time *t*, eq.;
$M_{a,t}$	total amount of perchlorate in the resin and aqueous phase in samples killed at time *t*, eq.;
MW	molecular weight of ClO_4^- - 99,450 mg /eq.;
n	coefficient of available perchlorate for the resin phase biodegradation;
$r_{a,t}$	rate of biodegradation of perchlorate in the aqueous phase at time *t*, eq./L-h;
$r_{d,t}$	rate of desorption of perchlorate from the resin phase in test samples at time *t*, eq./L-h;
$r_{r,t}$	rate of biodegradation of perchlorate in the resin phase at time *t*, eq./h;
SLV	superficial liquid velocity in the ion-exchange process, cm/min;
Δt	incremental time interval for numerical analysis, h;
V	volume of regeneration solution in the batch experiments, L;
V_{ma}	maximum specific perchlorate degradation rate in the aqueous phase, eq./mg VSS-h ;

V_{mr}	maximum specific perchlorate degradation rate in the resin phase exhibited during the initial rapid interval, eq.-L/mg VSS-h;
VSS	volatile suspended solids;
x_a	mole fraction of perchlorate at equilibrium in the aqueous phase;
x_{Cl}	mole fraction of chloride at equilibrium in the aqueous phase;
X_t	biomass concentration (VSS) in samples at time *t*, mg/L.
Y	yield of perchlorate-reducing biomass, mg VSS/mg ClO_4^-;
y_a	mole fraction of perchlorate at equilibrium in the resin phase;
y_{Cl}	mole fraction of chloride at equilibrium in the resin phase.

ACKNOWLEDGEMENTS

I offer my enduring gratitude to the faculty, staff and my fellow students at the UBC, who have inspired me to continue my work in this field. I owe particular thanks to my supervisor Dr. Deborah J. Roberts, for her motivation, guidance, encouragement, patience, and financial support. Her immense knowledge and enthusiasm and positive attitude towards science set a pattern for me. I could not have imagined having a better advisor and mentor for my Ph.D study. I would also like to thank my committee members Dr. Cigdem Eskicioglu, Dr. Eric Hall and Dr. Rehan Sadiq for their encouragement and insightful comments.

I wish to express my sincere thanks to Prof. Laura Hooker in Department of Biology and Dr. Kirsten Wolthers in Department of Biochemistry for their tutoring and guidance on experiments. I would also like to thank Laboratory Manager Russell LaMountain and laboratory technicians Matthew Gullen and Simon Oakley for their assistance of setting-up the laboratory.

I am grateful to laboratory technician Sarangi Ariyawansa and undergraduate research assistants Anthony King, Connor Wilson and Danny Trommelen for their help with the experiments. I also would like to thank my labmates Johanna Faccini and Rony Das for the support and fun. In addition, I wish to thank my colleagues and friends in UBC for their support and friendship.

Finally, but foremost, I am deeply grateful to my parents and wife for their love and understanding.

To my lovely wife

CHAPTER 1 INTRODUCTION

This chapter provides a general introduction to the research project, including the background, issues and objectives, which are necessary to understand the significance of the research problem. The structure of the thesis is described to provide a roadmap for readers.

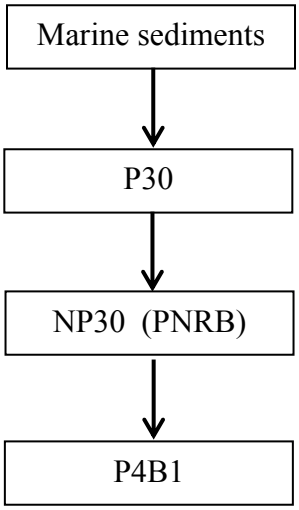
1.1 Background

Perchlorate (ClO_4^-) is an environmental contaminant that can cause irreversible damage to the growth and development of many species. It inhibits the transfer of iodide from the blood to the thyroid gland, which is required for the gland to produce hormones essential for metabolism and growth. Long-term disruptions in thyroid hormones may result in hypothyroidism and related changes in metabolism, decreased mental performance, and altered development (Wolff 1998). Perchlorate is both a naturally occurring and man-made compound. Most of the man-made perchlorate is used as the primary ingredient of solid rocket propellant. Perchlorate contamination has been found in groundwater, surfacewater, and soil in North America and Europe. In 2008, United States Environmental Protection Agency (USEPA 2011) proposed a health reference level (HRL) of 15 μg/L. The USEPA (2011) made a determination to regulate perchlorate in drinking water in 2011, after a long survey and review process started in 1998 when perchlorate was listed as a drinking-water contaminant candidate (USEPA 1998). In Canada, the contamination of perchlorate in Canadian waters is not viewed as a major issue. Perchlorate was detected in surface waters in the Great Lakes Basin at concentrations close to the detection limit (0.2 μg/L), possibly from fireworks and agricultural operations (Backus *et al.* 2005). In another study of groundwater contamination of a military training site, perchlorate was detected in the groundwater at concentrations as high as 67.1 μg/L (Bordeleau *et al.* 2008), which was far greater than the guideline (6 μg/L) set by Health Canada (2008).

The most widely used technology to remove perchlorate and nitrate from drinking water is ion-exchange. During this process, perchlorate, nitrate and other negatively charged ions are concentrated into a brine or resin which must be treated before discharging to the environment. Microbiological treatment is the most cost-effective way to treat these contaminants. The biggest difficulty is the high salt (typically >6%) in the brines or resins,

which inhibits the growth of many useful bacteria.

Recently a salt-tolerant (3-10% NaCl), perchlorate- and nitrate-reducing bacterial culture NP30 was enriched from marine sediments in Dr. Deborah Roberts' research laboratory. This culture has been successfully used to remove perchlorate and nitrate from synthetic regenerant brine in the laboratory (Aldridge *et al.* 2003; Cang *et al.* 2004; Patel *et al.* 2008). However, in a recent pilot plant project, the perchlorate removal by the culture, which was inoculated into a fluidized-bed reactor to treat ion-exchange regeneration brines contaminated with perchlorate and nitrate, was not stable. A study of the microbial communities in the pilot plant reactors allows a better understanding of the reasons for the poor performance of the culture in the pilot plant. Isolation and characterization of pure strains from the culture, which has not been accomplished to date may be necessary for stable reactor operation. In addition to the treatment of perchlorate-laden brines, the culture was shown to be very effective in the regeneration of perchlorate-laden resins in laboratory-scale batch reactors (Kashyap 2006). In order to scale up the biological regeneration process, a numerical model simulating the process would be very helpful. This thesis documents the study addressing these issues. The family tree of cultures used in the present study is schemed as follows:



1.2 Research objectives

The overall objective of the present research was to further understandings of and make improvements to the salt-tolerant perchlorate- and nitrate-reducing culture, and make the culture sustainable for industrial application. Thus, safe drinking water can be supplied economically to municipalities.

The specific objectives of this study were as follows:

- Construct a numerical model describing the biological regeneration of perchlorate-laden non-regenerable resins in batch reactors based on experimental results. Determine the key factors governing the process.
- Analyze the microbial community of the mixed culture inoculated in a pilot-plant fluidized-bed reactor (FBR) treating ion-exchange brines containing perchlorate and nitrate. Correlate the change of microbial community with water treatment results.
- Isolate a pure salt-tolerant, perchlorate-reducing strain from the mixed culture.
    - Characterize and identify the strain.
    - Determine the kinetic constants for growth and perchlorate degradation by the pure culture.
    - Analyze the salt-tolerance of the pure culture.
    - Investigate the nitrate effects on perchlorate reduction by the pure culture in batch reactors.

1.3 Thesis structure

After this general introduction, Chapter 2 provides a literature review of perchlorate contamination and treatment technologies. Chapters 3 to 6 document the detailed methods and results addressing the research objectives. Chapter 7 presents the final conclusions.

CHAPTER 2 LITERATURE REVIEW

Perchlorate is principally a man-made compound. Approximately 90% of the industrial perchlorate salt products are in the form of ammonium perchlorate, which is mainly used in munitions and as an oxidant in solid rocket fuels (Urbansky 1998; Motzer 2001). Other industrial application of perchlorate includes pyrotechnics, lubricating oil, fabric fixer and dyes etc. (Motzer 2001; USEPA 2005; Trumpolt *et al*. 2005). Natural perchlorate occurrence in Atacama Desert salts in Chile is widely known (Ericksen 1983). Recent measurements (Bao and Gu 2004) of the triple-oxygen isotope ratios in both manmade perchlorate and natural perchlorate in Atacama soils revealed that natural perchlorate has a unique oxygen isotope signature and suggested an atmospheric origin of the natural perchlorate source. The oxidation of volatile chlorine by O_3 and the formation of $HClO_4$ can be a sink (albeit a minor one) for atmospheric chlorine (Bao and Gu 2004).

Perchlorate is a powerful oxidant as a solid. In most environmental conditions it is highly stable because of its high activation energy (Urbansky 2002). It is highly soluble in water. In soils or sediments, less than 10% of the perchlorate will be absorbed, while the rest leaches into the aqueous phase (Urbansky and Brown 2003). Its fate in the environment is controlled by the hydrological conditions if there is no biological activity.

The adverse effect of perchlorate was first found in 1960s when seven cases of fatal aplastic anemia were reported after the usage of potassium perchlorate as a medicine to treat hyperthyroidism (Wolff 1998). Later studies found perchlorate binds to the sodium iodide symporter and consequently inhibits the uptake of iodide to the thyroid gland, which is required for the gland to produce hormones essential for metabolism and growth. Long-term disruptions in thyroid hormones may result in hypothyroidism and related changes in metabolism, decreased mental performance, and altered neuropsychological development (Wolff 1998; Brechner *et al*. 2000; Clark 2000).

The ecotoxicological effects of perchlorate have also been widely studied. Evidence has shown that perchlorate at environmentally relevant concentrations resulted in alterations of thyroid function in wild vertebrates and fish and alterations of metamorphosis in amphibians (Smith 2006). However, there is little evidence that perchlorate in environments has adverse effects on the growth, survival, and reproduction in those animals (Smith 2006).

2.1 Perchlorate contamination

Perchlorate was not regulated in ground water in the US until 1997 when a new analytical method was developed with the detection limit of 4μg/L (Motzer 2001). In 1998, perchlorate was listed as a contaminant candidate for drinking water by USEPA (1998). After that perchlorate contamination was found throughout the US. The major occurrences of perchlorate contamination in the US are associated with unregulated waste streams from the users and manufacturers of rocket fuel and explosives. The worst contamination was in Las Vegas, Nevada area, with perchlorate concentration ranging from 630 mg/L to 3,700 mg/L (Motzer 2001). In California, a statistical study shows that almost 6.5% of drinking water sources are contaminated with perchlorate, with concentrations ranging from 3.3 to 820 μg/L (CDPH 2007). Recently perchlorate was detected in milk, lettuce, spinach and melon with a mean concentration of 5.8, 10.6, 115 and 28.62 μg/L respectively (USFDA 2007). Studies on certain plant species, such as tobacco, forage and cereal crops, grown in perchlorate contaminated soils or irrigated with perchlorate contaminated water showed that perchlorate accumulates and persists in the final produce (Ellington *et al*. 2001; Tan *et al*. 2004).

It was believed that the occurrence of perchlorate in U.S. was limited primarily to military facilities and some perchlorate manufacturing sites (Urbansky 1998). However, more recent studies show that naturally-occurring perchlorate might be an important source for perchlorate in soils and ground water. Rajagopalan *et al*. (Rajagopalan *et al*. 2006) investigated perchlorate occurrence in groundwater from an area of 155 000 km^2 in northwest Texas and eastern New Mexico and attributed long-term atmospheric deposition to be the most likely source of perchlorate. Parker *et al*. (2008) also measured naturally-occurring perchlorate in ground water. Plummer *et al*. (2006) determined natural perchlorate in Middle Rio Grande Basin in north-central New Mexico, which also had an atmospheric origin. Rao *et al*. (2007) further confirmed that natural perchlorate is present in diverse unsaturated zones of the arid and semi-arid southwestern United States. Rajagopalan *et al*. (2009) evaluated perchlorate concentrations in wet deposition samples across US and detected perchlorate in all samples. Although perchlorate occurred at a very low concentration with a mean of 14.1 ±13.5 ng/L for all samples, the calculated annual net mass flux amounted to 51 000 kg, which is comparable to other known perchlorate release sources. Kounaves *et al*. (2010) discovered perchlorate in soil and ice in several Antarctic Dry Valleys,

which confirmed the global occurrence of perchlorate due to atmospheric deposition. Kounaves *et al*. (2010) further proposed that microbial reduction prevents the accumulation of perchlorate in ocean and wet areas.

In other research, by measuring the isotopic data ($\delta^{37}Cl$, $\delta^{18}O$, and $\Delta^{17}O$), Bohlke *et al*. (2009) confirmed the source of perchlorate in groundwater in Long Island, New York was from the Atacama fertilizer. Sturchio *et al*. (2011) examined the isotope ratios of chlorine and oxygen in perchlorate sampled from groundwater wells in the eastern San Bernardino Basin and found a minor contribution of perchlorate from the indigenous natural deposition compared to the predominant contribution from synthetic or agricultural sources.

In 2008, the USEPA (2008) proposed a health reference level (HRL) of 15 μg/L for perchlorate in drinking water based on a reference dose (RfD) of 0.7 μg/kg/day. In 2011, the USEPA (2011) has decided to regulate perchlorate in drinking water. While the USEPA is still working to establish a new regulatory standard, this HRL can be used at present for perchlorate treatment.

2.2 Technologies to treat perchlorate

The USEPA and Interstate Technology & Regulatory Council (ITRC) have both provided reviews of technologies to treat perchlorate in water and soil. Many technologies, such as ion-exchange, microbiological processes, activated carbon absorption, composting, i*n situ* bioremediation, permeable reactive barrier, phytotechnology, electrodialysis, reverse osmosis, nanofiltration/ultrafiltration, nanoscale bimetallic particles reduction and titanium(III) chemical reduction, have been tried for removal of perchlorate from water (USEPA 2005; ITRC 2005; ITRC 2008). By 2005, there were about 25 full scale and 27 pilot scale perchlorate treatment projects in U.S.A.. Among these, ion exchange was the most widely used technology for *ex situ* perchlorate removal, which amounts to 60% of the full scale projects. Microbiological processes were the most widely used technology for *in situ* bioremediation with a percentage of 37% of the pilot scale projects (USEPA 2005).

2.2.1 Abiotic technologies

Ion-exchange, activated carbon adsorption, membrane filtration and metal-based catalytic reduction are the most studied abiotic technologies for perchlorate removal.

2.2.1.1 Ion-exchange

The throughput of full scale ion-exchange systems reviewed ranged from 23 to 54,500 m^3/d. Among these projects, the influent and effluent perchlorate concentration was generally below 250 μg/L and 4 μg/L respectively except one case where the influent perchlorate ranged from 80 to 100 mg/L with effluent perchlorate below 2 mg/L (CEPA 2004).

Normally, in perchlorate contaminated ground water or surface water, anions such as SO_4^{2-}, NO_3^- and HCO_3^- exist at concentrations that are orders of magnitude higher than perchlorate as competing anions. The perchlorate selectivity is the most important feature of a resin to be considered for a perchlorate removal process.

Tripp and Clifford (2004) systematically studied the perchlorate selectivity of different resins and investigated different regeneration parameters, including temperature and regeneration brine concentration. Based on their results, the resins could be categorized into three groups according to their separation factors (α) of perchlorate relative to chloride: 1) low perchlorate selective resins, with the median separation factors <10.4; 2) medium perchlorate selective resins, with the median separation factors ranging from 81 to 373; and 3) high perchlorate selective resins, with the median separation factors >632.

The resin matrix and the functional group have significant effects on perchlorate selectivity. The polyacrylic resins are in the first category, due to their hydrophilic structure, while the polystyrene resins and polyvinylpyridine resins are within categories 2 or 3 (Tripp and Clifford 2004). Within the same matrix, the greater the size of the functional group, the higher the perchlorate selectivity is (Tripp and Clifford 2004). Assuming the same concentration of regenerant (1N NaCl), the authors calculated the efficiency of different resins, which is the ratio of bed volume (BV) of product to the BV of regenerant. Interestingly, although the perchlorate separation factors of the category 1 were very low, the efficiencies were as high as the highly perchlorate selective resins due to ease of regeneration. The medium selective resins have the lowest efficiency.

A practical example of using the category 1 resin (Purolite A-850) was reported by Lehman *et al*. (2008). The ion-exchange process was operated for ~250 BV before regeneration with brine for ~6 BV. The perchlorate in the brine was removed by microbial degradation and the brine was re-used for 20 cycles. During the 20 cycles, no breakthrough

of perchlorate was observed. Gu *et al*. (2005) also observed Purolite A-850 had the least adsorption for perchlorate among other strong-base anion-exchange resins. Another example was the removal of perchlorate in synthetic perchlorate solution using a polyacrylic resin (Purolite Macro-T) (Batista *et al*. 2000).

For the highly perchlorate selective resin, Ionac SR-7, a polystyrene resin with tripropylamine functional group, and Purolite A-530, a polystyrene resin with a mixture of trihexyl- and triethylamine bi-functional groups, there was not a significant breakthrough of perchlorate until a BV of over 10,000 and 30,000 respectively at normal flow rates of ~1 BV/min while treating ground water containing perchlorate of ~500 μg/L (Tripp and Clifford 2004). Ionac SR-7 was also tested by other researchers (Batista *et al*. 2000). The group in Oak Ridge National Laboratory has done extensive studies on resin Purolite A-530E and A-520E (Gu *et al*. 2001; Gu *et al*. 2003; Gu *et al*. 2005; B. Gu *et al*. 2007; Gu *et al*. 2011).

The application of medium-perchlorate-selectivity resins was reported once by Gu *et al.* (2005). This might be because the regeneration efficiency for this type of resin is relatively low. As suggested by Tripp and Clifford (2004), in order to increase the regeneration kinetics for the medium selective resins, regeneration at high temperature (60 ℃), could increase the efficiency by 100%.

In addition to the tests on some medium and high perchlorate selective resins, Xiong *et al*. (2007a) compared perchlorate adsorption by a class of polymeric ligand exchangers (PLEs), and an ion-exchange fiber (IXF). The results correspond with those by Tripp and Clifford (2004), showing polyacrylic resins had the lowest perchlorate selectivity. The PLEs used copper (II) as the ligand center to enhance perchlorate selectivity. The perchlorate selectivity of IXF is comparable to that of polystyrene resins, while it had much faster adsorption and desorption kinetics (with a sorption equilibrium time of <1.5 h), and much greater regeneration efficiency, because its unique structure greatly increases the site accessibility.

Typically, 1 N NaCl is used to regenerate the category 1 resins. For the complete removal of the perchlorate in regenerant brine, three technologies for brine treatment have been examined: 1) Catalytic chemical reduction, a process conducted under high pressure and temperature using ammonia as the reductant (Calgon Carbon Corporation 1999); 2) Ferrous chloride reduction, a process conducted under high pressure and temperature using

Fe^{2+} as the reductant (Gu *et al*. 2001); and 3) Microbial reduction using salt-tolerant culture (Cang *et al*. 2004; Lehman *et al*. 2008; Hiremath *et al*. 2006).

However, for the highly selective resins, the regeneration using NaCl brine is not effective (Tripp and Clifford 2004). The exhausted resin must be disposed of as a hazardous waste via fuel blending or incineration (ITRC 2005). Recently, some researchers have tried to regenerate the highly selective resins. Gu *et al*. (2001; 2003) developed a unique process to regenerate the highly selective resin A-530E by eluting the column with only 1 BV of $FeCl_3$-HCl regenerant solution. The perchlorate in the regeneration effluent could be concentrated up to 100 000 mg/L, which would favor recovery by precipitation as $KClO_4$ salts or destruction with $FeCl_2$ in a thermoreactor.

In addition to the traditional ion-exchange process, an electrically switched ion exchange (ESIX) film was also developed by depositing polypyrrole (PPy) on high surface area carbon nanotubes (Lin *et al*. 2006). It showed the potential to remove perchlorate from water.

2.2.1.2 Activated carbon adsorption

Activated carbon has been used to remove metals, volatile and non-volatile organic compounds, and inorganic anions from water. Experiments to determine the adsorption of perchlorate at different pH, measurement of the zeta potential of the carbon and Raman spectra analysis of the carbon before and after adsorption of perchlorate indicate that the perchlorate adsorption on the carbon is mostly due to the surface oxygenated functional groups instead of surface charge (Mahmudov and Huang 2010; Yoon *et al*. 2009a). pH was the most important factor affecting the adsorption process. The optimal adsorption of perchlorate on the carbon was usually observed at low pH (2-4), which was lower than the point of zero charge pH (pH_{PZC}) (Mahmudov and Huang 2010; Xu *et al*. 2011). At that low pH, the bituminous granular activated carbon (GAC) Filtrasorb 400 showed highest adsorption capacity for perchlorate (Mahmudov and Huang 2010). Nitrate was the only compound that demonstrated competitive inhibition of perchlorate adsorption among the anions (BrO_3^-, ClO_3^-, ClO_4^-, IO_3^-, NO_3^-, $H_2PO_4^-$, and SO_4^{2-}) tested. Removal of perchlorate using virgin GAC is not a cost-effective process as shown by Na *et al*. (2002).

In order to improve the adsorption capacity of GAC, some researchers have

developed tailored GAC, including GAC preloaded with iron-oxalic acid (Na *et al*. 2002), GAC preloaded with cationic surfactants including decyltrimethylammonium bromide (DTAB), tributylheptylammonium bromide (THAB), myristyltrimethylammonium bromide (MTAB), cetyltrimethylammonium chloride (CTAC), cetylpyridinium chloride (CPC), tallowalkyltrimethylammonium chloride (T-50), dicocoalkyldimethylammonium chloride (2C-75), and cetyltrimethyl ammonium bromide (CTAB) (Parette and Cannon 2005; Xu *et al*. 2011), and GAC thermally tailored with ammonia (Chen *et al*. 2005a; Chen *et al*. 2005b). The GAC preloaded with iron-oxalic acid had a perchlorate adsorption improvement of up to 42% over virgin GAC (Na *et al*. 2002). Although some of the surfactants leached out of the GAC tailored with cationic surfactants, which could be removed by a polishing adsorption bed using virgin carbon, the perchlorate adsorption capacity of the tailored carbons increased by up to 30 times (Na *et al*. 2002). The thermal tailoring featured flushing the GAC with N_2 while heating it in a furnace until the set temperature (500–800 ℃) was reached and then continuing flushing with NH_3 at the set temperature for 60 min (Chen *et al*. 2005a; Chen *et al*. 2005b). The bed volumes to 4 ppb perchlorate breakthrough treating a natural groundwater containing 70–78 ppb perchlorate, increased from 1100 BV for virgin carbon up to 4400 BV for carbon that was tailored at 700 ℃ (Chen *et al*. 2005b). The increased perchlorate adsorption corresponded linearly to the increased positive surface charges.

The biggest obstacle preventing the application of the tailored GAC for perchlorate removal is the competition of other anions in water with perchlorate. As evaluated by Patterson (2009), thiosulfate at a concentration of 1 mg/L or nitrate at a concentration of 30 mg/L in raw water could decrease the tailored GAC adsorption capacity by over 50%. Another issue is the regeneration of the exhausted GAC. One report showed a recovery of only 65-74% of the GAC's original adsorption capacity by chemically regenerating the GAC with sodium borohydride (Na *et al*. 2002). In another test, thermal reactivation with CO_2 or NH_3 at temperatures higher than 700 ℃ could effectively restore the adsorption capacity of the ammonia-tailored GAC, which would last for at least three cycles (Chen and Cannon 2005). Chemical regeneration using calcium thiosulfate was not an effective method to regenerate the virgin GAC (Chen 2005). The base/acid wash for the regeneration of perchlorate exhausted GAC was effective in small-scale, but failed in full scale (Chen 2005).

2.2.1.3 Membrane filtration

Reverse osmosis (RO), nanofiltration (NF) and ultrafiltration (UF) membranes have all been examined for perchlorate removal from water (Yoon *et al*. 2000; Yoon *et al*. 2005a; Yoon *et al*. 2009b). Size exclusion and then electrostatic exclusion are the two major mechanisms to remove perchlorate (Yoon *et al*. 2003a; Yoon *et al*. 2005a; Yoon *et al*. 2005b; Yoon *et al*. 2009b). A perchlorate rejection of >90%, 25-95% and 3-47% by RO, NF and UF respectively could be achieved at various pH (4-10) (Yoon *et al*. 2009b). The rejection of perchlorate by the membranes increased with increasing solution pH and with decreasing solution conductivity due to the increasingly negative membrane charge (Yoon *et al*. 2009b).

The performance of membrane filtration (especially NF and UF) is a balance between perchlorate rejection and water recovery. As shown by Yoon *et al*. (2005a), while increasing water recovery from 15% to 70%, the perchlorate rejection in the treatment of a river water by a NF significantly decreased from 55% to 20%. The authors further determined that inorganic scale formation, dominated by $CaCO_3$, was attributed to the rejection decrease. Antiscalant was effective to inhibit the scale formation.

2.2.1.4 Metal-based catalytic reduction

Although the contamination of perchlorate was not of concern until the 1990's, the catalytic reduction of perchlorate can be dated back to the 1940's using homogeneous catalyst osmium salts (Crowell *et al*. 1940). Later, molybdate with stannous ion (Haight and Sager 1952), molybdate with tungsten(V1) (Kolthoff and Hodara 1963), and molybdate with cadmium amalgams Cd(Hg) (Rechnitz and Laitinen 1961) were tested and had catalytic activities for perchlorate reduction in acidic solution. These early studies focused on developing a quantitative determination method for perchlorate (Rechnitz and Laitinen 1961). Heterogeneous catalytic reduction of perchlorate in acidic solutions on electrodes of Rh, Pt, WC, Al, Ti, Ir, Ru, Re and Tc has been widely observed since the 1970's, because perchloric acid and perchlorate solutions were used as inert and stable supporting electrolytes for many years (Láng and Horányi 2003).

Since 1997, perchlorate has been a contaminant of concern in drinking water, and more effort has been directed towards developing catalyts from less expensive metals that could reduce perchlorate at low concentration in mild conditions. Abu-Omar *et al*. (2000)

developed a stable rhenium complex that could catalyze perchlorate reduction for hundreds of cycles. However, the oxygen acceptor was a soluble organic sulfur reducing agent, which was not readily compatible with water purification systems.

Recently, nanoscale or microscale mono- or bi-metallic particles have been developed and shown to be very effective at perchlorate reduction. Nanoscale Fe^0 particles were very effective at perchlorate reduction, due to their great surface area and surface reactivity. Cao *et al*. (2005) reported that nanoscale Fe^0 particles (average diameter of 57 ±16 nm) at a dose of 10 g/L could reduce 90% perchlorate to chloride within 24 hours at 75 ℃. In another study, the nanoscale Fe^0 particles were stabilized with starch or sodium carboxymethyl cellulose (CMC), which improved the perchlorate reduction rate constant by 1.8 and 3.3 times respectively (Xiong *et al*. 2007b). The CMC stabilized Fe^0 particles (average diameter of 11.2 ±7.9 nm) at a dose of 1.8 g/L could reduce 90% perchlorate in both fresh water and simulated ion-exchange brine (6% NaCl) within 7 hours at 90-95 ℃. Adding another metal catalyst (Ag, Al, Cu, Co, Ni, Pd, or Re) to form nanoscale bimetallic particles did not improve or even worsen the activities of the nanoscale particles (Cao *et al*. 2005; Xiong *et al*. 2007b). However, the catalytic reduction of perchlorate by nanoscale Fe^0 particles at room temperature was very slow, as the half-life of perchlorate would be 18 days (Cao *et al*. 2005) or 3.5 days in CMC stabilized particles (Xiong *et al*. 2007b). Monometallic particles (Ti, Co, Ni, Pd, Cu, Zn and Sn) also showed some catalytic activity for perchlorate reduction at pH 3 and 2 atm H_2 gas (Wang *et al*. 2008).

A novel rhenium-palladium bimetal catalyst immobilized on activated carbon (Re/Pd-AC) showed great catalytic activity for perchlorate reduction by hydrogen at ambient temperature and pressure (Hurley and Shapley 2007; Choe *et al*. 2010; Zhang *et al*. 2011). Using the catalyst, at pH 2.7 (the optimal activity), the half-life of perchlorate was only 2 hours. Xu *et al*. (2010) synthesized a unique catalyst by hydrolyzing $FeSO_4$ on GAC and then reducing with $NaBH_4$to form iron compounds (mostly containing $FeOHSO_4$, Fe_2O_3 and Fe^0) supported on GAC. At ambient temperature and neutral pH, the catalyst could reduce perchlorate with a half-life of ~ 4 hours.

Electrodialytically assisted catalytic reduction of perchlorate was also studied by Wang and Huang (2008). An electrodialysis system using a stainless steel mesh coated with nano-sized bimetallic catalysts (Co–Rh, Co–Cr, Co–Ru and Pt–Ti) was successfully

developed to reduce perchlorate in ultra-low perchlorate concentrations (<1 ppm).

2.2.2 Biotic technologies

Perchlorate-reducing bacteria (PRB) have been shown to reduce perchlorate to chloride via using it as an electron acceptor. Biotic technologies feature stimulating the growth of indigenous PRB in perchlorate-contaminated sites (*in situ* bioremediation) or enrichment of PRB in engineered systems (*ex situ* biodegradation). Uptake of perchlorate by plants is another *in situ* bioremediation technology (phytoremediation).

2.2.2.1 *In situ* bioremediation

The indigenous PRB are widely present in perchlorate-contaminated sites, even in the 40-m deep vadose zone of an unlined pond receiving industrial wastewater from an ammonium perchlorate manufacturing plant (Gal *et al*. 2008). *In situ* bioremediation of perchlorate contaminated groundwater or soils features stimulating the growth of indigenous PRB within perchlorate-contaminated sites to reduce perchlorate to chloride via injecting electron donors into the sites.

Based on the different operation modes of the addition of electron donors, three different technologies, termed active, semi-passive and passive remediation, can be applied for *in situ* bioremediation (Stroo and Norris 2009). For the active bioremediation process, a certain portion of the contaminated ground water is continuously pumped and mixed with an electron donor solution and then injected back into the aquifer. Thus, continuous mixing of electron donor with perchlorate-contaminated groundwater is provided via recirculation. For the semi-passive process, the electron donor is added and mixed with the groundwater periodically, i.e., several times per month or even per year. The mixing of electron donor with groundwater is provided via recirculation and natural groundwater flow. For the passive remediation process, slow-release electron donor sources, such as emulsified vegetable oils (Borden and Lieberman 2009; Borden 2007), are directly injected into the aquifer or placed in walls or trenches built in the aquifer. The distribution of the electron donor in the aquifer is fully dependent on the natural groundwater flow. In a passive bioremediation process, emulsified vegetable oils were used as the electron donors and injected into a perchlorate-contaminated aquifer to form a biobarrier (Borden and Lieberman 2009; Borden 2007; Hunter 2002). Within 5 days of injection, perchlorate was reduced to below detection (<4

μg/L) from ~ 10,000 μg/L in all injection wells. The injection resulted in over 99% perchlorate reduction in downgradient monitoring wells even 18 months after injection.

With the recirculation decreasing from active to semi-passive to passive remediation, the capital and operation cost also generally decreases, while the adaptability to varying hydrological conditions also decreases (Stroo and Norris 2009). The passive process is limited to depths approximately <10–15 m below ground surface (bgs) based on costs (Stroo and Norris 2009). The active process, especially the horizontal flow treatment well (HFTW) system is more cost-effective for deeper aquifers (Hatzinger *et al*. 2009). The redox potential after the application of slow-release electron donors is hard to control and might produce undesired contaminants, such as hydrogen sulfide, manganese, iron and arsenic compounds (Hatzinger *et al*. 2009). Biofouling in injection wells is a significant issue for active remediation processes (Hatzinger *et al*. 2009). Thus, to select a most suitable process, site specific evaluation has to be performed.

The major problems for *in situ* bioremediation are: a) to effectively mix the electron donor with groundwater, b) to control biofouling inside pumping wells and c) to prevent production of secondary groundwater contaminants, such as manganese, iron, arsenic and hydrogen sulfide (Hatzinger *et al*. 2006; Hatzinger *et al*. 2009) . Through the operation of some projects, engineers and researchers have gained very important experience to deal with these problems.

Conservative tracer tests (Hatzinger *et al*. 2009) are usually needed to determine the mixing and diffusion conditions inside the aquifer. A laboratory microcosm study is very helpful to determine the optimal electron donor, which would provide the best performance and least biofouling and cost. Electron donors such as lactate (Hatzinger *et al*. 2006; Cox *et al*. 2009) and citric acid (Hatzinger *et al*. 2009) have been used. Compared to others, citric acid has the lowest potential for causing biofouling in wells.

In order to prevent production of secondary groundwater contaminants, the amount of electron donor to be added must be controlled carefully. As noticed in a project (Hatzinger *et al*. 2009), at the late stage of bioremediation when perchlorate was almost gone, the treated water had the “rotten egg” odor coming from hydrogen sulfide due to the reduction of sulphate. Some researchers have proposed to use the thermodynamic properties of electron donors to control specific microbial metabolisms (Van Trump and Coates 2009). For

example, hydroquinone as an electron donor could support nitrate and perchlorate reduction, but not sulfate reduction.

The operation mode of an *in situ* bioremediation system is also important to balance the performance and the operation costs. As demonstrated in a field project (Hatzinger *et al*. 2009), the intermittent operation of the bioremediation system coupled with small, frequent doses of chlorine dioxide greatly decreased the operation and maintenance costs associated with biofouling control. In order to stimulate the growth of indigenous PRB, the pH of the groundwater is another important factor governing the process. As tested in a demonstration project, when the pH is <5.9, the bioremediation won't function (Hatzinger *et al*. 2006; Cramer *et al*. 2004).

Another emerging *in situ* bioremediation process is monitored natural biological attenuation. As observed by Gal *et al*. (2008), without external carbon sources and electron donors, the indigenous PRB from the surface soil of a perchlorate-contaminated site could completely degrade ~ 70 mg/L perchlorate within 134 days. This suggests that natural perchlorate reduction in soils is an important mechanism of perchlorate attenuation. A rapid natural attenuation potential of perchlorate was also observed in saturated near-surface sediments (Tan *et al*. 2005). The natural organic matter (NOM) in the soil was believed to be the source of carbon and electron donors supporting growth of PRB (Gal *et al*. 2008).

In situ bioremediation was unequivocally validated recently via measuring isotopic ratio changes of perchlorate (Hatzinger *et al*. 2009). The invariant fractionation factor ratio $\varepsilon^{18}O/\varepsilon^{37}Cl$ for microbial degradation of perchlorate (Sturchio *et al*. 2007; Ader *et al*. 2008) was used to distinguish bioremediation processes from transport or mixing-related processes.

Phytoremediation of perchlorate via uptake by plants and rhizodegradation (Nzengung *et al*. 1999; Nzengung *et al*. 2009) has been evaluated and showed promising potential as a remediation process. In a batch study, poplar trees growing in hydroponic solution were shown to be capable of reducing ClO_4^- in solution by 50% in 30 days (Schnoor *et al*. 2002). In addition to the portion taken-up by leaves, 33% of ClO_4^- remaining in solution was recovered as chloride, which was not due to microbial conversion (Schnoor *et al*. 2002). However, the accumulation of perchlorate in leaves might recycle perchlorate back into the soils with senesced leaves in the winter due to the slow rate of phytodegradation. Thus, it is recommended that senesced leaves be collected and composted or

phytoremediation be designed to enhance rapid rhizodegradation (Yifru and Nzengung 2007).

2.2.2.2 *Ex situ* biodegradation

Ex situ biodegradation of perchlorate-contaminated groundwater using different reactors and inocula in different scales has been extensively studied. The throughput of full scale *ex situ* microbiological perchlorate reduction systems ranges from 190 to 28,887 m^3/d. The influent and effluent perchlorate concentration in these projects ranges from 35 μg/L to 4,624 mg/L (diluted brine with salt concentration <5%) and with effluent perchlorate ranging from 4 to 400μg/L (CEPA 2004; Coppola 2000).

Table 2-1 shows a summary of these studies. As shown in the table, fluidized-bed reactors (FBRs), membrane biofilm reactors (MBfRs), packed-bed reactors (PBRs), wetland bioreactors, bioelectrical reactors, microbial fuel cell (MFC) and sequential batch reactors (SBRs) have all been used to treat perchlorate at concentrations ranging from 50 μg/L in ground water to 1500 mg/L in a diluted perchlorate waste stream. Among them, PBRs are the most widely studied, followed by FBRs. These are also the only two types that have been used in full-scale projects (Evans *et al*. 2002; Zhang *et al*. 2005; McCarty and Meyer 2005).

The high efficiency of PBR could be seen from the short hydraulic retention time (HRT) of 56 min to degrade perchlorate in groundwater from 50-120 μg/L to less than 4 μg/L (Zhang *et al*. 2005) and a HRT of only 2.1 min to degrade ClO_4^- in synthetic water from 20 000 μg/L to less than 4 μg/L (Kim and Logan 2001). An FBR was also very efficient for removing perchlorate in groundwater from 52.5 μg/L to less than 6 μg/L within 12.2 min (Webster *et al*. 2009) and from ~2 600 μg/L to less than 4 μg/L within 12-23 min (McCarty and Meyer 2005). In addition to GAC, which is often used, sand, plastic and diatomaceous earth have been used as the supporting medium for FBRs and PBRs. Recently, crushed oyster shell was shown to be a good supporting medium (Sahu *et al*. 2009b), as it is an effective buffer and contains organic matter as the carbon source for bacterial growth. Zero-valent iron is another new supporting medium that has been tried recently (Son *et al*. 2006; Yu *et al*. 2007), with an intent to omit the need to supply electron donors continuously and to minimize organic residuals in the treated water.

