MICROBIOLOGICAL IMPROVEMENT OF THE PHYSICAL PROPERTIES OF SOILS

by

Ismail Karatas

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

ARIZONA STATE UNIVERSITY

December 2008

MICROBIOLOGICAL IMPROVEMENT OF THE PHYSICAL

PROPERTIES OF SOILS

by

Ismail Karatas

has been approved

October 2008

Graduate Supervisory Committee:

Edward Kavazanjian, Chair
Bruce Rittmann
Sandra Houston

ACCEPTED BY THE GRADUATE COLLEGE

ABSTRACT

The application of microbiological processes for improvement of the physical properties of soils offers the promise of sustainable, cost effective, non-disruptive solutions for a variety of geotechnical problems. Successful development and implementation of microbiological processes for ground improvement would have wide application to a variety of important geotechnical problems, including increasing shear strength and stiffness to enhance foundation bearing capacity and reduce associated settlements, mitigating the susceptibility to earthquake-induced liquefaction, reducing the swell potential beneath foundations and roadways, increasing shear strength to enhance slope stability, cementing soil to facilitate excavation and tunneling and to control soil erosion and scour, and reducing permeability for reducing under-seepage of levees and cut-off walls. Applications of microbiological processes may be especially useful near or beneath existing structures, where the application of traditional soil improvement techniques is limited because of the ground deformations and/or high cost associated with current technologies.

Microbiological processes that can be potentially employed for soil improvement include mineral precipitation, mineral transformation, and biofilms and biopolymers. Many of these processes are known to improve the engineering properties of soils on a geological time scale, and some of these processes are known to induce potentially beneficial effects in shorter time frames but in situations where the context renders these effects undesirable (e.g. clogging of treatment plant filters). The engineering challenge is to induce the desired process over a time frame of engineering interest in the location of interest.

Research at Arizona State University (ASU) on microbiological processes for soil improvement includes performing laboratory experiments to establish technologies for a number of candidate mechanisms, ultimately leading to field tests for mechanisms that look promising. The study presented herein includes a review of literature in microbiology, geology, and geotechnical engineering to identify potentially applicable microbial mechanisms to improve engineering behavior of soils and bench scale experiments to explore feasibility of one of the candidate microbial cementation mechanisms, denitrification. The results from the bench scale experiments suggest that microbial denitrification can be used to improve shear strength of granular soils and has the potential to become a sustainable and cost-effective ground improvement technology in granular soils.

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Edward Kavazanjian for the opportunity to work with him on this unique project. His vision and feedback has been instrumental in the success of this research. Ed has always been a great supervisor, a mentor, and a friend. He has impacted my personal and professional growth significantly since I met him six years ago and I look forward to working with him on other interesting projects.

I am also indebted to Dr. Bruce Rittmann for opening up his laboratory, sharing his expertise at every stage of my research, stepping in whenever I felt lost in between my test results, and for his constructive comments on this dissertation. His Advanced Environmental Biotechnology class was the break point for me to able to empathize with bacteria. I would like to thank Dr. Sandra Houston for accepting to be a part of my committee and for the opportunity to work with her throughout my graduate study.

I would like to thank Dr. Turan Durgunoglu, who introduced me to geotechnical engineering and advised me to go to graduate school, for all of his sincere support from the very first day that I met him. I am also indebted to my former professors at the University of Texas at Austin for "paving the road" for my professional career.

I am honored to acknowledge the help of many people throughout my research, including but not limited to, Dr. Ferran Garcia Pichel, Dr. Lynda Williams, Dr. Hilairy Hartnett, Dr. Claudia Zapata, Dr. Steve Van Ginkel, Dr. Randhir P. Deo, Prathap Paramesvaran, Cesar Torres, Andrew Kato Marcus, Dr. Ilsu Lee, Michal Ziv-El, Hyung Sool Lee, Dr. Absar Alum, Dr. Mohammed El-Zein, Dr. Mortaza Abbaszadegan, Peter Gougen, Jeffrey Long, Danny Clevenger, Hugo Beraldi, Sadik Kucuksari, Yunus Emre,

Cassandra Fowler, Nicole Bisacchi, Jack Kolopanis, Dr. Kenan Hazirbaba, Dr. Brady Cox, MinJae Jung, Eric Iglesias, and fellow graduate students at ASU Civil and Environmental Engineering and at ASU Biodesign Institute - Center for Environmental Biotechnology.

I would also like to take this opportunity to express my gratitude to Dr. Tarik Hadj-Hamou, Scott Brown, Katherine Roxlo, Paul Soltis, and Koray Karatas (who happens to be my brother, as well) for sharing their experience, priceless career advices, and support that definitely helped me in making career decisions.

This research has been a psychological as well as an intellectual challenge for me. I am thankful to my parents, my brother, my sister-in-law, and many other friends for the continuous support.

The material contained in this dissertation is based upon work supported in part by the Geomechanics and Geotechnical Systems program of the National Science Foundation Civil, Mechanical, and Manufacturing Innovation Division under Grant No. 0606678 "SGER: Biological Improvement of the Mechanical Properties of Soils." The author is grateful for this support.