Table 2-1 Summaries of *ex situ* biodegradation of perchlorate-contaminated water

Reactor	Support medium	HRT	Inoculum	Electron donor	Raw water	Performance	Reference
FBR i.d. 1.5 m × 6.4 m 55-190 L/min	GAC	~ 60-200 min	GAC containing Biosolids	50% acetic acid	Ground water	Pi 5 000 – 35 000; Po<350; Ni: ~ 1.9 mg/L	(Polk *et al*. 2002)
FBR (full-scale) i.d. 4.27 m × 4.6 m	GAC	12-23 min	NA	Ethanol	Ground water	Pi ~2 600, Po<4; Ni ~1.5, No<0.011	(McCarty and Meyer 2005)
FBR i.d 3.7 cm×5.72cm	GAC	154 min	Enrichment mixed culture	Acetate	Synthetic brine (6% NaCl)	Pi ~300 000, Po ~ 2 000; Ni: 200-900, No < 10	(Patel *et al*. 2008)
FBR i.d. 0.92 m ×5.2m	GAC	12.2 min	Indigenous organisms	Acetic acid	groundwater	Pi: 52.5, Po <6; Ni: 6.1, No<1	(Webster *et al*. 2009)
MBfR i.d. 14 cm×120 cm	Hollow-fiber membrane	55 min	Mixed culture	H_2	groundwater	Pi: 55, Po: 2; Ni: 24.4, No: <0.09; DOi ~8, DOo <0.1;	(Nerenberg *et al*. 2004)
MBfR i.d. 7.6 cm × 25.4 cm	Hollow silicone membrane	9.3-18.6 hrs	Soils from contaminated sites	H_2	Synthetic tap water spiked with perchlorate	Pi: 500, Po<4	(Padhye *et al*. 2007)
MBfR 11.7 mL. (i.d. 0.6cm)	Hollow-fiber membranes	4.52 d	Water in salt pond	H_2	IX brine (10% diluted)	Pi: 19 000, Po: 12000 Ni: 259, No: 112	(Chung *et al*. 2007)
MBfR (run as batch) i.d. 1.9 cm × 25 cm	Hollow-fiber membranes	53 hrs	Mixed culture	H_2	Synthetic water (0 or 1.25% NaCl)	Pi: ~20, Po: ~11.6	(Sahu *et al*. 2009a)
PBR i.d. 7.62 cm × 117 cm	Diatomaceous earth pellets	1.17-0.46 hr	Mixed culture containing *Wolinella* sp. HAP-1	Naturally occurring protein, peptides	Perchlorate waste stream was diluted with tap water	Pi: 1500 000, Po: 500 000 Po<100 000 (95%)	(Wallace *et al*. 1998)
PBR i.d. 2.5cm ×10 cm	Glass beads	22min	Enrichment mixed culture	H_2	Synthetic water	Pi: 740; 38±9% removal	(Miller and Logan 2000)
PBR i.d. 2.5 cm × 28 cm	Sand	EBCT 2.1 min 31 min	Pure strain KJ or mixed culture	acetate	Synthetic water	Pi ~20 000, Po<4	(Kim and Logan 2001)

Reactor	Support medium	HRT	Inoculum	Electron donor	Raw water	Performance	Reference
PBR i.d. 13.5cm×12.5 cm	Diatomaceous earth	<0.3 h	Pure strain Perc1ace	Acetate	Ground water	Pi: ~800, Po<4; Ni: ~25, No <1	(Losi *et al*. 2002)
PBR i.d. 2.5cm ×25 cm	Glass beads	1.5 min	Enrichment mixed culture	H_2	Groundwater	Pi: 73±2, P: 25±5 %removal Ni: 21±2, N: 10% removal	(Logan and LaPoint 2002)
PBR A 0.19 m^2×2.1m	Plastic or sand	EBCT 105 min	Pure strain KJ	acetic acid	Groundwater	Pi: 75, Po<4 Ni: 19.0	(Evans *et al*. 2002)
PBR i.d. 2.5 cm ×8 cm	GAC	EBCT 9 min	NA	acetate or ethanol	Surface water	Pi: 50, Po<4	(Brown *et al*. 2003)
PBR 0.61m×0.3m×2.1m(H)	Plastic medium	56 min	*Dechlorosoma* sp. KJ and indigenous bacteria	acetic acid	Ground water	Pi: 50-120, Po<4 Ni: 17.7-20	(Zhang *et al*. 2005)
PBR i.d. 2.5 cm ×30 cm	Zero-valent iron	2 days	Sludge from a wastewater treatment plant	Fe^0	Synthetic water	Pi: 16 000, Po<20	(Son *et al*. 2006)
PBR i.d. 3.8 cm ×60cm	Zero-valent iron	EBCT 0.15-3.8 hr	Soils of a rapid infiltration tertiary wastewater treatment	Fe	Tap water spiked with perchlorate	Pi: 30-600, Po<6; Ni: 20.8-26.1	(Yu *et al*. 2007)
PBR i.d. 50 cm ×99cm	GAC	EBCT 10 min	NA	Acetic acid	Groundwater	Pi: 34-88, Po < 2; Ni: 23-28, No <1	(Brown *et al*. 2008)
PBR i.d. 15 cm×70 cm	Plastic media	EBCT 8 hr	Sludge from a wastewater treatment plant	Acetate	Synthetic		(Choi and Silverstein 2008)
PBR i.d. 2.4 cm×14 cm	GAC or Glass beads	EBCT 23.5 min	Enrichment mixed culture	Acetate	Synthetic	Pi: 50, Po<1; DO_i < 1 mg/L	(Choi *et al*. 2008)
PBR i.d. 6.1 cm×35 cm	Crushed oyster shell	EBCT 7.5 h	Enrichment mixed culture	S^0	Synthetic water	Pi: 60-120, Po <4	(Sahu *et al*. 2009b)
PBR i.d. 76 cm×123 cm	Anthracite + graded gravel	HRT 3hr	Indigenous microorganisms	Acetic acid	Surface water spiked with ClO_4^-	Pi: 50, Po< 2	(Dugan *et al*. 2009)

Reactor	Support medium	HRT	Inoculum	Electron donor	Raw water	Performance	Reference
PBR i.d. 14 cm×42.5 cm	Plastic porous cylinder	EBCT 15 hr	Mixed culture	Acetate	Synthetic IX brine 0-10% NaCl	Pi: 6250 Ni: 1439 No N inhibition	(Chung *et al*. 2010)
PBR i.d. 5 cm × 30 cm	In layers: glass beads, iron fillings and sand	8 hrs	Sludge from a wastewater treatment plant	Fe^0	Synthetic water	Pi: 16 000, Po<15	(Arthur 2011)
Bioelectrical reactor	----------	3 days	Pure strain VDY	H_2 produced at cathode	Synthetic water	Volumetric loading 60 mg ClO_4^-/L-d	(Thrash *et al*. 2007)
Microbial Fuel Cell	----------	22 hrs	Mixed culture	Biocathode	Synthetic water	Volumetric loading 24 mg ClO_4^-/L-d	(Butler *et al*. 2010)
Wetland bioreactors $2 \times 0.61 \times 0.51 \times 0.43 m^3$ (L ×W ×H)	Coarse, aquarium-grade gravel	4 or 0.5 days (with acetic acid)	Indigenous mixed culture and plants	Acetic acid or no	Groundwater	Pi: 100, Po<0.5 Ni: 68	(Krauter 2001)
wetland bioreactors 2×1.9 m^3 2×4.2 m^3	Coarse, aquarium-grade gravel	17-20 hrs	Indigenous mixed culture and plants	NA	Groundwater	Pi: 5.8-14 , Po<0.5	(Krauter *et al*. 2005)
Upflow wetlands i.d. 15 cm×55 cm	Intact sediment	12 days	Intact sediment planted with or without *Bulrush*	NA	Aged tap water spiked with perchlorate	Pi: 32 000, Po<4; plant uptake 0–14.3%	(Tan *et al*. 2004)
SBR V=5L	----------	13-27 hrs per batch	Anaerobically-digested sludge	Acetate	Synthetic water	Pi: ~1200 000, Po<50 µg/L; Volumetric loading 45-90 mg ClO_4^-/L-h	(Nor *et al*. 2011)

*NA - not available.

Membrane biofilm reactors (MBfRs) inoculated with hydrogenotrophic PRB using hydrogen (H_2) gas as a sole electron donor are of interest to engineers and water utility owners because H_2 is non-toxic and relatively inexpensive. Using H_2 also eliminates the addition of organic carbon sources into the water. However, compared to FBRs and PBRs, the efficiency of MBfRs is relatively low, which could be seen from the relative longer HRT shown in Table 2-1. Except the study done by Nerenberg *et al*. (2004), in which an HRT of 55 minutes was needed to degrade from 55 μg/L to less than 2 μg/L, in other studies the HRT were all among 9 hours to 4.5 days. Two factors have caused this: one, the relative slow growth of hydrogenotrophic bacteria; and the other, the low solubility of H_2. The solubility of H_2 decreases with increasing salt concentration, which makes it even worse to treat perchlorate in brines (Sahu *et al*. 2009a).

The inocula that have been used in bioreactors include enrichment mixed cultures, pure cultures, anaerobic digester sludge, activated sludge, soils from contaminated sites and water from contaminated sites. After acclimation, these inocula functioned very well in the system. Whether the use of pure cultures might be better or worse than mixed cultures is totally case specific. As Yu *et al*. (2007) observed, an up-flow packed-bed reactor with zero-valent iron as the medium inoculated with a mixed culture inoculum exhibited much better performance for reduction of both perchlorate and nitrate than columns inoculated with a pure *Dechloromonas* sp. Strain HZ. In another study (Kim and Logan 2001), perchlorate was degraded from 20 000 μg/L to less than 4 μg/L within 2.1 minutes in a pure *Dechlorosoma* sp. strain KJ inoculated PBR compared to the 31 minutes in a mixed culture inoculated PBR. However, in a PBR treating groundwater containing 50-120 μg/L perchlorate (Zhang *et al*. 2005), the same strain KJ and indigenous microorganisms were inoculated together into the reactor; after 6 months' operation, strain KJ only constituted <1% of all cells in the biofilms in the reactor. Although different inocula were used, acetic acid is the most-widely used electron donor.

2.2.2.3 Biodegradation of perchlorate-contaminated soils

Bioremediation of perchlorate-contaminated soils has also been studied. Usually contaminated soils are excavated and *ex situ* remediated via composting. For example, ex situ bioremediation of soils after excavation from contaminated sites has been successful

with glycerin as the electron donor and carbon source and di-ammonium phosphate (DAP) as the nitrogen source (Evans *et al*. 2008). Some newly emerging technologies, including surface infiltration with water containing electron donor, subsurface injection of electron donor and gas-phase electron donor addition have also been tried (Cox 2009). Gaseous electron donor such as hydrogen or ethyl acetate is injected into the vadose zone to stimulate the growth of indigenous microorganisms to degrade perchlorate (Evans and Trute 2006). The potential of *in situ* bioremediation of perchlorate-contaminated sediments has also been studied (Batista *et al*. 2005).

2.2.2.4 Issues associated with biotic perchlorate treatment technologies

Biological processes are very effective for both *ex situ* and *in situ* treatment of perchlorate-contaminated groundwater and soils. However, some issues associated with biological processes compromise their application in some situations.

First, the effluent from a biological process has low dissolved oxygen and will probably contain high ammonia, soluble microbial products, sulfide and acetate (Dugan *et al*. 2009). If the water is to be used as a drinking water source, these products might cause unpleasant odors, increase the disinfectant dosage and produce more disinfection-by-products. Consequently, a downstream process is needed to normalize the dissolved oxygen and remove residual organics. For example, in a project of a lab-scale PBR treating perchlorate-contaminated groundwater, hydrogen peroxide was used as a post treatment to reoxygenate and oxidize residual organics and hydrogen sulfide (Brown *et al*. 2008). Second, the efficiency of a biological process decreases with temperature. Thus, in winter or during system upsets, a backup process such as ion-exchange might be needed; or temperature controlled bioreactors have to be used. The last, a biological process requires operators with good training. Daily real-time analyses of water quality and adjustment would be needed for successful operation (Dugan *et al*. 2009).

2.2.2.5 Newly emerging technologies

A novel ion exchange membrane bioreactor was built to test its potential to treat perchlorate and nitrate in drinking water (Matos *et al*. 2006). Perchlorate and nitrate anions were removed via exchanging with chloride anions when the polluted water ran through the water channel. In the other channel, perchlorate and nitrate were completely destroyed via

microbial degradation. There was no contact between biomass and the treated water, and no brine was produced as would be in the ion-exchange process.

Immobilization of a perchlorate reductase purified from Perc1ace on Ca-alginate was used to treat perchlorate in groundwater (Frankenberger 2003). In a most recent study, engineered vesicles were built to reduce perchlorate (Poust 2010). A triblock copolymer vesicle encapsulated with cell-free extracts containing perchlorate reductase and chlorite dismutase enzymes were constructed. The outer membrane porin OmpF implanted on the engineering vesicles allows for perchlorate transport into the vesicles. Then perchlorate is degraded to chloride inside the vesicles. These vesicles could be used in environments where growth of PRBs is inhibited (Poust 2010).

2.3 Perchlorate-reducing bacteria and cultures

It has been known since the 1960's that cell-free extracts obtained from nitrate-adapted cells of *Bacillus cereus* could reduce perchlorate (Hackenthal 1965). The first characterized pure PRB was *Vibrio dechloraticans* Cuznesove B-1168, a strain in γ-subclass of Proteobacteria isolated from a process of purification of perchlorate-contaminated industrial stream (Romanenko *et al*. 1976). From the early 1990's, when perchlorate contamination was widely discovered in US, more mixed and pure cultures were enriched and characterized (Attaway and Smith 1993; Wallace and Attaway 1994; Wallace *et al*. 1996; Rikken *et al*. 1996). By the middle 2000's, more than 56 perchlorate- or chlorate-reducing bacteria have been isolated (Coates and Achenbach 2004). Since then, more PRB have been identified in mixed and pure culture. Four new genera in the γ- Proteobacteria capable of reducing perchlorate were isolated from perchlorate-contaminated soils, which are *Aeromonas, Rahnella and Shewanella* (Kesterson *et al*. 2005). This increases the number of perchlorate-reducing genera in the γ- Proteobacteria to five together with the already-known *Dechloromarinus* and *Pseudomonas.* Recently, three novel strains *Pseudomonas stutzeri* A1, *Arthrobacter* A2 and *Arthrobacter* A3 were isolated from soils collected from a perchlorate-manufacturing factory in India. Although these strains could not grow anaerobically, they could perform perchlorate reduction in aerobic growth medium without addition of molybdenum, an important micronutrient required by all other perchlorate-reducing bacteria. (Shete *et al*. 2008). *Arthrobacter* is in the phylum of Actinobacteria, which is a new phylum

containing PRB, as previous PRB were all in the phylum of Proteobacteria (Coates and Achenbach 2004). Recently, more PRB species in the β-Proteobacteria outside of the *Dechloromonas* and *Azospira* genera were isolated, which include *Dechlorobacter hydrogenophilus, Propionivibrio militaris* (Thrash *et al*. 2010b), and *Burkholderia* sp. (Ghosh *et al*. 2011).

Table 2-2 lists all the pure perchlorate-reducing strains isolated or characterized. From Table 2-2, it is obvious PRB are ubiquitous in perchlorate-contaminated soils, groundwater, surface water, aquifer and sediments, and activated and digester sludge. The dominant PRB genera in the environment are *Azospira* and *Dechloromonas*. PRBs are phylogenetically diverse, with species in α, β, γ and ε subclass of phylum Proteobacteria, and phylum of Actinobacteria and Firmicutes. Even a species of Archaea in the phylum of Euryarchaeota was capable of perchlorate-respiration (Okeke *et al*. 2002). All known PRB are Gram-negative, except one thermophilic strain isolated from produced water from underground gas storage, two aerobic perchlorate-reducing strains (Shete *et al*. 2008), and three groups in Firmicutes (Hackenthal *et al.* 1964; Hackenthal 1965; Balk *et al.* 2008).

New mixed culture studies may suggest the existence of PRB with novel phenogenetic affiliation.. Autotrophic PRB cultures oxidizing Fe^0 have been established (Yu *et al*. 2006; Son *et al*. 2006). PRB that could grow chemolithotrophically on Fe^0, S^0 and H_2 were recently isolated from wastewater sludges (Ju *et al*. 2008). A probable new perchlorate-reducing genus in ε-Proteobacteria, *Sulfuricurvum,* was discovered in a sulfur-oxidizing PBR via microbial community study (Sahu *et al*. 2009b). Another genus *Clostridium* in the phylum Firmicutes might also contain PRB, as a species from which dominated in a salt-tolerant (<10% NaCl) PBR (Chung *et al*. 2010). A potential PRB might be found in the genus of *Burkholderia* in β-proteobacteria (Ghosh *et al*. 2011), which is a predominant species in a mixed enrichment PRB culture. A microbial community study in a Hydrogenotrophic MBfR also revealed the dominant species as *Sphingobacteria* (phylum Bacteroidetes) (Sahu *et al*. 2009a), which might be the evidence of a new phylum containing PRB.

Table 2-2 Perchlorate-reducing strains

Organism	Isolated from	Remark	Reference
α-Proteobacteria			
Paracoccus halodenitrficans		0-2.5% NaCl	(Okeke *et al*. 2002)
Azospirillum AJ2, ABL1, PMS1, PMS2, SN1A, SN1B, SN2	Aquifer and groundwater		(Waller *et al*. 2004)
Dechlorospirillum sp. cl-31-Sarno River	Surface water		(Vigliotta *et al*. 2010)
Azospirillum sp. cl-19-Sarno River	Surface water		(Vigliotta *et al*. 2010)
Magnetospirillum bellicus VDY^T	A cathodic chamber of a bioelectrical reactor	Heterotrophic and chemolithoautotrophic on H_2, CO_2	(Thrash *et al*. 2010a)
Dechlorospirillum anomalous WD	Swine waste lagoons	Microaerophilic	(Michaelidou *et al*. 2000)
β-Proteobacteria			
Azospira oryzae (*Dechlorosoma suillum*) PS^T	Soil and groundwater		(Achenbach *et al*. 2001; Bruce *et al*. 1999)
Azospira oryzae (*Dechlorosoma suillum*) type KJ, PDX	Primary digester sludge		(Logan *et al*. 2001b)
Azospira oryzae (*Dechlorosoma suillum*) GR-1	Activated sludge		(Rikken *et al*. 1996;Wolterink *et al*. 2005)
Azospira oryzae (*Dechlorosoma suillum*) JPLRND	Groundwater	N inhibits P	(Farhan and Hatzinger 2009; Sturchio *et al*. 2003)
Azospira oryzae (*Dechlorosoma suillum*) type: AH, Iso1, Iso2, SDGM			(Coates and Achenbach 2004)
Azospira (*Dechlorosoma*) Perc1ace	Biosolids	N inhibits P at beginning 0-2.5% NaCl	(Herman and Frankenberger 1999; Xu *et al*. 2003; Okeke *et al*. 2002)
Azospira (*Dechlorosoma*) PCC.	Activated sludge	Heterotrophic and chemolithoautotrophic on H_2, CO_2 Accumulate ClO_3^-	(Dudley *et al*. 2008)
Azospira (*Dechlorosoma*) cl-6-Sarno	Surface water		(Vigliotta *et al*. 2010)
Dechloromonas agitata CKB^T	Paper mill waste	Incapable of NO_3^- respiration 0-2% NaCl	(Achenbach *et al*. 2001)
Dechloromonas agitata type: FL2, FL8, FL9, CL, NM, MLC33, CL24plus, CL24			(Coates and Achenbach 2004)
Dechloromonas aromatica RCB	River sediments		(Coates *et al*. 2001)

Organism	Isolated from	Remark	Reference
Dechloromonas aromatica type: CCO, SIUL, MissR			(Coates and Achenbach 2004)
Dechloromonas hortensis MA-1^T	A garden soil		(Wolterink *et al*. 2005)
Dechloromonas sp. HZ	Enrichment culture in a bioreactor	Heterotrophic and chemolithoautotrophic on H_2, CO_2	(Zhang *et al*. 2002)
Dechloromonas EAB1, EAB2, EAB3, ABL2, PMC, RC1, RC2, RR, INS	Aquifer and groundwater		(Waller *et al*. 2004)
Dechloromonas JDS5 and JDS6	Soil and groundwater	Heterotrophic and chemolithoautotrophic on H_2, CO_2. Incapable of respiring atmospheric levels of O_2	(Shrout *et al*. 2005)
Dechlorobacter hydrogenophilus LT-1^T	Soil and groundwater	Heterotrophic (can use H_2 with acetate)	(Thrash *et al*. 2010b)
Propionivibrio militaris MP^T, CR	A cathodic chamber of a bioelectrical reactor	Heterotrophic (can use H_2 with acetate)	(Thrash *et al*. 2010b)
γ-Proteobacteria			
Aeromonas	Soil and groundwater		(Kesterson *et al*. 2005)
Citrobacter amalonaticus JB101	A sewage treatment facility	P prior to N	(Bardiya and Bae 2004)
Citrobacter farmeri JB109	A sewage treatment facility	P prior to N	(Bardiya and Bae 2004)
Citrobacter sp. IsoCock1	A enrichment hydrocarbon oxidizing mixed culture	0-10% NaCl	(Okeke *et al*. 2002)
Pseudomonas stutzeri A1	Soil	Obligate aerobic, perchlorate reduction in aerobic condition. Incapable of NO_3^- respiration	(Shete *et al*. 2008)
Rahnella	Soil and groundwater		(Kesterson *et al*. 2005)
Shewanella	Soil and groundwater		(Kesterson *et al*. 2005)
Vibrio dechloraticans Cuznesove B-1168			(Korenkov *et al*. 1976)
ε-Proteobacteria			
Wolinella succinogenes HAP-1	A municipal anaerobic digestor	P prior to N	(Wallace and Attaway 1994;Wallace *et al*. 1996)
Actinobacteria			
Arthrobacter A2, A3	Soil	Gram-positive. Obligate aerobic, perchlorate reduction in aerobic condition. Incapable of NO_3^- respiration	(Shete *et al*. 2008)

Organism	Isolated from	Remark	Reference
Euryarchaeota (Archaea)			
Haloferax denitrificans		0-2.5% NaCl	(Okeke *et al*. 2002)
Firmicutes			
Staphylococcus epidermidis		N inhibits P	(Hackenthal *et al*. 1964)
Bacillus cereus		P inhibits N in a non-competitive manner	(Hackenthal 1965)
Moorella perchloratireducens An10^T	Produced water from underground gas storage	Gram-positive. Thermophilic (optimal 55 to 60 ℃). Incapable of growing with >3% O_2 in gas phase. 0-4% NaCl	(Balk *et al*. 2008)
Un-identified strains			
IsoA, IsoB, IsoC	Sediments from a salt evaporation facility	0-2.5% NaCl	(Okeke *et al*. 2002)
IsoSol1	Marine sediments	0-2.5% NaCl	(Okeke *et al*. 2002)
PDC, PDD, PDE	Primary digester sludge		(Logan *et al*. 2001b)
KJ3, and KJ4	A perchlorate-degrading bioreactor		(Logan *et al*. 2001b)
D-8	Activated sludge		(Logan *et al*. 2001b)

2.3.1 Factors affecting microbiological perchlorate reduction

Microbial perchlorate degradation is generally accepted to proceed through the pathway: $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$ (Rikken *et al*. 1996). During the process, perchlorate is the electron acceptor being reduced to chlorate, then chlorite, and further to chloride. All known PRB can use chlorate as the electron acceptor, while several chlorate-reducing bacteria are not able to use the perchlorate as the electron accptor, such as strain PDA, PDB (Logan *et al*. 2001b) and ASK-1 (Wolterink *et al*. 2005). The common competitive eletron acceptors for perchlorate reduction are dissolved oxygen and nitrate.

2.3.1.1 Dissolved oxygen (DO) and redox potential

Most PRB are facultative anaerobes capable of growing on oxygen, while some are microaerophilic with no growth at atmospheric levels of oxygen (Michaelidou *et al*. 2000; Shrout *et al*. 2005). *Moorella perchloratireducens* Strain An10^T did not grow with oxygen in the gas phase exceeding 3% (Balk *et al*. 2008). And as mentioned before, two strains are obligate aerobes (Shete *et al*. 2008).

Dissolved oxygen < 2 mg/L in the water will be enough to inhibit perchlorate reduction for most PRB (Chaudhuri *et al*. 2002). However, perchlorate reduction by a mixed lactate enrichment culture from anaerobic digester sludge was observed with dissolved oxygen concentrations as high as 4.8 mg/L O_2 (Shrout and Parkin 2006). In a continuous-flow fixed bed biofilm reactor with granular activated carbon (GAC) as the supporting medium, Choi *et al*. (2008) observed that exposure to the DO in the influent would not negatively affect the operation of the reactor, due to the chemisorption of oxygen onto the GAC. In another MBfR, a DO of ~ 8 mg/L in the influent was successfully decreased to ~ 0.1 mg/L in the effluent with an empty bed contact time (EBCT) of 55 min, while degrading perchlorate from 55 μg/L to < 2 μg/L and nitrate from ~24.4 mg/L to < 0.09 mg/L (Nerenberg *et al*. 2004). Perchlorate degradation occurs at redox potentials from -220 mV to +180mV, but the extent of perchlorate degradation increased with decreasing redox potential (Shrout and Parkin 2006).

2.3.1.2 Nitrate

All PRB described to date could utilize nitrate as an alternative electron acceptor and reduce nitrate to nitrite and to nitrogen gas except strain *Dechloromonas agitata* CKB^T (Bruce *et al*. 1999) and the three aerobic perchlorate-reducing strains isolated recently (Shete *et al*. 2008). The inhibition of perchlorate reduction by nitrate has been widely observed in pure and mixed perchlorate reducing bacteria. For example, NO_3^- was always reduced at a faster rate than perchlorate by strain Perc1ace, although, perchlorate reduction did occur simultaneously with NO_3^- reduction (Herman and Frankenberger 1999). *Dechlorosoma suillum* strain PS would start perchlorate reduction only when nitrate was completely removed (Chaudhuri *et al*. 2002). Nitrate inhibition of perchlorate reduction was observed in a fixed film bioreactor treating synthetic perchlorate- and nitrate-contaminated solution even when the reactor was acclimated to perchlorate reduction for 10 months, especially when acetate was not in excess (Choi and Silverstein 2008). PRB in vadose soils collected from a perchlorate-contaminated site preferentially used nitrate instead of perchlorate as an electron acceptor (Nozawa-Inoue *et al*. 2011).

Although it is generally reasonable to assume nitrate inhibition of perchlorate reduction (Coates and Jackson 2009), exceptions have been discovered. Perchlorate was

found to inhibit nitrate reduction in a non-competitive manner by a strain of *Bacillus cereus* in a very early study done by Hackenthal *et al*. (1965). *Wolinella succinogenes* strain HAP-1 could reduce perchlorate and chlorate irrespective of the presence of nitrate (Wallace and Attaway 1994; Wallace *et al*. 1996). *Citrobacter amalonaticus* and *Citrobacter farmeri* preferred perchlorate to nitrate as electron acceptor. And after 150 h incubation, the total nitrate reduction was only approximately 20% by both the strains (Bardiya and Bae 2004). Nitrate reductase activity was competitively inhibited by chlorate in a phototrophically nitrate- and chlorate-reducing bacterium, *Rhodobacter sphaeroides* (Roldan *et al*. 1994).

Since perchlorate-reducing activity was first observed in nitrate-reducing strains and nitrate reduction was normally prior to or simultaneous with perchlorate reduction, perchlorate-reduction was believed to be conducted by nitrate reductase. Later studies have shown that in some strains, such as *Azospira* (*Dechlorosoma*) strains KJ and perc1ace and *Pseudomonas* strain PDA, nitrate and perchlorate are reduced by different enzymes (Xu *et al*. 2004; Giblin and Frankenberger 2001). An interesting study by Sun *et al*. (2009) showed the quite different chemotaxis response of three PRB to different electron acceptors (nitrate, perchlorate and sulfate). Strain CKB, which is incapable of growth by nitrate reduction, responded readily toward nitrate when grown with perchlorate. Strain RCB responded both to nitrate and perchlorate when grown with perchlorate, but only to nitrate when grown with nitrate. Strain PS responded only to perchlorate and chlorate but not nitrate when grown with perchlorate, but only to nitrate when grown with nitrate. This study suggests that separately-induced perchlorate and nitrate reductases are responsible for the perchlorate and nitrate reduction respectively when these strains are exposed to the electron acceptor. At least two different perchlorate reductases, which could or not reduce nitrate, may exist. As more exceptions are discovered, the effects of nitrate on perchlorate reduction need to be determined specifically for different cases, as stated by Coates and Jackson (2009).

2.3.1.3 Electron donor

Most PRB are heterotrophic and capable of growing on many kinds of electron donors such as simple organic acids and alcohols (Coates *et al*. 1999; Coates *et al*. 2001; Coates and Jackson, 2009), or aromatic hydrocarbons (Coates *et al*. 2001). *Dechloromonas* strains JDS5 and JDS6 (Shrout *et al*. 2005) and HZ (Zhang *et al*. 2002) can grow

chemolithoautrophically with H_2 as the electron donor and CO_2 as carbon source, while keeping their ability for heterotrophic growth. Strains *Dechlorobacter hydrogenophilus* LT-1^T and *Propionivibrio militaris* MP^T and CR can also use H_2 as electron donor, but could not assimilate CO_2 (Thrash *et al*. 2010b). Interestingly, the rate of perchlorate reduction by PRB in vadose soils was enhanced by hydrogen amendment and inhibited by acetate amendment (Nozawa-Inoue *et al*. 2011).

If acetate is used as the electron donor, the stoichiometric equation of perchlorate reduction is: $ClO_4^- + CH_3COO^- = Cl^- + HCO_3^- + CO_2 + H_2O$. In practical perchlorate treatment projects, the electron donor calculated using stoichiometric equations is not enough for optimal perchlorate reduction, especially when there are competing electron acceptors available (Shrout and Parkin 2006; Nozawa-Inoue *et al*., 2011). Usually a safety factor of 1.6-2 is needed (Shrout and Parkin 2006).

2.3.1.4 Salinity

The wide use of the ion-exchange process for *ex situ* perchlorate removal from drinking water creates an urgent need to completely remove perchlorate in regenerant brines. Microbial perchlorate reduction in high salt solutions is a possible cost-effective way to solve this problem.

When NaCl is > 2.5%, the perchlorate-reducing activity of most characterized PRB strains is significantly inhibited. The only known salt-tolerant strain was *Citrobacter sp.* IsoCock1, which could sustain 10% NaCl (Okeke *et al*. 2002). Another slightly salt-tolerant strain is *Moorella perchloratireducens* $An10^T$, which could sustain 0-4% NaCl (Balk *et al*. 2008).

Some researchers have tried to enrich salt-tolerant perchlorate-reducing cultures from activated sludge (Gingras and Batista 2002). However, these cultures were almost fully inhibited by 1.5% NaCl. A culture isolated from a saline lake-water enrichment could grow in saline solution with NaCl up to 11% (Logan *et al*. 2001a). The maximum growth rate at 5% NaCl was $0.060 \pm 0.003\ d^{-1}$. The culture developed in Dr. Roberts' research lab was capable of simultaneously reducing 100 mg/L ClO_4^- and 500 mg/L NO_3^- within 5 hours in a synthetic brine containing 3% NaCl and within 24 hours in a synthetic brine containing 6% NaCl (Aldridge *et al.* 2003; Cang *et al.* 2004). When the culture was used to treat real ion-

exchange spent brines in laboratory-scale batch reactors, it could reduce perchlorate in brines containing 3 or 5.2% NaCl to nondetectable limits with the addition of $MgCl_2$ to achieve a molar ratio of Mg^{2+}/Na^+ at 0.11 (Lin *et al.* 2007). At the same molar ratio of Mg^{2+}/Na^+, the culture could reduce perchlorate in real ion-exchange spent brines to below detection limits within 24 hours at different salt concentrations of up to 10% NaCl (Hiremath *et al*. 2006). In a bench-scale fluidized bed reactor, the culture was capable of reducing perchlorate in synthetic ion-exchange brines containing 6% NaCl (Patel *et al.* 2008).

2.3.1.5 Temperature and pH

Almost all known PRB grow optimally at ~ 30-35 ℃. Strain $An10^T$ (Balk *et al*. 2008), which was isolated from produced water from underground gas storage, is a unique thermophilic PRB, with the growth temperature ranging from 40 to 70 ℃ (optimal 55 to 60 ℃). Another exception is the culture NP30, which could degrade perchlorate at temperatures from 4-55 ℃ (Zuo 2008). The perchlorate degradation rate of NP30 increased with temperature and maximized at 55 ℃. All known PRB grow at neutral pH with some species growing at pH values as low as pH 5 (Coates and Achenbach 2004).

2.3.2 Perchlorate-reducing enzymes and encoding genes

Two sets of enzymes have been determined to be responsible for microbial perchlorate degradation through the pathway: $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$. The first two steps are catalyzed by perchlorate reductase. Chlorite dismutase catalyzes the disproportionation of chlorite to chloride and oxygen.

Kengen *et al.* (1999) purified and partially characterized a perchlorate reductase from strain GR-1. This enzyme is an oxygen-sensitive enzyme located in the periplasm and had an apparent molecular mass of 420 kDa, with subunits of 95 and 40 kDa in an $\alpha_3\beta_3$ composition. The K_m values for perchlorate and chlorate were 27 and <5 mM, respectively. Besides perchlorate and chlorate, nitrate, iodate, and bromate were also reduced at considerable rates (Kengen *et al*. 1999). Okeke and Frankenberger (2003) purified a perchlorate reductase from strain Perc1ace and presented the amino acid sequences of 22 tryptic peptides from the small subunit of the reductase. The enzyme has a different size than the one from GR-1. Wolterink *et al.* (2003) purified a chlorate reductase from the chlorate-reducing strain *Pseudomonas chloritidismutans*, which can only reduce chlorate and bromate. Differences were found in N-

terminal sequences, molecular weight, and subunit composition compared to perchlorate reductase of strain GR-1.

van Ginkel *et al.* (1996) purified a chlorite dismutase from strain GR-1. The specific activity of the enzyme was 2.0 mmol of chlorite per mg of protein per min. The enzyme had a molecular mass of 140 kDa. Coates *et al.* (1999) purified a chlorite dismutase from strain CKB. The specific activity of the enzyme was 1.928 mmol of chlorite per mg of protein per min. The enzyme was a homotetramer with a molecular mass of approximately 120 kDa. Recently more chlorite dismutase enzymes were purified and characterized, and their results were similar to these two (Stenklo *et al*. 2001; Bender *et al*. 2002).

The genes encoding perchlorate reductase were recently purified from two *Dechloromonas* species and characterized by Bender *et al*. (2005). The genes were organized as *pcrABCD.* The genes of *pcrAB* and *pcrD* were similar to subunits of nitrate reductase, selenate reductase, and chlorate reductase, all of which are type II members of the microbial dimethyl sulfoxide (DMSO) reductase family. The *pcrC* gene product was similar to a c-type cytochrome (Bender *et al*. 2005). The transcription of *pcrA* gene occurred only under anaerobic perchlorate-reducing conditions. The presence of oxygen completely inhibited *pcrA* expression regardless of the presence of perchlorate, chlorate, or nitrate (Bender *et al*. 2005). This study showed that the genes encoding perchlorate reductase were different from those encoding chlorate reductase, which were *clrABDC* (Bender *et al*. 2002).

The studies of the chlorite dismutase gene from *D. agitata* strain CKB and *Ideonella dechloratans* revealed that there was only one gene *cld* encoding chlorite dismutase (Bender *et al*. 2002; Thorell *et al*. 2003). Its expression was basal under aerobic conditions, but when the cells were grown under perchlorate reducing condition, its transcription was upregulated. In contrast, the chlorite dismutase gene expression was constitutive in the chlorate-reducing microorganisms *Pseudomonas* strain PDA and strain PK (Xu *et al*. 2004). In *D. agitata, the cld* gene was located upstream and transcribed in the same direction as the perchlorate reductase gene; while in *D. aromatica, the cld* gene was located downstream of the perchlorate reductase gene (Coates and Achenbach 2004).

2.3.3 Microbial ecology of PRB in bioreactors or natural consortia

Due to the availability of genetic and molecular tools, the microbial ecology in PRB

bioreactors has been extensively studied with the intent to identify the dominant microorganisms in bioreactors and the change of the organisms with water quality.

Zhang *et al*. (2005) used 16S-23S ribosomal intergenic spacer analysis (RISA) and fluorescence *in situ* hybridization (FISH) techniques to analyze the microbial communities in a pilot-scale perchlorate-reducing upflow bioreactor. The bioreactor was inoculated with a pure perchlorate-reducing culture *Dechlorosoma* sp. strain KJ. However, after 6 months' operation, strain KJ only constituted <1% of all cells in the biofilms in the reactor, except in the deepest portion it represented 3–5%. The predominant perchlorate-reducing species in the biofilm was an indigenous *Dechloromonas* strain. Its portion in the biofilms increased with the height, which suggested that perchlorate reduction occurred at the top-end in the bioreactor and nitrate was degraded first in the bottom. Nerenberg *et al*. (2008) determined *Dechloromonas* species as the dominant PRB in a hydrogen-based membrane biofilm reactor (MBfR) determined by denaturing gradient gel electrophoresis (DGGE) and FISH. In a recent study, the analysis of microbial communities of PRB growing on zero-valent iron was performed using a polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) technique and fatty acid methyl esters (FAMEs) and subsequent principal component analysis (PCA) (Son *et al*. 2011). FAME and PCA tests showed clear distinctions between iron-supported cultures and inoculum bacteria. DGGE patterns confirmed the iron-supported cultures were more similar to hydrogen-fed cultures compared to acetate-fed cultures.

In some recent studies, the specific genes or enzymes instead of microbial communities in the mixed culture were studied. Quantitative PCR (qPCR) assays targeting chlorite dismutase *(cld*) and perchlorate reductase subunit A (*pcrA*) genes were used to quantify these perchlorate-related genes in a perchlorate-reducing enrichment culture (De Long *et al*. 2010). The results showed that *cld* assays detected higher quantities of genes and transcripts than *pcrA* assays. The higher matches between *cld* primer sets and *cld* sequences in the cultures, and the existence of some chlorate-reducing bacteria in the culture were attributed to the discrepancy. Thus, the authors concluded the qPCR assays targeting *cld* and *pcrA* genes were more suitable for analysis of relative change of a culture instead of absolute quantities. A liquid chromatography-mass spectrometry-based proteomics approach for the detection of perchlorate-reducing enzymes was developed to measure the ability of microorganisms to degrade perchlorate (Bansal *et al*. 2011). Signature peptides derived from

cld, *pcrA* and perchlorate reductase subunit B (*PcrB*) were selected as biomarkers of perchlorate presence and biodegradation. The biomarker peptides were detected at perchlorate concentrations as low as 0.1 mM and at different time points both in pure cultures and within perchlorate-reducing environmental enrichment consortia.

In summary, the wide use of ion-exchange processes to remove perchlorate from drinking water creates an urgent need to develop methods for the regeneration or treatment of perchlorate-laden ion-exchange resins and regenerant brines. A biological process would be a promising approach. Although many mixed and pure perchlorate-reducing cultures have been enriched and isolated, only a few salt-tolerant perchlorate-reducing cultures have been documented. Only one pure salt-tolerant perchlorate-reducing strain has been isolated and characterized. The mixed culture NP30, developed in Dr. Roberts' lab, is the only culture that has been documented to treat real ion-exchange spent brines. Although NP30 could reduce perchlorate in real ion-exchange spent brines within a reasonable time in laboratory batch reactors (Hiremath *et al.* 2006), its performance in scaled-up continuous-flow reactors was not stable. The improvement of understanding of salt-tolerant, perchlorate-reducing cultures via studies on mixed culture NP30 and especially on isolation and characterization of pure strains would greatly promote the ion-exchange process for perchlorate treatment in drinking water.

CHAPTER 3 EXPERIMENTAL AND NUMERICAL ANALYSIS OF BIOLOGICAL REGENERATION OF PERCHLORATE LADEN ION-EXCHANGE RESINS IN BATCH REACTORS

The biological regeneration of exhausted single-use ion-exchange resin involves the addition of the resin to a synthetic medium containing chloride ions and salt-tolerant, perchlorate and nitrate-reducing bacteria. The salt is required to initiate the desorption of perchlorate from the resins. The salt-tolerant bacteria degrade the perchlorate ions in solution as electron acceptors, using the acetate supplied as a carbon source and electron donor (Cang *et al*. 2004; Lehman *et al*. 2008; Hiremath *et al*. 2006; Lin *et al*. 2007). This chapter presents initial results for the biological regeneration of resin laden with perchlorate and the development of a numerical approach to the analysis and design of the biological batch system that can be used to treat exhausted single-use ion-exchange resins.

The objectives of the research were as follows:

- Conduct bench scale experiments to generate kinetic and equilibrium data to calibrate the numerical simulations.
- Develop a conceptual model based on:
  - ✓ Desorption kinetics of the perchlorate from the loaded resin.
  - ✓ Degradation of perchlorate by the salt-tolerant culture.
  - ✓ Bacterial growth with respect to acetate and perchlorate loading.
- Conduct a sensitivity analysis to determine the most sensitive parameters for further experimental study.