TABLE OF CONTENTS

Page

LIST OF TABLES xi

LIST OF FIGURES xii

CHAPTERS

1. INTRODUCTION 1

1.1. General 1

1.2. Scope and Organization 3

2. BACKGROUND 5

2.1. Interaction Between Microorganisms and Minerals 5

2.2. Previous Research Relevant to the Microbial Improvement of the Physical Properties of Soils 8

2.3. Conclusion 16

3. MICROBIAL MECHANISMS POTENTIALLY APPLICABLE FOR SOIL IMPROVEMENT 17

3.1. Introduction 17

3.2. Mineral Precipitation 17

3.2.1. Microbial Mineral Precipitation Mechanisms 17

3.2.2. Application to Mitigation of Liquefaction Potential 21

3.3. Mineral Transformation 25

3.2.1. Microbial Mineral Transformation Mechanisms 25

3.3.2. Application to Swelling Soils 25

3.4. Biopolymers and Biofilms 27

CHAPTER Page

3.4.1. Previous Studies with Biofilms and Biopolymers 27

3.4.2. Application to Groundwater Control 29

3.5. Conclusion 30

4. CALCIUM CARBONATE PRECIPITATION THROUGH DENITRIFICATION 33

4.1. Introduction 33

4.2. Carbonate Equilibrium 33

4.2.1. pH, Alkalinity, Acidity, and Carbonate Content 34

4.2.2. Calcium Carbonate Precipitation 36

4.3. Denitrification 40

4.4. Stoichiometric Analysis 45

4.4.1. Example Reaction Energetics for Dentrification 46

4.4.2. Stoichiometric Analysis of Denitrification with Different Electron Donors 54

4.4.3. Stoichiometric Analysis of Denitrification under Limited Growth Conditions 62

4.4.4. Summary 63

5. BATCH REACTOR EXPERIMENTS 65

5.1. Introduction 65

5.2. Batch Reactor 66

5.3. Methods 67

5.3.1. Microorganism and Growth Medium 67

5.3.2. Liquid Medium Preperation and Inoculation Technique 69

CHAPTER Page

5.3.3. Analytical Methods 73

5.4. Results and Discussion 75

5.4.1. Denitrification Experiments 75

5.4.2. Precipitation Experiments 83

5.4.2.1. Effect of Phosphorus 84

5.4.2.2. Nitrite Accumulation 91

5.4.2.3. Nitrous Oxide Accumulation 108

5.4.2.4. Calcium Carbonate Precipitation 113

5.4.3. X-ray Diffraction Analysis 124

5.5. Summary of Resuts and Field Application Strategies 127

6. EFFECT OF MICROBIAL CEMENTATION ON THE SHEARING BEHAVIOR OF GRANULAR SOILS AND SOIL-COLUMN EXPERIMENTS 132

6.1. Experience with Ex-situ Artificial Cementation Experiments 132

6.2. Previous Research on Microbial Cementation 136

6.3. Soil Column Experiments 140

6.3.1. Closed System Soil Column Experiments – Methods 140

6.3.2. Closed System Soil Column Experiments – Results 143

6.3.3. Experiments with Bender Elements 149

6.4. Conclusion 150

7. CONCLUSION 152

7.1. Summary of Results 152

7.2. Future Research 157

CHAPTER Page

REFERENCES ..... .159

APPENDIX

A – STOICHIOMETRIC ANALYSIS OF DENITRIFICATION ..... .173

B – BATCH REACTOR TEST RESULTS ..... .187

C – ASTM 20/30 OTTAWA SAND PRODUCT SHEET ..... .199

LIST OF TABLES

Table Page

4-1. Polymorphs of calcium carbonate ..... .38

4-2. Steps of denitrification..... .43

4-3. Reaction energetics for denitrification of glucose (ε=0.55) ..... .53

4-4. Reaction energetics and stoichiometric analysis of denitrification (ε=0.55)..... .57

4-5. Calcium carbonate precipitation controlled by production of alkalinity..... .61

5-1. Nutrient composition for DN-N19 liquid medium ..... .82

5-2. Composition of DN-N9, DN-N10, and DN-N13 liquid medium ..... .86

5-3. Factors that may cause nitrite accumulation during denitrification ..... .92

5-4. Composition of DN-N13 and DN-N21 liquid medium ..... .105

5-5. Factors that may cause nitrous oxide accumulation during denitrification..... .111

5-6. Summary of results from calcium carbonate precipitation experimens ..... .114

5-7. Composition of DN-N17 and DN-N19 liquid medium ..... .119

5-8. Comparison of results from stoichiometric analysis and batch experiments ..... .123

LIST OF FIGURES

Figure Page

2-1. Consolidated Undrained triaxial shear strength tests by Karimi (Martin et al., 1996; Karimi, 1998; Mitchell and Santamarina, 2005) .12

2-2.a. Shear strength envelope from CU and CD triaxial tests (Perkins et al., 2000) .13

2-2.b. Secondar compression experiments on Ottawa sand (Perkins et al., 2000) .14

2-3. Scanning electron micrograph of silica sand cemented with calcium carbonate precipitated through microbial ureolysis (Whiffin, 2004) .15

3-1. Closed system carbonate equilibrium .20

3-2. Comparative sizes of microorganisms and soil particles (Mitchell and Santamarina, 2005) .27

4-1. Relative abundance of polymorphs of calcium carbonate (Swada, 1997) .39

5-1. Bacterial growth phases (Maier et al., 2000) .67

5-2. Same bacterial growth represented in three different ways (Maier et al., 2000) .68

5-3. Batch reactors – 150-mL glass serum bottles and 2.0-L glass bottles .70

5-4. Gas chromatography before and after purging reactors with N_2 .72

5-5. Effect of denitrification on pH and alkalinity (DN-N3: nutrient broth) .78

5-6. Effect of continuous denitrification on pH and alkalinity (DN-N3: nutrient broth) .79

5-7. Denitrification experiment with L-glutamic acid (DN-N19) .85

5-8. Incomplete denitrification-precipitation experiment with L-glutamic acid (DN-N9) .87

5-9. Incomplete denitrification-precipitation experiment with acetic acid (DN-N10) .88

Figure Page

5-10. Incomplete denitrification-precipitation experiment with L-glutamic acid and phosphates (DN-N13) ..... .90

5-11. Nitrite-Nitrous Acid equilibrium ..... .100

5-12. Growth inhibition due to nitrous acid for *P. fluorescens* (Almeida et al., 1995) ..... .101

5-13. Effect of initial nitrate concentrations on denitrification with L-glutamic acid .103

5-14. Effect of total dissolved solids in denitrification with L-glutamic acid ..... .106

5-15. Nitrate reductase activity for *Pseudomonas denitrificans* (Radcliffe, 1969) .... .107

5-16. Nitrous oxide accumulation during denitrification-precipitation experiment with nutrient broth (DN-N18) ..... .109

5-17. Nitrous oxide accumulation during denitrification-precipitation experiment with L-glutamic acid (DN-N21) ..... .110

5-18. Effect of calcium carbonate precipitation on geochemistry (initial Ca^{2+} concentration: 90 mM; electron donor: nutrient broth) ..... .116

5-19. Effect of calcium carbonate precipitation on geochemistry (initial Ca^{2+} concentration: 100 mM; electron donor: L-glutamic acid) ..... .120

5-20. Effect of calcium carbonate precipitation on geochemistry (initial Ca^{2+} concentration: 20 mM; electron donor: L-glutamic acid) ..... .121

5-21. Sediment collected on filter paper – DN-N17 ..... .124

5-22. X-ray diffraction test results – DN-N9 sediment ..... .125

5-23. X-ray diffraction test results – DN-N17 sediment ..... .126

6-1. Change in unconfined compressive strength with cement content (Rad, 1983) . .133

Figure Page

6-2. Effect of cementation on cohesion and friction angle (Rad, 1983) ..... .134

6-3. Shear wave velocity measured during monotonic triaxial tests (DeJong, 2006). .138

6-4. Change in unconfined compressive strength with microbially induced calcium carbonate precipitation (Whiffin et al., 2007) ..... .139

6-5. Acrylic cylinders used in closed system soil column tests ..... .142

6-6. Air dried contents of soil column tests ..... .145

6-7. Scanning electron micrograph of weakly cemented Ottawa sand ..... .146

6-8. Closed view of intergranular contact shown in Figure 6-7 ..... .147

6-9. Elemental analysis of the section shown in Figure 6-8 ..... .148

CHAPTER 1

INTRODUCTION

1.1. General

Leonardo Da Vinci was not only an artist but also a physicist, a mathematician, a biologist, a geologist, an architect, and an engineer. He was the proto-typical "Renaissance" man. His portfolio included the design of advanced combat devices and war vehicles, design of canals, churches, fortresses, studies on reflection of light, elements of mechanics, and, of course, his famous paintings. Yet, he found time to cut cadavers to study and sketch anatomy and physiology, regardless of the Pope's interdiction. Of course, he was not the only "Renaissance" man, but one of the many that could qualify as scientist/engineer/artist. As technology advanced and our knowledge expanded from Leonardo's time, scientists and engineers began to specialize in one area.