Experimental investigations confirmed that perchlorate-selective ion-exchange resins laden with perchlorate could be regenerated in a batch process using a salt-tolerant bacterial enrichment culture in 6% NaCl solution. The numerical model developed using a two-site perchlorate desorption module coupled with a modified Monod microbial metabolism for 'direct' resin phase and aqueous phase biodegradation fit with the experimental data well. The analysis revealed that the biodegradation process probably includes degradation of perchlorate in both a concentrated liquid film immediately on or around the resin and in the bulk aqueous phase. Model sensitivity analyses revealed that the initial mass of organisms added, the maximum degradation rate and the half saturation constant in the liquid film

around the resin had the largest effect on the time to reach complete degradation. The perchlorate selectivity coefficient with respect to chloride also had a significant effect on the regeneration time. Higher selectivity coefficients resulted in shorter regeneration times. Because the majority of the perchlorate was degraded in the liquid film, the resin desorption constant, resin amount and the aqueous phase degradation coefficients had the least effects on the process. Since many of the parameters are controlled by the nature of the resin and the culture itself, the regeneration process can be maximized by using resins with higher selectivity for perchlorate with high concentrations of biomass (> 1,500 mg VSS/L).

3.1 Materials and methods

The experimental work documented in this chapter were performed by Vikram Kashyap in the University of Houston.

3.1.1 Resin exhaustion

The perchlorate-selective resins used in the experimental part of this study were Sybron Chemical's Ionac SR-7 and Rohm and Haas Amberlite IRA-996. Both are macroporous polystyrenedivinylbenzene polymers. The major different characteristics between these two resins are the ClO_4^-/Cl^- separation factor and the loading capacity, which are shown in Table 3-1. The higher the ClO_4^-/Cl^- separation factor, the stronger the perchlorate is adsorbed onto the resin and the more difficult the desorption process will be. Table 3-1 shows that the ClO_4^-/Cl^- separation factor of IRA-996 resin is about 60% smaller than SR-7 resin, while its loading capacity is larger. The resins were placed in a 25.4 mm I.D. downflow column and saturated with a solution containing 2 mg/L ClO_4^- in 6% NaCl. The NaCl solution was used to ensure that the perchlorate ions would only occupy a fraction of the sites as is typical during the treatment of groundwater containing other ions such as nitrate, bicarbonate, and sulfate. The exhaustion solution was placed in a carboy at a level above that of the column so as to obtain gravity flow. The flow rate was controlled to 30 mL/min (2 min of EBCT) by using a valve at the inlet to the column.

Table 3-1 Characteristics of the tested strong base anion (SBA) resins

Resin Name	Functionality	ClO_4^-/Cl^- Separation Factor	Loading capacity (eq./g)
Ionac SR-7	Tripropyl Amine	1300	0.00206
Amberlite IRA-996	Triethyl Amine	800	0.00312

3.1.2 Synthetic medium

A synthetic medium based on general marine medium composition with the addition of a group of trace metals as developed by Cang *et al*. (2004), was used for culture development and maintenance. The medium contained 21.8 g/L $MgCl_2 \cdot 6H_2O$, 1.4 g/L $CaCl_2 \cdot 2H_2O$, 0.72 g/L KCl, 60 g/L NaCl, 0.3 mL/L Resazurin (0.1%), 205.5 mg/L $CH_3COONa \cdot 3H_2O$, 0.3 g/L $NaHCO_3$, 5 mL/L 67 mM $Na_2S \cdot 9H_2O$, 1 mL/L phosphate solution (50 g/L KH_2PO_4), 1 mL/L mineral solution (which contained 50 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 g/L $ZnCl_2$, 0.3 g/L H_3BO_3, 1.5 g/L $FeCl_2 \cdot 4H_2O$, 10 g/L $CoCl_2 \cdot 6H_2O$, 0.03 g/L $MnCl_2 \cdot 6H_2O$, and 0.03 g/L $NiCl_2 \cdot 6H_2O$). Sulfate was omitted purposely to avoid the development of sulfate-reducing bacteria. Sodium acetate was used as the sole electron donor and perchlorate desorbing off the resin acted as the electron acceptor. The initial concentration of sodium acetate was kept as high as 2 g/L to make sure the sodium acetate would not be a limiting factor. Lin *et al*. (2007) demonstrated that the stable activity of the culture in high salt solutions required the addition of magnesium to achieve a Mg/Na ratio of 0.11 (mol/mol) so the Mg/Na ratio was kept at 0.11 mol/mol in this study. This ratio is very typical of the ratio of these ions in seawater which may explain the requirement by this culture. During the preparation of synthetic medium, general anaerobic culture techniques were followed (Hungate 1950). Synthetic medium was boiled on a hot plate for ~2 min to remove dissolved oxygen and cooled under a flush of nitrogen gas in an ice bath before being dispensed into experimental cultures.

3.1.3 Inoculum source

The inoculum was obtained from the salt-tolerant, perchlorate-degrading culture developed by Cang *et al*. (2004). Previous molecular analysis revealed that the dominant perchlorate- and nitrate-reducing bacteria in the culture were *Azoarcus* and *Halomonas* separately (Zuo *et al*. 2009). The culture has been maintained using daily spikes of 100 mg/L perchlorate and a weekly sequencing batch reactor operation using a 50% medium replacement after settling the biomass. Since the synthetic medium used in the parent culture contained perchlorate and was at 3% NaCl, the biomass drawn for the experiments was centrifuged at 1000 rpm (275 ×g) for 2 minutes and washed twice with 6% synthetic medium to remove any residual perchlorate, and finally resuspended in synthetic medium to get a

VSS concentration of 500 mg/L in each 75 mL serum bottle.

3.1.4 Resin regeneration batch test

The effluent histories obtained during exhaustion were used to calculate the resin loadings which were in turn used to calculate a mass of resin that would give a perchlorate equilibrium concentration of 2 mg/L in solution. The calculated amounts of each resin type were added to a 75 mL serum bottle along with the synthetic medium and the perchlorate reducing inoculum to make a volume of 50 mL. The control bottles contained resin and synthetic medium without inoculum to establish desorption of perchlorate off of the resin. Multiple test samples were set up containing synthetic medium, exhausted resin and the inoculum. The bottles were placed on a shaker at 120 cycles per min for a period of 48 hours at room temperature (20 ℃). Triplicate test bottles were sampled at each time period. Test samples were taken out from the bottles immediately after the test bottles were acidified (with HCl) to get a pH of lower than 4 to kill the organisms before each sampling. Then the bottles were put back on the shaker for 48 hours and then sampled again. Those new samples were called killed samples or new equilibrium samples. Thus each test replicate was sampled twice to get two perchlorate concentrations with one showing the concentration of perchlorate in the aqueous phase at the time of the first sampling and another showing the new equilibrium concentration after the culture was killed and resins were allowed to re-equilibrate. The controls were run with sampling intervals the same as the test samples to estimate the rate of desorption.

3.1.5 Evaluation of performance of regenerated resin

After regeneration, the resins were separated from the biomass by rinsing them with DI water in a filter funnel using 150 mm diameter paper filters. The resins were then sterilized using methanol and re-rinsed with DI water to remove any traces of methanol. The resins were then dried in an oven at 30 ℃ for one day with periodic mixing to ensure that the resin dried evenly. Approximately 3 mL of fresh or regenerated resin was packed in chromatographic columns having an I.D. of 1 cm. A peristaltic pump was used to pump the exhaustion solution containing 5 mg/L of ClO_4^- at a constant flow rate of 4 mL/min, to give an empty bed contact time (EBCT) of 0.75 min and a superficial liquid velocity (SLV) of ~ 5

cm/min. The high initial ClO_4^- concentration and a higher flow rate were used to accelerate the ClO_4^- sorption and minimize the time to achieve perchlorate removal as well as waste generation. Effluent samples were collected at regular intervals until ~ 50% breakthrough of ClO_4^- was observed.

3.1.6 Model development

A numerical model incorporating the principles of desorption kinetics and equilibrium driving force for perchlorate desorption from the resin and microbial degradation was developed. The objective of the modeling process was the prediction of the conditions which would allow the shortest reaction time for the complete removal of the perchlorate from the aqueous phase and thus resin in batch reactors. In this research, the complete removal of perchlorate was considered to be achieved when the perchlorate concentration was less than 50 μg/L (the detection limit of the instrument) in new equilibrium samples. The parameter values determined for the Ionac SR-7 resin were used to develop the model and these were applied to IRA 996 for model validation. The major assumptions were: 1) there was no lag time before biodegradation began; The experimental results showed that during the first 30 minutes, the biodegradation of perchlorate already started. Thus, the lag time must have been less than 30 minutes. Since no test samples were taken during the first 30 minutes, in the model, the lag time was assumed to be zero. 2) Although the perchlorate- and nitrate-reducing bacteria (PNRB) inoculum was a mixed culture, it was dealt with as a composite one in previous kinetics studies without any efforts to separate it. Thus, in this model, the 'direct' biodegradation was attributed to the mixed culture and effective PNRB was attracted onto the resin surface at the same probability for non-effective bacteria. 3) the biomass added in the medium did not affect the desorption of perchlorate off the resin.

The desorption profile determined experimentally for the Ionac SR-7 resin (Figure 3-1) demonstrates that desorption kinetics were rapid at the beginning, but when the aqueous perchlorate concentration was about 60% of the equilibrium concentration, the rapid desorption kinetics stopped. After that, a comparatively slower desorption phase started. This second rate was much slower than could be predicted with a single kinetic model and suggests that the resins have more than 1 type of binding site. A 2-site driving force equation was developed to model the desorption kinetics found in the control samples (Equation 1).

The difference between the final equilibrium concentration and the aqueous phase concentration at any time was used as the driving force, which allows the incorporation of the concepts of kinetic limitations and end point equilibrium.

$$\frac{dC_{a,t}}{dt} = \begin{cases} k_{des1}\left(C_{e,a,0} - C_{a,t}\right) & \text{if } C_{a,t} \le f\ C_{e,a,0} \\ k_{des2}\left(C_{e,a,0} - C_{a,t}\right) & \text{if } C_{a,t} > f\ C_{e,a,0} \end{cases} \quad (1)$$

where, $C_{a,t}$ (eq./L) is the perchlorate concentration in the aqueous phase in control samples at time *t*; $C_{e,a,0}$ (eq/L) is the final equilibrium concentration of perchlorate in control samples; k_{des1} and k_{des2} (h^{-1}) are the desorption kinetic constants exhibited during and after the initial rapid interval respectively; f is the coefficient for switching between the 2-site desorption coefficients. In control samples, Equation 1 can be used alone to calculate the concentration of the anion in the aqueous phase at each time period.

In test samples the equilibrium concentration is changing continually. The perchlorate desorption rate in test samples is expressed using Equation 2.

$$r_{d,t} = \begin{cases} k_{des1}\left(C_{e,a,t} - C_{a,b,t}\right) & \text{if } C_{a,b,t} \le f\ C_{e,a,0} \\ k_{des2}\left(C_{e,a,t} - C_{a,b,t}\right) & \text{if } C_{a,b,t} > f\ C_{e,a,0} \end{cases} \quad (2)$$

where, $r_{d,t}$ (eq./L-h) is the rate of desorption of perchlorate from the resin phase in test samples at time of *t*; $C_{e,a,t}$ (eq/L) is the equilibrium concentration of perchlorate in samples killed at time *t*; $C_{a,b,t}$ (eq/L) is the perchlorate concentration in the aqueous phase in test samples at time *t*. The equilibrium concentration can be calculated using the degree of affinity of the anion (perchlorate) with respect to chloride ion for the resin. This is expressed as a function of separation factor (α) and can be determined using Equation 3 (Clifford 1982).

$$\alpha = \frac{y_a x_{Cl}}{y_{Cl} x_a} \quad (3)$$

where, x_a is the mole fraction of perchlorate at equilibrium in the aqueous phase; x_{Cl} is the mole fraction of chloride at equilibrium in the aqueous phase; y_a is the mole fraction of perchlorate at equilibrium in the resin phase; y_{Cl} is the mole fraction of chloride at equilibrium in the resin phase.

Because the concentration of the perchlorate in the resin phase is not directly measurable but the total concentrations of either the chloride or the perchlorate are either known or calculable, the following relationships are defined (Equations 4-6) and substituted into equation 3.

$$x_{Cl} = \frac{C_{e,Cl}}{C_T} \quad (4)$$

$$x_a = \frac{C_{e,a,t}}{C_T} = 1 - \frac{C_{e,Cl}}{C_T} \quad (5)$$

$$y_a = 1 - y_{Cl} \quad (6)$$

where, $C_{e,Cl}$ (eq./L) is the equilibrium concentration of chloride in the aqueous phase; C_T (eq./L) is the total anionic equilibrium concentration in the aqueous phase. Rearranging equation 3, $C_{e,Cl}$ and $C_{e,a,t}$ are correlated to the separation factor (α) as:

$$\alpha = \frac{y_a}{1-y_a}\frac{C_{e,Cl}}{C_{e,a,t}} \quad (7)$$

At equilibrium, the fraction of the amount of anion in the resin phase (y_a) can be calculated using Equation 8.

$$y_a = \frac{M_{a,t} - C_{e,\mathrm{a,t}}V}{L_a G} \quad (8)$$

where, $M_{a,t}$ (eq.) is the total amount of perchlorate in resin and aqueous phase in samples killed at time t, L_a (eq./g) is the loading capacity of air-dried resin; G (g) is the amount of air-dried resin used in batch experiments; V (L) is the volume of regeneration solution in batch experiments. Substituting Equation 8 into Equation 7 and solving for $C_{e,a,t}$ and $M_{a,t}$, gives Equations 9 and 10. When t = 0, $M_{a,t}$ is the initial total mass of perchlorate on resin, and $C_{e,a,t}$ is the equilibrium concentration of perchlorate in control samples.

$$M_{a,t} = C_{e,\mathrm{a,t}}V + L_a G \frac{\alpha C_{e,\mathrm{a,t}}}{C_{e,Cl} + \alpha C_{e,\mathrm{a,t}}} \quad (9)$$

$$C_{e,a,t} = \sqrt{\frac{M_{a,t}C_{e,Cl}}{\alpha V} + \left(\frac{L_a G - M_{a,t}}{2V} + \frac{C_{e,Cl}}{2\alpha}\right)^2} - \left(\frac{L_a G - M_{a,t}}{2V} + \frac{C_{e,Cl}}{2\alpha}\right) \quad (10)$$

In the regeneration process, the chloride concentration in the aqueous phase was highly concentrated so any change in concentration of chloride from the initial to completion is insignificant and can be ignored in the model. Thus, $C_{e,Cl}$ was equal to 1.2748 eq./L (6% NaCl) at all times. Since the equilibrium concentration of perchlorate was known, the initial amount of perchlorate on the resin can also be calculated using Equation 9. Using Equation 2 combined with equations 10 and 15, the desorption rate in test samples at any time can be determined.

The next requirement of the model is to calculate the amount of perchlorate removed at any time interval due to biodegradation. The experimental results suggested there might

be a 'direct' biodegradation of perchlorate in the resin phase. The model used the Monod rate law to simulate the 'direct' biodegradation of perchlorate in the resin phase. As it is impossible to measure the concentration of perchlorate in the aqueous film, the total amount of perchlorate on the resin phase was used as the substrate. In the model (Equations 11 and 12), the ratio of total perchlorate anions left on the resin at time *t* to the initial total anions was used to express the limitation of the availability of anions. n was the limitation coefficient determined by an automated parameter estimation algorithm.

$$r_{r,t} = \frac{V_{mr} M_{a,r,t} X_t}{K_{s,r} + M_{a,r,t}} \left(\frac{M_{a,r,t}}{M_{a,0}}\right)^{n} \quad (11)$$

$$M_{a,r,t} = M_{a,t} - C_{a,t} V \quad (12)$$

where, $r_{r,t}$ (eq./h) is the rate of biodegradation of perchlorate in the resin phase at time *t*; V_{mr} (eq.-L / mg VSS-h) is the maximum specific perchlorate degradation rate in the resin phase exhibited during the initial rapid interval; $M_{a,r,t}$ (eq.) is the total amount of perchlorate in the resin phase at time *t*; X_t (mg/L) is the biomass concentration (VSS) in samples at time *t*; K_{Sr} (eq.) is the half saturation constant of perchlorate degradation in the liquid film of the resin phase; *n* is the coefficient of available perchlorate for resin phase biodegradation.

In addition to the 'direct' biodegradation of perchlorate from the resin, the perchlorate in the aqueous phase would also be subject to biodegradation. The Monod rate law was used again to simulate the aqueous phase biodegradation (Equation 13).

$$r_{a,t} = \frac{V_{ma} X_t C_{a,b,t}}{K_{s,a} + C_{a,b,t}} \quad (13)$$

where, $r_{a,t}$ (eq./L-h) is the rate of biodegradation of perchlorate in aqueous phase at time *t*; V_{ma} (eq. / mg VSS-h) is the maximum specific perchlorate degradation rate in aqueous phase; K_{Sa} (eq./L) is the half saturation constant of perchlorate degradation in aqueous phase.

The concentration of biomass (*X*) at any time (*t*) was calculated using the yield of biomass (*Y*), which was determined as 0.06 mg VSS/mg ClO_4^- in previous research (Zuo, 2008) (Equation 14).

$$\frac{dX_t}{dt} = Y\left(\frac{r_{r,t}}{V} + r_{a,t}\right)(MW) \quad (14)$$

where, *Y* (mg VSS / mg ClO_4^-) is the yield of perchlorate-reducing biomass; *MW* is the molecular weight of ClO_4^- (99,450 mg /eq.). After biodegradation, the change rate of total amount of perchlorate available in the resin and aqueous phase at any specific time is

equivalent to the sum of the perchlorate degradation rate in 'direct' and aqueous biodegradation. (Equation 15).

$$\frac{dM_{a,t}}{dt} = -\left(r_{r,t} + r_{a,t}V\right) \quad (15)$$

The total amount of perchlorate is distributed in both the resin and aqueous phases as a function of the separation factor (α). When the test samples were killed, the biodegradation stopped. The residual perchlorate desorbed off the resin and the system attained to a new equilibrium. The new equilibrium concentration of perchlorate ($C_{e,a,t}$) in samples killed at any time (t) can be calculated using Equation 10, since the total residual perchlorate is determined using Equation 15.

The difference between the new equilibrium concentration of perchlorate ($C_{e,a,t}$) and the aqueous phase concentration ($C_{a,b,t}$) is the driving force for the desorption of perchlorate in biodegradation samples. The desorption rate can be calculated using Equation 2 (above). The change of perchlorate concentration in aqueous phase in biodegradation samples ($C_{a,b,t}$) is the total result of perchlorate desorbed from the resin and biodegraded in the aqueous phase, which is expressed by Equation 16.

$$\frac{dC_{a,b,t}}{dt} = r_{d,t} - r_{a,t} \quad (16)$$

The mathematical model was a system of ordinary differential equations with given initial values (at t=0), which was resolved using Euler's algorithm as an approximation in MATLAB® 7.1.

Parameters k_{des1}, k_{des2}, f, K_{Sr}, K_{Sa}, V_{mr}, V_{ma} and n were estimated using the scatter search method (SSm), a global optimization method developed by Rodriguez-Fernandez *et al*. (2006) and Egea *et al*. (2007). As a hybrid stochastic deterministic global optimization method with robustness and reduced computational time, the SSm method is especially good for nonlinear dynamic models. The interrelationship between the estimated parameters was measured using the parameter correlation matrix. The elements (R_{ij}) in the correlation matrix are the approximate correlation coefficients between i-th and j-th parameter, defined by Equation 17.

$$R_{ij} = \frac{C_{ij}}{\sqrt{C_{ii}C_{jj}}} \quad (17)$$

where, C is the covariance matrix of the sensitivity functions with respect to the parameters

defined by Equation 18.

$$C_{jk} = \frac{1}{N}\left[\sum_{i=1}^{N}\left(x_{ij} - \overline{x_j}\right)\left(x_{ik} - \overline{x_k}\right)\right] \quad (j, k=1,..., K) \tag{18}$$

where, $x_{ij(k)}$ is the sensitivity function of *i*th response (*i*=1,..., *N*) with respect to the *j*(*k*)th parameter (*j*(*k*)=1,..., *K*) estimated at $\hat{p}$, i.e., $\frac{\partial y_i}{\partial p_j}(\hat{p})$. They were arranged in an $N \times K$ matrix. Here, *K*=8 (the total number of parameters to be estimated) and *N*=3. The three responses chosen for sensitivity analysis were the perchlorate concentration in the aqueous phase in control samples at time *t* ($C_{a,t}$), the equilibrium concentration of perchlorate in samples killed at time *t* ($C_{e,a,t}$), and the perchlorate concentration in the aqueous phase in test samples at time *t* ($C_{a,b,t}$). If the correlation coefficient between two parameters is equal to 1, the two parameters are linearly dependent and the parameters are uncertain.

Euler's algorithm is sensitive to the increment. In order to get an appropriate increment Δt, a sensitivity test for the increment was done by comparing the sensitivity function with respect to four different increments (Δt = 0.1, 0.05, 0.01, and 0.005 hr). For model validation, the model was applied to the Amberlite IRA-996 resin using the α specific for this resin.

3.2 Results and discussion

3.2.1 Experimental results

An initial experimental study was performed as a proof of concept. Ionac SR-7 and Amberlite IRA-996 resins were exhausted with perchlorate and then used in batch regeneration study tests. The perchlorate concentrations from control (resin in medium), and test (resin in medium plus inoculum) samples of Ionac SR-7 resins are presented using the scattered dots in Figure 3-1 a. The results from the control samples show that the perchlorate desorbed from the exhausted resin very rapidly over the first 2 hours and then equilibrium was slowly reached by about 40 hours. Perchlorate was not detectable in the aqueous phase in test samples after 5 hours. The new equilibrium samples (48 hours after the culture were killed) showed that perchlorate was left on the resin for up to 40 hours. These results suggest that the rate of desorption of the perchlorate from the exhausted resin might be the rate limiting step during the slow desorption phase.

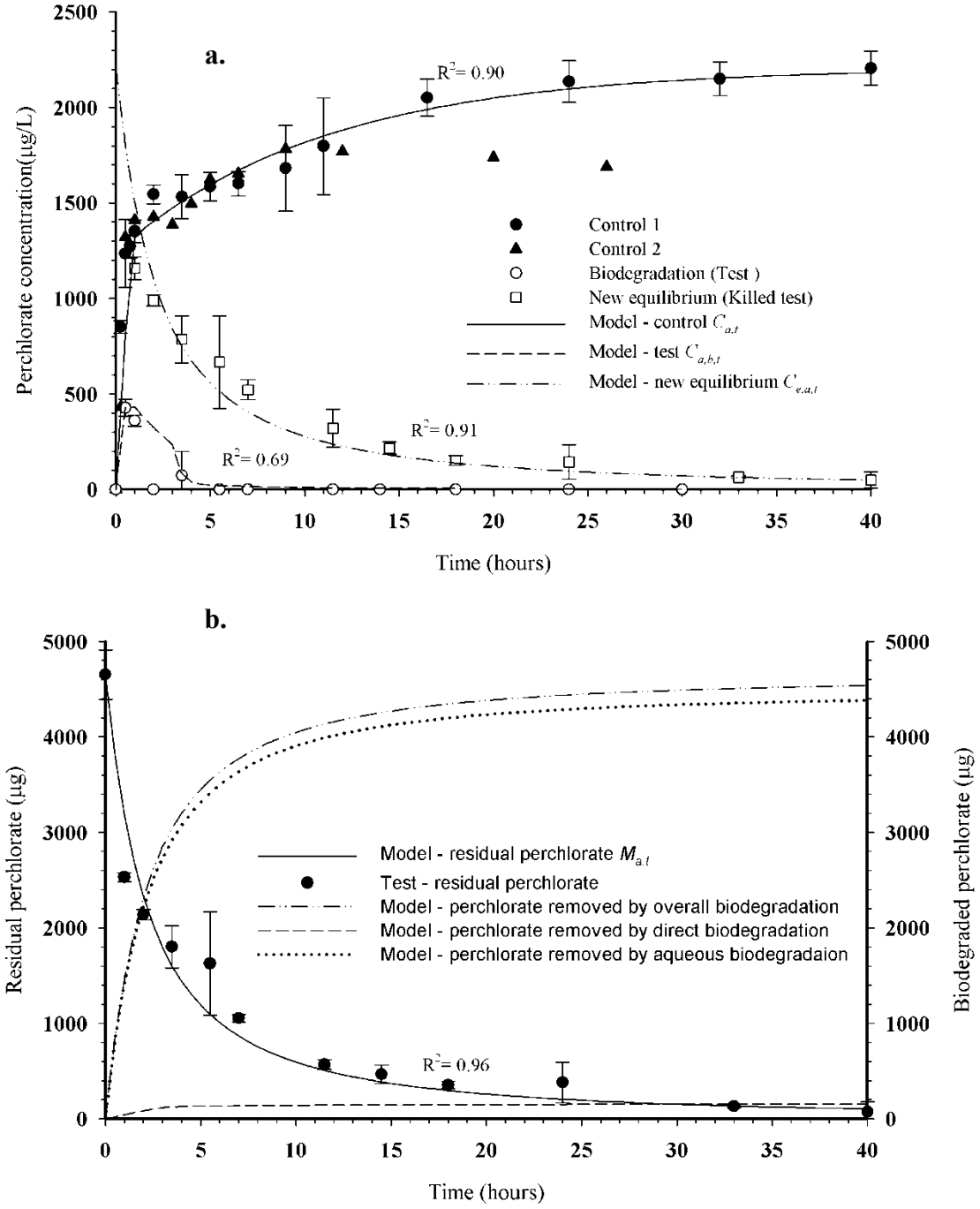


Figure 3-1 Batch bioregeneration of perchlorate-exhausted Ionac SR-7

a.) Experimental and model-predicted perchlorate concentrations in the aqueous phase; b.) Calculated and model predicted perchlorate mass balance. Error bars represent the standard deviations of triplicate tests. Data of Control 1 were from a triplicate experiment. Data of Control 2 were from a different batch experiment with no replicate and were not used to calculate the R^2 value.

The mass of perchlorate left on the resins at any time in the test cultures was calculated from the new equilibrium concentrations in the killed samples using Equation 9. The scattered dots in Figure 3-1 b show the calculated masses. The figure shows that the mass removal curve follows a similar curve to the new equilibrium aqueous phase concentrations. The amount of perchlorate degraded in test samples is much greater in the initial phase than can be explained by desorption alone. This suggests that the rate of biodegradation in this initial stage was not limited by the desorption of perchlorate from the resins, which is the opposite of the conclusions from the last phase of the batch system.

It is interesting to notice that the biodegradation kinetics expressed in the regeneration process were faster than the PNRB culture could achieve even if all of the perchlorate on the resin had been in the aqueous phase at the beginning (based on (Zuo 2008)). In that study, the Monod model was used to describe the perchlorate degradation by the PNRB culture. At a biomass concentration of 5473 mg VSS/L, the half saturation constant of perchlorate degradation in aqueous phase K_{Sa} and the maximum degradation rate V_{ma} were determined to be 26.39 $\pm$2.16 mg ClO_4^-/L and 0.0043 $\pm$0.00041 hr^{-1}. In the regeneration experiment of SR-7 resin, the average initial and final (t = 40 hr) ClO_4^- concentration would be 92.82 mg/L and 2.09 mg/L separately, if all perchlorate anions had been dissolved into the 50 mL synthetic medium. The required degradation time would be 88.76 hours with a biomass concentration of 500 mg VSS/L using the kinetics parameters determined in Zuo (2008). For the IRA-996 resin, the time would be 83.47 hours. But the actual regeneration time was about half of this. Thus, there could be a 'direct' biodegradation mechanism occurring in the biological regeneration process. Since most bacteria can only utilize substrate when it is dissolved in water, there might be a liquid film on the resin surface where free perchlorate anions are available for biodegradation in a concentrated form. Figure 3-2 shows the probable conceptual mechanism for this. As shown in the figure, there are many micro pores on the resin surface. In the aqueous phase, a liquid film is formed on the surface of this micro porous resin. Perchlorate anions are desorbed freely into the liquid film, and most of them are limited in the layer of liquid film because of the attraction by the resins. Only a few of the free anions can diffuse into the bulk liquid phase. When PNRB inoculum is added into the solution, the bacteria will be attracted onto the resin surface, because the resins are positively charged and the bacterial cell surface is negatively charged. The free anions in the

liquid film are now available for bacterial degradation. This forms the ‘direct’ biodegradation of perchlorate on resins.

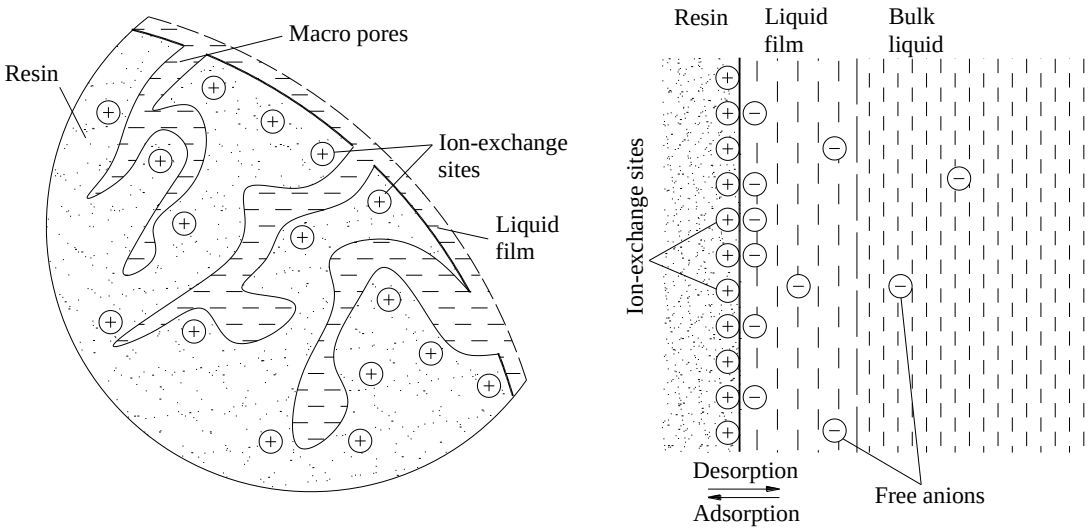


Figure 3-2 Conceptual diagram of ion-exchange anion resin microstructure and the anions desorption mechanism

The mass balance curves in Figure 3-1 b show that the ‘direct’ biodegradation also exhibited much faster kinetics during the initial step and relatively slower kinetics in the later stages. This may be because during the initial step, there were lots of free anions on or near the surface, which were readily degraded by bacteria. It would take much longer for bacteria to get into the micro pores so the free perchlorate anions in the aqueous film in the micro pores would be degraded more slowly. There would also be many micro pores so tiny that bacteria cannot get into them. The free perchlorate anions in the liquid film of those micro pores can only be degraded after they are fully diffused into the surface aqueous film. The availability of the free perchlorate anions affected the rate of the ‘direct’ biodegradation.

Figure 3-3 a shows the results of bioregeneration of Amberlite IRA-996 resin. The results were similar to those for Ionac SR-7 resin. Perchlorate desorbed from resins rapidly during the initial two hours and then relatively slowly achieved an equilibrium in the control samples. Because the perchlorate separation factor relative to chloride for IRA-996 resin was smaller than Ionac SR-7 resin, it took approximately half of the time (20 hours) to reach

equilibrium. And the time (35 hours) for complete perchlorate degradation for this resin was also shorter than Ionac SR-7 resin (40 hours). The residual perchlorate mass on this resin was also calculated. Figure 3-3 b shows results that were similar to the Ionac SR-7 resin. Again the amount of perchlorate degraded in test samples was much more than the perchlorate that could desorb from the resins in control samples at the each time interval.

The success of the regeneration process was checked by comparing the performance of the regenerated resin to fresh resin in exhaustion studies. Figure 3-4 shows that the regenerated IRA-996 resin performed identically to fresh IRA-996 resin. The results were similar for the Ionac SR-7 resin (data not shown). The regeneration process returned the ion exchange capabilities of the resin to the fresh state.

3.2.2 Model performance

Because most models are sensitive to the incremental time steps used, a sensitivity test for four different increments Δt of 0.1, 0.05, 0.01, and 0.005 h (not shown) was performed. The results showed that the correlation coefficient between Δt of 0.01 h and 0.005 h was 0.996, which means there was almost no variance added to the sensitivity function when the time increment was less than 0.01 h. An increment of 0.01 h was used in all numerical calculations.

The optimal values of the resin parameters k_{des1} , k_{des2} , and f were determined using the SSm method for both resins, whereas the values of the biodegradation parameters V_{mr} , K_{Sr} , V_{ma} , K_{Sa} , and n were estimated for SR-7 resin and then applied to IRA-996 resin. The optimal values of f for the SR-7 and IRA-996 were 0.58 and 0.57, respectively, and the optimal value of n was 0.8 for both resins. The other parameters are shown in the shaded cells in Table 3-2. The parameter correlation matrix for SR-7 resin in Table 3-3 shows that as expected, the three desorption parameters k_{des1} , k_{des2} , and f are highly correlated (r = 0.99) because they are related to properties of the resin. As expected, the three resin phase biodegradation parameters V_{mr} , K_{Sr} , and n and aqueous phase biodegradation parameters (V_{ma} and K_{Sa}) are also highly correlated (r = 1) because they are integral properties of the biological system. The correlation coefficient between the three group parameters (resin phase biodegradation, and aqueous phase biodegradation) is approximately 0.5, indicating that the groups are not correlated and therefore independent.

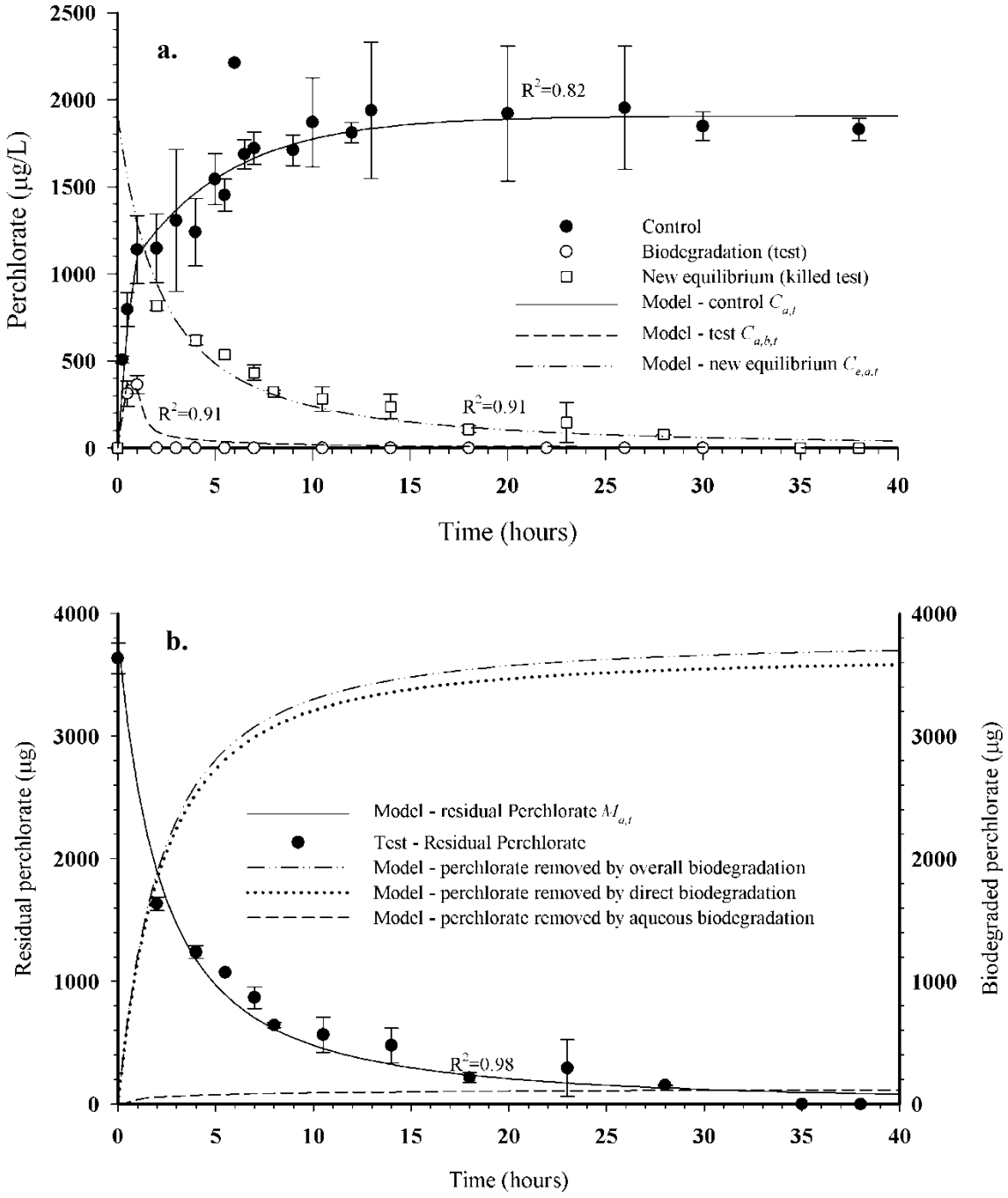


Figure 3-3 Batch bioregeneration of perchlorate-exhausted Amberlite IRA-996

a.) Experimental and model-predicted perchlorate concentrations in the aqueous phase; b.) Calculated and model predicted perchlorate mass balance. Error bars represent the standard deviations of triplicate tests.

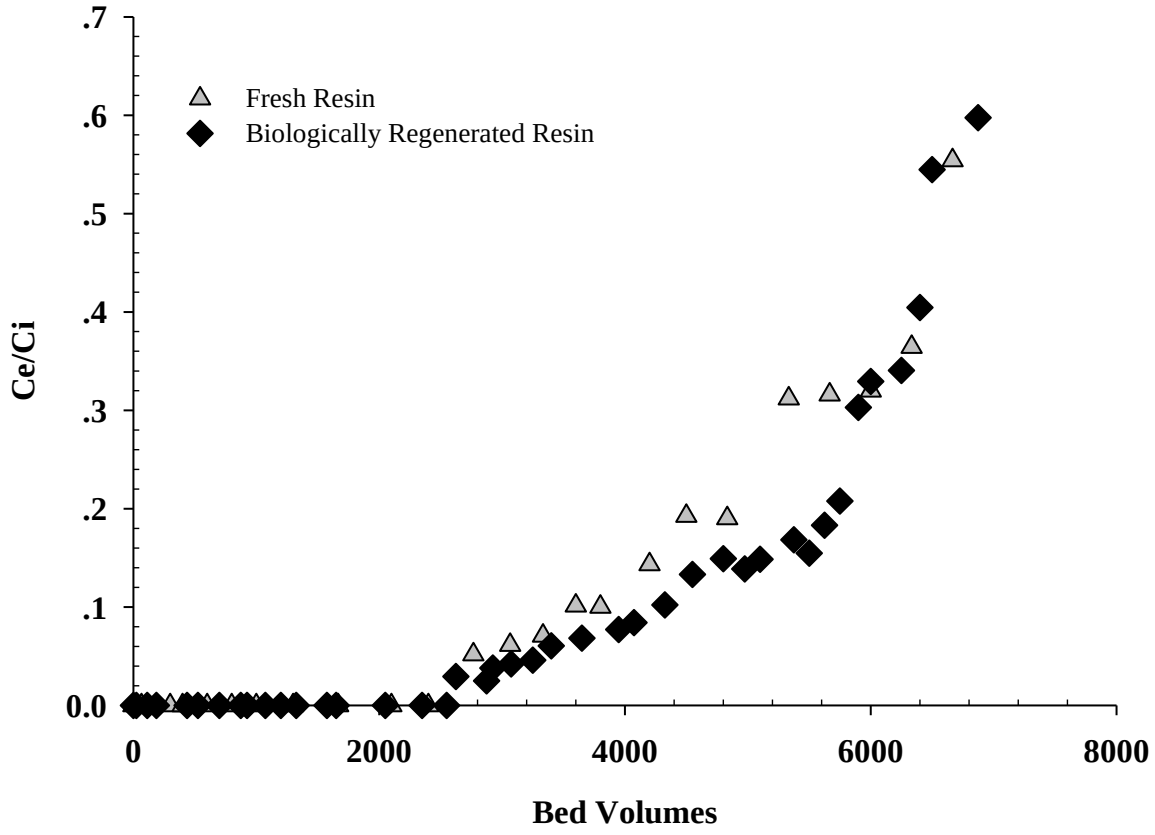


Figure 3-4 Exhaustion profiles of biologically regenerated and fresh IRA 996 resin

A concentrated exhaustion solution was used to minimize bed volumes during regeneration.