In civil engineering, the period from 1930 to 1990 saw the development of geotechnical engineering as a distinct field of study in engineering mechanics and increasing specialization in the study of geo techniques. Developments in geotechnical engineering in recent years, such as the use of microorganisms for groundwater remediation and the use of Micro Electrical Mechanical Systems (MEMS) and nano-materials for sensors and sensing, have reversed this specialization trend, resulting in a "new" interdisciplinary approach to geotechnical engineering for integrating ideas and techniques from other disciplines to develop creative solutions to complex problems. The development of microbiological processes for improvement of the physical properties of soil is one of the more recent manifestations of this trend. The aim of this study is to explore the feasibility of potential applications in one interdisciplinary area:

the application of microbiology to geotechnical engineering. It has been known for many years that microorganisms play various significant roles in the evolution of earth, particularly in geological processes. Interactions between minerals and microorganisms have been studied extensively by microbiologists and geologists, though not by geotechnical engineers. Collaborative interdisciplinary research among microbiologists, chemists, geologists, and geotechnical engineers is required to realize of the potential of microbiological soil improvement technologies.

The engineering challenges in developing beneficial applications of these processes involve identifying the appropriate processes and inducing the desired process over a time frame of engineering interest in the location of interest. Successful development and implementation of microbiological processes for soil improvement would have wide application to a variety of important geotechnical problems, including stabilizing slopes, controlling soil erosion and scour, reducing under-seepage of levees and cut-off walls, increasing the bearing capacity of shallow foundations, facilitating dewatering, excavation, and tunneling in cohesionless soils, and remediating the potential for seismic settlement and liquefaction. Use of microbiological processes can be especially useful near or beneath existing structures, where the application of traditional soil improvement techniques is limited because of the ground deformations and/or high cost associated with alternative techniques.

The successful application of microbiological processes to soil improvement will likely depend on a variety of factors, including the type of microbial metabolism desired, interactions with other microbes present in the environment, soil type, available nutrients,

depth below ground surface, pH, temperature, pressure, concentration of ions, and the availability of oxygen and other oxidants and require understanding of microbiology, chemistry, geology, groundwater hydrology, and geotechnical engineering.

1.2. Scope and Organization

Current research at Arizona State University (ASU) includes performing bench-scale experiments to establish candidate technologies for a number of microbial mechanisms, ultimately leading to field tests for mechanisms that look promising based upon the bench scale experiments. However, the scope of this study is limited to reviewing relevant literature in microbiology, geology, environmental biotechnology, and geotechnical engineering to identify potentially applicable mechanisms and perform batch reactor and soil column tests to evaluate the feasibility of one of the microbial mechanisms for ground improvement: namely, cementation of granular soils through microbial denitrification. The goals of the study presented herein are to:

(a) Present a brief overview of interaction between microorganisms and soil minerals, *Chapter 2*;

(b) Summarize findings from previous research relevant to microbial improvement of the physical properties of soils, *Chapter 2*;

(c) Identify microbial mechanisms potentially applicable as a soil improvement technology and discuss potential geotechnical engineering applications, *Chapter 3*;

(d) Perform a stoichiometric evaluation on one of the candidate microbial cementation mechanisms (i.e., denitrification) and present a detailed biogeochemical evaluation, *Chapter 4;*

(e) Perform bench-scale experiments to evaluate the effects of various biogeochemical factors and to serve as proof of concept for the candidate microbial mechanism, *Chapter 5*;

(f) Discuss the effects of the candidate microbial mechanism on the shearing properties of soils through laboratory test results presented in the literature and discuss the results from ASU soil column tests, *Chapter 6*; and

(g) Identify future areas of research required for practical implementation of the candidate soil improvement mechanism, *Chapter 7*.

Detailed biogeochemical analysis and the results from all the batch reactor laboratory tests are presented in Appendices A and B, respectively.

CHAPTER 2

BACKGROUND

2.1. Interaction between Microorganisms and Minerals

Geomicrobiology is the study of the role of microorganisms in geological processes and the interactions between minerals and microorganisms. It is an interdisciplinary science that requires understanding of microbial physiology, microbial ecology, geochemistry, and sedimentary geology. Microorganisms take part in reduction-oxidation (redox) reactions, gaining energy by reducing or oxidizing chemicals and use this energy for growth, maintenance, mobility, and other activities. Through these reactions, microorganisms may directly precipitate/dissolve/transform minerals or indirectly affect their formation by changing the geochemistry of the environment and producing extracellular polymeric substances. One of the earliest geomicrobiologists, Winogradsky, in the second half of the 19th century discovered that the microbe *Beggiatoa* could oxidize elemental sulfur and that *Leptothrix ochracea* promoted oxidation of $FeCO_3$ to ferric oxide (Ehrlich, 2002). Subsequent researchers also found that not only do microorganisms catalyze redox reactions, but they may also precipitate and/or dissolve minerals, both directly and indirectly. A few years after Winogradsky's study, Nadson (1903) discovered that microbes play a role in calcium carbonate ($CaCO_3$) precipitation. The results of studies by Bryner et al. (1954) indicated that acidophilic (i.e., grows well in acidic medium) iron-oxidizing bacteria can promote the leaching of metals from various metal sulfide ores. In recent years, bioremediation, the use of microbiological mechanisms to transform or immobilize environmental contaminants, has attracted a lot of attention in geoenvironmental engineering. Bioremediation has become an accepted

remedy for soil and groundwater contaminated with hydrocarbons, especially with benzene, toluene, ethylbenzene, and xylene (BTEX). Bioremediation processes include natural attenuation, biostimulation, and bioaugmentation. Natural attenuation relies upon native microorganisms to degrade and transform contaminants. Monitored natural attenuation (MNA) has become the preferred remedy for soil and groundwater contaminated with many types of hydrocarbons, especially with BTEX contaminants. Biostimulation is a process in which environmental conditions are modified to enhance natural microbiological attenuation. Bioaugmentation is a process in which the subsurface environment is amended with exotic (i.e. non-native) microorganisms to degrade and/or immobilize harmful chemical constituents. Biostimulation is used in practice to remediate chlorinated hydrocarbons and other biostimulation and bioaugmentation remediation processes are now being implemented with increasing frequency.

Until recently, the application of microbiological processes to improve the mechanical properties of soil for engineering purposes (e.g., increasing shear strength, decreasing compressibility, decreasing hydraulic conductivity) remained largely unexplored, despite the role these processes play in many geologic and anthropogenic processes that are potentially beneficial. For instance, microbially induced mineral precipitation is known to create cemented soils naturally on a geologic time frame (Ehrlich, 2002), but its potential to improve soil over a time frame of engineering interest has not been widely investigated. However, observations of clogging of filters and drainage media in dams, landfills, and at mine sites and the development of mineral

"scale" in soil and on drainage pipes demonstrate that these phenomena can occur within a time frame of engineering interest. By harnessing geomicrobiological processes, engineering solutions can be devised for temporary and/or permanent geotechnical engineering problems, including enhancing foundation bearing capacity, reducing susceptibility to earthquake-induced liquefaction, reducing the swell potential beneath foundations and roadways, enhancing slope stability, facilitating excavations and tunneling, and reducing permeability for groundwater control.

Successful development and implementation of microbiological processes for soil improvement may be especially useful near or beneath existing structures, where the application of traditional soil improvement techniques is limited because of the ground deformations and/or high cost associated with alternative technologies. Ivanov and Chu (2008) estimate that the material cost for soil grouting using chemicals (e.g., lignosulphites, sodium silicates, phenoplasts) can vary from \$2 to \$72 per m^3 of soil, whereas, if waste materials are used as carbon source for microbial growth, the material cost for cementation through microbiological processes can be as low as \$0.5 to \$9.0 per m^3 of soil. In addition to material costs, the selection process for an alternative technology will include consideration of installation costs and costs associated with the potential environmental and health risks.