The results from the numerical model are plotted as lines in Figure 3-1 and 3-3. The use of two desorption constants (k_{des1}= 1.954 h^{-1} and k_{des2} = 0.087 h^{-1} in Figure 3-1, k_{des1}= .052 h^{-1} and k_{des2} = 0.179 h^{-1} in Figure 3-3), allowed the good prediction of both the initial rapid desorption rate and the slower second rate (R^2= 0.94 and 0.84 for SR-7 and IRA-996 resin separately). It is possible that the slower desorption phenomenon is due to more than one slow rate (due to several different strong perchlorate exchange sites) and therefore would require multiple desorption rate equations in the model. The model does capture the final equilibrium concentration effectively. The same initial rapid desorption constant for both resins suggests that there were similar rapid exchange sites on both resins, since both resins were macroporous polystyrenedivinylbenzene polymers.

Table 3-2 Summary of model sensitivity analysis

Parameters	Unit	Value	Ionac SR-7 resin		IRA-996 resin	
			Reg.T* (h)	Equ.C** (ug/L)	Reg.T* (h)	Equ.C** (ug/L)
G	g	1	39.22	2208.3	34.00	1906.5
		4	42.82	2249.3	37.87	1943.7
		8	43.87	2256.3	39.18	1950.1
α		300	93.42	8892.6	60.21	4888.6
		800	55.50	3537.9	34.00	1906.5
		1300	39.22	2208.3	23.57	1184.2
		1800	30.55	1605.0	18.11	858.8
k_{des1}	h^{-1}	0.150	39.22	2208.3	34.04	1906.5
		1.052	39.22		34.00	
		1.954	39.22		34.00	
		2.856	39.22		34.00	
k_{des2}	h^{-1}	0.040	39.84	2208.3	35.45	1906.5
		0.087	39.22		34.92	
		0.179	38.15		34.00	
		0.225	37.68		33.59	
X_0	mg VSS/L	500	39.22	2208.3	34.00	1906.5
		1000	19.98		17.50	
		1500	13.40		11.79	
$V_{mr}/10^{-7}$	eq.-L/mg VSS-h	0.881	81.03	2208.3	68.40	1906.5
		1.881	39.22		34.00	
		2.881	25.87		22.63	
$K_{sr}/10^{-4}$	eq.	0.540	14.84	2208.3	13.01	1906.5
		1.540	39.22		34.00	
		2.540	62.68		53.57	
$V_{ma}/10^{-7}$	eq./mg VSS-h	3.437	39.27	2208.3	34.13	1906.5
		5.437	39.22		34.00	
		7.437	39.20		33.94	
$K_{sa}/10^{-4}$	eq./L	0.413	39.16	2208.3	33.84	1906.5
		1.413	39.22		34.00	
		2.413	39.28		34.15	
NaCl conc.		3%	23.02	1133.5	20.19	979.2
		6%	39.22	2208.3	34.00	1906.5
		9%	52.39	3259.3	44.49	2812.3

* Reg. T = Regeneration time. **Equ. C = Equilibrium perchlorate concentration in control samples.
In the sensitivity analysis for a specific parameter, other parameters were set at the default values, which are shown in the dark cells. The responses obtained for the default values are also shown in the dark cells.

Table 3-3 Parameter correlation matrix for SR-7 resin

	k_{des1}	k_{des2}	V_{mr}	K_{Sr}	V_{ma}	K_{Sa}	n	f
k_{des1}	1.0000	0.9985	0.5311	-0.5311	0.5566	-0.5565	-0.5309	1.0000
k_{des2}		1.0000	0.5761	-0.5761	0.5109	-0.5108	-0.5760	0.9987
V_{mr}			1.0000	-1.0000	-0.4083	0.4085	-1.0000	0.5342
K_{sr}				1.0000	0.4083	-0.4085	1.0000	-0.5342
V_{ma}					1.0000	-1.0000	0.4085	0.5536
K_{sa}						1.0000	-0.4086	-0.5534
n							1.0000	-0.5341
f								1.0000

The calculated biodegradation and new equilibrium curves shown in Figure 3-2 and Figure 3-3 fit well with the experimental data (R^2= 0.96 and 0.97 for SR-7 and IRA-996 resin separately). The same biodegradation constants were used for both resins suggesting that the biological regeneration process was not affected by resin characteristics.

3.2.3 Estimation of resin regeneration parameters by model sensitivity analysis

The purpose of the sensitivity analyses was to investigate the effects of different parameters on the process and to aid in reactor design and operational decisions. The parameters included in the sensitivity analysis are divided into three groups: 1) resin parameters: the mass of resin (G) added to the batch reactors, the perchlorate separation factor relative to chloride (α), and the perchlorate desorption coefficients (k_{des1} and k_{des2}); 2) biological parameters: the initial concentration of biomass (X_0), the maximum specific perchlorate degradation rate in resin and aqueous phase (V_{mr} and V_{ma}), and the half saturation constant in resin and aqueous phase (K_{sr} and K_{sa}), and 3) medium parameters: salt concentration in medium. The analyses were conducted by varying one parameter and maintaining the others constant as the default value. In order to compare the effects of all the parameters, the regeneration time, which was the time required to reach a new equilibrium concentration of less than 50 μg/L, was selected as the major response. The equilibrium concentration in control samples was selected as another response for comparing the effect of resin parameters. In the analyses, all the parameters were assumed to be independent, although, the biological kinetic parameters in either resin or aqueous phase were highly correlated. Table 3-2 presents a summary of the results.

Group 1: resin parameters. The resin parameters in group 1 did affect the time to

complete resin regeneration. Adding more resin (G) resulted in slightly longer times to complete regeneration. As presented in the table, when the mass of resin was increased from 1 g to 8 g, the regeneration time increased from 39 hours to 44 hours for Ionac SR-7, and from 34 hours to 39 hours for IRA-996. The increase in regeneration time with additional resin over 1 g was linear 0.67 or 0.77 h per gram of SR-7 or IRA-996 resin, respectively, with R^2 values of 0.94 and 0.98, respectively. This suggests that although the addition of more resin resulted in a slightly longer time to complete regeneration, the increased time is a small fraction of the overall batch time and would provide significant savings in reactor space.

The perchlorate separation factor relative to chloride (α) is a measure of the resin selectivity for perchlorate, as seen in Table 3-2. With the increase of perchlorate separation factor, the equilibrium perchlorate concentration decreased greatly, which caused more perchlorate to be retained in the resin phase. In the model, the 'direct' resin phase perchlorate biodegradation is the dominant perchlorate removal mechanism. More perchlorate in the resin phase would accelerate perchlorate biodegradation. When the perchlorate separation factor was increased from 300 to 1800, the regeneration time was shortened by 67.3% and 69.9% for Ionac SR-7 and IRA-996 resin respectively. It should be noted that comparing the experimental data of two different resins, the regeneration time for IRA-996 resin with smaller separation factor (α = 800) was shorter than the Ionac SR-7 resin with larger separation factor (α = 1300). This is because the initial total amount of perchlorate on the two resins was different. The initial perchlorate was about 4640 μg on the Ionac SR-7 resin and 3778 μg on the IRA-996 resin. If the initial perchlorate amount increased to 4640 μg for IRA-996 resin, the regeneration time (40 hours) calculated with the model would be slightly longer than for Ionac SR-7 resin (39 hours). This suggests that the total amount of perchlorate on the resins is more important than the perchlorate selectivity of the resin for a biological regeneration process for different resins. The amount of perchlorate on the resin is a function of the selectivity factor for perchlorate over all other ions in the water being treated.

As expected, the perchlorate desorption coefficients (k_{des1} and k_{des2}) did not affect the equilibrium concentration. They only affected the time to reach equilibrium. The higher the desorption coefficients were, the faster equilibrium was reached. The change of desorption coefficients had little effect on the regeneration time. As presented in Table 3-2, the

regeneration times for different initial rapid desorption constants (k_{des1}) were almost the same for both resins. Increasing k_{des2} resulted in slightly faster regeneration times. This is because at the end of the regeneration process, the aqueous phase degradation is more dominant than the 'direct' resin phase degradation. At this time in the process, more perchlorate in the aqueous phase accelerates the degradation. This result was not as significant as changing the separation factor or the mass of resin added.

Group 2: biological parameters. Since the biological parameters do not affect the perchlorate desorption from the resin, the equilibrium concentration of perchlorate did not change as shown in Table 3-2. Because the resin phase biodegradation dominates the majority of the early regeneration process, the change of the maximum specific perchlorate degradation rate in aqueous phase (V_{ma}) and the half saturation constant in aqueous phase (K_{sa}) only slightly changed the regeneration time. The change of the maximum specific perchlorate degradation rate in resin phase (V_{mr}) and the half saturation constant in resin phase (K_{sr}) had much larger effects on the regeneration time. With the increase of V_{mr} from 0.881×10^{-7} to 2.881×10^{-7} eq.-L/ mg VSS-h, the regeneration time was shortened by about 68% for both resins. With the decrease of K_{sr} from 2.540×10^{-4} to 0.540×10^{-4} eq., the regeneration time was shortened by about 67% for IRA-996 resin and 75.7% for Ionac SR-7 resin. An increase in initial biomass added also greatly shortened the regeneration time as shown in Table 3.

Group 3: salt concentration in medium. Higher salt concentrations in the medium would cause more perchlorate to desorb into the aqueous phase. As presented in Table 3-2, the equilibrium perchlorate concentration increased with the salt concentration. At the same time, the regeneration time also increased with salt concentration. This is because when more perchlorate is present in the bulk aqueous phase less is left in the concentrated liquid layer used for the "direct" degradation and this would slow down the regeneration process. This is similar to the effects of the perchlorate separation factor.

CHAPTER 4 CHARACTERIZATION OF MICROBIAL POPULATIONS IN PILOT-SCALE FLUIDIZED-BED REACTORS TREATING PERCHLORATE- AND NITRATE-LADEN BRINE

In this pilot study, an ion-exchange process incorporated with biological brine treatment inoculated with the PNRBculture was used to treat perchlorate contaminated drinking water in two FBRs. The reactors, which treated an ion-exchange regenerant brine (6% NaCl) containing 500 ±84 mg-N/L nitrate and 4.6 ±0.6 mg/L perchlorate, were operated in series in continuous flow mode for 107 days after an acclimation period of 65 days. Pilot operation data suggest that complete denitrification was achieved after 70 days of operation, but significant perchlorate removal was not observed. In order to reveal the factors causing the unsatisfactory performance of the process, microbial ecology of the culture in the FBRs was analyzed using DGGE and FISH.

DGGE has been widely applied to the analysis of 16S rRNA genes from environmental samples. Individual bands in DGGE gels can be excised, reamplified and sequenced to give an indication of phylogenetic affiliation of the community members. Once the identity of an organism associated with any particular band has been determined, any shifts in the components of a microbial community due to environmental changes can be rapidly assessed. It is commonly used in molecular microbial ecology to study microbial population composition and dynamics (Villanueva *et al*. 2004; Hamamura *et al*. 2006; Shrout *et al*. 2006).

FISH utilizes fluorescently labeled DNA probes to detect and localize the presence or absence of specific DNA sequences in samples. FISH has been successfully used to investigate microbial communities in marine sediments (Pernthaler *et al*. 2002; Mahmoud *et al*. 2005; Tang *et al*. 2005). In the present study, oligonucleotide probes targeting 16S rRNA *of Rhodobacteraceae, Halomonas, Marinobacter, Dechloromarinus, Azoarcus* and *Denitromonas* were developed in this work or obtained from literatures if they were available. The specificities of the probes and hybridization protocols were determined using pure cultures and then the probes were used to elucidate the cultures and biomass samples from the pilot plant. The composition of microbial communities and the key perchlorate- and nitrate-reducing genera in the communities were determined. The differences in the

compositions between the inoculum culture and the pilot plant were analyzed.

4.1 Materials and methods

4.1.1 Pilot-plant

The groundwater treated by the pilot plant contained 93 μg/L perchlorate and 11.3 mg-N/L nitrate. The pilot plant consisted of two parallel ion-exchange columns (Φ10 cm ×1.52 m) and two serial fluidized bed reactors (FBRs) as the biological brine treatment system. The ion-exchange columns were packed with polyacrylic resin (Purolite A850, strong base anion resin) and operated using up-flow exhaustion and down-flow regeneration in the same configuration as documented in Lehman *et al*. (2008). The FBRs were packed with a uniformly graded granular activated carbon (16×20 mesh sized GAC, Calgon Carbon Corporation). The recirculation lines of the FBRs were located at the top of the GAC to make sure no GAC particles recirculated. The two FBRs were sized based on the perchlorate and nitrate reduction rate observed in the bench-scale tests conducted by the authors as reported by Patel *et al*. (2008). The bench-scale results suggested that FBR1 would be adequate to demonstrate the desired removal of perchlorate and nitrate from the ion-exchange brine. However, due to uncertainties associated with the scale-up issues and acclimation of the GAC particles, a smaller reactor named FBR2 was used as a polishing column for perchlorate removal that was not achieved by FBR1. The dimensions and operating parameters of the FBRs are listed in Table 4-1.

Table 4-1 Dimensions and operation parameters of the FBRs

Parameter	Unit	FBR1	FBR2
Diameter	cm (inch)	10 (4)	5 (2)
Depth	m (ft)	2.44 (8)	2.44 (8)
Depth of packed bed	m (ft)	0.91 (3)	1.1 (3.5)
Bed expansion	%	30-50	30-50
GAC mass	kg	3.5	1
Recycle flow	mL/min	1600	1000

The carbon used in the FBRs was inoculated with the culture developed by the University of Houston, initially in the laboratory (Patel *et al*. 2008) and then moved to the

pilot plant. Some fresh inoculum from the lab parent culture was supplemented to the FBRs as well. The acclimation of the FBRs was conducted in two stages. During the first stage, the reactors were acclimated independently in batch recycle mode. In other words, there was no inflow or outflow, the brine was recycled using the recycle loop. Nitrate, perchlorate and acetate were spiked intermittently during the acclimation. In the second stage, the acclimation was carried out in the flow through mode. Fresh brine was passed through the reactors and acetate was injected continuously in the feed line. After acclimation, the FBRs were operated continuously in series with the intention of complete removal of nitrate in FBR1 and perchlorate removal in FBR2. After 107 days' operation, the system was stopped. In operation, the FBRs were fed with the brine generated from the ion-exchange process containing 500 ± 84 mg-N/L nitrate and 4.6 ± 0.6 mg/L perchlorate. Magnesium and micronutrients were added to each reactor independently according to the recommendations from bench-scale testing (Patel *et al*. 2008). Acetate was continuously added to the reactors in the feed of the system. The amount of acetate added to the reactor was about 1.5 times the stoichiometric requirement for the complete reduction of nitrate and perchlorate present in the feed. However, due to fluctuations of flow-rate and the concentration of the acetate feed solution, the actual acetate concentration fed to the reactor fluctuated. The average acetate concentration fed to the reactor during the entire phase of the study was 2170 ± 500 mg/L. Perchlorate, nitrate, nitrite, sulfate, chloride, chlorate, chlorite, and acetate were determined as described by Lehman *et al*. (2008). The reactors were open to the atmosphere and the DO levels at the top of the reactor were occasionally measured to make sure they were below 0.5 mg/L.

4.1.2 Biomass sampling and fixing

Biomass samples from each FBR column were first taken during the acclimation phase. At the end of pilot plant operation, samples were taken again from the carbon at the top of each column and from a mix of carbon and supernatant after they were removed from the columns. The inoculum was also sampled as a control. All biomass samples were fixed with phosphate buffered saline (3% NaCl) solution (PBS) containing 4% formaldehyde immediately after sampling (after (Amann *et al*. 1990)).

4.1.3 DGGE

DNA from the FBR pilot plant samples and the inoculum culture was extracted using the Promega wizard genomic DNA purification kit (Promega Corp. Madison, WI). PCR primers for the DGGE were 16S 341F (CGC CCG CCG CGC CCC CGC CCC CCG CCG CCG CCC CCG CCT ACG GGA GGC AGC AG) and 16s 907F (CCG TCA ATT CMT TTG AGT TT) (Marchesi *et al*. 1998; Schäfer and Muyzer 2001) and were loaded at 40 pmol/50 µL. The PCR was carried out with initial heating at 95 ℃ for 10 minutes, 30 cycles of 94 ℃ for 1 minute, annealing at 50 ℃ for 1 minute, extension at 72 ℃ for 2 minutes. The final extension period was at 72 ℃ for 30 minutes. The PCR master mix was obtained from Promega (Madison, WI) and the reaction was carried out in a PerkinElmer GeneAmp PCR System 2400 (PerkinElmer Life and Analytical Science, Inc., Waltham, Ma.).

DGGE was performed in the CBS (Del Mar, CA) DGGE system (DGGE-2401-110). The PCR products were loaded to a 6% polyacrylamide gel using a denaturant gradient of 30% to 70% denaturant. The running buffer was 0.5 × TAE (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2EDTA). Samples of 40 µL (~1200 ng DNA) of the PCR products were loaded into each well in the gel. Since the well only held a 20 µL sample, initially one half of the 40 µL samples were loaded into the well, then the electrode was connected to the upper reservoir and the gel was run at 100 V for about 5 minutes, the rest of the samples were then loaded into each well.

Electrophoresis was performed at a constant voltage of 100 V and temperature of 60 ^{o}C for 22 hours. After electrophoresis, the gel was incubated in 0.5 mg/L ethidium bromide solution for 30 minutes, rinsed in DI water for 30 minutes, and then photographed with UV transilluminator.

Bands of interest from the DGGE gels were excised and sequenced using the ABI 3130xl Genetic Analyzer and ABI BigDye v3.1 terminator chemistry in both directions. The consensus sequences developed from the two sequences obtained were then searched against the National Center for Bioinformatics (NCBI) database using the BLAST tool.

4.1.4 FISH probe design

The 16s rRNA sequences obtained from DGGE analysis in this and previous studies (Zuo 2008) were compared to the NCBI database. FISH probes were designed by selecting

consensus subsets of the sequences after sequence alignment of all known strains of the target genera. The short sequences selected were compared to the NCBI database and were determined acceptable if they did not result in a 100% match to organisms from different genera. The probe sequences and target organisms are presented in Table 4-2. In addition to the probes that were designed in this research probe sequences targeting *Roseobacter, Archaeabacteria,* and a universal 16s rRNA sequence were obtained from Probebase (Loy *et al*. 2003, 2007). All oligonucleotide probes were purchased from Sigma-Aldrich (Ontario, CA).

The specificity of the probes was tested by performing hybridization tests against pure cultures of organisms representing genera that are suspected of being present in the culture (*Marinobacter hydrocarbonoclasticus, Halomonas halodenitrificans, Dechloromarinus agitate, Roseobacter denitrificans,* Strain PNRB-1) plus negative control organisms, (*Clostridium sordellii, Micrococcus roseus, Methanosarcina mazei and Escherichia coli*). No control organisms could be purchased for *Azoarcus* or *Denitromonas*. Strain PNRB-1 is a putatively pure perchlorate-reducing culture isolated from the inoculum culture. The DGGE band sequence alignment resulted in 95% match with both *Azoarcus* and *Denitromonas* 16s rDNA sequences. This culture was used as a positive control for the *Azoarcus* and *Denitromonas* probes.

Table 4-2 FISH Probe Sequences

Probe Name	Targeted bacteria	Sequence	Formamide (%)	T_H (°C)	Reference
Mb115	*Marinobacter*	ATGCTTAGGAATCTGCCCAGTAGTG	5	60	This research
Halo429	*Halomonas*	CTTTCAGTGGGGAAGAAAGCCTT	10	53	This research
Dcl464	*Dechloromarinus*	CGAGTCTTGACGTTAACTTTAG	0	42	This research
Azo634	*Azoarcus*	GCTGCGTTACTCAGAAAG	0	48	This research
Den650	*Denitromonas*	AGTTTCCTCTCCGAACAA	30	48	This research
G Rb	*Roseobacter*	GTCAGTATCGAGCCAGTGAG	5	53	ProbeBase[1]
Univ1390	All bacteria	GACGGGCGGTGTGTACAA	0	53	ProbeBase[1]
Arch915	All Archaea	GTGCTCCCCCGCCAATTCCT	30	53	ProbeBase[1]

[1]http://www.microbial-ecology.net/probebase/

Based on the study of Hames and Higgins (1995), the optimal hybridization temperature is about 5 ℃ below its denaturing temperature. This was used as the start temperature for each probe developed in this study. If the oligonucleotide probes hybridized with non-specific organisms, the temperature was decreased at 5 ℃ intervals. The formamide

concentration in the hybridization buffer was increased in 5% intervals from 0 to 40% at each temperature. The optimal hybridization conditions obtained from this specificity test are presented in Table 4-2. Under the conditions listed in Table 4-2, all of the probes were specific to the organisms in the intended genera.

4.1.5 FISH analysis

All samples were hybridized with each probe using the hybridization protocol determined in the probe specificity test. The hybridization procedure was based on Amann *et al*. (1990). The hybridization buffer consisted of 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% (W/V) SDS and the concentration of formamide as listed in Table 4-2. The hybridization buffer was filtered through a 0.2 μm membrane filter before use. A sample of 200 μL was combined with 20 μL of probe (2 μg/mL) and 1 mL preheated hybridization buffer in a 2 mL centrifuge tube and mixed by vortexing. The tubes were put into an oven and hybridized for 4 hours at the temperature listed in Table 4-2 for each probe. The samples were then centrifuged at 10,000 g for 2 minutes, the supernatants were carefully discarded and the pellets were resuspended in 1 mL hybridization buffer by vortexing at maximum speed for 30 seconds. The samples were incubated for 10 minutes at the hybridization temperature again. Then the step of centrifuging, resupending and incubating was repeated for four times. During the second time, 140 μL of DAPI (4',6-diamidino-2-phenylindole) solution was added in the samples before incubation. A sample of 15 μL of the final resuspended sample was dropped onto microscope slides and covered with a cover slip. These samples were observed using an epifluorescence microscope (Zeiss, Germany) using a 100× oil objective. Images were recorded with a Zeiss Axioimager black and white camera at the wavelength specific for DAPI and each probe fluor (FITC or TRITC).

The number of cells stained with DAPI and each individual probe were counted in at least 6 randomly selected microscopic fields. The sum of all counts was tallied and the percentage of cells stained with individual probes was compared to the total cells (DAPI stained).

4.2 Results and discussion

4.2.1 Pilot plant performance

Figure 4-1 a and b present the nitrate and perchlorate removal performance in both FBRs during the operation stage. The nitrate removal gradually increased except for two incidents due to pump failure on the 26th day and pipe clogging on the 50th day. The system recovered soon after the problems were corrected. During the operation process, nitrite production was detected in the first 70 days (Figure 4-1 c). After that, no nitrite was detected in the effluent suggesting complete denitrification. Figure 4-1 also reveals that there was no noticeable perchlorate reduction during the first 70 days of operation. This might be due to the competitive inhibition caused by the presence of nitrate and competitive or toxic inhibition of nitrite in the reactor. After complete denitrification was observed, the perchlorate concentration in the influent was increased from 4 mg/L to 15 mg/L within 80th day to 90th day intentionally to promote the growth of perchlorate reducers. At the 91st day, 70 mg/L of perchlorate was applied. At this high level of perchlorate in the feed, about 20-30% perchlorate was reduced in each FBR with the result of a total of 40% reduction from the overall biological system. This suggested that the perchlorate reducers were in both bioreactors, but they were inhibited when perchlorate was too low compared to nitrate. The acetate concentration in the feed and effluent from both FBRs in Figure 4-1 d showed the acetate consumption profile was similar to the nitrate degradation profile and during the whole operation process, acetate was present in excess in the FBRs. The system was not run until full perchlorate reduction was observed due to time and funding limitations.

4.2.2 DGGE analysis

The DGGE gel image is shown in Figure 4-2. The lack of observable bands in lanes S4 and S7 (samples from the top carbon of both FBR columns) suggest that no PCR products were isolated from the carbon at the top of either reactor at the completion of the pilot plant trial. Because the FBR columns were operating up-flow, this indicates that the carbon has not been fully colonized and the organisms in the column were distributed with a vertical concentration gradient decreasing from bottom to top. There were no PCR products isolated from the sample of supernatant from final carbon mixture in FBR 2 (lane S8) suggesting that the biomass was indeed on the carbon and not in the aqueous phase.

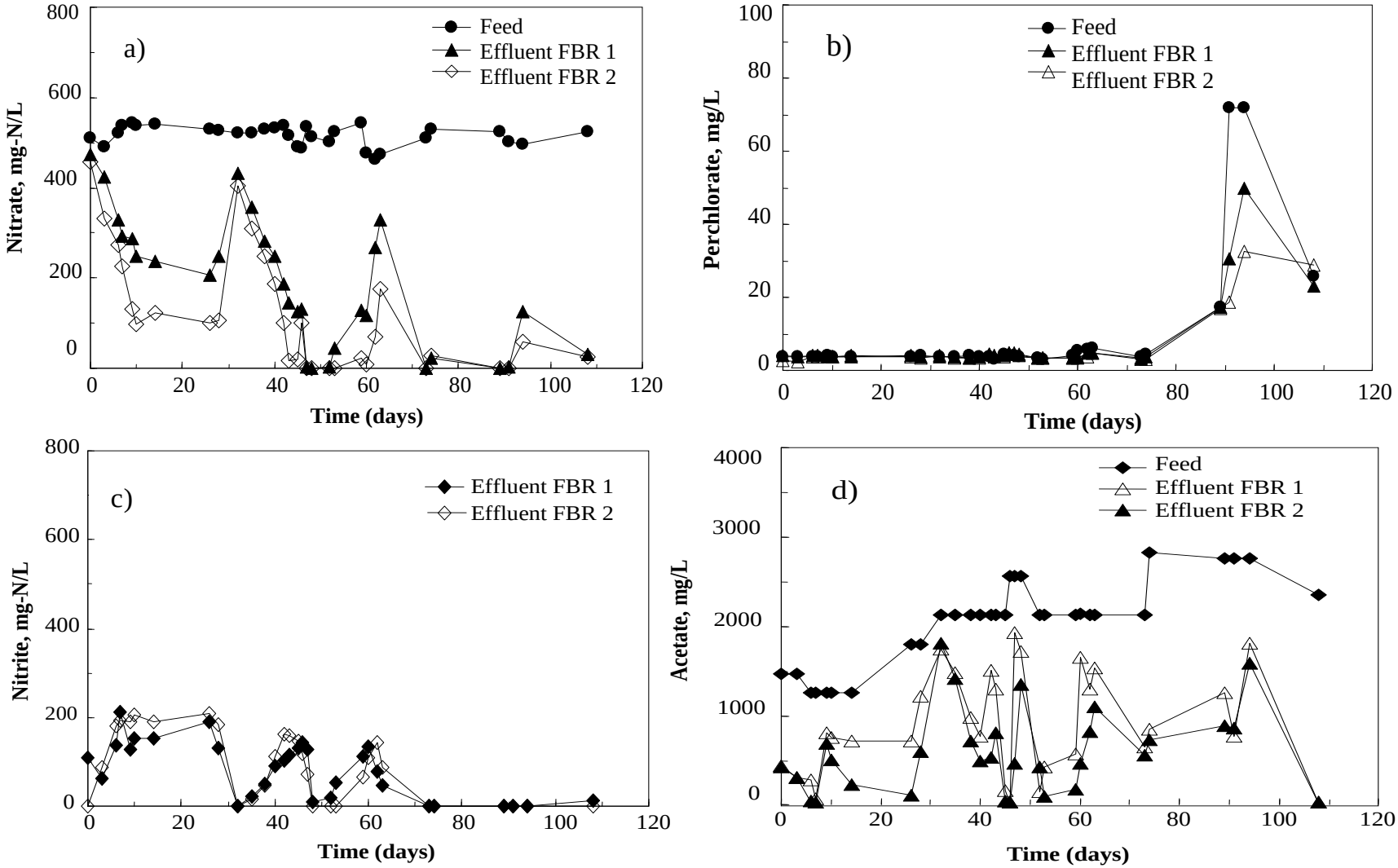


Figure 4-1 a) Nitrate, b) perchlorate, c) nitrite, and d) acetate concentrations in the FBRs

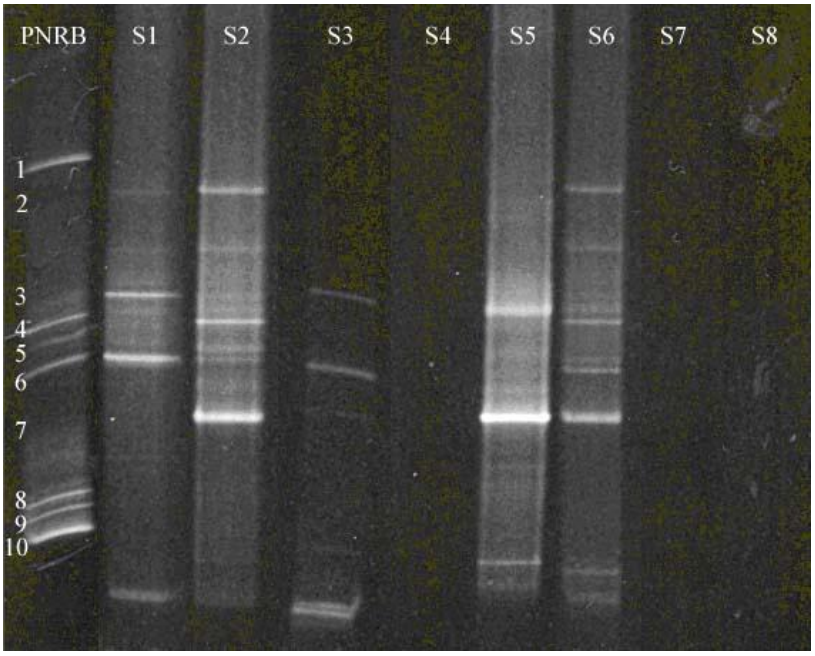


Figure 4-2 Photograph of DGGE gel

PNRB - the inoculum culture; S1 - acclimation biomass from FBR1; S2 - acclimation biomass from FBR1; S3 - final mixture of carbon from FBR1; S4 - carbon from the top of FBR1; S5 – acclimation biomass from FBR2; S6 - final mixture of carbon from FBR2; S7 - carbon from the top of FBR2; S8 - supernatant from the mix of carbon.

The inoculum culture (lane PNRB) had seven major bands, and may have electrophoresed slightly differently than the other lanes. The other lanes had bands in similar patterns but not exactly in the same places as lane PNRB. The figure also shows that the DGGE patterns of the pilot plant samples were different from each other indicating that the microbial communities in the pilot plant samples changed with operation time and the position in the bioreactors. Both reactors were inoculated in the same manner and run identically. By the end of acclimation the DGGE patterns in biomass samples from FBR1 (lanes S1 and S2) and FBR2 (lane S5) were slightly different. Even within the acclimation samples from FBR1, the DGGE patterns were quite different (lane S1 and S2). This could be due to heterogeneous samples.

A new band (band 7), that does not seem to be present in the inoculum culture, emerged in lanes S2, S5 and S6 (acclimation biomass from both reactors and final biomass from FBR2). The sequence of this band matched closest (95% similarity) to sequences from organisms in the genera *Azoarcus* and *Denitromonas*. Similar sequences to band 7 were found in the culture when only perchlorate was fed as the sole electron acceptor (Zuo *et al*.

2009). The sequence also matches the sequence obtained from Strain PNRB-1, putative pure culture obtained in our lab.

Halomonas (represented by bands 8, 9 and 10, as determined by previous DGGE analysis (Zuo *et al*. 2009), was lost in all pilot plant samples. This organism may be incapable of forming a biofilm and would therefore wash out of the system. *Marinobacter* (represented by bands 4, 5, and 6), another nitrate reducer, was quite prevalent in the acclimation biomass from FBR1 (lane S1) but weak in a second sample of acclimation biomass from this column (lane S2). A very weak band was observed in the acclimation sample from the FBR2 (lane S5) and was absent in the final mix of carbon from this column (lane S6). The DGGE analysis is a good tool to reveal overall changes in the composition of the culture, but cannot be used for quantitative purposes.

4.2.3 FISH probe design and specificity test

The probes designed for this study are documented in Table 4-2 and were all tested against both positive and negative control organisms. No type strains were available for the Azo634 and Den650 probes. They were, instead, tested against a laboratory culture that is in the final stage of purification. This culture was isolated from the parent culture using anaerobic techniques and solid agar roll tubes or flat bottles containing the synthetic medium used to maintain the parent cultures with perchlorate as the sole electron acceptor. When colonies were picked, grown in liquid culture on perchlorate and transferred onto another solid plate, two different colonies appeared indicating that it was not a pure culture. The isolation process has been attempted several times, but every time the results showed that it was not a pure culture. One possible reason is that the perchlorate-reducing bacterium is an obligate anaerobe and another organism has to grow with it to use the oxygen it produces when it reduces perchlorate. Previous DGGE tests showed that the gene sequences of one of the organisms in this co-culture matched over 95% with *Azoarcus* and *Denitromonas*. No species of either of these two genera have been reported to reduce perchlorate. Recently species of *Azoarcus* have been found to be capable of denitrification and desulfurization (Juteau *et al*. 1999; Liu *et al*. 2006; Zhou *et al*. 1995; Chen *et al*. 2008; Springer *et al*. 1998; Song *et al*. 2001). Most of the reported *Azoarcus* species could grow in medium with 2% NaCl, but none grew in medium with 5% or more NaCl. *Denitromonas* are less well studied.

Some were found in an anaerobic wastewater treatment UASB bioreactor (Zhuang *et al*. 2005), while others were detected in an aerobic SBR bioreactor for the treatment of *tert*-butyl alcohol (Etchebehere *et al*. 2003). There have been no reports of salt tolerance of members of the *Denitromonas* genera. This is the first observation of *Azoarcus* and *Denitromonas* in saline (6% NaCl) solution.

Two oligonucleotide probes Azo634 and Den650 were designed for this co-culture targeting at *Azoarcus* and *Denitromonas* genera separately. The probe specificity tests showed that these two probes hybridized with this co-culture only, and no negative controls. The ratio of cell numbers of *Azoarcus* to *Denitromonas* in the co-culture was about 7:3, confirming this culture was not pure but a co-culture of *Azoarcus* and *Denitromonas.*

4.2.4 FISH analysis

Figure 4-3 shows an example of the photomicrographs of the FISH analyses when samples were probed with DAPI and Halo429. After counting the number of organisms fluorescing from the probe and DAPI, the percentage of organisms targeted by Halo429 to DAPI was obtained.

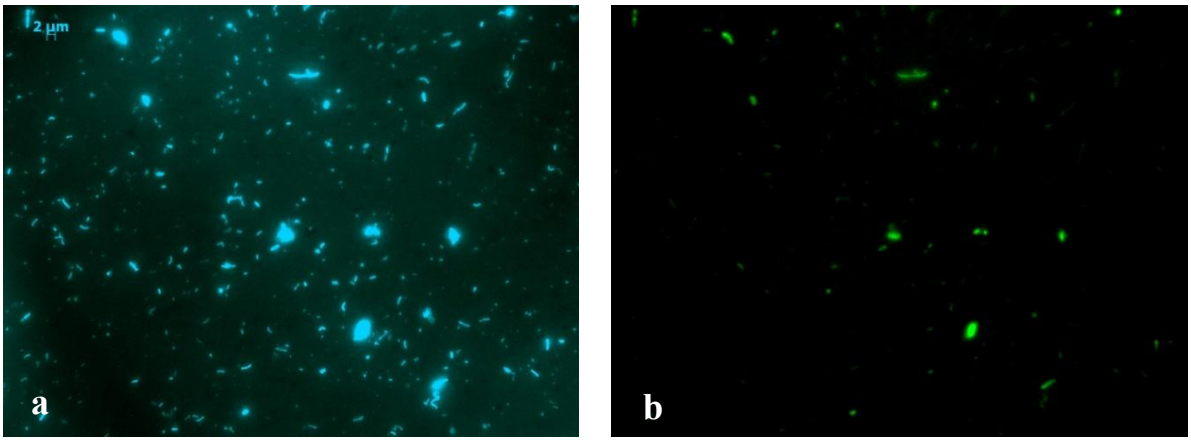


Figure 4-3 Photomicrographs of samples of final mixture of carbon from FBR2 (S2), that were a) stained with DAPI and b) probed with Halo429

A summary of the FISH results is presented in Table 4-3. It is immediately noticeable that there were organisms observed with both DAPI and hybridization probes in the samples from the tops of the column and in the supernatant (sample S4, S7 and S8), where no DNA was detected in DGGE analysis. This suggests that FISH was more effective for analysis of the composition of these cultures. The total percentages of bacteria probed with Mb115,

Halo429, Dcl464, G Rb, Azo634 and Den650 in these samples (S4, S7 and S8) (57.0%, 36.8% and 33.4% respectively) was a small fraction of the cells probed with Univ1390. In these samples the total number of organisms was low (<50/field on microscope) and the sampling process may not have enabled a full profile of the samples. In the quantitative analysis of the microbial composition of the cultures, these three samples were ignored. The table shows that only a small percentage (not greater than 8.6%) of the population probed with the *Archaea* probe (Arch915) in all samples, and there were more *Archaea* in the samples from the pilot plant than the inoculum culture. The DGGE primers were designed for Bacteria and not Archaea so these organisms would not be represented in the DGGE. The sum of the number of organisms probed with Univ1390 and Arch915 was always less than 100% (from 72.3% to 87.5%) of those stained with DAPI. This might be because the efficiency of the hybridization is less than 100%. The probe binds with the rRNA. When the cells are dead and part of the rRNA is hydrolyzed, the oligonucleotide probes would not hybridize with the rRNA. Meanwhile, the genes can still be stained with DAPI. Another possible reason could be that 23% to 27% of the cells in the samples do not probe with these two "universal" probes used in this study.