Although cost is an important item in the decision process regarding the use of an alternative microbiological technology, efficiency and risks associated with various microbiological soil improvement processes need to be assessed prior to finalizing the

decision tree. Thus, extensive laboratory and field investigation is required to identify the associated risks and challenges with microbial soil improvement technologies.

2.2. Previous Research Relevant to Microbial Improvement of Physical Properties of Soils

Mitchell and Santamarina (2005) published an overview of biological considerations in geotechnical engineering and discussed the interaction between microorganisms and geological processes. Mitchell and Santamarina (2005) highlighted that only a limited number of studies address the effect of microbial activity on the shear strength and stiffness of soils. However, some data in the literature are related to the effect of microbial activity on the hydraulic conductivity on soils. Most of the work that may be relevant to microbiological improvement of physical properties of soils (e.g., microbial mineral precipitation/plugging, biofilms, and biopolymers) is related to either bioremediation applications or to the efforts to enhance oil recovery from the petroleum reservoirs.

In addition to the work on the bioremediation of hydrocarbons discussed previously, several researchers have studied the feasibility of capturing inorganic contaminants through mineral precipitation and containment of contaminated groundwater using biobarriers. For example, Warren et al. (2001) studied solid phase capture of uranium dioxide (UO_2), strontium (Sr), and copper (Cu) through biomineralization (i.e., direct or indirect formation of insoluble precipitates by microorganism) in the laboratory and concluded that calcium carbonate precipitation promoted by bacterial hydrolysis of urea was an effective method of capturing Sr.

Cunningham et al. (1991) performed a series of laboratory tests to assess the effect of biofilm accumulation on porous media hydrodynamics and found that the intrinsic permeability of different sizes of glass spheres and sands decreased by up to 98 percent, stabilizing at 1 to 5 percent of the original value (i.e., the value with no biofilm) within a few days.

Komlos et al. (1998) performed laboratory experiments to examine the effects of thick biofilms in porous media under radial flow conditions using *Pseudomonas fluorescens*, facultative anaerobic bacteria capable of denitrification. Through bacterial inoculation and nutrient addition, Komlos et al. (1998) reported the formation of a biobarrier that resulted in a decrease in horizontal hydraulic conductivity of the porous media from 6.7×10^{-2} cm/s to 1.7×10^{-2} cm/s (a 75 percent reduction) over 24 hours. Dutta et al. (2005) built an *in-situ* biobarrier at a site near Albuquerque, New Mexico, USA, to remediate groundwater contaminated with nitrate. The stimulated indigenous bacteria were used to contain and remediate groundwater through denitrification. Even though the biobarrier did not completely halt the flow of contaminants, the reduction in the hydraulic conductivity of the subsoils resulted in a formation of an active treatment zone, and nitrate concentrations dropped from 275 mg/L to less than maximum contaminant level (MCL) for safe drinking water (10 mg-N/L; US EPA, 2001) over a period of approximately 300 days. Dutta et al. (2005) also reported problems with biofouling around the well screens used to monitor this process within four months from the start of the field study.

The oil industry has been interested in microbiological mechanisms that result in plugging of geological formations in order to reduce the hydraulic conductivity of the layers surrounding oil bearing strata and to improve the efficiency of oil extraction. A series of laboratory tests were conducted at the University of Calgary, Canada, to evaluate the plugging of sintered glass bead cores using vegetative and starved bacteria by MacLeod et al. (1988). The results of the experiments indicated, under the same injection conditions (500 pore volumes of *Klebsiella pneumoniae* suspension), bacteria starved for 2 weeks reduced core permeability by 71 percent, whereas the use of vegetative cultures resulted in a reduction in core permeability by 99 percent. Based on the data of respiratory activity and Deoxyribonucleic Acid (DNA) – derived cell density with respect to core depth, MacLeod et al. (1988) also concluded that, while the vegetative cultures were somewhat more effective at plugging in the short term, the general starvation of the bacterial cultures prior to core injection can improve penetration and may provide a new bacterial plugging technique for petroleum reservoirs.

Research on the impact of biopolymers on the geotechnical properties of compacted soils was conducted by Karimi (1998), who performed hydraulic conductivity and triaxial shear strength tests on compacted specimens of Bonnie silt mixed with xanthan gum, a commercially available biopolymer. The results of permeability tests indicated that the hydraulic conductivity of Bonnie silt was reduced by two orders of magnitude when mixed with 0.3 percent xanthan gum by weight at a water content greater than the optimum moisture content of the silt and that this effect lasted for at least six months (Martin et al., 1996). Karimi (1998) also performed consolidated-undrained

(CU) triaxial tests on compacted samples of Bonnie silt mixed with xanthan gum solution and observed a change in the shear strength of soil samples with age (i.e., the duration after the hydration of the compacted silt mixed with xanthan gum). The maximum deviatoric stress measured by Karimi (1998) during the CU triaxial tests are presented in Figure 2-1. The maximum deviatoric stress is defined as the difference between the major and minor principal stress and is proportional to the shear strength of a soil specimen in triaxial tests. The CU triaxial tests performed on compacted Bonnie silt samples mixed with 0.3 percent xanthan gum by weight resulted in improved shear strengths up to 30 percent within a week (Fig. 2-1). The measured maximum deviatoric stress (and thus the shear strength) reached a constant value for samples aged more than 20 days. Note that "gum 1%" solution in Figure 2-1 corresponds to a xanthan gum content of 0.3 percent by weight (Karimi, 1998).

Perkins et al. (2000) performed triaxial shear strength tests on dense Ottawa Sand specimens to evaluate the effect of biofilms on the shearing properties of granular soils. *Klebsiella oxytaca* was introduced into the soil specimen with a nutrient solution. Perkins et al. (2000) used ultra-micro-bacteria (UMB) (i.e., vegetative cells that shrink and revert to a low metabolic rate when subjected to starvation) to ensure homogeneous distribution of microorganisms. The growth of the biofilm was facilitated via periodic flow of a nutrient solution through the sample prior to the application of loading. Perkins et al. (2000) concluded that the biofilm had a negligible influence on the shear strength and stiffness of the Ottawa sand based on CU and consolidated-drained (CD) triaxial tests (Fig. 2-2a), but that it increased the creep deformation (i.e., time-dependent

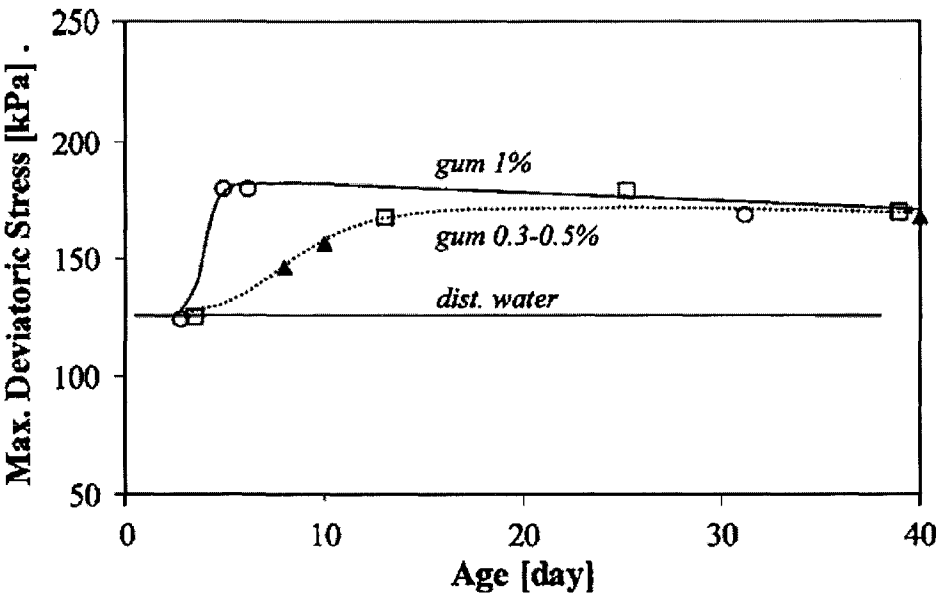


Fig. 2-1. Consolidated-undrained Triaxial shear strength tests performed by Karimi (Martin el al., 1996; Karimi, 1998; Mitchell and Santamarina, 2005)

deformation under constant load) (Fig. 2-2b). The "average creep slope" plotted in Fig. 2-2b represents the average of the slope of the vertical strain vs. log-time curves from two secondary compression experiments. Furthermore, Perkins et al. (2000) reported that the hydraulic conductivity of the sand was reduced by an order of magnitude by the biofilm. Although Perkins et al. (2000) performed direct and plate counts to evaluate the population of the microorganisms at the end of the laboratory experiments, no data were provided on the distribution of biofilm throughout the sample (e.g., images from scanning electron microscopy, SEM).

Cabalar and Canakci (2005) performed a series of laboratory tests on sand mixed with different ratios of xanthan gum. Cabalar and Canakci (2005) state that direct shear

tests showed an increase in "average shear strength at failure" from 30 kPa to 190 kPa when the xanthan gum content of the sample was increased from 1 percent to 5 percent. However, neither the normal stress nor load-deformation data were provided by Cabalar and Canakci (2005). Furthermore, because no data were provided on the baseline shearing strength of the sand (i.e., the shear strength of the sand with no xanthan gum), there is no way to assess whether the addition of xantham gum initially resulted in an increase or decrease in shear strength.

Researchers at the Delft University of Technology and GeoDelft Institute (GeoDelft) in the Netherlands also have been studying improvement of soil properties using microbiological processes. The two processes that are being studied at GeoDelft are biogrout, an *in situ* cementation process controlled by microorganisms that degrade

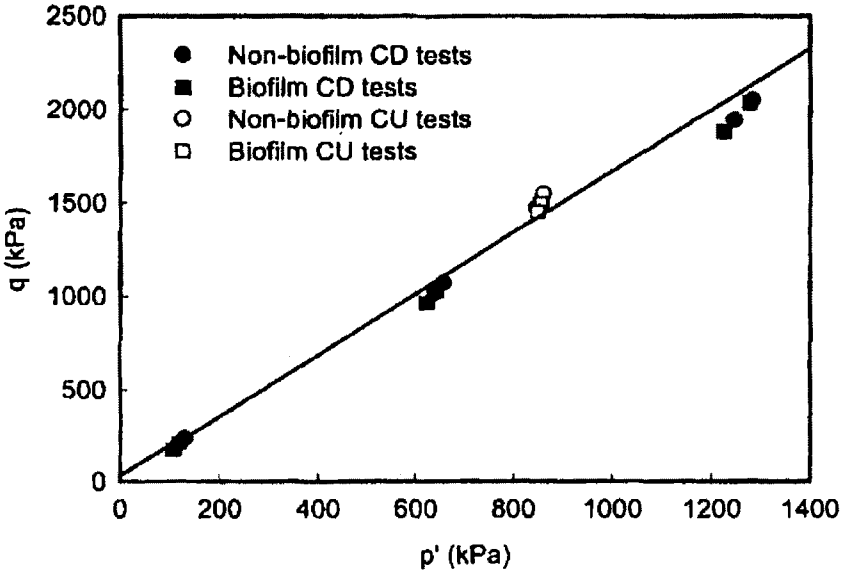


Fig. 2-2a. Shear strength envelope from CU and CD Triaxial (Perkins et al., 2000)

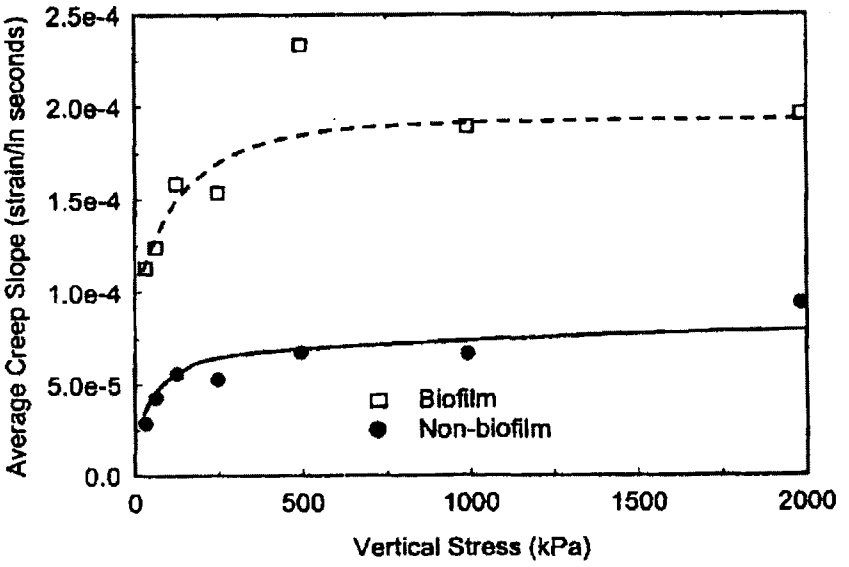


Fig. 2-2b. Secondary compression experiments on Ottowa sand (Perkins et al., 2000)

urea, and bioseal, a sealing process which locates and seals leaks in water retaining soil/fractured rock layers. Research work initially carried out by Whiffin (2004) at Murdoch University in Western Australia led to development of the GeoDelft biogrout testing program. Whiffin (2004) studied the effects of microbial precipitation of calcium carbonate through hydrolysis of urea on the physical properties of sands. Whiffin (2004) injected an aerated solution of urea, calcium, and urea-hydrolyzing bacteria into sand specimens to induce calcium carbonate precipitation (Fig. 2-3) and reported that the P-wave measurements indicated an increase in cementation and shear strength with increasing concentration of hydrolyzed urea. Whiffin (2004) then performed triaxial shear strength tests on Dutch Koolschhijn sand injected with urea, calcium, and urea-

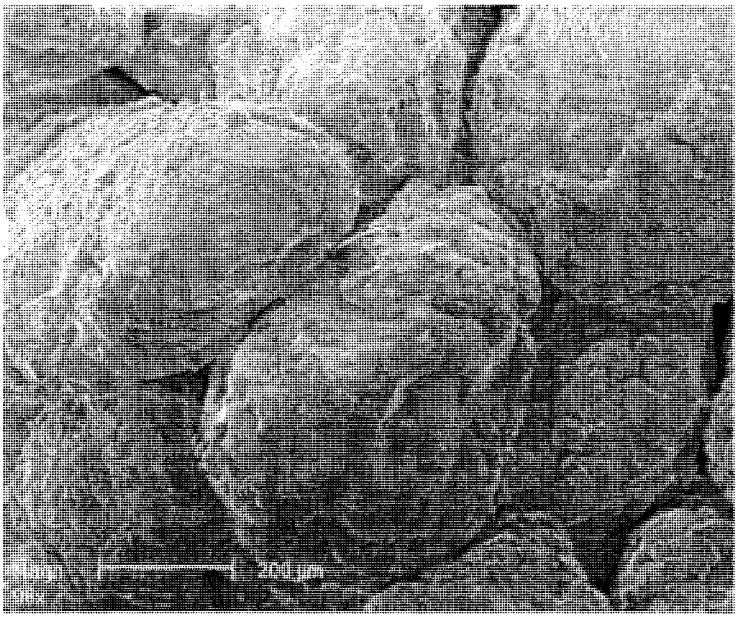


Fig. 2-3. Scanning electron micrograph of silica sand cemented with calcium carbonate precipitated through microbial ureolysis (Whiffin, 2004)

hydrolyzing bacteria and reported that shear strength increased by a factor of 8 and stiffness increased by a factor of 3 without a significant change in pore volume.