Table 4-3 FISH results

Organism	% Total DAPI with							
	Mb115	**Halo429**	**Dcl464**	**GRb**	**Azo634**	**Den650**	**Univ1390**	**Arch915**
Inoculum culture	7.2	13.5	4.6	5.3	30.4	24.4	87.1	0.4
S1 - acclimation biomass from FBR1	0.6	18.4	39.0	1.6	29.0	11.0	78.0	8.6
S2 - acclimation biomass from FBR1	8.4	19.5	28.2	3.4	26.3	5.3	75.0	3.0
S3 – final mixture of carbon from FBR1	5.3	33.1	27.5	5.2	13.6	8.9	74.9	1.7
S4 - carbon from the top of FBR1	2.6	34.1	19.7	0.0	0.0	0.6	75.5	1.8
S5 - acclimation biomass from FBR2	4.5	29.8	32.4	2.6	25.5	5.3	85.7	3.7
S6 - final mixture of carbon from FBR2	6.3	47.7	23.8	3.4	20.5	2.7	65.6	6.7
S7 - carbon from the top of FBR2	2.3	12.0	17.4	1.2	1.4	2.5	67.0	5.8
S8 - supernatant from the mix of carbon from FBR2	0.7	11.6	16.1	0.0	0.0	5.0	84.1	2.8

Table 4-3 shows that *Roseobacter* (targeted by the probe G Rb) was a minority (less

than 5.3%) genus in both inoculum and pilot plant samples. This corresponds to the results of previous DGGE analysis that *Roseobacter* only appeared when the culture was fed with both perchlorate and nitrate and when there was excess phosphorus in the medium. *Marinobacter* (targeted by the probe Mb115) and *Halomonas* (targeted by the probe Halo429) were the two major nitrate reducers determined by previous DGGE analysis (Zuo *et al*. 2009). In this study *Marinobacter* was a minority (less than 8.4%) genus in both inoculum and pilot plant samples. *Halomonas,* which was not detected in all pilot plant samples by DGGE, was the major nitrate reducer found in both the inoculum culture and pilot plant samples (Table 4-3). The percentage of *Halomonas* in pilot plant samples was much greater than in the inoculum culture. The percentage of *Halomonas* gradually increased with the time of operation from the initial 18.4% to 33.1% in FBR1, and from 29.8% to 47.7% in FBR2. This indicates that this population was responding to the nitrate fed to the reactors.

Azoarcus and *Denitromonas* which were the dominant putative perchlorate-reducing genera in the inoculum culture amounted to 54.8% of all organisms. At the beginning of the acclimation stage, the total percentage of these two organisms decreased to 40% in sample S1. Then it decreased to 31.6% in FBR1 (S2) and 30.8% in FBR2 (S5) by the end of the acclimation stage. At the end of operation stage, this population had decreased to 22.5% in FBR1 (S3) and 23.2% (S6) in FBR2. There was, however, a larger percentage of *Azoarcus* in the final mixed biomass from FBR2 (S6) than in the final mixed biomass from FBR1 (S3). This corresponds to the findings that most of the perchlorate reduction was in FBR2 as shown in Figure 4-1 d. *Azoarcus* also survived the pilot plant start-up process better than *Denitromonas*. The percentage of *Denitromonas* in the cultures decreased from 24% in the parent culture to 9% or 2% in FBR1 or FBR2, respectively.

Dechloromarinus (targeted by the probe Dcl464) represented only 4.6% of total organisms in the inoculum culture, but increased in pilot plant samples during the acclimation stage and stayed at a reasonable percentage during the operation stage. The only characterized species of *Dechloromarinus, D. chlorophilus* could only reduce chlorate and nitrate but not perchlorate (Bruce 1999). Thus, the strain could have survived in the inoculum by metabolizing chlorate or nitrate although it has never been found in the laboratory cultures. While in the FBRs, this strain became more representative because it might form a biofilm.

In summary, this chapter shows that the dominant perchlorate-reducing bacteria in the inoculum were *Azoarcus* and *Denitromonas*, which could not be isolated by solid plate cultivation. The dominant nitrate-reducing bacterium of the inoculum was *Halomonas*. This is different from all-known isolated PRB in environment where *Dechloromonas* and *Azospira* are the dominant genera (Coates *et al*. 1999). The FISH test clearly showed that the percentages of *Azoarcus* and *Denitromonas* in the pilot plant decreased with time, and the number of *Halomonas* increased during the operation. This indicates a shift towards nitrate reduction in the pilot plant system, which corresponds to the actual operation data. Because the nitrate concentration was much higher than the perchlorate in the brine, the reaction time in the FBR1 system was too short for the culture to fully degrade nitrate, which would be required to make FBR2 more favorable to perchlorate degradation.

DGGE failed to detect *Halomonas,* the major nitrate-reducing bacterium detected using FISH. It also failed to detect *Azoarcus*, the major perchlorate-reducing bacterium in the inoculum culture. Thus, FISH is a more effective test for monitoring this culture than DGGE. FISH is also less time-consuming than DGGE, costs less and uses fewer hazardous chemicals.

CHAPTER 5 *MARINOBACTER MULTIRESPIRO* SP. NOV., A NEW SALT-TOLERANT PERCHLORATE-REDUCING BACTERIUM

Previous molecular analysis of the cultures (Chapter 4) in a pilot plant revealed that with the operation time increasing, more nitrate-reducing bacteria grew in the reactor, which suggested that perchlorate-reducing bacteria were not competitive in the system compared to nitrate-reducing bacteria. Other studies also showed that nitrate was an inhibitor for perchlorate-reducing bacteria (Chaudhuri et al. 2002). In order to overcome the inhibition, two approaches could be used: One would be to increase the hydraulic retention time of the nitrate reduction process to make nitrate almost fully degraded before the waste streams enter into the perchlorate degradation process; The other is to improve the culture to make perchlorate degradation more resilient to the nitrate concentration or (i.e. change the regulation of perchlorate reductase and chlorite dismutase genes so they are expressed constitutively). While the first approach is a temporal correction, the second one is a permanent measure. Furthermore, an increase in the hydraulic retention time would greatly increase the capital cost of full-scale projects. Thus, the second approach to improve the culture to overcome the nitrate inhibition was chosen.

In order to implement the approach, isolation and characterization of the major microbial components from the mixed culture is a prerequirement. The study on the mixed culture in Chapter 4 led to the conclusions that the major perchlorate-reducing genera in the enrichment cultures were *Denitromonas* and *Azoarus*. Attempts to obtain these organisms in pure culture were unsuccessful to date. In the present study, multiple isolation methods were used in combination to isolate a pure salt-tolerant perchlorate-reducing strain.

5.1 Methods

5.1.1 Parent culture, medium and cultivation

The mixed culture NP30 has been fed with perchlorate and nitrate as the electron acceptors and acetate as the electron donor and carbon source in a laboratory-scale batch reactor for more than 10 years as described previously (Zuo 2008). In brief, the 1.5 L parent culture was spiked daily with 100 × concentrated stock solution containing 100 × 815.5 mg/L KNO_3, 100 ×117.5 mg/L NH_4ClO_4 (or 100 ×618.4 mg/L $NaClO_4$), and 100 ×1225 (or 2040)

mg/L $CH_3COONa \cdot 3H_2O$ to achieve ~500 mg/L NO_3^-, ~100 (or 500) mg/L ClO_4^-, and 1.5 × the stoichiometric requirement for acetate. Once each week the culture was operated as a sequencing batch reactor and after 1 hour of settling 1 L of supernatant was removed and replaced with 1 L of fresh medium. The parent culture feed medium contained (per litre) NH_4Cl, 0.48 g; NaCl, 30 g; $MgCl_2 \cdot 6H_2O$, 11g; $CaCl_2 \cdot 2H_2O$, 1.4 g; KCl, 0.72 g; KH_2PO_4, 0.05 g; $NaHCO_3$, 0.3 g; $Na_2S \cdot 9H_2O$, 0.05 g and 1 mL mineral solution. The mineral solution contained (per litre): $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 10 g; $ZnCl_2$, 0.05 g; H_3BO_3, 0.3 g; $FeCl_2 \cdot 4H_2O$, 1.5 g; $CoCl_2 \cdot 6H2O$, 10 g; $MnCl_2 \cdot 6H_2O$, 0.03 g and $NiCl_2 \cdot 6H_2O$, 0.03 g.

The selective agar medium for isolation of the pure culture contained (per litre): $NaClO_4$, 0.62 g; $CH_3COONa \cdot 3H_2O$, 1.24 g; NaCl, 30 g; $MgCl_2 \cdot 6H_2O$, 11 g and plate count agar, 12.5g. The basal perchlorate (P) medium for cultivation was the same as the medium used for maintaining the parent culture and also included 0.25g yeast extract, and 0.25g peptone per litre. The basal nitrate (N) medium was identical to the P medium except that 0.62 g $NaClO_4$ was replaced by 0.68 g $NaNO_3$. The perchlorate and nitrate (PN) medium was prepared using the same method as P medium with 0.68 g $NaNO_3$ supplemented and $CH_3COONa \cdot 3H_2O$ increased to 2.48 g. The blank medium for washing cells was prepared in the same method as the P medium except that $NaClO_4$; $CH_3COONa \cdot 3H_2O$, yeast extract and peptone were omitted.

The basal and blank media were boiled, cooled and transferred into serum bottles (50 mL) or Hungate tubes (10 mL) under a nitrogen atmosphere. Then the serum bottles or Hungate tubes containing the media were sealed with butyl rubber stoppers. The media were sterilized by autoclaving at 121 ℃ for 30 min. Cultures were incubated on a rotary shaker at 110 rpm at 35 ℃ if not specified otherwise.

5.1.2 Isolation of pure strains

The mixed culture was first serially diluted using a 10-fold series in Hungate tubes containing P medium under anaerobic conditions. After incubation, the last growth positive tube was selected as the parent sub-culture for isolation. Then a 10-fold dilution series of the parent sub-culture was inoculated into agar shake tubes (of the same medium) after the method of Evans and Kloos (1972). Three colonies P4, P5 and P6 picked from the bottom of the tubes were selected as putative pure cultures and transferred back to the P medium to

confirm their capability for perchlorate reduction. Culture purity was checked using microscopic observation and polymerase chain reaction (PCR) - denaturing gradient gel electrophoresis **(**DGGE). Additionally, these putative pure cultures were streaked on agar plates containing the selective agar medium and incubated aerobically. After differentiable colonies formed on the plates, different single colonies were picked, streaked on new plates, and incubated again. Then the pure colonies were washed off the plates and transferred back into Hungate tubes containing the P medium. One pure strain was successfully isolated and grown in serum bottles in the P medium for further characterization.

5.1.3 Cell and colony morphology

On P medium at 35 ℃, colony morphology was noted, and cell morphology was determined by means of Gram staining and bright-field microscopy (Zeiss AxioVision 600). Motility was determined by phase-contrast microscopy. The temperature range for growth was determined in the PN medium incubated for 10 days at temperatures from 15 to 45 ℃ at an interval of 5 ℃. The optimal pH for growth was determined using the PN medium with the pH adjusted to 4.1, 5.1, 6.1, 7.1, 8.1, 9.1 and 10.1 respectively using 1 M HCl or 1M NaOH solution.

A response surface experiment (Montgomery 2009) was designed to determine the optimal salt (NaCl) and magnesium concentrations for the strain. The detailed method is documented in Chapter 6.

5.1.4 Alternative electron donors and acceptors

Perchlorate reduction coupled with alternative electron donors was tested for the pure strain using P medium with the acetate replaced by yeast extract, glucose, ethanol (1 mM) or H_2 (10% head space, balanced with 30% CO_2 and 60% N_2). The utilization of alternative electron acceptors coupled with acetate was also tested using P medium with the perchlorate replaced by nitrate, sulphate, thiosulphate (0.5 mM) and O_2 (growing aerobically). The medium for the aerobic test was not boiled before sterilization. The pure culture (50 mL) cultivated in PN medium was used as the inoculum for these tests. The culture estimated to be in mid-log phase was harvested by centrifugation at 3500 × g for 5 minutes, washed twice with the blank medium, and resuspended in 2 mL blank medium before inoculation.

5.1.5 Analytical techniques

Perchlorate (>5 mg/L) was measured using a perchlorate selective electrode PER 1502 (pHoenix Electrode Co., Houston, TX). Perchlorate with low concentration (<5 mg/L) was analyzed with an ion chromatograph (ICS-3000, Dionex, Sunnyvale, CA) equipped with an AS16 and an AS20 columns. Nitrate was measured using the nitrate test kit equipped with an Orion 9512BNWP ammonia ion selective electrode (Thermo Fisher Scientific). Cell growth was monitored by OD_{600}. The OD_{600} was correlated to VSS using the equation: VSS (mg/L) = OD_{600} ×893.1+68.89, determined for this strain in the basal PN medium. Volatile suspended solids (VSS) were measured according to the Standard Methods (APHA *et al*. 2005).

5.1.6 Cellular fatty acid composition

The cellular fatty acid composition of the pure strain P4B1was analyzed using the fatty acid methyl ester (FAME) analysis method MIDI RCLIN6 by a commercial lab (Keystone Labs, Alberta, Canada).

5.1.7 DGGE

DNA of the cultures was extracted using the Promega wizard genomic DNA purification kit (Promega Corp. Madison, WI). The DNA was amplified by a PCR as previously described in Chapter 4 in a Mastercycler® ep Thermal Cycler (Eppendor AG, Hamburg, Germany). DGGE was performed in the CBS (Del Mar, CA) DGGE system (DGGE-2401-110) based on the method developed by Zuo *et al*. (2009). The PCR products were loaded to a 6% polyacrylamide gel using a denaturant gradient of 30% to 70% denaturant. The running buffer was 0.5 ×TAE (20 mMTris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2EDTA). Samples of 15~20 μL (~600 ng DNA) of the PCR products were loaded into each well in the gel. Electrophoresis was performed at a constant voltage of 100V and temperature of 60 ℃ for 22 hours. After electrophoresis, the gel was incubated in 1×SYBR® safe DNA gel-staining solution for 30 minutes and then photographed with a UV transilluminator (Alpha Innotech, San Leandro, CA).

5.1.8 16S rRNA gene sequencing and phylogentic analysis

The PCR product was purified using the ExoSAP-IT kit (USB Corporation,

Cleveland, Ohio) and then sequenced by a commercial laboratory (MIDI Labs). The identity of the 16S rRNA gene sequence was analyzed using the Basic Local Alignment Search Tool (BLAST). Similar sequences were picked and aligned using the CLUSTAL W multiple sequence alignment algorithm (Thompson *et al*. 1994) in the software of BioEdit 7.0 (Hall 1999). The phylogenetic tree of the selected sequences was constructed using the PhyML 3.0 program (Guindon and Gascuel 2003) based on the maximum likelihood algorithm in the software of SeaView 4.2 (Gouy *et al*. 2010). The reference 16S rRNA sequences are from the following GenBank accession numbers: *Marinobacter. koreensis* DD-M3 (DQ325514), *M. lutaoensis* T5054 (AF288157), *M. gudaonensis* SL014B61A (DQ414419), *M. bryozoorum* PTG4-16 (EU603452), *M. algicola* DG893 (AY258110), *M. sediminum* R65 (AJ609270), *M. maritimus* (AJ704395), *M. lipolyticus* SM-19 (NR_025671), *M. guineae* (AM503093), *M. aquaeolei* VT8 (NR_027551), *M. daepoensis* SW-156 (NR_025800), *M. hydrocarbonoclasticus* P210 (GU370082), *M. alkaliphilus* ODP1200D-1.5 (AB125941), *M. litoralis* SW-45 (NR_028841), *M. excellens* KMM 3809 (NR_025690), *M. vinifirmus* FB1 (DQ235263) and *M. bacchus* FB3 (DQ282120).

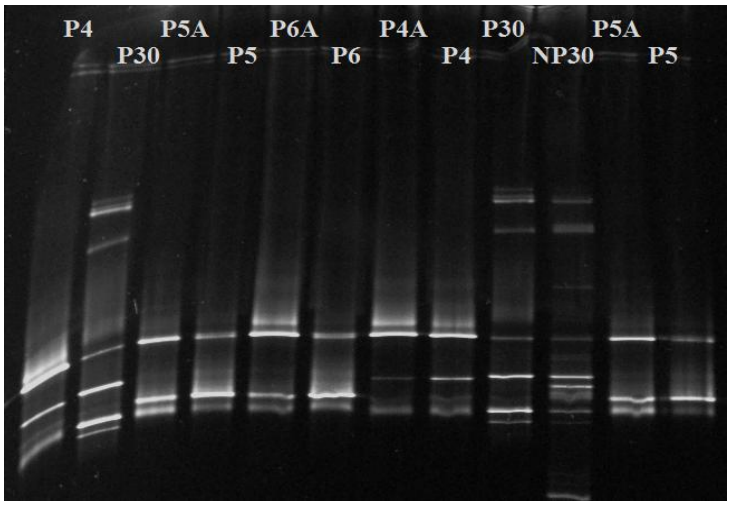
5.2 Results

5.2.1 Enrichment and isolation

Many previous attempts have been made to isolate pure cultures from the parent culture NP30. Many colonies could be isolated by streaking samples of the parent culture onto many different types of agar, under aerobic and anaerobic conditions. None of the colonies obtained could use perchlorate as an electron acceptor once transferred back to liquid perchlorate medium. The methods reported in the present study were eventually successful. The following documents the results of the successful isolation.

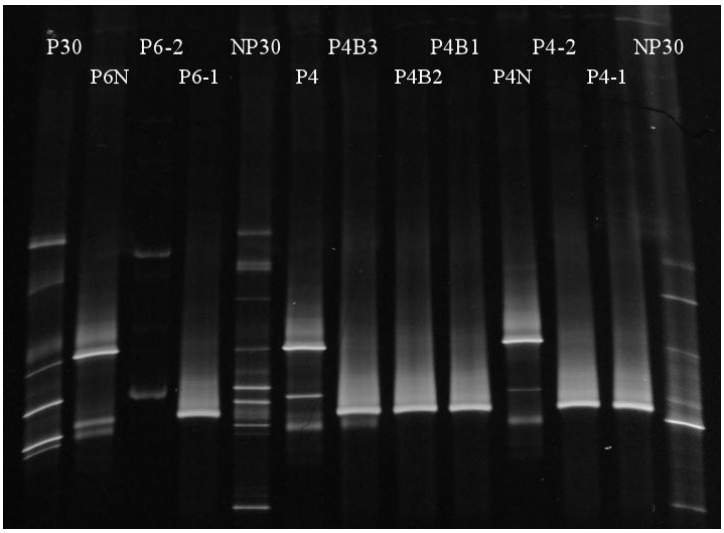
First, a mixed sub-culture was obtained via diluting the parent culture in Hungate tubes containing the aqueous medium. The sub-culture was then serially diluted in test tubes containing the selective agar medium and incubated under aerobic conditions at 35 ℃. After 2 days' incubation, some colonies appeared at the top of the solid medium in the test tube. These would be aerobic cultures and not of interest to this study. After 5 days some single small colonies appeared at the bottom of the solid medium. Because there was no oxygen at the bottom, the colonies were assumed to be anaerobic bacteria and of interest to this study.

Considerable effort was taken to pick a single colony from the bottom of the 10^{-4}, 10^{-5} and 10^{-6} dilution tubes, and to transfer these colonies back into the P medium. After 1 week's incubation, all of the new sub-cultures could reduce perchlorate. These three cultures (named P4, P5 and P6 respectively) were chosen for further study. PCR-DGGE was used to determine the purity of these putative pure cultures. The results of DGGE (Figure 5-1) revealed that the putative pure cultures were possibly not pure. P4 had three bands and P5 and P6 had two bands at the same position, which suggests that P5 and P6 might be identical mixed cultures. P6 was chosen to represent P5 and P6 in further study. P4 and P6 were then streaked on the selective agar plates and incubated both anaerobically and aerobically at 35 ℃. After 2 days' incubation, many white colonies appeared on both aerobic and anaerobic plates. Some single colonies were picked randomly from these plates and streaked again on the selective agar plates. After 2 days' incubation, the white colonies appeared again and were washed off and transferred back into the P medium. Within 1 week, perchlorate reduction was observed in the tubes inoculated from the aerobically grown colonies, while no perchlorate reduction was observed in the anaerobically grown colonies after more than 1 month's incubation. DNA was extracted from the cultures showing perchlorate reduction. PCR-DGGE was used again to check their purity. As shown in Figure 5-2, three pure strains (P4B1, P4B2 and P6-1) were obtained after this step. Their DNA bands appeared at the same position on the graph, which suggests that these strains might be identical to each other. The strain P4B1 was selected for further characterization.



- P4, P5 and P6: three putative pure cultures.
- P4A, P5A and P6A: the 1st generation sub-culture of P4, P5 and P6 respectively
- NP30 and P30: the parent culture.

Figure 5-1 Photographs of DGGE bands of three putative pure cultures



- P4N and P6N: newly transferred P4 and P6;
- P6-1, P6-2: two putative cultures isolated from P6;
- P4-1, P4-2: two putative cultures isolated from P4;
- P4B1, P4B2 and P4B3: three putative cultures isolated from P4B .

Figure 5-2 Photographs of DGGE bands of new isolates

5.2.2 Cell and colony morphology

Strain P4B1 is a non-fermentative, Gram-negative (Figure 5-3), facultative aerobe. Cells of strain P4B1 are 0.2 - 0.4 × 1 - 4 μm rods and are motile. When growing on selective agar plates aerobically, colonies were white, smooth and 1~3 mm in diameter. On anaerobic plates with acetate as the electron donor and perchlorate as the electron acceptor, colonies were smaller (0.5~2 mm diameter).

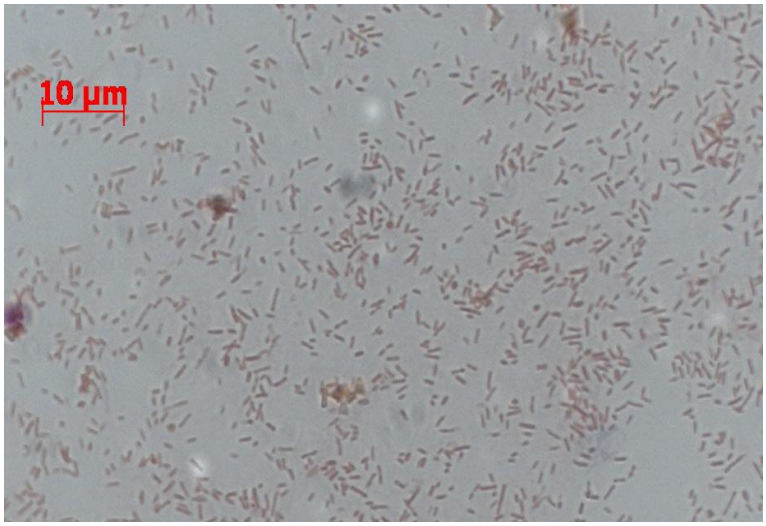


Figure 5-3 Gram-stain of strain P4B1

5.2.3 Optimal temperature, pH and salinity

Figure 5-4 shows an example of the perchlorate degradation and growth of strain P4B1 grown at 35 ℃. The culture grew and metabolized the first allotment of electron acceptors more slowly than it did for the spike feeds supplied after the initial components of the medium were exhausted. This may be a result of the culture removing residual oxygen in the initial feed or achieving a critical mass to allow maximum degradation. The tendency of the culture to remove perchlorate before nitrate is most obvious in the second feed shown in Figure 5-4.

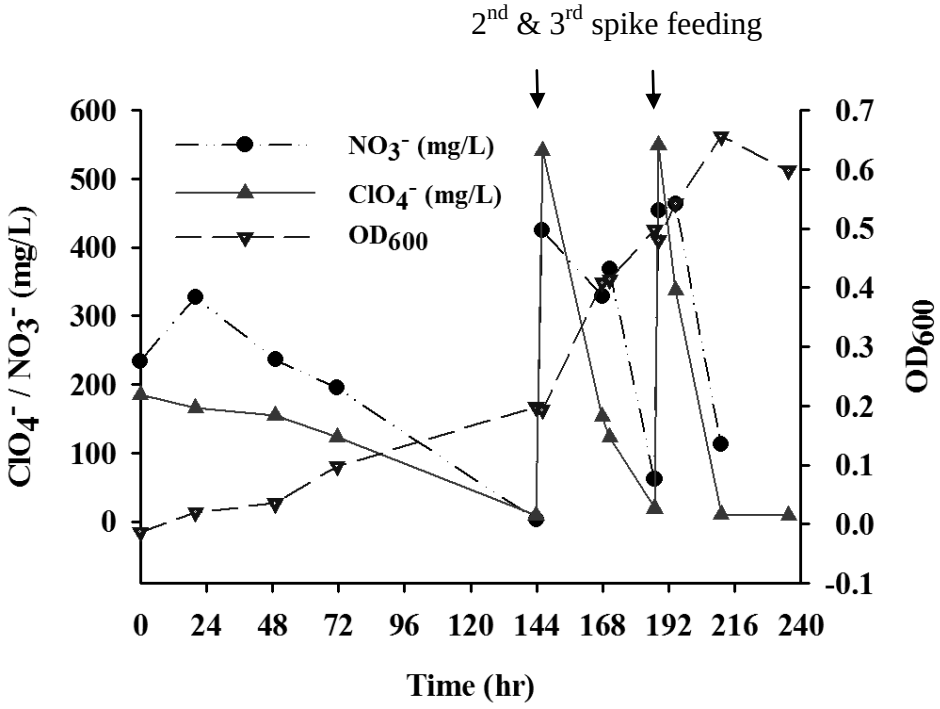


Figure 5-4 Perchlorate/ nitrate degradation and growth of strain P4B1 in PN medium

The cultures were grown at 35 ℃, 3% NaCl and a Mg^{2+}/Na^{+} molar ratio of 0.11. In the 2nd and 3rd spike feedings, the cultures were spiked with a 100x concentrated solution pf perchlorate, nitrate and acetate.

P4B1 grew over a temperature range of 15-40 ℃ (Figure 5-5). The specific perchlorate degradation rate and the specific growth rate were calculated using data from the log-phase growth period within the initial sub-culture phase. The specific perchlorate degradation rate increased almost linearly with temperature from 15-35 ℃ and decreased significantly at 40 ℃. A similar result was also observed for the parent culture P30, whose perchlorate degradation rate also peaked at 35 ℃ (Zuo 2008). However, the specific growth rate reached a plateau between 20 and 35°C. An ANOVA test showed there were significant

differences in the specific perchlorate reduction rates at different temperatures except for the rate at 25°C compared with the ones at 20, 30 and 40 ℃; the rate at 35°C compared with the one at 30 ℃; and the rate at 20°C compared with the one at 40 ℃. Although the ANOVA test on the the specific growth rates at different temperatures showed there were no significant differences between them, the trend of the change was obvious.

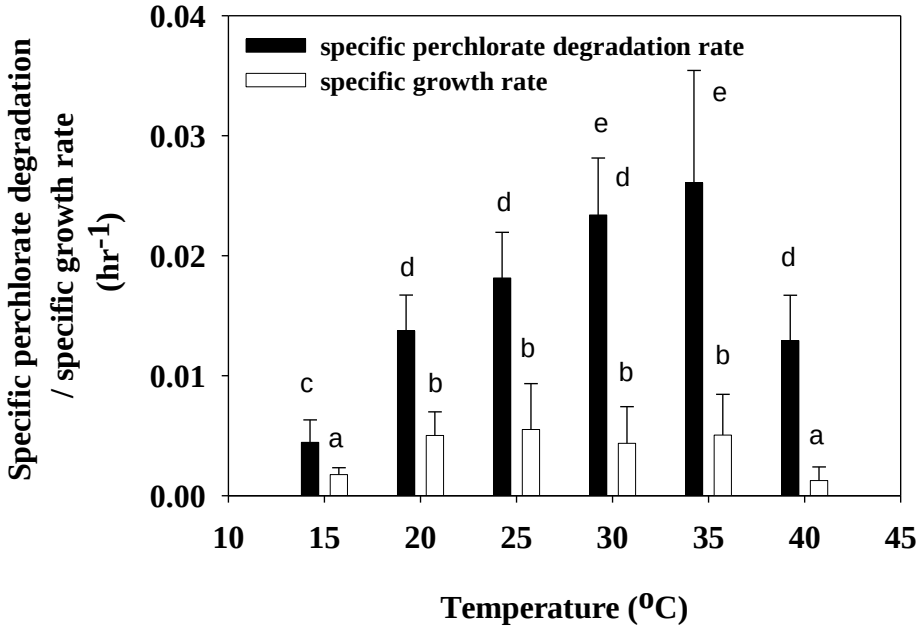


Figure 5-5 Temperature effects on specific perchlorate degradation and growth rate of strain P4B1 in PN medium

The culture was grown in 3% NaCl and a Mg^{2+}/Na^{+} molar ratio of 0.11. The letters above each bar indicate the statistical grouping ($p>0.05$) determined by an all pairwise multiple comparison using the Holm-Sidak method.

The difference of the optimal temperature between perchlorate degradation and growth might be because the activities of assimilative enzymes, each with its own maximum temperature and thus a plateau is seen for growth between 25 to 35°C, while the activity of perchlorate reductase as a single system is more obviously affected by temperature showing greatest activity at 35 ℃. No growth was observed for inoculum incubated at 45 ℃.

P4B1 grew over a pH range of 5.1 - 9.1 (Figure 5-6). No obvious growth was observed for inoculum incubated at pH 4.1 and 10.1. Although the differences of the specific growth rates at different pH were not significant as shown by the ANOVA test, the change was obvious and the optimal growth was observed at pH 7.1-8.1.

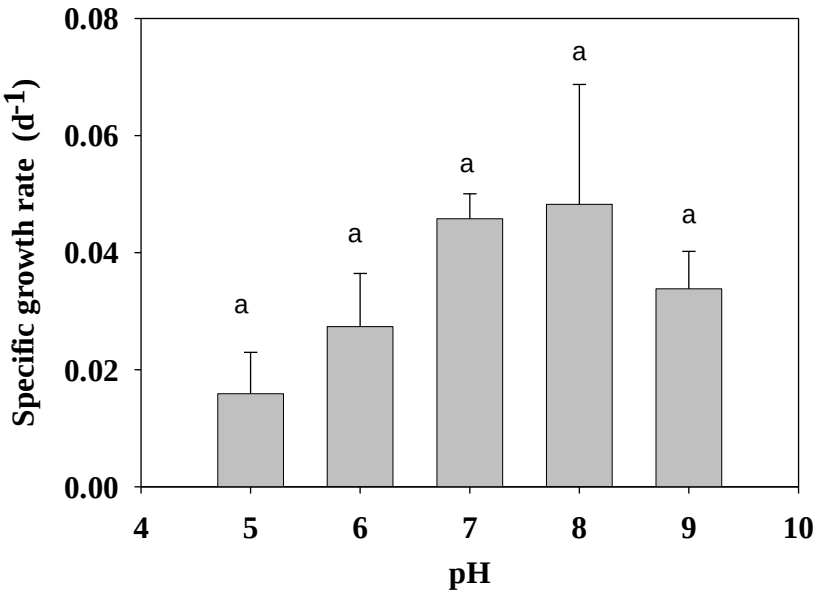


Figure 5-6 pH effects on the specific growth rate of strain P4B1 in PN medium

The culture was grown in 3% NaCl and a Mg^{2+}/Na^{+} molar ratio of 0.11 at 35 ℃. The letters above each bar indicate the statistical grouping (p>0.05) determined by an all pairwise multiple comparison using the Holm-Sidak method.

Strain P4B1 could grow in a salinity range of 1.8 – 10.2% NaCl. The optimal growth was observed at 1.8% NaCl (Figure 5-7). The ANOVA test confirmed the significant differences between the specific perchlorate degradation rate at 1.8% NaCl with the ones at 6% and 10.2% NaCl.

5.2.4 Electron donor and acceptor

Strain P4B1 is typically grown in defined medium with acetate as the sole electron donor, sodium perchlorate as the sole electron acceptor and ammonium as the sole nitrogen source. However, when the biomass was very low, the addition of yeast extract was needed for obvious growth. When yeast extract and peptone were supplied, the culture could fully degrade perchlorate within 120 hours after a new transfer. When yeast extract and peptone were not available, neither growth nor perchlorate degradation was observed over 192 hours' incubation (Figure 5-8). Similar results were also observed in the salt-tolerant tests. This might be because the strain needs a small organic nitrogen source which could be provided by the decay of cells when large amounts of cell mass are available. The parent culture typically was operated with around 2 g/L VSS and so required no organic supplements.

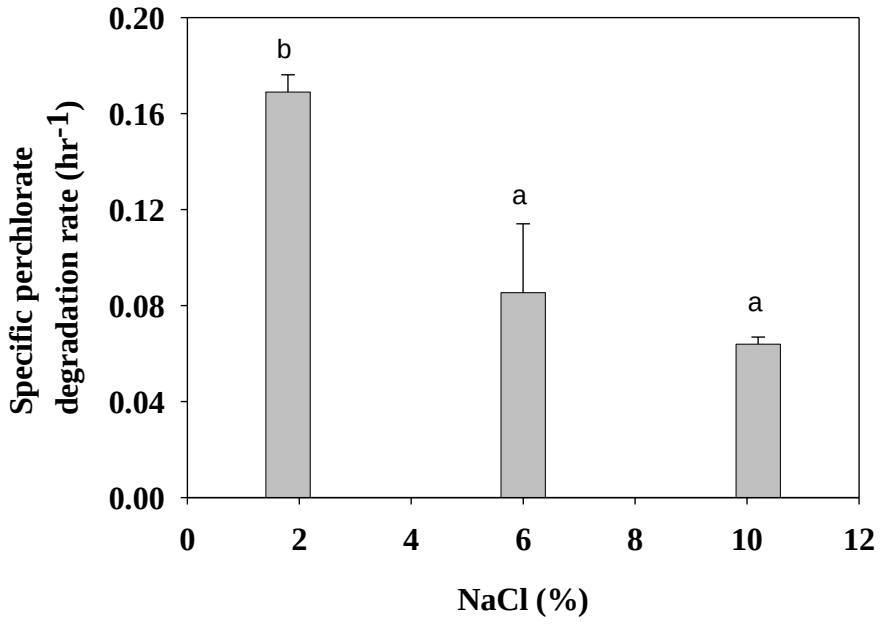


Figure 5-7 NaCl effects on the specific perchlorate degradation rate of strain P4B1 in PN medium

The culture was grown at 35 ℃ and a Mg^{2+}/Na^{+} molar ratio of 0.11. The letters above each bar indicate the statistical grouping (p>0.05) determined by an all pairwise multiple comparison using the Holm-Sidak method.

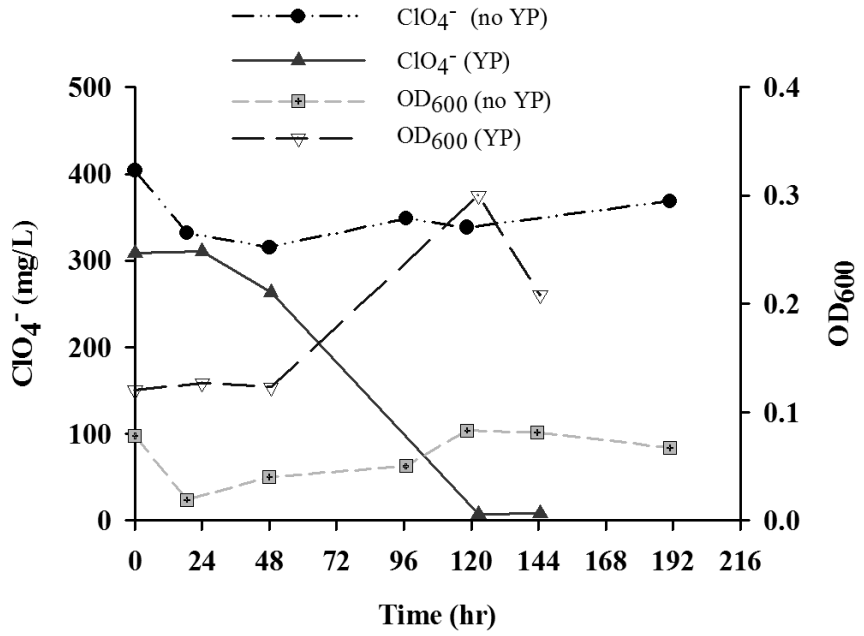


Figure 5-8 Effect of yeast extract and peptone (YP) on perchlorate degradation and biomass growth of strain P4B1 in P medium

The culture was grown in 3% NaCl and a Mg^{2+}/Na^{+} molar ratio of 0.11 at 35 ℃.

In addition to acetate as carbon and electron sources, strain P4B1 also grew on, and reduced perchlorate with other simple organics such as ethanol, peptone and yeast extract. Glucose and H_2 were not utilized as electron donors for perchlorate reduction.
With acetate (5 mM) as the electron donor, strain P4B1 could use nitrate or oxygen as alternative electron acceptors to perchlorate, but not sulphate or thiosulfate. As shown in Figure 5-9, both the growth rate and the biomass yield of cells grown on oxygen were the highest compared to cells grown on perchlorate and nitrate. Cells grown on nitrate had the lowest growth rate and biomass yield. The Gibbs free-energy changes ($\Delta G^{0'}$) of stoichiometric reactions of acetate with different electron acceptors: oxygen, perchlorate (ClO_4^-/Cl^-) and nitrate (NO_3^-/N_2) are -844, -822 and -792 kJ/mol acetate respectively (Rikken *et al*. 1996). Based on these data, oxygen is the most favorable thermodynamically, followed by perchlorate, and nitrate is the least favorable. The order of the growth rate (OD_{600} in Figure 5-9) observed in this study meets with these thermodynamic data. When nitrate was the sole electron acceptor, strain P4B1 could grow without the addition of yeast extract and peptone regardless of cell density. When nitrate or perchlorate was the sole electron acceptor, strain P4B1 could degrade them with no lag-time. Strain P4B1 did not grow by fermentation of glucose.

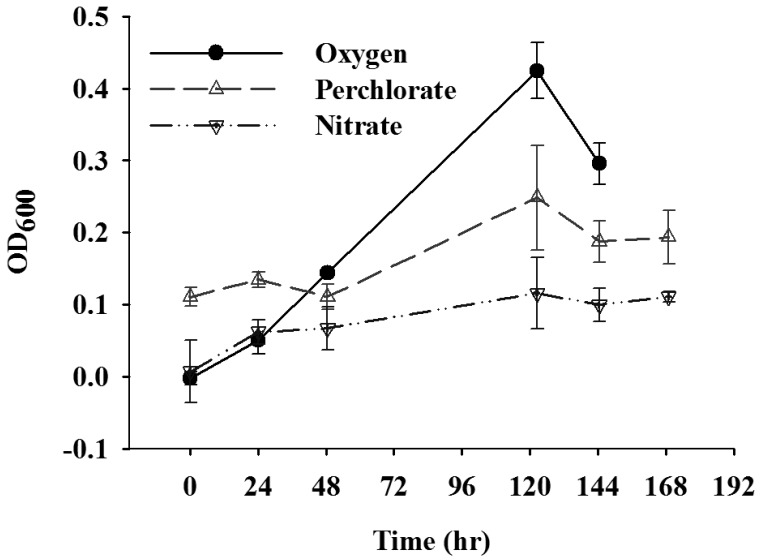


Figure 5-9 Comparison of P4B1 growing on acetate (5mM) with different electron acceptors: O_2, perchlorate (3.5mM) and nitrate (3.5mM)

The culture was grown in 3% NaCl and a Mg^{2+}/Na^+ molar ratio of 0.11 at 35 ℃.

5.2.5 Cellular fatty acid compositions

As shown in Table 5-1, the predominant cellular fatty acids of strain P4B1 were $C_{16:0}$ (29.83%), $C_{18:1}$ ω9c (22.41%), $C_{16:1}$ ω9c (16.06%), $C_{12:0}$ 3-OH (11.43%) and $C_{12:0}$ (9.09%). The profile of fatty acid composition of strain P4B1 is similar to other type strains of different *Marinobacter* species as $C_{16:0}$, $C_{18:1}$ ω9c and $C_{16:1}$ ω9c are also the major fatty acids for other strains. However, important differences were also detected. Compared to other strains, strain P4B1 exhibited the highest portion of $C_{12:}$0, $C_{12:}$0 3-OH, C16$_{:0}$ and $C_{16:1}$ ω9c.