GeoDelft's biosealing process involves a consortium of microorganisms that form a bioslime and an insoluble iron sulfide (FeS) precipitate that accumulate around a leak. While the biogrouting work reported by GeoDelft involves only bench-scale laboratory tests, GeoDelft's biosealing technology has actually been tested in the field in 2004 and was used on a major infrastructure project in 2005, construction of the Aquaduct Ringvaart Haarlemmermeer, a part of the high-speed rail link (GeoDelft, 2006).

Recently, DeJong et al. (2006) evaluated the effects of calcium carbonate precipitation induced microbially through urea hydrolysis on the shearing properties of

loose sands. DeJong et al. (2006) observed an increase in shear wave velocity from approximately 200 m/s to 540 m/s due to microbial treatment and reported that the microbially cemented soils displayed a similar shearing response to gypsum cemented soils under undrained conditions.

2.3. Conclusion

The application of microbiological processes to improve the mechanical properties of soil for engineering purposes (e.g., increasing shear strength, decreasing compressibility, decreasing hydraulic conductivity) remained largely unexplored until recently. The studies presented in this chapter suggest that interaction of microorganisms and minerals can potentially result in improvement of the engineering properties of soils. The pathways through which microorganisms can affect the engineering properties of soils and possible geotechnical engineering applications are discussed in a subsequent chapter.

CHAPTER 3

MICROBIAL MECHANISMS POTENTIALLY APPLICABLE FOR SOIL IMPROVEMENT

3.1. Introduction

Mechanisms for potential applications of microbiology to geotechnical engineering can be divided into three main categories: mineral precipitation, mineral transformation, and biopolymers and growth of biofilms. Each one of these groups of microbial mechanisms is described in detail in the subsequent sections. Examples of potential geotechnical engineering applications for each one of these categories are also presented.

3.2. Mineral Precipitation

3.2.1. Microbial Mineral Precipitation Mechanisms

Sediments undergo compaction and cementation when forming sedimentary rocks over a geologic time scale. The most common type of cements that bind granular soil particles at the grain contacts or fill in the voids between the grains in sedimentary rocks are silica, calcium carbonate, clay, and iron-bearing mineral based cements (Krynine and Judd, 1957). Sitar et al. (1980) indicate that, in cemented sands, the cement may either be present at the time of deposition or precipitate at a later stage after deposition. Among the different cement types, calcium carbonate is the most common throughout sedimentary formations. One issue with carbonate cementation is that it may be weakened after deposition as a result of changes in the pH of the environment. On the other hand, silica cement is the strongest and the most resistant to weathering among the various common geological cementation agents (Sitar et al. 1980). Clay cement is also

reported to be strong when dry (unsaturated), but can be easily affected by moisture fluctuations, displaying brittle or ductile behavior depending on the degree of saturation.

More than 60 types of minerals (e.g., carbonates, silicates, oxides, sulfides, phosphorites) have been reported to be formed directly or indirectly by organisms under a wide variety of conditions (Lowenstam and Weiner, 1989). These minerals can be deposited at many different locations inside and outside cells. Thus, theoretically, any of the minerals that act as cementing agents in sedimentary rocks can be formed by microorganisms if suitable conditions are met.

Carbonate precipitation is perhaps the most widely studied among the minerals formed by microorganisms. Although it was thought that only certain types of microorganisms are able to induce calcium carbonate at early stages of the research (Drew, 1911; Drew, 1914), almost all geomicrobiologists today agree that microorganisms can precipitate calcium carbonate through a variety of pathways. Some microorganisms directly mediate carbonate precipitation intracellularly (e.g., *Agromatium exaliferum*; Buchanan and Gibbons, 1974) or extracellularly (e.g., coccolithophores; Lewin, 1965). Many microorganisms can induce indirect carbonate precipitation extracellularly through metabolic processes that affect the geochemistry of the pore fluid by increasing either the total carbonate content or the pH or both. Boquet et al. (1973) cultured soil bacteria on solid media and reported that crystal formation is a function of the medium used; under suitable conditions, most bacteria can form calcite (calcium carbonate) crystals. Anaerobic and aerobic oxidation of an organic compound results in production of CO_2. The produced CO_2 dissolves in water in proportion to the partial

pressure of the CO_2 gas. In a closed system, where there is no exchange between the produced/consumed CO_2 gas and the atmosphere, the dissolved CO_2 dissociates into carbonic acid, bicarbonate, and carbonate, as shown in Figure 3-1. If the medium is a well-buffered neutral or alkaline environment, a portion of CO_2 produced as a result of oxidation of an organic compound transforms into carbonate (Figure 3-1) and then precipitates if an adequate amount of appropriate cations, such as Ca^{2+}, is present.

Precipitation is enhanced if the pH increases due to microbial production of alkalinity, which can occur in several ways. For instance, organic nitrogen may be released from organic compounds in the form of ammonia (NH_3). This includes organic nitrogen in urea, which releases NH_3 by ureolysis. Protonation of NH_3 generates alkalinity (OH^-) and leads to an increase in pH: $H_2O + NH_3 \rightarrow NH_4^+ + OH^-$ (Krumbein, 1979; Stocks-Fischer et al., 1999; Fujita et al., 2000; Hammes et al., 2003; Whiffin, 2004). In oxygen deficient environments, nitrate (NO_3^-) can be used as an electron acceptor by many bacteria (i.e., denitrification), producing N_2 gas, CO_2, and alkalinity: $NO_3^- + 1.25CH_2O \rightarrow 0.5N_2 + 1.25CO_2 + 0.75H_2O + OH^-$. Sulfate ($SO_4^{2-}$) can also be used as an electron acceptor under anaerobic conditions by microorganisms. In sulfate reduction, sulfate-reducing bacteria, such as *Desulfovibrio* spp. and *Desulfotomaculum* spp., oxidize organic compounds while reducing sulfate to produce H_2S, CO_2, and alkalinity: $SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2CO_2 + 2OH^-$ (Abd-el Malik and Rizk, 1963a, 1963b). In landfills, methane formation from acetic acid adds CO_2 and removes the acidity of acetic acid while adding CO_2: $CH_3COOH \rightarrow CH_4 + CO_2$ (Brune et al., 1991; Fleming et al., 1999; Cooke et al., 2001; Rowe et al., 2002).