Table 5-1 Cellular fatty acid composition of strain P4B1 and some *Marinobacter* species

	1	2	4	5	6	7	8
Saturated fatty acids							
$C_{10:0}$	1.06	1.0	ND	ND	ND	0.57	1.5
$C_{12:0}$	9.09	8.4	4.5	5.58	2.8	7.89	8.3
$C_{12:0}$ 3-OH	11.43	10.3	ND	10.68	3.4	9.94	11.3
$C_{14:0}$	2.83	1.7	2.3	0.81	1.3	2.60	ND
$C_{15:0}$	ND	0.3	0.5	ND	ND	2.24	1.0
$C_{16:0}$	29.83	25.7	26.0	17.45	21.2	22.63	28.5
$C_{16:0}$ N alcohol	0.21	ND	ND	ND	7.1	ND	ND
$C_{16:0}$ 10-methyl	0.25	ND	ND	ND	2.5	2.84	4.0
$C_{17:0}$	0.5	0.6	3.5	1.36	0.9	3.78	3.6
$C_{18:0}$	1.86	1.9	5.4	4.24	5.6	1.44	2.7
$C_{19:0}$ cyclo ω10c /19 ω6	ND	ND	ND	4.91	ND	ND	ND
Unsaturated fatty acids							
$C_{16:1}$ ω5c	0.28	0.4	ND	ND	ND	0.46	ND
$C_{16:1}$ ω7c	3.26*	8.0	6.0	ND	7.3	ND	ND
$C_{16:1}$ ω7c/15 iso 2-OH	ND	ND	ND	0.97	ND	ND	ND
$C_{16:1}$ ω9c	16.06	9.7	11.3	3.67	6.4	11.62	10.5
$C_{17:1}$ ω8c	0.40	0.5	1.3	0.53	ND	4.54	2.9
$C_{18:1}$ ω7c	ND	ND	1.0	0.78	5.4	ND	2.3
$C_{18:1}$ ω9c	22.41	30.4	36.7	47.51	20.3	19.82	13.9
$C_{18:3}$ ω6c (6,9,12)	0.53	ND	ND	ND	8.5	ND	ND

Strains: 1, strain P4B1 (this study); 2, *M. vinifirmus* FB1 (Liebgott *et al*. 2006); 4, *M. excellens* KMM 3809 (Gorshkova *et al*. 2003); 5, *M. bryozoorum* KMM 3840 (Romanenko *et al*. 2005); 6, *M. gudaonensis* SL014B61A (J. Gu *et al*. 2007); 7, *M. aquaeolei* VT8 (Huu *et al*. 1999) ; 8, *M. lipolyticus* SM-19 (Martin *et al*. 2003). ND, not detected. *, $C_{16:1}$ ω7c or $C_{16:1}$ ω6c

5.2.6 Phylogenetic analysis

Analysis of the 16S rRNA gene sequence (1502 bases) of strain P4B1 indicates that it is a member of the *γ-Proteobacteria.* As shown in Figure 5-10, it forms a coherent cluster with species of the genus *Marinobacter*. Strain P4B1 has a high level of sequence similarity

with the type strain of *M. bacchus* (99.3%), *M. vinifirmus* (98.8 %) and M. *alkaliphilus* (97.6%). The similarity of strain P4B1 with other species of *Marinobacter* is 92.0 - 95.9%.

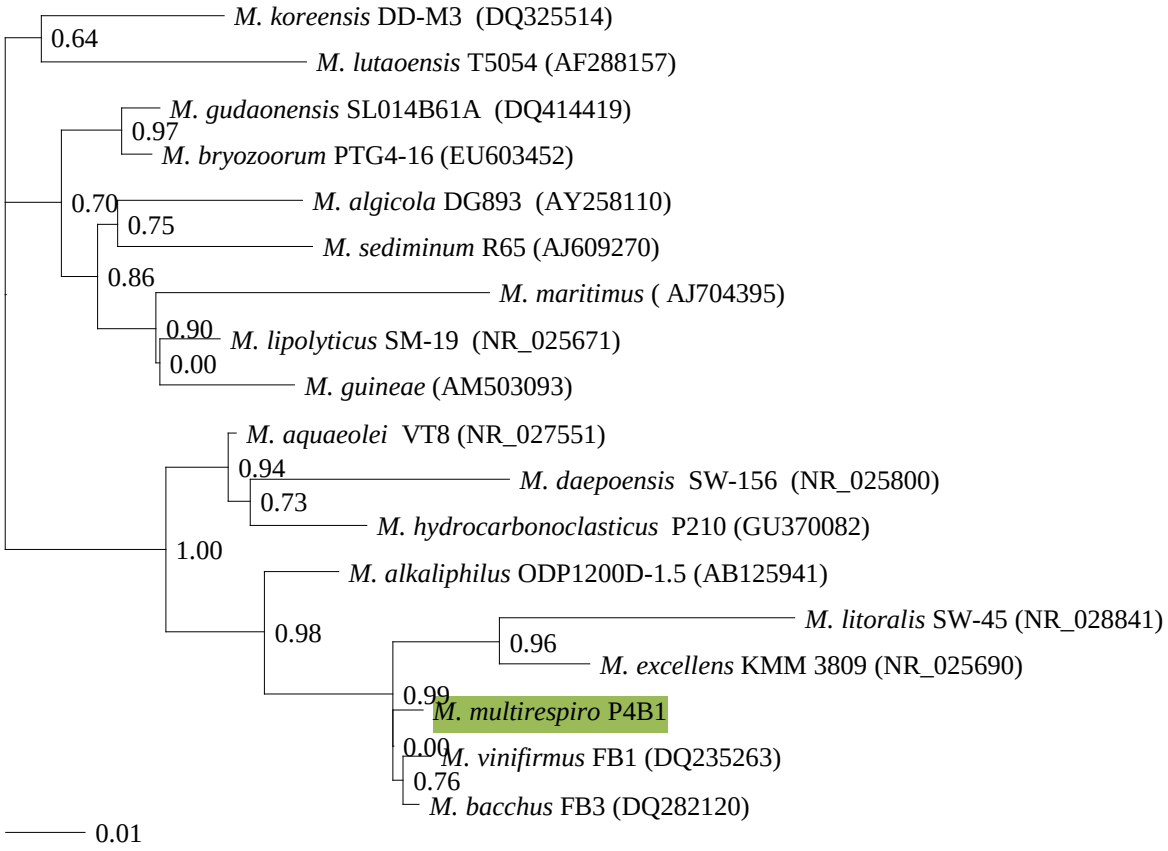


Figure 5-10 16S rDNA-based phylogenetic dendrogram (maximum likelihood algorithm) showing the position of strain P4B1 within the radiation of its related taxa in the *Marinobacter*

GenBank accession numbers are shown in parentheses. The scale bar represents 0.1% divergence. The numbers at nodes represent the approximate likelihood ratio test (aLRT) support for the branches.

M. bacchus type strain FB3 was isolated from an evaporation pond of wine wastewater and proposed as a new species by Liebgott PP *et al*. (2005) (unpublished). However, the characterization of the species has not been published. Based on the information provided by the authors, strain FB3 could ferment glucose. This is the significant difference between it and the strain P4B1, which could not ferment glucose. *M. vinifirmus* type strain FB1 is strictly aerobic, heterotrophic and did not grow under anaerobic

conditions on basal medium with acetate, citrate, succinate or glucose as carbon and energy sources (Liebgott *et al*. 2006). This differentiates it from strain P4B1, which is a facultative anaerobe. The alkaliphilic growth (optimal pH 8.5-9) of *M. alkaliphilus* is a distinctive physiological property (Takai *et al*. 2005).

5.3 Discussion

5.3.1 Perchlorate reducing bacteria

Researchers have been attempting to produce salt-tolerant cultures of PRB. Some success has been met with mixed cultures. For example, Gingras and Batista (2002) tried to enrich a salt-tolerant perchlorate-reducing culture from activated sludge; however, these cultures were almost fully inhibited by 1.5% NaCl. Logan *et al.* (2001a) produced a mixed culture enriched from a saline lake-water that could grow with NaCl up to 11%. Recently, more mixed cultures have been enriched and show potential perchlorate-reducing activities in saline solutions (Ahn *et al*. 2009; Chung *et al*. 2009; Van Ginkel *et al*. 2010).

Currently more than 80 perchlorate-reducing bacteria have been isolated in pure culture. Except for P4B1, the putative *Marinobacter* strain documented here, the only known salt-tolerant strain is *Citrobacter sp.* IsoCock1, which could sustain 10% NaCl (Okeke *et al*. 2002). Both *Marinobacter* and *Citrobacter* are members of γ-Proteobacteria. *Marinobacter* has been found as the predominant organism in the mixed culture (Zuo *et al*. 2009) and autohydrogenotrophic membrane biofilm reactors treating perchlorate and nitrate in ion-exchange brines (Ahn *et al*. 2009; Van Ginkel *et al*. 2010). Other putative salt-tolerant perchlorate-reducing strains were found in the genera of *Pseudomonas* (Sahu *et al*. 2009a), *Clostridium* and *Rhodocyclaceae* (Chung *et al*. 2009), which were determined to be the dominant strains in salt-tolerant perchlorate-reducing mixed cultures. All of these pure or putative salt-tolerant PRB are not members of the β subclass of Proteobacteria, where dominant PRB previously studied belong.

5.3.2 Genus of *Marinobacter*

The genus *Marinobacter*, a subgroup of γ-Proteobacteria, comprises moderate halophilic bacteria from marine-related environments. Since first proposed by Gauthier *et al*. (1992) in 1992, thirty-five (35) species have been isolated and/or characterized at the time of

this writing. Most of the species were isolated from habitats associated with seawater, marine sediment or salterns. The three exceptions are type strains of *M. vinifirmus* (Liebgott *et al*. 2006), *M. bacchus* (unpublished) and *M. bryozoorum* (Romanenko *et al*. 2005), which were from wine-barrel-decalcification wastewater, an evaporation pond of wine wastewater and a marine invertebrate *Bryozoa* specimen. While the majority of the species investigated are mesophilic, thermophilic and psychrophilic species were also isolated from a coastal hot spring (Shieh *et al*. 2003) and sea-ice of the arctic basin (Zhang *et al*. 2008). Except for a few strict aerobes: *M. vinifirmus*, *M. zhanjiangensis* (Zhuang *et al*. 2009), *M. litoralis* (Yoon *et al*. 2003b) and *M. lutaoensis* (Shieh *et al*. 2003), most of the recognized strains are facultative anaerobes capable of respiration of nitrate. In addition to nitrate, respiration on arsenate was also observed for *M. santoriniensis* (Handley *et al*. 2009). Strain P4B1 is the first *Marinobacter* that has been shown to reduce perchlorate. The recognition of bacteria capable of reducing these oxyanions creates a potential for biotreatment or bioremediation in high salt solutions.

The phenotypic characteristics of strain P4B1 and other related species in *Marinobacter* are shown in Table 5-2. Nitrate reduction to nitrite is a common characteristic shared by most species of *Marinobacter* listed, while nitrite reduction to N_2 is only shared by strain P4B1 and *M. alkaliphilus* ODP1200D-1.5 (Takai *et al*. 2005). As shown in Table 5-2, the optimal salinity for P4B1 is 1.8% NaCl, while the others prefer higher NaCl contents. The RNA data suggest that P4B1 could be one of three species of *Marinobacter* (>98% match), the phenotypic data suggest that it is neither of these three species and so we propose strain P4B1 should be placed in the genus of *Marinobacter* as the type strain of a distinct novel species, for which the name *Marinobacter multirespiro* sp. nov. is proposed.

Table 5-2 Phenotypic characteristics of strain P4B1 and some related *Marinobacter* species

	1	2	3	4	5	6	7	8
Nitrate reduction to nitrite	+	-	+	+	+	+	+	-
Nitrite reduction to N_2	+	-	+	ND	-	-	-	-
Temperature for growth (℃)								
Range	15-40	15-45	10-50	10-41	7-42	10-45	13-50	15-40
Optimum	25	20-30	30-35	20-25	ND	ND	30	37
NaCl for growth (%)								
Range	1.8-10.2	0-20	0-21	1-15	1-18	0-15	0-20	1-15
Optimum	1.8	5-6	2.5-3.5	ND	ND	2-3	5	7.5
pH for growth								
Range	5.1-9.1	ND	6.5-11.4	6-10	ND	6.0-9.5	5-10	5-10
Optimum	7.1-8.1	6.5-8.4	8.5-9.0	7.5	ND	7.5-8.0	7.3	7.5
Substrate								
Acetate	+	+	+	ND	-	+	+	ND
Glucose	-	-	+	+	-	+	-	+
Ethanol	+	ND	+	ND	ND	+	ND	ND
Yeast extract	+	+	+	ND	ND	ND	ND	ND
Electron acceptors								
Perchlorate	+	ND	ND	ND	ND	ND	ND	ND
Sulfate	-	-	-	ND	ND	ND	ND	ND
Thiosulfate	-	-	-	ND	ND	ND	ND	ND
Oxygen	+	+	+	+	+	+	+	+

Strains: 1, strain P4B1 (this study); 2, *M. vinifirmus* FB1 (Liebgott *et al*. 2006); 3, *M. alkaliphilus* ODP1200D-1.5 (Takai *et al*. 2005); 4, *M. excellens* KMM 3809 (Gorshkova *et al*. 2003); 5, *M. bryozoorum* KMM 3840 (Romanenko *et al*. 2005); 6, *M. gudaonensis* SL014B61A (J. Gu *et al*. 2007); 7, *M. aquaeolei* VT8 (Huu *et al*. 1999) ; 8, *M. lipolyticus* SM-19 (Martin *et al*. 2003).

CHAPTER 6 KINETIC ANALYSIS OF A PERCHLORATE-REDUCING BACTERIUM: EFFECTS OF SODIUM, MAGNESIUM AND NITRATE

The pure perchlorate-reducing culture P4B1 isolated in Chapter 5 could degrade perchlorate prior to or simultaneously with nitrate. This property is a revolutionary advantage compared to the mixed culture. Application of this pure culture in the treatment of real perchlorate- and nitrate-laden ion-exchange spent brines would eliminate the inhibition of nitrate, which is the major goal of the present research. In order to apply the pure culture in real projects, the kinetics of perchlorate reduction and the optimal growth conditions must be well studied. While most parameters (temperature, pH, salinity) for the optimal growth of the P4B1 had been determined in Chapter 5, this chapter focuses on the interactions of Mg^{2+} and Na^{+} on growth and perchlorate reduction of P4B1. Lin *et al*. (2007) showed that addition of Mg^{2+} and keeping the molar ratio of Mg^{2+} to Na^{+} (Mg^{2+}/Na^{+}) at ~0.11 would benefit NP30 in high salt solution. One objective of this study was to determine whether the NaCl concentration and the molar Mg^{2+}/Na^{+} ratio have similar effects on the pure culture P4B1.

The inhibition of perchlorate reduction by nitrate reduction has been widely observed in pure and mixed perchlorate-reducing bacteria. However, some strains preferred perchlorate to nitrate as the eletron acceptor. *Bacillus cereus* was the first known bacterium that showed perchlorate inhibition of nitrate reduction in a non-competitive manner (Hackenthal *et al*., 1965). Nitrate had no effect on perchlorate and chlorate reduction by *Wolinella succinogenes* strain HAP-1 (Wallace and Attaway 1994; Wallace *et al*. 1996). The nitrate reduction by a nitrate- and chlorate-reducing bacterium, *Rhodobacter sphaeroides* was completely inhibited by chlorate (Roldan *et al*. 1994). Two perchlorate-reducing strains *Citrobacter amalonaticus* and *Citrobacter farmeri* degraded nitrate much slower than perchlorate (Bardiya and Bae 2004). However, the mechanisms of the effects of perchlorate on nitrate reduction for these strains have not been discovered yet. The last objective of this chapter was to reveal the possible mechanism of the preferred perchlorate reduction by strain P4B1. The understanding of this mechanism is crucial to applications of this pure culture, as the nitrate to perchlorate ratio in real ion-exchange spent brines would be quite different from the experimental conditions and would have distinct effects on the culture.

6.1 Methods

6.1.1 Batch kinetics experiments

The media used for cultivation of the pure culture and other tests were described previously in Chapter 5. Batch studies with acetate as the sole electron donor and perchlorate or nitrate (or both) as the electron acceptor(s) were performed to determine the effects of nitrate on perchlorate reduction. These studies were performed in Hungate tubes containing 9 mL P, N or PN medium and 1 mL washed inoculum (initial OD600 ~ 0.030). The Hungate tubes were incubated on a rotary shaker at 110 rpm at 20 ℃. All experiments were performed in quadruplicate. The analytical techniques documented in Chapter 5 were also used here. The VSS and dry weight (DW) of the culture were determined after each batch was done.

6.1.2 Salt-tolerance test

A response surface with central composite design methodology (Montgomery 2009) was used to design an experiment to determine the optimal NaCl concentration and Mg^{2+}/Na^{+} ratio for P4B1. As shown in Table 6-1, a total of 10 conditions was used in the experiment, with the two factors at different levels, i.e., 4 factorial points, 2 center points and 4 axis points. The test was performed with two different media: one with perchlorate as the sole electron acceptor; and the other with perchlorate and nitrate as the electron acceptors. Other nutrients in the test media were identical to the basal medium. The experiments were run in duplicate batches in Hungate tubes containing 10 mL medium and 0.1 mL washed inoculum (initial VSS ~ 120 mg/L) at 35 ℃.

After the experiments were run, the initial specific perchlorate and nitrate reduction rate for each run was calculated and analyzed using the software Design-Expert® 8.0. The sequential model sum of squares was analyzed first to determine the significant model terms. Then a regression model was constructed incorporating the significant factors. The model adequacy was checked via lack-of-fit test and R-squared analysis.

Table 6-1 Design of experiments for salt-tolerance test

Experiment run	Levels of Factors			
	NaCl		Mg^{2+} / Na^{+}	
	Coded	Actual (g/L)	Coded	Actual
1	-1	30	-1	0.04
2	1	90	-1	0.04
3	-1	30	1	0.18
4	1	90	1	0.18
5	0	60	0	0.11
6	0	60	0	0.11
7	0	60	-1.414	0.01
8	0	60	1.414	0.21
9	-1.414	18	0	0.11
10	1.414	102	0	0.21

Other ingredients in the medium were identical to the basal medium.

6.1.3 Perchlorate and nitrate reduction by washed cell suspensions

Acclimated cells (200 mL) estimated to be in mid-log phase in the P or N medium were washed twice with 1X saline phosphate buffer solution (SPBS) containing 8.7 mM $Na_2HPO_4 \cdot 7H_2O$, 1.3 mM KH_2PO_4 and 450 mM NaCl (pH=7.6) and resuspended in 15 mL SPBS. The activity of perchlorate and nitrate reduction by the washed cell suspension was assayed anaerobically in stoppered Hungate tubes by monitoring the oxidation of reduced methyl viologen (MV) at 578 nm and 20 ℃, a method developed by Kengen et al. *(50)*. Three milliliters (3 mL) assay mixture consisting of 50 mM Tris-Cl^- buffer (pH 7.5), 0.5 mM MV and 2mM perchlorate (or nitrate) was transferred into a Hungate tube and flushed with N_2 gas before sealing. The MV was prereduced by adding a small amount of a dithionite solution (0.2 M) until an absorbance of 1.5 was reached. The assay reaction was started by the addition of 0.5 mL of the washed cell suspension.

6.1.4 Kinetic model parameters estimation

The data obtained from the batch experiments were used for the kinetic study. The standard Monod equation was used to model the substrate utilization (Eq. 1) and the biomass growth (Eq. 2):

$$-\frac{dS}{dt} = \frac{V_m S}{K_S + S} X \qquad (1)$$

$$\frac{dX}{dt} = Y\frac{dS}{dt} = Y\frac{V_m S}{K_S + S} X \qquad (2)$$

where, S is the substrate (perchlorate) concentration (mg/L); t is the reaction time (hr); V_m is the maximum specific substrate utilization rate (hr^{-1}); K_S is the half saturation coefficient (mg/L); X is the biomass concentration (mg VSS/L); Y is the biomass yield.

The two equations were combined as a system of ordinary differential equations with given initial values (at t=0). The numeric solutions were resolved using Euler's algorithm with a step of 0.1 hr as an approximation in MATLAB® 7.1. The parameters of the Monod equation K_S, V_m, and Y were estimated using the scatter search method (SSm), a global optimization method developed by Rodriguez-Fernandez *et al*. (2006) and Egea *et al*. (2007). As a hybrid stochastic deterministic global optimization method with robustness and reduced computational time, the SSm method is especially good for nonlinear dynamic models.

6.2 Results

6.2.1 Kinetics of perchlorate reduction

The kinetics of perchlorate reduction were examined in batch cultures using perchlorate and acetate as the sole electron acceptor and donor, respectively. A typical result showing perchlorate reduction and biomass growth is presented in Figure 6-1. As shown in the figure, there was no lag phase when the inoculum was first transferred into the medium. However, the specific perchlorate reduction rate after the 2nd spike feeding with perchlorate and acetate was much higher than that for the first transfer. The data after the 2nd spike feeding was used to determine the kinetic parameters of perchlorate reduction for this analysis. The determined maximum specific perchlorate reduction rate (V_m) and half saturation coefficient (K_s) were 0.050 ±0.007 mg ClO_4^-/mg VSS-hr and 22 ±12 mg ClO_4^-/L at 20 ℃ during degradation of the second spike. The biomass yield was 0.1 ±0.04 mg VSS/mg ClO_4^-.

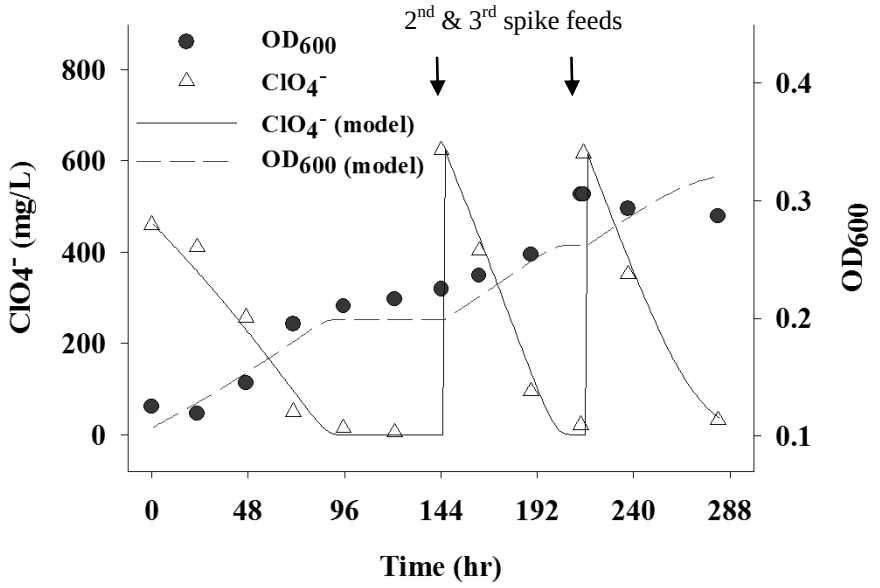


Figure 6-1 Typical perchlorate degradation and biomass growth of P4B1 at 20 ℃

In the 2nd and 3rd spike feedings, a 100× stock solution of perchlorate and acetate was fed to the culture.

6.2.2 Salt-tolerance test

The culture showed quite similar responses to salt shock in P and PN medium. In P medium, perchlorate was degraded to different extents in all experimental runs within the initial 20 hours. In PN medium, perchlorate was also degraded in all experimental runs except the run 10, which had the highest NaCl concentration as shown in Table 6-1. Perchlorate degradation was detected after 44 hours. The initial specific perchlorate degradation rate was calculated by dividing the perchlorate degraded within the initial 20 hours (or 44 hours) by the initial biomass (120 mg VSS/L). The nitrate degradation in PN medium in runs 5-8 (Table 6-1) showed obvious lag stages within the initial 20 hours (data not shown). After the initial 20 hours, nitrate degradation started at its maximum rate. For those tests, the initial specific nitrate degradation rate was calculated by dividing the nitrate degraded within 20-44 hours by the initial biomass (120 mg VSS/L). For other tests, the calculation was similar to the initial specific perchlorate degradation rate. Figure 6-2 presents the results of initial specific perchlorate (nitrate) degradation rate when the NaCl concentration and Mg^{2+}/Na^{+} ratio were varied. The p-values of all the regressed models were much less than 0.05 and the R^2 values were among 0.68-0.85, which indicate that the models fit the experimental data quite well.

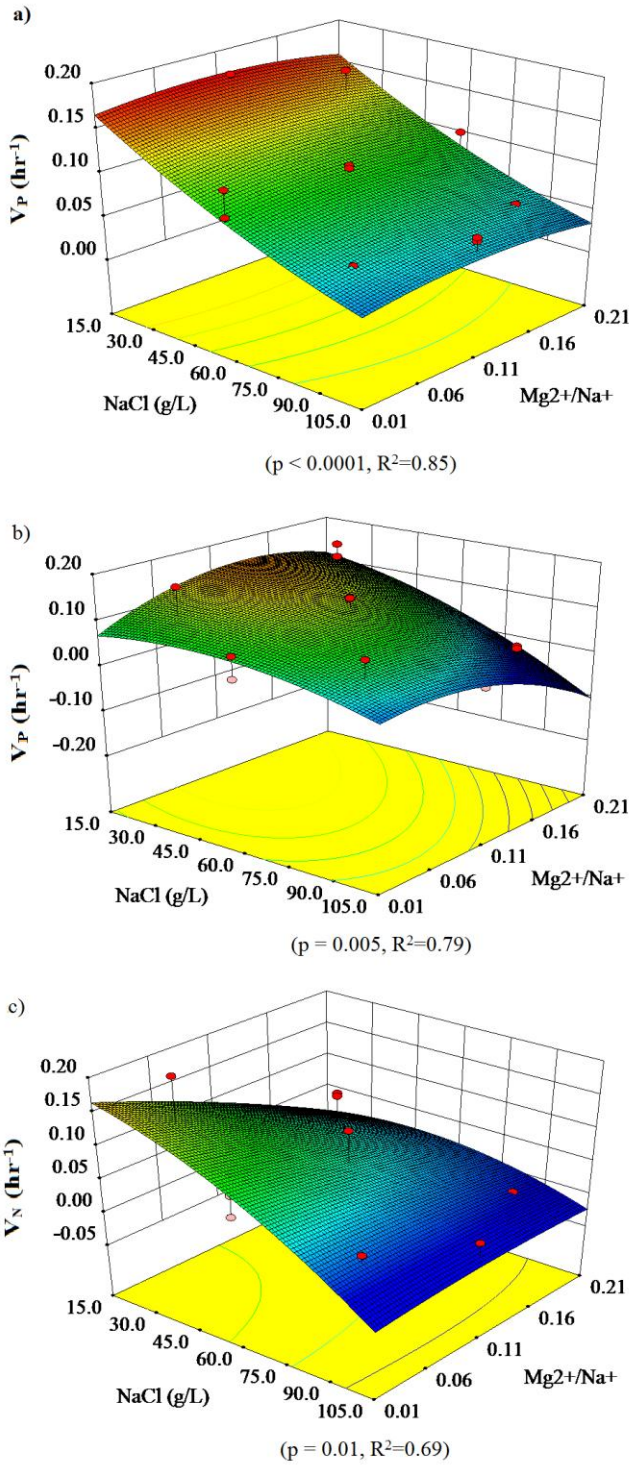


Figure 6-2 Effects of NaCl concentration and Mg^{2+}/Na^{+} ratio on the initial specific perchlorate (V_P, $mgClO_4^-/mgVSS\text{-}hr$) and nitrate ($V_N$, $mgNO_3^-/mgVSS\text{-}hr$) degradation rate in a) P medium, b) medium and c) PN medium

When perchlorate and nitrate were both available as electron acceptors (Figure 6-2 a), the NaCl concentration had significant effects on the perchlorate degradation rate. The initial specific perchlorate degradation rate increased from 0.06 hr^{-1} to 0.13 hr^{-1} when the NaCl concentration decreased from 10.2% to 1.8%. Although the Mg^{2+} concentration had a much smaller effect, an optimal ratio of Mg^{2+}/Na^{+} around 0.11 was consistently observed at the different NaCl concentrations. This optimal Mg^{2+}/Na^{+} ratio was previously observed in the parent mixed culture (Hiremath *et al*. 2006; Lin *et al*. 2007). When perchlorate and nitrate were both available as electron acceptors, the effects of NaCl concentration and Mg^{2+}/Na^{+} ratio on the initial specific perchlorate degradation rate (Figure 6-2 b) were similar to the ones in P medium. An optimal ratio of Mg^{2+}/Na^{+} around 0.11 was observed at lower NaCl concentrations (<60 g/L). With NaCl concentrations increased from 60 to 102 g/L, the optimal ratio of Mg^{2+}/Na^{+} shifted gradually from 0.11 to 0.06. The initial specific nitrate degradation rate in PN medium showed quite different responses to NaCl concentration and Mg^{2+}/Na^{+} ratio (Figure 6-2 c). Both NaCl concentration and Mg^{2+}/Na^{+} ratio had significant effects on nitrate reduction. With the increase of either factor of the two, nitrate degradation rate decreased significantly. There is no optimal Mg^{2+}/Na^{+} ratio observed for nitrate reduction.

6.2.3 Effects of nitrate on perchlorate reduction

When the culture was transferred from PN medium into N medium, nitrate was degraded without any lag. However, after a 2nd spike feed, nitrate degradation was insignificant, as shown in Figure 6-3 a). Figure 6-3 b) shows the perchlorate and nitrate degradation in the PN medium. After the first transfer, perchlorate reduction started immediately but there was a lag for nitrate reduction. After the 2nd spike feed, nitrate reduction was delayed slightly until the perchlorate was almost depleted from the medium. This shows that nitrate reduction was inhibited by perchlorate for P4B1.

Figure 6-4 shows the degradation of perchlorate by the culture transferred from N to P medium and the degradation of nitrate by the culture transferred from P to N medium. The initial reduction rates are identical and follow zero order kinetics to around 350 mg/L but perchlorate reduction follows first order kinetics below 350 mg/L while nitrate reduction continues to follow zero order kinetics below 20 mg/L.

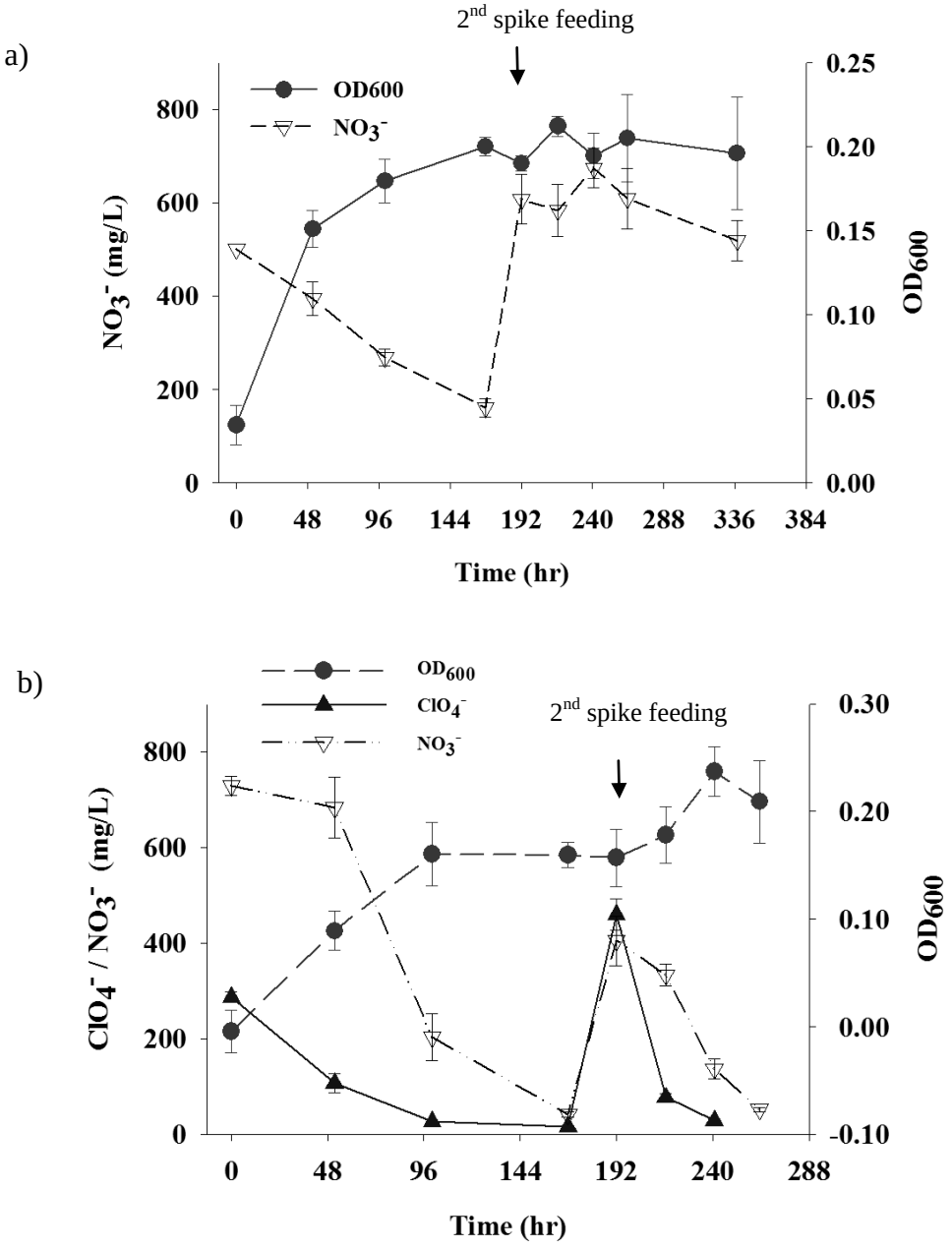


Figure 6-3 Perchlorate/nitrate degradation and biomass growth of strain P4B1 in a) N medium and b) PN medium at 20 ℃

In the 2nd spike feedings, a stock solution of nitrate and acetate was fed to the culture grown in N medium; and a 100× stock solution of perchlorate, nitrate and acetate was fed to the culture grown in PN medium.

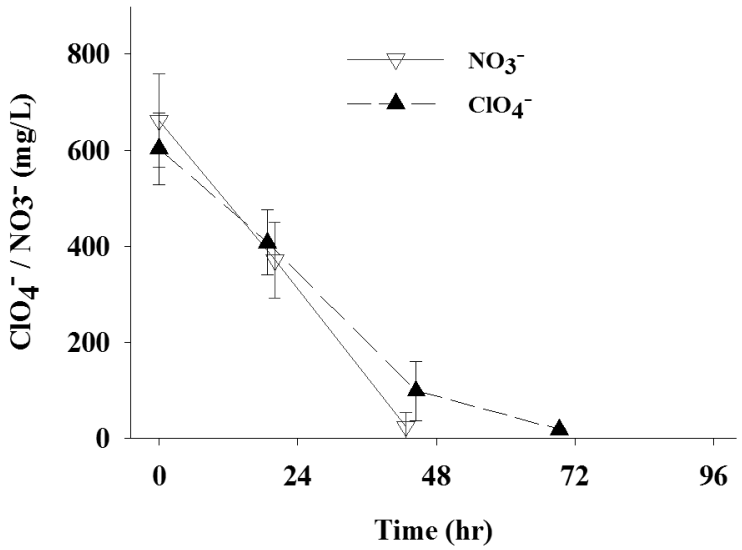


Figure 6-4 Perchlorate degradation of P4B1 acclimated to nitrate and nitrate degradation of P4B1 acclimated to perchlorate

The inoculum was first grown in PN medium and inoculated to P or N medium. After the 2nd spike feeding, the culture grown in P (or N) medium was harvested, washed and transferred back to N (or P) medium.

When a nitrate-acclimated culture was fed perchlorate (~600 mg/L), the perchlorate was degraded to ~ 50 mg/L within 98 hours, although the perchlorate reduction rate within the first 20 hours was much faster. The calculated specific nitrate reduction rate was 0.034 ±0.025 mg NO_3^-/mg VSS-hr, which is much less than the maximum specific perchlorate degradation rate. This indicates that the nitrate-acclimated culture lost some perchlorate-reducing activity.

This might suggest that perchlorate is needed for the nitrate reduction, i.e., the culture P4B1 degraded nitrate via the enzyme of perchlorate reductase and perchlorate is needed for the expression of perchlorate reductase genes.

6.2.4 Perchlorate and nitrate reduction by washed cell suspensions

Further evidence to support the theory that nitrate was reduced by the perchlorate reductase of this culture was obtained by studying the reduction of perchlorate and nitrate coupled with the oxidation of methyl viologen (MV) by washed cell suspensions. Figure 6-5 shows that when washed cell suspensions were transferred into the MV assay solutions containing perchlorate, the perchlorate reduction started immediately as seen from the instantaneous decrease in the absorbance of MV at 578 nm (A_{578}) no matter the origin of the

washed cells (perchlorate-fed or nitrate-fed). Nitrate reduction exhibited a 30 minute lag phase and a much slower degradation rate no matter the origin of the cells. Although, the perchlorate-reducing activity of the nitrate-fed culture was still high, the nitrate-reducing activity was relatively deteriorated in the washed cell suspensions.

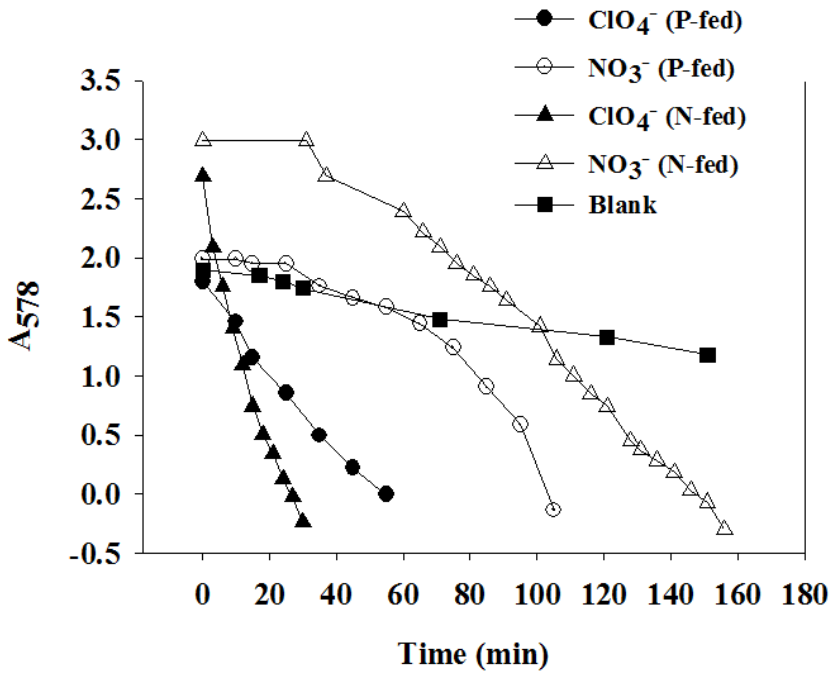


Figure 6-5 Perchlorate/nitrate degradation of washed cell suspensions obtained from perchlorate- (P-) fed or nitrate- (N-) fed cultures

The decreasing absorbance of MV at 578 nm indicates oxidation by enzymes while they reduce the substrates. The blank contains the assay solution without the cells.

6.3 Discussion

6.3.1 Kinetics of perchlorate reduction

Table 6-2 shows the comparison of the perchlorate reduction kinetics of this pure culture with those of other reported perchlorate-reducing bacteria. In order to provide results that could be more easily compared to other data, the biomass data were converted to dry weight (DW) in the table using the conversion factor of 0.17 mg VSS/mg DW determined in this study.