In addition to these mechanisms, Ehrlich (2002) lists "removal of CO_2 from bicarbonate containing solutions" as one of the microbial mechanisms that may lead to calcium carbonate precipitation through dissociation of bicarbonate into carbonic acid and carbonate according to Figure 3-1. The most well-known process that may result in carbonate precipitation through this mechanism is oxygenic photosynthesis. In principle, all autotrophs (organisms that need CO_2 as source of carbon) may precipitate carbonate by removing CO_2, unless they also generate acids (e.g., sulfide-oxidizing bacteria, nitrifying bacteria). Moore (1983) reported that cyanobacteria and algae deposited calcareous nodules and crusts on sub-aqueous levees in the Flathead Lake delta in Montana through this mechanism (removal of CO_2 from bicarbonate containing solutions through oxygenic photosynthesis). However, oxygenic photosynthesis mainly depends on light as source of energy, which limits the depth at which these microorganisms live. Because sunlight can only penetrate up to couple of millimeters below ground surface,

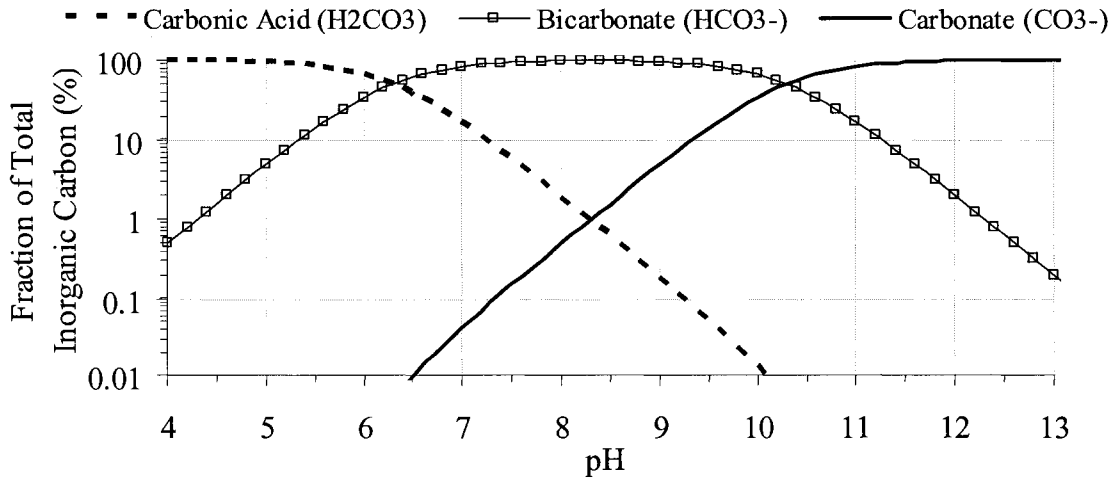


Fig. 3-1. Closed system carbonate equilibrium

oxygenic photosynthesis is only limited to the formation of soil crust.

In addition to calcium carbonates, microorganisms can also induce precipitation of other minerals that may potentially cement soil particles. For instance, precipitation of iron sulfide (FeS) as a result of microbial reduction of sulfate to sulfide has been reported in the laboratory and in the field (Rickard, 1969; Donald, 1998; Popa and Kinkle, 2000; Fortin et al., 2002; GeoDelft, 2006). Sulfate-reducing bacteria have also been reported to be involved in precipitation of silicates. Birnbaum and Wireman (1984) and Birnbaum et al. (1989) attributed the precipitation of silica to the modification of the pH as a result of sulfate-reducers' metabolic activity (Guidry and Chafetz, 2003).

In summary, there are a variety of mineral precipitation mechanisms that can change the mechanical properties of soil. For instance, carbonate precipitation can result in cementation within soil with a potential increase in shear strength and stiffness. Furthermore, based upon geologic evidence, microbially induced carbonate precipitation is expected to be long lasting, suggesting that soil improvement due to this mechanism may be assumed to be permanent (i.e. irreversible or very slowly reversible) for engineering purposes. Ideally, based on the site characteristics, the optimal microbial mineral precipitation mechanism would be identified through a screening process and then applied in the field.

3.2.2. Application to Mitigation of Liquefaction Potential

One of the scenarios as to what happened to Bronze Age towns Sodom and Gomorrah, places believed to be located on the edge of the Dead Sea, is related to the phenomenon of earthquake-induced soil liquefaction, where saturated cohesionless soils lose their

ability to support structures and, often, slope failures occur when slopes underlain by liquefiable soils are subjected to strong earthquake shaking. Cecil (2001) reports that a group of geologists traveled to the Middle East to investigate possible locations of these two towns. Geological investigations revealed that the towns were built close to the edge of Dead Sea, and the site was underlain by intensely stratified alternating loose sand, fine silt, and clays deposited during different seasons. Subsequently, Haigh and Madabhushi (2002) of the Cambridge University built and tested a centrifuge model of the buildings and the subsoil based on the geological investigations. Haigh and Madabhushi (2002) subjected the model to a simulated earthquake and concluded that liquefaction-induced lateral spreading after an earthquake of these layered deposits is a plausible explanation for the disappearance of Sodom and Gomorrah, with displacements of hundreds of meters being possible even on fairly gentle slopes.

Regardless of whether these cities were destroyed by brimstone and fire because of the sins of their inhabitants or destroyed by earthquakes, earthquake-induced liquefaction has been affecting the lives of human beings for a very long time. In 1964, one-third of the city of Niigata, Japan, subsided as much as 2 m as a result of seismic settlement following soil liquefaction (NOAA, 2005). The 1971 San Fernando Earthquake caused over $3 billion property damage (AIR, 2006), including a liquefaction-induced slide of the top 30 ft of the Lower San Fernando Dam. The dam was very close to completely failing, and 80,000 people living downstream of the dam were immediately ordered to evacuate (ABAG, 2003). The 1989 Loma Prieta Earthquake was responsible for 62 deaths, 3,757 injuries, and more than $6 billion property damage

(Marshall et al., 1989), most of which was caused by severe ground shaking, landsliding, and liquefaction (Montgomery, 1990).

Many of the current technologies to improve the behavior of soils susceptible to earthquake-induced liquefaction result in large ground deformations, making them unsuitable for use in developed areas. Furthermore, techniques suitable for use in developed areas, e.g., compaction grouting, generally incur high costs. Microbiologically induced mineral precipitation offers the potential for significant improvement in liquefaction resistance due to development of intergranular cementation with little to no ground deformation. Potentially liquefiable sand deposits near and/or along the shores of water bodies, a common situation along the west coast of the US and many other liquefaction-prone areas, may provide a suitable environment for microbiologically induced cementation technologies because of the presence of elevated cation concentration and/or anaerobic conditions.

Depending on the characteristics of the subsurface environment, one or more of the microbial carbonate precipitation mechanisms described earlier may be applicable in coastal environments, provided that appropriate engineering measures are taken to stimulate the appropriate microbial mechanism. For instance, if denitrification is found to be feasible for microbial carbonate precipitation based on laboratory screening studies using sites-specific groundwater and soil samples, nitrate (NO_3^-), nutrients, Ca^{2+}, or other chemical agents, as needed, can be introduced to the ground through wells screened along the thickness of the potentially liquefiable layers to promote the growth of indigenous denitrifying microorganisms. Laboratory screening studies may help to optimize the

amount of electron acceptor (i.e., NO_3^- in this case), electron donor, carbon source, nitrogen source, and other nutrients and salts required for growth and will result in a list of agents that can be injected into the ground for biostimulation and carbonate precipitation. This biostimulation approach can be applied for the promotion of the growth of sulfate reducing microorganisms as well. If urea hydrolyzing enzyme, urease, is detected during laboratory feasibility studies, urea can also be amended to the ground at desired amounts in a solution to promote microbial carbonate precipitation through ureolysis. Because the production of urease by soil microorganisms is retarded by lack of oxygen (McCarty and Bremner, 1991), air sparging may be required to accelerate microbial mineral precipitation when ureolysis is employed below groundwater table. However, if urease is supplied to the soil, air sparging may not be needed as hydrolysis of urea by urease is not affected by oxygen concentration.