Table 6-2 Kinetic parameters of perchlorate reducing bacteria culture

Culture	Electron donor	V_m $\left(\frac{\text{mg } ClO_4^-}{\text{mg DW} \cdot \text{hr}}\right)$	K_s (mg ClO_4^-/L)	μ_m (hr^{-1})	Y $\left(\frac{\text{mg DW}}{\text{mg } ClO_4^-}\right)$	Temp (℃)	Reference
Marinobacter sp. P4B1[#]	Acetate	(0.050 ±0.007)	22.53±12.23	0.0047±0.0017	(0.098±0.044)	20	This study
		0.015±0.002			0.33±0.15		
Dechlorosoma sp. KJ	Acetate	0.055±0.004	470±290	0.20±0.07	NA	24	(Logan *et al*. 2001b)
Dechlorosoma sp. PDX	Acetate	0.017±0.002	45±19	0.24±0.03	NA	24	(Logan *et al*. 2001b)
Azospirillum sp. SN1A	Acetate	0.19	2.2 ±2.8	0.069 ±0.009	0.36 ±0.02	~22	(Waller *et al*. 2004)
Azospirillum sp. ABL1	Acetate	0.23	4.8 ±0.9	0.086 ±0.007	0.38 ±0.10	~22	(Waller *et al*. 2004)
Dechloromonas sp. INS	Acetate	0.18	18.0 ±5.2	0.067 ±0.007	0.37 ±0.03	~22	(Waller *et al*. 2004)
Dechloromonas sp. RC1	Acetate	0.25	12.0 ±2.4	0.085 ±0.005	0.34 ±0.02	~22	(Waller *et al*. 2004)
Dechlorosoma sp. PCC	Acetate	0.18	76.6			~22	(Dudley *et al*. 2008)
Dechlorimonas sp. JM	H_2	0.089±0.007*	14.85±5.94			Room	(Miller and Logan 2000)
Dechloromonas sp. HZ	H_2	0.009	8.9			NA	(Yu *et al*. 2006)
Dechloromonas sp. PC1	H_2	0.129	0.15		0.23	22	(Nerenberg *et al*. 2006)
Mixed culture	Acetate	0.02	<0.1	0.006	0.2	~27	(Wang *et al*. 2008)
P30 (mixed)[#]	Acetate	(0.031 ±0.001)	4.17 ±0.13			Room	(Zuo 2008)
NP30 (mixed)[#]	Acetate	(0.0043 ±0.0004)	26.39 ±2.16			Room	(Zuo 2008)
Mixed culture	H_2	0.12	567.3			30	(Cheong *et al*. 2010)
Mixed culture ADS	Acetate 0%	(0.0346 ±0.0013)	119.3			21-25	(Park and Marchand 2006)
	Acetate 3%	(0.0187 ±0.0013)	295			21-25	(Park and Marchand 2006)
Mixed culture RDS	Acetate 0%	(0.0378 ±0.0008)	72.8			21-25	(Park and Marchand 2006)
	Acetate 3%	(0.0073 ±0.0004)	300			21-25	(Park and Marchand 2006)
Mixed culture	Acetate	0.67	193.8				(Nor *et al*. 2011)

Data in '()' are calculated in volatile suspended solids (VSS), instead of dry weight (DW).

* Calculate from original data.

Compared to the parent culture NP30, the V_m of this pure culture was 10 times greater than NP30, although both had similar Ks values. The mixed culture P30, which was the parent culture of NP30 (Zuo *et al*. 2009), had a slightly lower V_m than this pure culture. The V_m of this pure culture was about half of that of the strain PDX (Logan *et al*. 2001b) and a mixed culture (Wang *et al*. 2008), which is a group of the lowest V_m within heterotrophic cultures. The cultures (SN1A, ABL1, INS and RC1) isolated and enriched from perchlorate-contaminated sites (Waller *et al*. 2004) had the highest maximum specific perchlorate reduction rates. The pure culture PCC which was isolated from activated sludge of a sewage treatment plant also showed very high V_m. When compared to the autotrophic hydrogen-oxidizing cultures, the V_m of this pure culture was much less than that exhibited by JM and PC1, and slightly lower than a mixed culture isolated and enriched from an anaerobic digester (Cheong *et al*. 2010), but similar to the pure culture HZ. All of the other cultures shown in Table 2 were all grown in fresh water media (except the parent cultures P30 and NP30). Many of these cultures would be inactive in 3% NaCl. As noticed in the salt-tolerance test, when NaCl was decreased to 1.8%, the observed maximum specific perchlorate degradation rate would be 0.13 mg ClO_4^-/mg VSS-hr (0.022 mg ClO_4^-/mg DW-hr), which would be slightly higher than strain PDX. The tests were all performed at 3% NaCl because this is the lowest concentration of NaCl effective for treatment of perchlorate contaminated ion-exchange brines or resins.

The biomass yield (Y) of this pure culture was 0.6 ± 0.2 mg DW/mg ClO_4^-, which was similar to other heterotrophic organisms and less than autotrophic hydrogen-oxidizing organisms, as shown in Table 6-2. The maximum specific growth rate (μ_m) of this pure culture was 0.005 ± 0.002 hr^{-1}, which was much lower (2.1-7.5%) than other reported cultures listed in Table 6-2. One exception is a mixed culture enriched from anaerobic sludge of a wastewater treatment plant (Wang *et al*. 2008), whose μ_m was similar to this culture. The need to expend energy to balance the salt content is most likely the cause of the low growth rate of this culture.

6.3.2 Benefits of Mg^{2+} to salt-tolerance and biomass growth

The requirement for a large amount of Mg^{2+} for optimal growth was widely observed for halophiles isolated from the Dead Sea (Mack *et al*. 1993), where the dominant cation is

Mg^{2+} instead of Na^{+}. Two groups of prokaryotes are defined on the basis of their Mg^{2+} requirements. One group shows high (0.5 M) Mg^{2+} requirements for optimal growth. Another group grows optimally at 0.1-0.3 M Mg^{2+} (Mack *et al*. 1993). For the first group, the optimal Mg^{2+}/Na^{+} is generally greater than 0.1. For example, *Halobacterium sodomense* grew optimally at 2M NaCl and 0.6-1.2 M Mg^{2+}, a Mg^{2+}/Na^{+} ratio of 0.3-0.6 (Oren 1983). For the second group, the optimal Mg^{2+}/Na^{+} is generally less than 0.1. An example is *Rhodovibrio sodomensis,* a photoheterotrophically anoxygenic purple bacterium. The optimal growth was found at 12% (2M) NaC1 and 0.05-0.1 M Mg^{2+}, i.e., a Mg^{2+}/Na+ ratio of 0.025-0.05 (Mack *et al*. 1993).

The requirement for higher than normal concentrations of Mg^{2+} for optimal growth was also observed for other moderately salt-tolerant bacteria. *Desulfothermus naphthae,* isolated from anoxic marine sediments, grows optimally at 0.36 M Na^{+} and 0.025 M Mg^{2+}, a Mg^{2+}/Na^{+} ratio of 0.07 (Kuever *et al*. 2005). *Desulfocell halophia*, a halophilic sulfate-reducing bacterium isolated from surface sediment of the Southern arm of the Great Salt Lake, grew optimally at 4-5% NaCl and 0.002-0.1M Mg^{2+}, a broad range of optimal Mg^{2+}/Na^{+} ratios from 0.0058 to 0.12 (Brandt *et al*. 1999). Another example is *Methylarcula marina*, a moderately halophilic facultatively methylotrophic bacterium isolated from a coastal saline habitat. The optimal Mg^{2+}/Na^{+} ratio was not determined. However, it was observed that the divalent cations Mg^{2+} and Ca^{2+} at concentrations as low as 50 mM added to the 0.5 M NaCl washing solution prevented cell lysis (Doronina *et al*. 2000). Comparison of these results with those of the present research indicates that each organism has an optimal Mg^{2+}/Na^{+} ratio.

Despite these findings of the requirement for Mg^{2+}, the physiological mechanism underlying this is not fully clear. For most salt-tolerant bacteria, K^{+} or amino acids and occasionally both were found to be concentrated inside the cells in response to the increased osmotic stress (Vreeland *et al*. 1983). Relatively, Mg^{2+} has been less observed to balance the osmotic stress. One example was *Halobacterium halobium* and related organisms, when grown in 4M NaCl + 0.01M KCl medium, the intracellular ions were determined to be 3.5 M KCl, 1.0 M NaCl, and O.l M $MgCl_2$ (Kushner 1985), which has a Mg^{2+}/Na^{+} of 0.1. However, whether keeping this ratio in the medium would optimize the cell growth was not documented. Another study made by Hurst *et al*. (1974) proved the positive relationship

between the presence of Mg^{2+} and salt tolerance for *Staphylococcus aureus.* They found a good correlation between loss of salt tolerance and loss of cellular Mg suggesting intracellular osmotic balancing. Another possible mechanism for Mg requirement could be that there were some sites, like teichoic acid in the cell wall, where Mg^{2+} is bound for cell support. Hoover and Gray (1977) found that a surface pool of Mg^{2+} was necessary for the proper functioning of *S. aureus*.

The presence of Mg^{2+} might also be important to the activities of some enzymes. As Shafea (2003) noticed, the activity of the nitrate reductase extracted from *Chlorella fusca*, a unicellular chlorophycean algae, was enhanced by Mg^{2+} at all Mg^{2+}/Na^{+} ratios applied (0.33-1.0). In the culture studied in the present research there were differences in the effect of NaCl and Mg^{2+} addition and these differences might be because the optimal conditions for perchlorate reductase are different from other assimilatory enzymes suggesting there is a NaCl dependent magnesium requirement for the perchlorate reductase enzyme. To the best of our knowledge no similar observation for bacteria has been previously published.

6.3.3 Nitrate effects on perchlorate reduction

Nitrate is known to inhibit the perchlorate-reducing activities of many cultures including the parent culture NP30 from which P4B1 was isolated (Hiremath *et al*. 2006; Zuo 2008; Lin *et al*. 2007). However, nitrate inhibition of perchlorate reduction was not observed for this culture P4B1. Instead, perchlorate was observed to inhibit nitrate reduction.

Based on all the results, the most plausible explanation of the effect of perchlorate on nitrate reduction is that perchlorate is needed for nitrate reduction, i.e., the culture P4B1 degrades nitrate via the enzyme perchlorate reductase and perchlorate is required for the expression of perchlorate reductase genes. Immediately after the perchlorate- and nitrate-fed culture was transferred to N medium, the perchlorate reductase that still actively existed inside the bacteria contributed to the reduction of nitrate and the growth of the culture. However, due to the lack of perchlorate, the expression of the genes encoding the perchlorate reductase decreased and perchlorate reductase levels decreased. Eventually, nitrate reduction stopped and even perchlorate reduction activity decreased. The analogous phenomenon was also observed for some nitrate-reducers (Attaway and Smith 1993). Nitrate inhibited perchlorate reduction in these cultures and repeated cultivation on perchlorate caused the loss

of perchlorate- and nitrate-reducing ability, suggesting that nitrate reductase was responsible for perchlorate reduction.

Since perchlorate-reducing activity was first observed in nitrate-reducing strains and nitrate reduction normally occurred prior to or simultaneous with perchlorate reduction, perchlorate-reduction was believed to be conducted by nitrate reductase. Later studies have shown that in some strains, such as *Azospira* (*Dechlorosoma*) strains KJ and perc1ace and *Pseudomonas* strain PDA, nitrate and perchlorate are reduced by different enzymes, which are also induced separately (Xu *et al*. 2004; Giblin and Frankenberger 2001). The separately-induced enzymes were also detected in some nitrate- and chlorate-reducing microorganisms, such as *Proteus mirabilis* (Oltmann *et al*. 1976), and *Rhodobacter capsulatus* (Roldan *et al*. 1994).

An interesting study by Sun *et al*. (2009) showed the quite different chemotaxis response of three dissimilatory PRB to different electron acceptors (nitrate, perchlorate and sulfate). Strain CKB, which is incapable of growth by nitrate reduction, responded readily toward nitrate when grown with perchlorate. Strain RCB responded both to nitrate and perchlorate when grown with perchlorate, but only to nitrate when grown with nitrate. Strain PS responded only to perchlorate and chlorate but not nitrate when grown with perchlorate, and only to nitrate when grown with nitrate. This study suggests that separately-induced perchlorate and nitrate reductases are responsible for the perchlorate and nitrate reduction respectively when these strains are exposed to the electron acceptor, and at least two different perchlorate reductases, which may or may not reduce nitrate, exist. A perchlorate reductase that could not reduce nitrate might be because of the inhibition of nitrite instead of nitrate as shown by Attaway and Smith (1993). Chaudhuri *et al*. (2002) also observed nitrate was reduced to nitrite by *Dechloromonas agitata* strain CKB concomitantly with perchlorate reduction, and nitrite accumulated in the medium and reduced the perchlorate reduction rate.

The results presented in this study suggest P4B1 has a different enzyme. The perchlorate reductase could reduce both perchlorate and nitrate; however, it is only induced by perchlorate.

In summary, this chapter shows that strain P4B1 could utilize perchlorate and grow in the presence of 1.8 to 10.2% NaCl. Lower NaCl concentrations allowed faster perchlorate reduction and growth rates. The addition of Mg^{2+} to the culture showed significant effects on

perchlorate reduction and biomass growth when perchlorate was the sole electron acceptor. A molar Mg^{2+}/Na^{+} ratio of ~0.11 optimized perchlorate degradation and cell growth. When perchlorate and nitrate were both present in the culture, P4B1 preferred perchlorate to nitrate as the electron acceptor. The results suggest that a perchlorate reductase of P4B1 is active in both perchlorate and nitrate reduction. However, the perchlorate reductase is only induced by perchlorate. When nitrate was used as the sole electron acceptor, the strain eventually lost the ability to reduce nitrate. The maximum specific substrate utilization rate (V_m) and the half saturation coefficient (K_S) for P4B1 determined in this study were 0.050 ± 0.007 mg ClO_4^-/mg VSS-hr and 22 ± 12 mg ClO_4^-/L respectively.

CHAPTER 7 CONCLUSIONS

The biological regeneration of perchlorate-laden non-regenerable ion-exchange resins was successfully demonstrated with two perchlorate-selective resins. The regeneration process is governed by biological activities. The following conclusions were made from this study:

- ✓ Perchlorate-selective ion-exchange resins exhausted with perchlorate could be regenerated in a batch process using a salt-tolerant bacterial enrichment culture in 6% NaCl solution.
- ✓ The numerical model coupling two-site perchlorate desorption module with a modified Monod microbial metabolism for resin phase (a concentrated liquid film on or around the resin) and aqueous phase biodegradation fit with the experimental data well (R^2 = 0.82-0.97).
- ✓ The biodegradation process probably includes degradation of perchlorate in both resin phase and in the bulk aqueous phase, while the majority of the degradation occurred in resin phase.
- ✓ The initial mass of organisms added and the kinetics of the culture in the liquid film around the resin had the largest effect on the time to reach complete degradation.
- ✓ The perchlorate selectivity coefficient with respect to chloride also had a significant effect on the regeneration time. Higher selectivity coefficients resulted in shorter regeneration.
- ✓ Since many of the parameters are controlled by the nature of the resin and the culture itself, the regeneration process can be maximized by using resins with higher selectivity for perchlorate with high concentrations of biomass (> 1,500 mg VSS/L).

In a pilot-plant, NP30 was inoculated to two FBRs to treat ion-exchange regenerant brines containing perchlorate and nitrate. Complete denitrification was achieved after 70 days operation of the FBRs, but the perchlorate removal rate was not significant until the later stages of the process. The microbial community analysis of the mixed culture in the FBRs indicated:

- ✓ Both DGGE and FISH revealed that the dominant perchlorate-reducing bacteria in the inoculum were *Azoarcus* and *Denitromonas*, which could not be isolated by solid plate cultivation. The dominant nitrate-reducing bacterium of the inoculum was *Halomonas*.
- ✓ The FISH test clearly showed that the percentages of *Azoarcus* and *Denitromonas* in the pilot plant decreased with time, and the number of *Halomonas* increased during the operation. This indicates a shift towards nitrate reduction in the pilot plant system, which corresponds to the actual operation data.
- ✓ Because the nitrate concentration was much higher than the perchlorate in the brine, the reaction time in the FBR1 system was too short for the culture to fully degrade nitrate, which would be required to make FBR2 more favorable to perchlorate degradation.
- ✓ FISH is a more effective test for monitoring this culture than DGGE. FISH is also less time-consuming than DGGE, costs less and uses fewer hazardous chemicals.

A pure salt-tolerant perchlorate- and nitrate- reducing bacteria, strain P4B1, was successfully isolated from the mixed culture NP30. The following properties of P4B1 were observed:

- ✓ The cells are non-fermentative, Gram-negative, motile rods. P4B1 grew optimally at 25 ℃, in 1.8% NaCl and at pH 7.1 -8.1.
- ✓ It could use perchlorate, nitrate and oxygen as electron acceptors. When perchlorate and nitrate were used as electron acceptors simultaneously, perchlorate was degraded prior to nitrate.
- ✓ Phylogenetic analysis showed that it belongs to the genus *Marinobacter*. Based on this and phenotypic characteristics (such as FAME), it is proposed to represent a novel species of the genus *Marinobacter*, with the name *Marinobacter multirespiro* sp. nov..

The kinetics of perchlorate reduction by P4B1and the effects of sodium, magnesium and nitrate on perchlorate reduction were studied. The following conclusions were made from this study:

- ✓ Strain P4B1 could utilize perchlorate and grow in the presence of 1.8-10.2% NaCl. The perchlorate reduction and biomass growth rates increased with the

decreasing of NaCl concentrations. A molar Mg^{2+}/Na^{+} ratio of ~0.11 optimized the perchlorate degradation and cell growth when perchlorate was the sole electron acceptor.

- ✓ A perchlorate reductase of P4B1 is active in both perchlorate and nitrate reduction. However, the perchlorate reductase is only induced by perchlorate. When nitrate was used as the sole electron acceptor, the strain eventually lost the ability to reduce nitrate.
- ✓ The maximum specific substrate utilization rate (V_m) and the half saturation coefficient (K_S) for P4B1 determined in this study are 0.05 ±0.007 mg ClO_4^-/mg VSS-hr and 22 ±12 mg ClO_4^-/L respectively.

The direct biological regeneration of perchlorate-exhausted resins demonstrated in this study is an attractive approach. It combines resin regeneration and brine treatment into a single process. The contact of resins with bacterial culture not only eliminates one unit process, but also greatly increases the efficiency of bioregeneration.

The pure culture P4B1 isolated in the present research is the first salt-tolerant perchlorate-reducing culture that has been characterized. The culture has the unique property of perchlorate reduction prior to nitrate reduction. The application of this pure culture in the treatment of real perchlorate-laden ion-exchange spent brines would overcome the inhibition of nitrate that occurred with the mixed culture, thus would make the treatment process much more stable. The combination of ion-exchange with biological process using this culture, either for brine treatment or direct resin regeneration, provides an economic and sustainable technology to remove perchlorate in drinking water.

REFERENCES

Abu-Omar, M., McPherson, L. D., Arias, J., and Bereau, V. M. (2000). "Clean and efficient catalytic reduction of perchlorate." *Angew. Chem.*, 112(23), 4480-4483.

Achenbach, L. A., Michaelidou, U., Bruce, R. A., Fryman, J., and Coates, J. D. (2001). " *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position." *Int. J. Syst. Evol. Microbiol.*, 51(2), 527-533.

Ader, M., Chaudhuri, S., Coates, J. D., and Coleman, M. (2008). "Microbial perchlorate reduction: A precise laboratory determination of the chlorine isotope fractionation and its possible biochemical basis." *Earth Planet. Sci. Lett.*, 269(3-4), 605-613.

Ahn, C., Oh, H., Ki, D., Van Ginkel, S., Rittmann, B., and Park, J. (2009). "Bacterial biofilm-community selection during autohydrogenotrophic reduction of nitrate and perchlorate in ion-exchange brine." *Appl. Microbiol. Biotechnol.*, 81(6), 1169-1177.

Aldridge, L., Gillogly, T., Lehman, G., Clifford, D. A., Roberts, D. J., and Lin, X. (2003). *Treatability of perchlorate in groundwater by ion-exchange technology, phase II pilot plant study.* AWWA Research Foundation, Denver, CO, USA.

Amann, R. I., Krumholz, L., and Stahl, D. A. (1990). "Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology." *J. Bacteriol.*, 172(2), 762-770.

APHA, AWWA and WEF (2005). *Standard methods for the mxamination of water & wastewater*, 21st Edition. Eaton, A. D., Clesceri, L. S., Rice, E. W., and Greenberg, A. E. eds. American Public Health Association (APHA), Washington, DC, USA.

Arthur, R. D. (2011). "Process control factors for continuous microbial perchlorate reduction in the presence of zero-valent Iron " PhD thesis, Auburn University, Auburn, AL.

Attaway, H., and Smith, M. (1993). "Reduction of perchlorate by an anaerobic enrichment culture." *J. Ind. Microbiol.*, 12 408-412.

Backus, S.M., Klawuun, P., Brown, S., D'sa, I., Sharp, Surette, C., and Williams, D.J. "Determination of perchlorate in selected surface waters in the Great Lakes Basin by HPLC/MS/MS." *Chemosphere*, 61, 834–843.

Balk, M., van Gelder, T., Weelink, S. A., and Stams, A. J. M. (2008). "(Per)chlorate reduction by the thermophilic bacterium *Moorella perchloratireducens* sp. nov., isolated from underground gas storage." *Appl. Environ. Microbiol.*, 74(2), 403-409.

Bansal, R., Crawford, R. L., and Paszczynski, A. J. (2011). "Peptide biomarkers as evidence of perchlorate biodegradation." *Appl. Environ. Microbiol.*, 77(3), 810-820.

Bao, H., and Gu, B. (2004). "Natural perchlorate has a unique oxygen isotope signature." *Environ. Sci. Technol.*, 38(19), 5073-5077.

Bardiya, N., and Bae, J. (2004). "Role of *Citrobacter amalonaticus* and *Citrobacter farmeri* in dissimilatory perchlorate reduction." *J. Basic Microbiol.*, 44(2), 88-97.

Batista, J. R., MF, X., and Vieira, A. R. (2000). "The removal of perchlorate from waters using ion-exchange resins." *Perchlorate in the Environment*, E. T. Urbansky, ed., Kluwer Academic/Plenum Publishers, New York, NY, USA, Chapter 13.

Batista, J. R., Papelis, L., Kesterson, K., and Amy, P. (2005). "Potential for bioremediation of the perchlorate-contaminated sediments in the Las Vegas Wash area, Henderson, Nevada." *Remediation*, 15(4), 73-87.

Bender, K. S., O'Connor, S. M., Chakraborty, R., Coates, J. D., and Achenbach, L. A. (2002). "Sequencing and transcriptional analysis of the chlorite dismutase gene of *Dechloromonas agitata* and its use as a metabolic probe." *Appl. Environ. Microb.*, 68(10), 4820-4826.

Bender, K. S., Shang, C., Chakraborty, R., Belchik, S. M., Coates, J. D., and Achenbach, L. A. (2005). "Identification, characterization, and classification of genes encoding perchlorate reductase." *J.Bacteriol.*, 187(15), 5090-5096.

Bohlke, J. K., Hatzinger, P. B., Sturchio, N. C., Gu, B., Abbene, I., and Mroczkowski, S. J. (2009). "Atacama perchlorate as an agricultural contaminant in groundwater:isotopic and chronologic evidence from Long Island, New York." *Environ. Sci. Technol.*, 43(15), 5619-5625.

Bordeleau, G., Martel, R., Ampleman, G., and Thiboutot, S. (2008). "Environmental impacts of training activities at an air weapons range." *J. Environ. Qual.*, 37, 308–317.

Borden, R. C. (2007). "Concurrent bioremediation of perchlorate and 1,1,1-trichloroethane in an emulsified oil barrier." *J. Contam. Hydrol.*, 94(1-2), 13-33.

Borden, R. C., and Lieberman, M. T. (2009). "Passive bioremediation of perchlorate using emulsified edible oils." *In Situ Bioremediation of Perchlorate in Groundwater,* H. F. Stroo, and C. H. Ward, eds., Springer New York, 155-175.

Brandt, K. K., Patel, B. K. C., and Ingvorsen, K. (1999). "*Desulfocella halophila* gen. nov., sp. nov., a halophilic, fatty-acid-oxidizing, sulfate-reducing bacterium isolated from sediments of the Great Salt Lake." *Int. J. Syst. Bacteriol.,* 49(1), 193-200.

Brechner, R. J., Parkhurst, G. D., Humble, W. O., Brown, M. B., and Herman, W. H. (2000). "Ammonium perchlorate contamination of Colorado River drinking water is associated with abnormal thyroid function in newborns in Arizona." *J. Occup. Environ. Med.,* 42(8), 777-782.

Brown, J., Lauderdale, C., Estavo, G., Ettori, A., Shih, W., Poust, S., Walker, S., Raskin, L., Upadhyaya, G., Li, X., and Morgenroth, E. (2008). "Direct fixed-bed biological perchlorate destruction demonstration." *Rep. No. ESTCP Project ER-0544,* Carollo Engineers, Walnut Creek, CA, USA.

Brown, J. C., Snoeyink, V. L., Raskin, L., and Lin, R. (2003). "The sensitivity of fixed-bed biological perchlorate removal to changes in operating conditions and water quality characteristics." *Water Res.,* 37(1), 206-214.

Bruce, R. A. (1999). "The microbiology and bioremediative potential of (per)chlorate reducing bacteria." PhD thesis, Southern Illinois University,Carbondale, IL, USA.

Bruce, R. A., Achenbach, L. A., and Coates, J. D. (1999). "Reduction of (per)chlorate by a novel organism isolated from paper mill waste." *Environ. Microbiol.,* 1(4), 319-329.

Butler, C. S., Clauwaert, P., Green, S. J., Verstraete, W., and Nerenberg, R. (2010). "Bioelectrochemical perchlorate reduction in a microbial fuel cell." *Environ. Sci. Technol.,* 44(12), 4685-4691.

Calgon Carbon Corporation. (1999). "Executive summary, pilot study conducted by Calgon Carbon Corporation at JPL." Calgon Carbon Corporation, US.

Cang, Y., Roberts, D. J., and Clifford, D. A. (2004). "Development of cultures capable of reducing perchlorate and nitrate in high salt solutions." *Water Res.,* 38(14-15), 3322-3330.

Cao, J., Elliott, D., and Zhang, W. (2005). "Perchlorate Reduction by Nanoscale Iron Particles." *J. Nanopart. Res.,* 7(4), 499-506.

CDPH. (2007). "History of perchlorate in California drinking water." http://www.cdph.ca.gov/certlic/drinkingwater/Pages/Perchloratehistory.aspx (02/16, 2009).

CEPA. (2004). "Perchlorate contamination treatment alternatives (draft)." Office of Pollution Prevention and Technology Development, Department of Toxic Substances Control, California Environmental Protection Agency, US.

Chaudhuri, S. K., O'Connor, S. M., Gustavson, R. L., Achenbach, L. A., and Coates, J. D. (2002). "Environmental factors that control microbial perchlorate reduction." *Appl. Environ. Microb.*, 68(9), 4425-4430.

Chen, C., Ren, N., Wang, A., Yu, Z., and Lee, D. J. (2008). "Simultaneous biological removal of sulfur, nitrogen and carbon using EGSB reactor". *Appl. Microbiol. Biotechnol.*, 78(6), 1057-1063.

Chen, W. (2005). *Tailoring and regeneration of granular activated carbon for perchlorate removal.* PhD thesis, The Pennsylvania State University, US.

Chen, W., and Cannon, F. S. (2005). "Thermal reactivation of ammonia-tailored granular activated carbon exhausted with perchlorate." *Carbon*, 43(13), 2742-2749.

Chen, W., Cannon, F. S., and Rangel-Mendez, J. R. (2005a). "Ammonia-tailoring of GAC to enhance perchlorate removal. I: Characterization of NH3 thermally tailored GACs." *Carbon*, 43(3), 573-580.

Chen, W., Cannon, F. S., and Rangel-Mendez, J. R. (2005b). "Ammonia-tailoring of GAC to enhance perchlorate removal. II: Perchlorate adsorption." *Carbon*, 43(3), 581-590.

Cheong, S. -., Lee, S. -., Lee, J. -. C., -Suh, W., Nam, J. -., and Shin, H. -. (2010). "**A** comparative study of perchlorate degradation in acute toxicity and chronic toxicity." *Water Sci. Technol.*, 62(5), 1143-1148.

Choe, J. K., Shapley, J. R., Strathmann, T. J., and Werth, C. J. (2010). "Influence of rhenium speciation on the stability and activity of Re/Pd bimetal catalysts used for perchlorate reduction." *Environ. Sci. Technol.*, 44(12), 4716-4721.

Choi, H., and Silverstein, J. (2008). "Inhibition of perchlorate reduction by nitrate in a fixed biofilm reactor." *J. Hazard. Mater.*, 159(2-3), 440-445.

Choi, Y. C., Li, X., Raskin, L., and Morgenroth, E. (2008). "Chemisorption of oxygen onto activated carbon can enhance the stability of biological perchlorate reduction in fixed bed biofilm reactors." *Water Res.*, 42(13), 3425-3434.

Chung, J., Nerenberg, R., and Rittmann, B. E. (2007). "Evaluation for biological reduction of nitrate and perchlorate in brine water using the hydrogen-based membrane biofilm reactor." *J. Environ. Eng.*, 133(2), 157.

Chung, J., Shin, S., and Oh, J. (2010). "Biological reduction of nitrate and perchlorate in brine water using up-flow packed bed reactors." *J. Environ. Sci. Heal. A*, 45(9), 1109.

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

Clark, J. J. J. (2000). "Toxicology of Perchlorate." *Perchlorate in the Environment*, E. T. Urbansky, ed., Kluwer Academic/Plenum Publishers, New York, USA, Chapter 3.

Clifford, D. (1982). "Multicomponent ion-exchange calculations for selected ion separations." *Ind. Eng. Chem. Fund.*, 21(2), 141-153.

Coates, J. D., and Achenbach, L. A. (2004). "Microbial Perchlorate Reduction: Rocket-Fuelled Metabolism." *Nat. Rev. Micro.*, 2(7), 569-580.

Coates, J. D., and Jackson, W. A. (2009). "Principle of perchlorate treatment." *In Situ Bioremediation of Perchlorate in Groundwater*, H. F. Stroo, and C. H. Ward, eds., Springer, New York, USA, 29-52.

Coates, J. D., Chakraborty, R., Lack, J. G., O'Connor, S. M., Cole, K. A., Bender, K. S., and Achenbach, L. A. (2001). "Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*." *Nature*, 411(6841), 1039-1043.

Coates, J. D., Michaelidou, U., Bruce, R. A., O'Connor, S. M., Crespi, J. N., and Achenbach, L. A. (1999). "Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria." *Appl. Environ. Microb.*, 65(12), 5234-5241.

Coppola, E. N. (2000). "Optimization of perchlorate biodegradaion: performance and cost assessment for the Thiokol prototype (final report)." Applied Research Associates, Inc., Albuquerque, NM, USA.

Cox, E., Krug, T., and Bertrand, D. (2009). "Comparative Demonstration of Active and Semi-Passive In Situ Bioremediation Approaches for Perchlorate-Impacted

Groundwater (Longhorn Army Ammunition Plant)." *Rep. No. ESTCP Project ER-0219,* Geosyntec Consultants, Atlanta, GA, USA.

Cox, E. E. (2009). "Development of *in situ* bioremediation technologies for perchlorate." *In Situ Bioremediation of Perchlorate in Groundwater,* H. F. Stroo, and C. H. Ward, eds., Springer New York, USA, 15-27.

Cramer, R. J., Yates, C., Hatzinger, P., and Shaw, J. D. (2004). "Field Demonstration of In Situ Perchlorate Bioremediation at Building 1419." *Rep. No. NOSSA-TR-2004-001,* Naval Surface Warfare Center, Indian Head DIV, MD, USA.

Crowell, W. R., Yost, D. M., and Roberts, J. D. (1940). "The catalytic effect of osmium compounds on the reduction of perchloric acid by hydrobromic acid." *J. Am. Chem. Soc.,* 62(8), 2176-2178.

De Long, S. K., Kinney, K. A., and Kirisits, M. J. (2010). "qPCR assays to quantify genes and gene expression associated with microbial perchlorate reduction." *J. Microbiol. Methods,* 83(2), 270-274.

Doronina, N., Trotsenko, Y., and Tourova, T. (2000). "*Methylarcula marina* gen. nov., sp. nov. and *Methylarcula terricola* sp. nov.: novel aerobic, moderately halophilic, facultatively methylotrophic bacteria from coastal saline environments." *Int. J. Syst. Evol. Microbiol.,* 50(5), 1849-1859.

Dudley, M., Salamone, A., and Nerenberg, R. (2008). "Kinetics of a chlorate-accumulating, perchlorate-reducing bacterium." *Water Res.,* 42(10-11), 2403-2410.

Dugan, N. R., Williams, D. J., Meyer, M., Schneider, R. R., Speth, T. F., and Metz, D. H. (2009). "The impact of temperature on the performance of anaerobic biological treatment of perchlorate in drinking water." *Water Res.,* 43(7), 1867-1878.

Egea, J., Rodr ́guez-Fern ́andez, M., Banga, J., and Mart ́ı, R. (2007). "Scatter search for chemical and bio-process optimization." *J. Global Optim.* 37(3), 481-503.

Ellington, J. J., Wolfe, N. L., Garrison, A. W., Evans, J. J., Avants, J. K., and Teng, Q. (2001). "Determination of perchlorate in tobacco plants and tobacco products." *Environ. Sci. Technol.,* 35(15), 3213-3218.

Ericksen, G. E. (1983). "The Chilean nitrate deposits." *Am. Sci.,* 71(4), 366-374.

Etchebehere, C., Cabezas, A., Dabert, P., and Muxi, L. (2003). "Evolution of the bacterial community during granules formation in denitrifying reactors followed by molecular, culture-independent techniques." *Water Sci. Tech.,* 48(6), 75-79.

Evans, J. B., and Kloos, W. E. (1972). "Use of shake cultures in a semisolid thioglycolate medium for differentiating Staphylococci from Micrococci." *Appl. Microbiol.,* 23(2), 326-331.

Evans, P., Chu, A., Liao, S., Price, S., Moody, M., Headric, D., Min, B., and Logan, B. (2002). "Pilot testing of bioreactor for perchlorate-contaminated groundwater treatment." *Remediation of Chlorinated and Recalcitrant Compounds—Proceedings of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds (Monterey, CA; May 2002),* A. R. Gavaskar, and A. S. C. Chen, eds., Battelle Press, Columbus, OH, USA.

Evans, P. J., Lo, I., Moore, A. E., Weaver, W. J., Grove, W. F., and Amini, H. (2008). "Rapid full-scale bioremediation of perchlorate in soil at a large brownfields site." *Remediation,* 18(2), 9-25.

Evans, P. J., and Trute, M. M. (2006). "In situ bioremediation of nitrate and perchlorate in vadose zone soil for groundwater protection using gaseous electron donor injection technology." *Water Environ. Res.,* 78 2436-2446.

Farhan, Y. H., and Hatzinger, P. B. (2009). "Modeling the biodegradation kinetics of perchlorate in the presence of oxygen and nitrate as competing electron acceptors." *Bioremediation J.,* 13(2), 65.

Frankenberger, W. (2003). "Perchlorate removal in groundwater by perchlorate reductases from the perchlorate respiring bacterium, perc1ace." *Rep. No. WRC Project W-950,* University of California Water Resources Center, CA, USA.

Gal, H., Ronen, Z., Weisbrod, N., Dahan, O., and Nativ, R. (2008). "Perchlorate biodegradation in contaminated soils and the deep unsaturated zone." *Soil Biol. Biochem.,* 40(7), 1751-1757.

Gauthier, M. J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P., and Bertrand, J. (1992). "*Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium." *Int. J. Syst. Bacteriol.,* 42(4), 568-576.

Ghosh, A., Pakshirajan, K., Ghosh, P. K., and Sahoo, N. K. (2011). "Perchlorate degradation using an indigenous microbial consortium predominantly *Burkholderia* sp." *J. Hazard. Mater.,* 187(1-3), 133-139.

Giblin T., and Frankenberger W.T. (2001). "Perchlorate and nitrate reductase activity in the perchlorate-respiring bacterium perc1ace." *Microbiol. Res.,* 156, 311-315.

Gingras, T. M., and Batista, J. R. (2002). "Biological reduction of perchlorate in ion exchange regenerant solutions containing high salinity and ammonium levels." *J. Environ. Monitor.,* 4, 96-101.

Gorshkova, N. M., Ivanova, E. P., Sergeev, A. F., Zhukova, N. V., Alexeeva, Y., Wright, J. P., Nicolau, D. V., Mikhailov, V. V., and Christen, R. (2003). "*Marinobacter excellens* sp. nov., isolated from sediments of the Sea of Japan." *Int. J. Syst. Evol. Microbiol.,* 53(6), 2073-2078.

Gouy, M., Guindon, S., and Gascuel, O. (2010). "SeaView version 4: a multiplatform fraphical user interface for sequence alignment and phylogenetic tree building." *Mol. Biol. Evol.,* 27(2), 221-224.

Gu, B., Bohlke, J. K., Sturchio, N. C., Hatzinger, P. B., Jackson, A., Beloso, A. D. J., Heraty, L., Bian, Y., Jiang, X., and Brown, G. M. (2011). "Applications of selective ion exchange for perchlorate removal, recovery and environmental forensics." *Ion Exchange and Solvent Extraction: A Series of Advances, Vol. 20.* A. K. SenGupta, eds. CRC Press, USA, 117-144.

Gu, B., Brown, G. M., and Chiang, C. (2007). "Treatment of perchlorate-contaminated groundwater using highly selective, regenerable ion-exchange technologies." *Environ. Sci. Technol.,* 41(17), 6277-6282.

Gu, B., Brown, G. M., Maya, L., Lance, M. J., and Moyer, B. A. (2001). "Regeneration of perchlorate (ClO_4^-)-loaded anion exchange resins by a novel tetrachloroferrate ($FeCl_4^-$) displacement technique." *Environ. Sci. Technol.,* 35(16), 3363-3368.

Gu, B., Ku, Y., and Brown, G. M. (2003). "Treatment of perchlorate-contaminated groundwater using highly selective, regenerable ion-exchange technology: a pilot-scale demonstration." *Fedl. Fac. Environ. J.,* 14(1), 75-94.

Gu, B., Ku, Y., and Brown, G. M. (2005). "Sorption and desorption of perchlorate and U(VI) by strong-base anion-exchange resins." *Environ. Sci. Technol.,* 39(3), 901-907.

Gu, J., Cai, H., Yu, S., Qu, R., Yin, B., Guo, Y., Zhao, J., and Wu, X. (2007). "*Marinobacter gudaonensis* sp. nov., isolated from an oil-polluted saline soil in a Chinese oilfield." *Int. J. Syst. Evol. Microbiol.,* 57(2), 250-254.

Guindon, S., and Gascuel, O. (2003). "A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood." *Syst. Biol.,* 52(5), 696-704.

Hackenthal, E. (1965). "Die reduktion von perchlorat durch bakterien—II: Die identität der nitratreduktase und des perchlorat reduzierenden enzyms aus *B. cereus*." *Biochem.Pharmacol.,* 14(9), 1313-1318, IN7-IN8, 1319-1324.

Hackenthal, E., Mannheim, W., Hackenthal, R., and Becher, R. (1964). "Die reduktion von perchlqrat durch bakterien. *I. Untersuchungen* an intakten zellen." *Biochem. Pharmacol.,* 13(12), 195-206.

Haight, G. P., and Sager, W. F. (1952). "Evidence for preferential one-step divalent changes in the molybdate-catalyzed reduction of perchlorate by stannous ion in sulfuric acid solution." *J. Am. Chem. Soc.,* 74(23), 6056-6059.

Hall, T. A. (1999). "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT." *Nucl. Acid. S.,* 41 95-98.

Hamamura, N., Olson, S. H., Ward, D. M., and Inskeep, W. P. (2006). "Microbial population dynamics associated with crude-oil biodegradation in diverse soils." *Appl. Environ. Microbiol.,* 72(9), 6316-6324.

Hames, B. D., and Higgins, S. J. (1995). *Gene Probes 1: A Practical Approach.* Oxford University Press, USA.

Handley, K. M., Hery, M., and Lloyd, J. R. (2009). "*Marinobacter santoriniensis* sp. nov., an arsenate-respiring and arsenite-oxidizing bacterium isolated from hydrothermal sediment." *Int. J. Syst. Evol. Microbiol.,* 59(4), 886-892.

Hatzinger, P. B., Bahlke, J. K., Sturchio, N. C., Gu, B., Heraty, L. J., and Borden, R. C. (2009). "Fractionation of stable isotopes in perchlorate and nitrate during in situ biodegradation in a sandy aquifer." *Environ. Chem.,* 6(1), 44-52.

Hatzinger, P. B., Diebold, J., Yates, C. A., and Cramer, R. J. (2006). "Field demonstration of *in situ* perchlorate bioremediation in groundwater." *Perchlorate: Environmental Occurrence, Interactions and Treatment,* B. Gu and J. D. Coates, eds., Springer, USA, 311-341.

Health Canada. (2008). "*Perchlorate and Human Health*." http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/perchlorate-eng.php (02/16, 2009).

Herman, D. C., and Frankenberger, W. T. (1999). "Bacterial reduction of perchlorate and nitrate in water." *J. Environ. Qual.*, 28(3), 1018-1024.

Hiremath, T., Roberts, D. J., Lin, X., Clifford, D. A., Gillogly, T., and Lehman, G. (2006). "Biological treatment of perchlorate in spent ISEP ion-exchange brine." *Environ. Eng. Sci.*, 23(6), 1009-1016.

Hoover, D. G., and Gray, R. J. H. (1977). "Function of cell wall teichoic acid in thermally injured *Staphylococcus aureus*." *J. Bacteriol.*, 131(2), 477-485.

Hungate, R. E. (1950). "The anaerobic mesophilic cellulolytic bacteria." *Microbiol. Mol. Biol. Rev.*, 14(1), 1-49.

Hunter, W. J. (2002). "Bioremediation of chlorate or perchlorate contaminated water using permeable barriers containing vegetable oil." *Curr. Microbiol.*, 45(4), 287-292.

Hurley, K. D., and Shapley, J. R. (2007). "Efficient heterogeneous catalytic reduction of perchlorate in water." *Environ. Sci. Technol.*, 41(6), 2044-2049.

Hurst, A., Hughes, A., Collins-Thompson, D. L., and Shah, B. G. (1974). "Relationship between loss of magnesium and loss of salt tolerance after sublethal heating of *Staphylococcus aureus*." *Can. J. Microbiol.*, 20(8), 1153-1158.

Huu, N. B., Denner, E. B. M., Ha, D. T. C., Wanner, G., and Stan-Lotter, H. (1999). "*Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well." *Int. J. Syst. Bacteriol.*, 49(2), 367-375.

ITRC. (2008). "*Remediation Technologies for Perchlorate Contamination in Water and Soil*." Interstate Technology & Regulatory Council, Perchlorate Team., Washington, DC, USA.

ITRC. (2005). "*Perchlorate: Overview of Issues, Status, and Remedial Options*." Interstate Technology & Regulatory Council, Perchlorate Team, Washington, DC, USA.

Ju, X., Sierra-Alvarez, R., Field, J. A., Byrnes, D. J., Bentley, H., and Bentley, R. (2008). "Microbial perchlorate reduction with elemental sulfur and other inorganic electron donors." *Chemosphere*, 71(1), 114-122.

Juteau, P., Larocque, R., Rho, D., and LeDuy, A. (1999). "Analysis of the relative abundance of different types of bacteria capable of toluene degradation in a compost biofilter." *Appl. Microbiol. Biotechnol.,* 52(6), 863-868.

Kashyap, V. (2006). "Biological regeneration of perchlorate loaded strong base anion exchange resins." M.Sc. thesis, University of Houston, Houston, TX.

Kengen, S. W. M., Rikken, G. B., Hagen, W. R., Ginkel, C. G. v., and Stams, A. J. M. (1999). "Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain GR-1." *J. Bacteriol.,* 181(21), 6706-6711.

Kesterson, K. E., Amy, P. S., and Batista, J. R. (2005). "Limitations to natural bioremediation of perchlorate in a contaminated Site." *Bioremediation J.,* 9(3), 129.

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

Kolthoff, I. M., and Hodara, I. (1963). "Effect of tungsten(VI) on molybdenum(VI)-catalyzed reduction waves of chlorate, nitrate and perchlorate." *J. Electroanal. Chem.,* 5(3), 165-170.

Korenkov, V. N., Romanenko, V. I., Kuznetsov, S. I., and Voronov, J. V. (1976). "Process for purification of industrial waste waters from perchlorates and chlorates." US Patent (3943055).

Kounaves, S. P., Stroble, S. T., Anderson, R. M., Moore, Q., Catling, D. C., Douglas, S., McKay, C. P., Ming, D. W., Smith, P. H., Tamppari, L. K., and Zent, A. P. (2010). "Discovery of natural perchlorate in the Antarctic dry valleys and its global implications." *Environ. Sci. Technol.,* 44(7), 2360-2364.

Krauter, P. W. (2001). "Using a wetland bioreactor to remediate ground water contaminated with nitrate (mg/L) and perchlorate (µg/L)." *Int. J. Phytoremediation,* 3(4), 415.

Krauter, P., Daily Jr., B., Dibley, V., Pinkart, H., and Legler, T. (2005). "Perchlorate and nitrate remediation efficiency and microbial diversity in a containerized wetland bioreactor." *Int. J. Phytoremediation,* 7(2), 113-128.

Kuever, J., Rainey, F. A., and Widdel, F. (2005). "Genus III. *Desulfothermus* gen. nov." *Bergey's Manual of Systematic Bacteriology, second edition, vol. 2 (The Proteobacteria), part C (The Alpha-, Beta-, Delta-, and Epsilonproteobacteria),* D. J.

Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity, eds., Springer, New York, USA, 955-956.

Kushner, D. J. (1985). "The Halobacteriaceae." *The bacteria.* C. R. Woese, and R. S. Wolfe., eds., Academic Press, New York, USA, 171-214.

L áng, G. G., and Hor ányi, G. (2003). "Some interesting aspects of the catalytic and electrocatalytic reduction of perchlorate ions." *J. Electroanal. Chem.*, 552 197-211.

Lehman, S. G., Badruzzaman, M., Adham, S., Roberts, D. J., and Clifford, D. A. (2008). "Perchlorate and nitrate treatment by ion exchange integrated with biological brine treatment." *Water Res.*, 42(4-5), 969-976.

Liebgott, P., Casalot, L., and Labat, M. (2005). " *Marinobacter bacchus* sp. nov., a novel bacterium isolated from an evaporation pond of wine wastewater." (unplished). http://www.ncbi.nlm.nih.gov/nuccore/DQ282120.1 (09/10, 2011)

Liebgott, P., Casalot, L., Paillard, S., Lorquin, J., and Labat, M. (2006). "*Marinobacter vinifirmus* sp. nov., a moderately halophilic bacterium isolated from a wine-barrel-decalcification wastewater." *Int. J. Syst. Evol. Microbiol.*, 56(11), 2511-2516.

Lin, X., Roberts, D. J., Hiremath, T., Clifford, D. A., Gillogly, T., and Lehman, G. (2007). "Divalent cation addition (Ca^{2+}or Mg^{2+}) stabilizes biological treatment of perchlorate and nitrate in ion-exchange spent brine." *Environ. Eng. Sci.*, 24 725-735.

Lin, X. (2004). "Development of Cultures Capable of Perchlorate Degradation in Ion-Exchange Spent Brine." PhD thesis, University of Houston, Houston, TX.

Lin, Y., Cui, X., and Bontha, J. (2006). "Electrically controlled anion exchange based on polypyrrole and carbon nanotubes nanocomposite for perchlorate removal." *Environ. Sci. Technol.*, 40(12), 4004-4009.

Liu, B., Zhang, F., Feng, X., Liu, Y., Yan, X., Zhang, X., Wang, L., and Zhao, L. (2006). "*Thauera* and *Azoarcus* as functionally important genera in a denitrifying quinoline removal bioreactor as revealed by microbial community structure comparison." *FEMS Microbiol. Ecol.*, 5 274-286.

Logan, B. E., and LaPoint, D. (2002). "Treatment of perchlorate- and nitrate-contaminated groundwater in an autotrophic, gas phase, packed-bed bioreactor." *Water Res.*, 36(14), 3647-3653.

Logan, B. E., Wu, J., and Unz, R. F. (2001a). "Biological perchlorate reduction in high-salinity solutions." *Water Res.*, 35(12), 3034-3038.

Logan, B. E., Zhang, H., Mulvaney, P., Milner, M. G., Head, I. M., and Unz, R. F. (2001b). "Kinetics of perchlorate- and chlorate-respiring bacteria." *Appl. Environ. Microb.*, 67(6), 2499-2506.

Losi, M. E., Giblin, T., Hosangadi, V., and Frankenberger, W. T. (2002). "Bioremediation of perchlorate-contaminated groundwater using a packed bed biological reactor." *Bioremediation J.*, 6(2), 97.

Loy, A., Horn, M., andWagner, M. (2003) "ProbeBase - an online resource for rRNA-targeted oligonucleotide probes". *Nucleic Acids Res.*, 31, 514-516.

Loy, A., Maixner, F., Wagner, M., and Horn, M. (2007) "ProbeBase - an online resource for rRNA-targeted oligonucleotide probes: new features". *Nucleic Acids Res.*, 35, D800-D804.

Mack, E., Mandelco, L., Woese, C., and Madigan, M. (1993). "*Rhodospirillum sodomense*, sp. nov., a Dead Sea *Rhodospirillum* species." *Arch. Microbiol.*, 160(5), 363-371.

Mahmoud, K. K., Leduc, L. G., and Ferroni, G. D. (2005). "Detection of *Acidithiobacillus ferrooxidans* in acid mine drainage environments using fluorescent in situ hybridization (FISH)." *J. Microbiol. Methods*, 61(1), 33-45.

Mahmudov, R., and Huang, C. P. (2010). "Perchlorate removal by activated carbon adsorption." *Sep.Purif. Technol.*, 70(3), 329-337.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., and Wade, W. G. (1998). "Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA." *Appl. Environ. Microbiol.*, 64(2), 795-799.

Martin, S., Marquez, M. C., Sanchez-Porro, C., Mellado, E., Arahal, D. R., and Ventosa, A. (2003). "*Marinobacter lipolyticus* sp. nov., a novel moderate halophile with lipolytic activity." *Int. J. Syst. Evol. Microbiol.*, 53(5), 1383-1387.

Matos, C. T., Velizarov, S., Crespo, J. G., and Reis, M. A. M. (2006). "Simultaneous removal of perchlorate and nitrate from drinking water using the ion exchange membrane bioreactor concept." *Water Res.*, 40(2), 231-240.

McCarty, P. L., and Meyer, T. E. (2005). "Numerical model for biological fluidized-bed reactor treatment of perchlorate-contaminated groundwater." *Environ. Sci. Technol.,* 39(3), 850-858.

Michaelidou, U., Achenbach, L. A., and Coates, J. D. (2000). "Isolation and characterization of two novel (per)chlorate-reducing bacteria from swine waste lagoons." *Perchlorate in the Environment,* E. T. Urbansky, ed., Kluwer Academic/Plenum Publishers, New York, USA, Chapter 25.

Miller, J. P., and Logan, B. E. (2000). "Sustained perchlorate degradation in an autotrophic, gas-phase, packed-bed bioreactor." *Environ. Sci. Technol.,* 34(14), 3018-3022.

Montgomery, D. C. (2009). *Design and Analysis of Experiments.* John Wiley & SOns, Inc, USA.

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

Na, C., Cannon, F. S., and Hagerup, B. (2002). "Perchlorate removal via iron-preloaded GAC and borohydride regeneration." *Journal AWWA,* 94(11), 90-102.

Nerenberg, R., Kawagoshi, Y., and Rittmann, B. E. (2006). "Kinetics of a hydrogen-oxidizing, perchlorate-reducing bacterium." *Water Res.,* 40(17), 3290-3296.

Nerenberg, R., Rittman, B. E., Gillogly, T. E., Lehman, G. E., and Adham, S. S. (2004). "Perchlorate reduction using a hollow-fiber membrane biofilm reactor: kinetics, microbial ecology, and pilot-scale studies." *In Situ and On-Site Bioremediation—2003. Proceedings of the Seventh International In Situ and On-Site Bioremediation Symposium (Orlando, FL; June 2003).* V. S. Magar, and M. E. Kelley, eds., Battelle Press, Colombus, C08.

Nerenberg, R., Kawagoshi, Y., and Rittmann, B. E. (2008). "Microbial ecology of a perchlorate-reducing, hydrogen-based membrane biofilm reactor." *Water Res.,* 42(4-5), 1151-1159.

Nor, S. J., Lee, S. H., Cho, K., Cha, D. K., Lee, K. I., and Ryu, H. W. (2011). "Microbial treatment of high-strength perchlorate wastewater." *Bioresour. Technol.,* 102(2), 835-841.

Nozawa-Inoue, M., Jien, M., Yang, K., Rolston, D. E., Hristova, K. R., and Scow, K. M. (2011). "Effect of nitrate, acetate, and hydrogen on native perchlorate-reducing

microbial communities and their activity in vadose soil." *FEMS Microbiol.Ecol.*, 76(2), 278-288.

Nzengung, V. A., Lieberman, M. T., Stroo, H. F., and Evans, P. J. (2009). "Emerging technologies for perchlorate bioremediation." *In Situ Bioremediation of Perchlorate in Groundwater*, H. F. Stroo, and C. H. Ward, eds., Springer New York, USA, 219-243.

Nzengung, V. A., Wang, C., and Harvey, G. (1999). "Plant-mediated transformation of perchlorate into chloride." *Environ. Sci. Technol.*, 33(9), 1470-1478.

Okeke, B. C., and Frankenberger, J.,William T. (2003). "Molecular analysis of a perchlorate reductase from a perchlorate-respiring bacterium Perc1ace." *Microbiol. Res.*, 158(4), 337-344.

Okeke, B. C., Giblin, T., and Frankenberger, W. T.,Jr. (2002). "Reduction of perchlorate and nitrate by salt tolerant bacteria." *Environ. Pollut.*, 118(3), 357-363.

Oltmann, L. F., Reijnders, W. N. M., and Stouthamer, A. H. (1976). "The correlation between the protein composition of cytoplasmic membranes and the formation of nitrate reductase A, chlorate reductase C and tetrathionate reductase in *Proteus mirabilis* wild type and some chlorate resistant mutants." *Arch. Microbiol.*, 111(1), 37-43.

Oren, A. (1983). "*Halobacterium sodomense* sp. nov., a Dead Sea *Halobacterium* with an extremely high magnesium requirement." *Int. J. Syst. Bacteriol.*, 33(2), 381-386.

Padhye, L., Rainwater, K., Jackson, W. A., and Morse, A. (2007). "Kinetics for a membrane reactor reducing perchlorate." *Water Environ. Res.*, 79 140-146.

Parette, R., and Cannon, F. S. (2005). "The removal of perchlorate from groundwater by activated carbon tailored with cationic surfactants." *Water Res.*, 39(16), 4020-4028.

Park, C., and Marchand E.A. (2006). "Modelling salinity inhibition effects during biodegradation of perchlorate." *J. Appl. Microbiol.*, 101(1), 222-233.

Parker, D. R., Seyfferth, A. L., and Reese, B. K. (2008). "Perchlorate in groundwater: a synoptic survey of 'Pristine' Sites in the coterminous united states." *Environ. Sci. Technol.*, 42(5), 1465-1471.

Patel, A., Zuo, G., Lehman, S. G., Badruzzaman, M., Clifford, D. A., and Roberts, D. J. (2008). "Fluidized bed reactor for the biological treatment of ion-exchange brine containing perchlorate and nitrate." *Water Res.*, 42(16), 4291-4298.

Patterson, J. (2009). *Evaluation of tailored granular activated carbon to remove perchlorate in the presence of oxyanions.* PhD thesis, The Pennsylvania State University, USA.

Pernthaler, A., Pernthaler, J., and Amann, R. (2002). "Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria." *Appl. Environ. Microbiol.*, 68(6), 3094-3101.

Plummer, L. N., Bohlke, J. K., and Doughten, M. W. (2006). "Perchlorate in pleistocene and holocene groundwater in North-Central New Mexico." *Environ. Sci. Technol.*, 40(6), 1757-1763.

Polk, J., Onewokae, C., Guarini, W. J., Murray, C., Tolbert, D. E., and Togna, A. P. (2002). "Army success story: ex-situ biological treatment of perchlorate-contaminated groundwater." *Fedl. Fac. Environ. J.*, 13(2), 85-94.

Poust, S. K. (2010). "Engineered vesicles for perchlorate degradation." PhD thesis, University of Illinois at Urbana-Champaign, .USA.

Rajagopalan, S., Anderson, T. A., Fahlquist, L., Rainwater, K. A., Ridley, M., and Jackson, W. A. (2006). "Widespread presence of naturally occurring perchlorate in high plains of Texas and New Mexico." *Environ. Sci. Technol.*, 40(10), 3156-3162.

Rajagopalan, S., Anderson, T., Cox, S., Harvey, G., Cheng, Q., and Jackson, W. A. (2009). "Perchlorate in wet deposition across North America." *Environ. Sci. Technol.*, 43(3), 616-622.

Rao, B., Anderson, T. A., Orris, G. J., Rainwater, K. A., Rajagopalan, S., Sandvig, R. M., Scanlon, B. R., Stonestrom, D. A., Walvoord, M. A., and Jackson, W. A. (2007). "Widespread natural perchlorate in unsaturated zones of the Southwest United States." *Environ. Sci. Technol.*, 41(13), 4522-4528.

Rechnitz, G. A., and Laitinen, H. A. (1961). "A study of the molybdenum catalyzed reduction of perchlorate." *Anal. Chem.*, 33(11), 1473-1477.

Rikken, G. B., Kroon, A. G. M., and Ginkel, C. G. v. (1996). "Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation." *Appl. Microbiol. Biotechnol.*, 45 420-426.

Rodriguez-Fernandez, M., Egea, J., and Banga, J. (2006). "Novel metaheuristic for parameter estimation in nonlinear dynamic biological systems." *BMC Bioinformatics,* 7(1), 483.

Roldan, M. D., Reyes, F., Moreno-Vivian, C., and Castillo, F. (1994). "Chlorate and nitrate reduction in the phototrophic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*." *Curr. Microbiol.,* 29(4), 241-245.

Romanenko, V., Korenkov, V., and Kuznetsov, S. (1976). "Bacterial decomposition of ammonium perchlorate. " *Mikrobiologiya,* 45 204-209.

Romanenko, L. A., Schumann, P., Rohde, M., Zhukova, N. V., Mikhailov, V. V., and Stackebrandt, E. (2005). "*Marinobacter bryozoorum* sp. nov. and *Marinobacter sediminum* sp. nov., novel bacteria from the marine environment." *Int. J. Syst. Evol. Microbiol.,* 55(1), 143-148.

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

Sahu, A. K., Conneely, T., NuÌˆsslein, K. R., and Ergas, S. J. (2009b). "Biological perchlorate reduction in packed bed reactors using elemental sulfur." *Environ. Sci. Technol.,* 43(12), 4466-4471.

Schäfer, H., and Muyzer, G. (2001). "Denaturing gradient gel electrophoresis in marine microbial ecology." *Methods in Microbiology,* John H Paul, ed., Academic Press, 425-468.

Schnoor, J. L., Parkin, G. F., Just, C. L., and van Aken, B. (2002). "Phytoremediation and bioremediation of perchlorate at the Longhorn Army Ammunition Plant." The University of Iowa, US.

Shafea, A. A. (2003). "Growth and nitrate reductase activity of *Chlorella fusca* cells as affected by long term salinity." *Biologia Plantarum,* 46(3), 423-427.

Shete, A., Mukhopadhyaya, P., Acharya, A., Aich, B., Joshi, S., and Ghole, V. (2008). "Aerobic reduction of perchlorate by bacteria isolated in Kerala, South India." *J. Appl. Genet.,* 49(4), 425-431.

Shieh, W. Y., Jean, W. D., Lin, Y., and Tseng, M. (2003). "*Marinobacter lutaoensis* sp. nov., a thermotolerant marine bacterium isolated from a coastal hot spring in Lutao, Taiwan." *Can. J. Microbiol.,* 49 244-252.

Shrout, J. D., and Parkin, G. F. (2006). "Influence of electron donor, oxygen, and redox potential on bacterial perchlorate degradation." *Water Res.,* 40 1191-1199.

Shrout, J. D., Struckhoff, G. C., Parkin, G. F., and Schnoor, J. L. (2006). "Stimulation and molecular characterization of bacterial perchlorate degradation by plant-produced electron donors." *Environ. Sci. Technol.,* 40(1), 310-317.

Shrout, J. D., Scheetz, T. E., Casavant, T. L., and Parkin, G. F. (2005). "Isolation and characterization of autotrophic, hydrogen-utilizing, perchlorate-reducing bacteria." *Appl.Microbiol.Biotechnol.,* 67(2), 261-268.

Smith, P. N. (2006). "The ecotoxicology of perchlorate in the environment." *Perchlorate: Environmental Occurrence, Interactions and Treatment,* B. Gu, and J. D. Coates, eds., Springer US, 153-168.

Son, A., Lee, J., Chiu, P. C., Kim, B. J., and Cha, D. K. (2006). "Microbial reduction of perchlorate with zero-valent iron." *Water Res.,* 40(10), 2027-2032.

Son, A., Schmidt, C. J., Shin, H., and Cha, D. K. (2011). "Microbial community analysis of perchlorate-reducing cultures growing on zero-valent iron." *J. Hazard. Mater.,* 185(2-3), 669-676.

Song, B., Palleroni, N., Kerkhof, L., and Haggblom, M. (2001). "Characterization of halobenzoate-degrading, denitrifying *Azoarcus* and *Thauera* isolates and description of *Thauera chlorobenzoica* sp. nov." *Int. J. Syst. Evol. Microbiol.*, 51(2), 589-602.

Springer, N., Ludwig, W., Philipp, B., and Schink, B. (1998). "*Azoarcus anaerobius* sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium." *Int. J. Syst. Bacteriol.,* 48(3), 953-956.

Stenklo, K., Thorell, H. D., Bergius, H., Aasa, R., and Nilsson, T. (2001). "Chlorite dismutase from *Ideonella dechloratans*." *J.Biol.Inorg.Chem.,* 6(5-6), 601-607.

Stroo, H. F., and Norris, R. D. (2009). "Alternatives for *in situ* bioremediation of perchlorate." *In Situ Bioremediation of Perchlorate in Groundwater,* H. F. Stroo, and C. H. Ward, eds., Springer New York, USA, 79-90.

Sturchio, N. C., Bahlke, J. K., Beloso, , Abelardo D., Streger, S. H., Heraty, L. J., and Hatzinger, P. B. (2007). "Oxygen and chlorine isotopicfractionation during perchlorate biodegradation: laboratory results and implications for forensics and natural attenuation studies." *Environ. Sci. Technol.,* 41(8), 2796-2802.

Sturchio, N. C., Hatzinger, P. B., Arkins, M. D., Suh, C., and Heraty, L. J. (2003). "Chlorine isotope fractionation during microbial reduction of perchlorate." *Environ. Sci. Technol.*, 37(17), 3859-3863.

Sturchio, N. C., Hoaglund, J. R., Marroquin, R. J., Beloso, A. D., Heraty, L. J., Bortz, S. E., and Patterson, T. L. (2011). "Isotopic mapping of groundwater perchlorate plumes." *Ground Water*, In press.

Sun, Y., Gustavson, R., Ali, N., Weber, K., Westphal, L., and Coates, J. (2009). "Behavioral response of dissimilatory perchlorate-reducing bacteria to different electron acceptors." *Appl.Microbiol.Biotechnol.*, 84(5), 955-963.

Takai, K., Moyer, C. L., Miyazaki, M., Nogi, Y., Hirayama, H., Nealson, K. H., and Horikoshi, K. (2005). "*Marinobacter alkaliphilus* sp. nov., a novel alkaliphilic bacterium isolated from subseafloor alkaline serpentine mud from Ocean Drilling Program Site 1200 at South Chamorro Seamount, Mariana Forearc." *Extremophiles*, 9(1), 17-27.

Tan, K., Anderson, T. A., Jones, M. W., Smith, P. N., and Jackson, W. A. (2004). "Accumulation of perchlorate in aquatic and terrestrial plants at a field scale." *J. Environ. Qual.*, 33(5), 1638-1646.

Tan, K., Anderson, T. A., and Jackson, W. A. (2005). "Temporal and spatial variation of perchlorate in streambed sediments: results from in-situ dialysis samplers." *Environ. Pollut.*, 136(2), 283-291.

Tan, K., Jackson, W. A., Anderson, T. A., and Pardue, J. H. (2004). "Fate of perchlorate-contaminated water in upflow wetlands." *Water Res.*, 38(19), 4173-4185.

Tang, Y. Z., Gin, K. Y. H., and Lim, T. H. (2005). "High-temperature fluorescent in situ hybridization fordetecting *Escherichia coli* in seawater samples, using rRNA-targeted oligonucleotide probes and flow cytometry." *Appl. Environ. Microbiol.*, 71(12), 8157-8164.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Nucleic Acids Res.*, 22(22), 4673-4680.

Thorell, H. D., Stenklo, K., Karlsson, J., and Nilsson, T. (2003). "A gene cluster for chlorate metabolism in *Ideonella dechloratans*." *Appl. Environ. Microb.*, 69(9), 5585-5592.

Thrash, J. C., Van Trump, J. I., Weber, K. A., Miller, E., Achenbach, L. A., and Coates, J. D. (2007). "Electrochemical stimulation of microbial perchlorate reduction." *Environ. Sci. Technol.*, 41(5), 1740-1746.

Thrash, J. C., Ahmadi, S., Torok, T., and Coates, J. D. (2010a). " *Magnetospirillum bellicus* sp. nov., a novel dissimilatory perchlorate-reducing Alphaproteobacterium isolated from a bioelectrical reactor." *Appl. Environ. Microbiol.*, 76(14), 4730-4737.

Thrash, J. C., Pollock, J., Torok, T., and Coates, J. (2010b). "Description of the novel perchlorate-reducing bacteria *Dechlorobacter hydrogenophilus* gen. nov., sp. nov. and *Propionivibrio militaris*, sp. nov." *Appl. Microbiol. Biotechnol.*, 86(1), 335-343.

Tripp, A. R., and Clifford, D. (2004). "Selectivity considerations in modeling the treatment of perchlorate using ion-exchange processes." *Ion Exchange and Solvent Extraction. A Series of Advances, Volume 16,* A. K. SenGupta, J. A. Marinsky, and Y. Marcus, eds., CRC Press, New York, USA.

Trumpolt, C. W., Crain, M., Cullison, G. D., Flanagan, S. J. P., Siegel, L., and Lathrop, S. (2005). "Perchlorate: sources, uses, and occurrences in the environment." *Remediation,* 16(1), 65-89.

Urbansky, E. T., and Brown, S. K. (2003). "Perchlorate retention and mobility in soils." *J. Environ. Monit.*, 5(3), 455-462.

Urbansky, E. T. (1998). "Perchlorate chemistry:implications for analysis and remediation." *Bioremediation J.*, 2(2), 81-95.

Urbansky, E. T. (2002). "Perchlorate as an Environmental Contaminant." *Environ. Sci. Pollut. R.*, 9(3), 187-192.

USEPA. (1998). "*Drinking water contaminant candidate list*." *Rep. No. EPA/600/F-98/002,* United States Environmental Protection Agency, Washington DC.

USEPA. (2005). "*Perchlorate treatment technology update*." *Rep. No. EPA 542-R-05-015,* United States Environmental Protection Agency, Washington DC.

USEPA. (2008). "Interim drinking water health advisory for perchlorate." *Rep. No. EPA 822-R-08-025,* United States Environmental Protection Agency, Washington DC.

USEPA. (2011). "Drinking water: regulatory determination on perchlorate." *Federal Register*, 76(29), 7762-7767.

USFDA. (2007). "2004-2005 Exploratory Survey Data on Perchlorate in Food." http://www.cfsan.fda.gov/~dms/clo4data.html (02/16, 2009).

van Ginkel, C. G., Rikken, G. B., Kroon, A. G. M., and Kengen, S. W. M. (1996). "Purification and characterization of chlorite dismutase: a novel oxygen-generating enzyme." *Arch. Microbiol.*, 166(5), 321-326.

van Ginkel, S. W., Lamendella, R., Kovacik Jr., W. P., Santo Domingo, J. W., and Rittmann, B. E. (2010). "Microbial community structure during nitrate and perchlorate reduction in ion-exchange brine using the hydrogen-based membrane biofilm reactor (MBfR)." *Bioresour. Technol.*, 101(10), 3747-3750.

Van Trump, J. I., and Coates, J. D. (2009). "Thermodynamic targeting of microbial perchlorate reduction by selective electron donors." *ISME J*, 3(4), 466-476.

Vigliotta, G., Motta, O., Guarino, F., Iannece, P., and Proto, A. (2010). "Assessment of perchlorate-reducing bacteria in a highly polluted river." *Int. J. Hyg. Environ. Health*, 213(6), 437-443.

Villanueva, L., Navarrete, A., Urmeneta, J., White, D. C., and Guerrero, R. (2004). "Combined phospholipid biomarker-16S rRNA gene denaturing gradient gel electrophoresis analysis of bacterial diversity and physiological status in an intertidal microbial Mat." *Appl. Environ. Microbiol.*, 70(11), 6920-6926.

Vreeland, R. H., Mierau, B. D., Litchfield, C. D., and Martin, E. L. (1983). "Relationship of the internal solute composition to the salt tolerance of *Halomonas elongata* ." *Can. J. Microbiol.*, 29(4), 407-414.

Wallace, W., and Attaway, H. (1994). "Perchlorate degradation by an anaerobic bacterium HAP-1." *American Society for Microbiology General Meeting 1994*, Abstract Q189, p.421.

Wallace, W., Beshear, S., Williams, D., Hospadar, S., and Owens, M. (1998). "Perchlorate reduction by a mixed culture in an up-flow anaerobic fixed bed reactor." *J. Ind. Microbiol. Biotechnol.*, 20(2), 126-131.

Wallace, W., Ward, T., Breen, A., and Attaway, H. (1996). "Identification of an anaerobic bacterium which reduces perchlorate and chlorate as *Wolinella succinogenes*." *J. Ind. Engineering,* 16, 68-72.

Waller, A. S., Cox, E. E., and Edwards, E. A. (2004). "Perchlorate-reducing microorganisms isolated from contaminated sites." *Environ. Microbiol.*, 6(5), 517-527.

Wang, C., Lippincott, L., and Meng, X. (2008). "Kinetics of biological perchlorate reduction and pH effect." *J. Hazard. Mater.*, 153(1-2), 663-669.

Wang, D. M., and Huang, C. P. (2008). "Electrodialytically assisted catalytic reduction (EDACR) of perchlorate in dilute aqueous solutions." *Sep.Purif. Technol.,* 59(3), 333-341.

Wang, D. M., Shah, S. I., Chen, J. G., and Huang, C. P. (2008). "Catalytic reduction of perchlorate by H_2 gas in dilute aqueous solutions." *Sep.Purif. Technol.,* 60(1), 14-21.

Webster, T. S., Guarini, W. J., and Wong, H. S. (2009). "Fluidized bed bioreactor treatment of perchlorate-laden groundwater to potable standards." *Journal AWWA*, 101(5), 137-151.

Wolff, J. (1998). "Perchlorate and the thyroid gland." *Pharmacol. Rev.*, 50(1), 89-105.

Wolterink, A. F. W. M., Schiltz, E., Hagedoorn, P., Hagen, W. R., Kengen, S. W. M., and Stams, A. J. M. (2003). "Characterization of the chlorate reductase from *Pseudomonas chloritidismutans*." *J. Bacteriol.*, 185(10), 3210-3213.

Wolterink, A., Kim, S., Muusse, M., Kim, I. S., Roholl, P. J. M., van Ginkel, C. G., Stams, A. J. M., and Kengen, S. W. M. (2005). "*Dechloromonas hortensis* sp. nov. and strain ASK-1, two novel (per)chlorate-reducing bacteria, and taxonomic description of strain GR-1." *Int. J. Syst. Evol. Microbiol.*, 55(5), 2063-2068.

Xiong, Z., Zhao, D., and Harper, W. F. (2007a). "Sorption and desorption of perchlorate with various classes of ion exchangers: a comparative study." *Ind. Eng. Chem. Res.*, 46(26), 9213-9222.

Xiong, Z., Zhao, D., and Pan, G. (2007b). "Rapid and complete destruction of perchlorate in water and ion-exchange brine using stabilized zero-valent iron nanoparticles." *Water Res.*, 41(15), 3497-3505.

Xu, J., Gao, N., Deng, Y., Sui, M., and Tang, Y. (2011). "Perchlorate removal by granular activated carbon coated with cetyltrimethyl ammonium bromide." *J.Colloid Interface Sci.*, 357(2), 474-479.

Xu, J., Gao, N., Deng, Y., Sui, M., and Tang, Y. (2011). "Perchlorate removal by granular activated carbon coated with cetyltrimethyl ammonium chloride." *Desalination*, 275 (1-3), 87-92.

Xu, J., Gao, N., Tang, Y., Deng, Y., and Sui, M. (2010). "Perchlorate removal using granular activated carbon supported iron compounds: Synthesis, characterization and reactivity." *J. Environ. Sci.*, 22(11), 1807-1813.

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

Xu, J., Trimble, J. J., Steinberg, L., and Logan, B. E. (2004). "Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA." *Water Res.*, 38(3), 673-680.

Yifru, D. D., and Nzengung, V. A. (2007). "Uptake of perchlorate by vegetation growing at field sites in arid and subhumid climates." *Remediation*, 17(4), 53-68.

Yoon, I., Meng, X., Wang, C., Kim, K., Bang, S., Choe, E., and Lippincott, L. (2009a). "Perchlorate adsorption and desorption on activated carbon and anion exchange resin." *J. Hazard. Mater.*, 164(1), 87-94.

Yoon, J., Amy, G., Chung, J., Sohn, J., and Yoon, Y. (2009b). "Removal of toxic ions (chromate, arsenate, and perchlorate) using reverse osmosis, nanofiltration, and ultrafiltration membranes." *Chemosphere*, 77(2), 228-235.

Yoon, J., Yoon, Y., Amy, G., Cho, J., Foss, D., and Kim, T. (2003a). "Use of surfactant modified ultrafiltration for perchlorate (ClO_4^-) removal." *Water Res.*, 37(9), 2001-2012.

Yoon, J., Yoon, Y., Amy, G., and Her, N. (2005a). "Determination of perchlorate rejection and associated inorganic fouling (scaling) for reverse osmosis and nanofiltration membranes under various operating conditions." *J. Environ. Eng.*, 131(5), 726-733.

Yoon, J., Shin, D., Kim, I., Kang, K. H., and Park, Y. (2003b). "*Marinobacter litoralis* sp. nov., a moderately halophilic bacterium isolated from sea water from the East Sea in Korea." *Int. J. Syst. Evol. Microbiol.,* 53(2), 563-568.

Yoon, Y., Amy, G., and Liang, S. (2000). "Effect of zeta potential on perchlorate rejection by negatively charged nanofiltration membranes." *AWWA 2000 Inorganic Contaminants Workshop,* Albuquerque, NM, USA.

Yoon, Y., Amy, G., Cho, J., and Pellegrino, J. (2005b). "Systematic bench-scale assessment of perchlorate rejection mechanisms by nanofiltration and ultrafiltration membranes." *Sep. Sci. Technol.,* 39(9), 2105-2135.

Yoon, Y., Amy, G., and Yoon, J. (2005c). "Effect of pH and conductivity on hindered diffusion of perchlorate ions during transport through negatively charged nanofiltration and ultrafiltration membranes." *Desalination,* 177(1-3), 217-227.

Yu, X., Amrhein, C., Deshusses, M. A., and Matsumoto, M. R. (2007). "Perchlorate reduction by autotrophic bacteria attached to zerovalentiron in a flow-through reactor." *Environ. Sci. Technol.,* 41(3), 990-997.

Yu, X., Amrhein, C., Deshusses, M. A., and Matsumoto, M. R. (2006). "Perchlorate reduction by autotrophic bacteria in the presence of zero-valent iron." *Environ. Sci. Technol.,* 40(4), 1328-1334.

Zhang, H., Logan, B. E., Regan, J. M., Achenbach, L. A., and Bruns, M. A. (2005). "Molecular assessment of inoculated and indigenous bacteria in biofilms from a pilot-scale perchlorate-reducing bioreactor." *Microb. Ecol.,* 49(3), 388-398.

Zhang, D., Li, H., Xin, Y., Chi, Z., Zhou, P., and Yu, Y. (2008). "*Marinobacter psychrophilus* sp. nov., a psychrophilic bacterium isolated from the Arctic." *Int. J. Syst. Evol. Microbiol.,* 58(6), 1463-1466.

Zhang, H., Bruns, A., and Logan, B. E. (2002). "Perchlorate reduction by a novel chemolithoautotrophic, hydrogen-oxidizing bacterium." *Environ. Microbiol.,* 4(10), 570-576.

Zhang, Y., Hurley, K. D., and Shapley, J. R. (2011). "Heterogeneous catalytic reduction of perchlorate in water with Re-Pd/C catalysts derived from an oxorhenium(V) molecular precursor." *Inorg. Chem.,* 50(4), 1534-1543.

Zhou, J., Fries, M. R., Chee-Sanford, J. C., and Tiedje, J. M. (1995). "Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov." *Int. J. Syst. Bacteriol.,* 45(3), 500-506.

Zhuang, D., Chen, Y., Zhang, Y., Tang, S., Wu, X., Tan, Z., Li, W., and Cui, X. (2009). "*Marinobacter zhanjiangensis* sp. nov., a marine bacterium isolated from sea water of a tidal flat of the South China Sea." *Antonie Van Leeuwenhoek,* 96(3), 295-301.

Zhuang, W., Tay, J., Yi, S., and Tay, S. T. (2005). "Microbial adaptation to biodegradation of tert-butyl alcohol in a sequencing batch reactor." *J.Biotechnol.,* 118(1), 45-53.

Zuo, G., Roberts, D. J., Lehman, S. G., Jackson, G. W., Fox, G. E., and Willson, R. C. (2009). "Molecular assessment of salt-tolerant, perchlorate- and nitrate-reducing microbial cultures." *Water Sci. Technol.,* 60(7), 1745-1756.

Zuo, G. (2008). "Kinetics and Microbial Ecology of Two Salt-Tolerant Perchlorate-Reducing Enrichment Cultures." PhD thesis, University of Houston, TX, USA.