If the promotion of the growth of the desired indigenous microbial community is not successful, bioaugmentation can be applied. When an anaerobic subsoil environment with high sulfate concentration is encountered, sulfate reducing microorganisms, e.g., *Desulfovibrio desulfuricans*, with appropriate nutrient solution (and salts, if needed) can be introduced to the ground. Similarly, *Bacillus pasteurii* or *Pseudomonas denitrificans* can be introduced if calcium carbonate precipitation through ureolysis or denitrification, respectively, seems feasible.

3.3. Mineral Transformation

3.3.1. Microbial Mineral Transformation Mechanisms

Microbial mechanisms play an important role in weathering of minerals and the geologic cycle. For instance, some bacteria and fungi play an important role in mobilization of silica (Si) in nature (Ehrlich, 2002). Microbial metabolisms cause the mobilization of silica through solubilization by metabolically produced complexing ligands, acids, and alkalinity. In addition, Kim et al. (2004) report that microorganisms can promote the transformation of smectite to illite through reduction of structural Fe(III) to Fe(II), which leads to potassium (K^+) uptake into the inter-layers. Smectite refers to a family of clay minerals composed primarily of hydrated sodium-calcium-aluminum silicate. Smectite minerals are the predominant cause of excessive swell (expansion) potential in soils. Illite refers to potassium-rich clay minerals that have much lower swell potential than smectite; the swell potential of illitic soils is not usually of engineering concern. Kim et al. (2004) report that microbial transformation of smectite to illite occurred at ambient conditions within 14 days in laboratory experiments in which Fe(III)-rich smectite was incubated with *Shewanella oneidensis*. This transformation typically requires 4 to 5 months at a temperature of 300°C to 350°C and a pressure of 100 MPa in the absence of microbial activity (Kim et al., 2004). These findings suggest that microbial processes may be used to mitigate swell potential in some expansive soils.

3.3.2. Application to Swelling Soils

While most work to date on use of microbial processes for soil improvement have focused on granular soils, the mitigation of swelling (expansive) soils is one area where

microbial processes may improve the performance of fine grained soils. The total cost of damage due to swelling soils, one of the least publicized geologic hazards, were $2 to $7 billion in the U.S. in 1987 (Jones and Jones, 1987) and may reasonably be considered to be at least twice as much today. Currently, pre-wetting of the site and *ex situ* lime treatment of expansive soils are among the most common geotechnical approaches used to mitigate the potential for swell in a fine grained soil, along with expensive structural measures such as post-tensioning of foundation slabs. In iron-rich expansive soils, promoting iron-reducing microbial activity to reduce Fe(III) to Fe(II) and possibly transform smectite to illite may provide an alternative solution to mitigating swell (expansion) potential of soils and could provide significant advantages over the existing remedies in terms of both cost and environmental impact.

Similar to lime improvement, microbial improvement of fine grained soil is likely to be initially applied *ex situ*. Nutrients and salts containing potassium can be added to stockpiled soil to stimulate the iron-reducing microorganisms. The nutrients and salts can be introduced in solution to promote their distribution through the soil. As suction draws the pre-wetting solution into the soil, it will carry the required nutrients and cations with it. In the absence of indigenous iron-reducing microbial metabolic activity, the soil could be augmented with *Shewanella oneidensis* or some other iron-reducing microorganism along with a source of nutrient. The application of this technology *in situ* may be limited due to the size of the iron-reducing bacteria relative to the pore size of fine grained soils, which may limit the ability of the bacteria to penetrate the soil. A

comparison of the size of microorganisms and soil particle/pore size by Mitchell and Santamarina (2005) is presented in Figure 3-2.

3.4. Biopolymers and Biofilms

3.4.1. Previous Studies with Biopolymers and Biofilms

A number of investigators have investigated the impact of biopolymers on saturated hydraulic conductivity with respect to the potential for forming hydraulic barriers, or bio-barriers, to contaminant transport. Khachatoorian et al. (2003) performed a series of permeability tests to evaluate "plugging" of fine sand by biopolymer slurry impregnation using five different biopolymers. The results of these tests demonstrated a permeability decrease of up to 14 orders of magnitude in a fine sand in less than two weeks.

Biofilms form on a wide variety of surfaces, including living tissues, medical

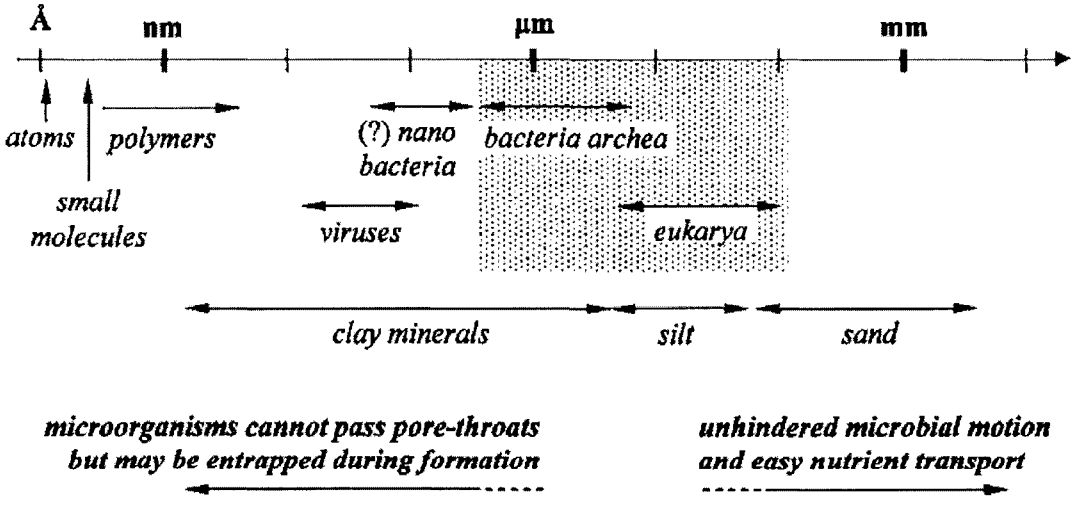


Fig. 3-2. Comparative sizes of microorganisms and soil particles (Mitchell and Santamarina, 2005)

devices, industrial or potable water system piping, natural aquatic systems, soil particles, and geosynthetics. According to Donlan (2002), the solid-liquid interface between a surface and an aqueous medium provides an ideal environment for the attachment and growth of microorganisms. Many case histories of clogging of filters in dams, landfills, and water treatment plants due to growth of biofilms have been reported. For instance, in October 1985 an investigation was carried out to evaluate the reason for the clogging of the subsurface drains at the Ergo Tailings Dam (ETD) in South Africa. The aggregate and geotextile drains clogged only six months after they were put in service. Legge et al. (1985) concluded that the geotextile filter for the drains was clogged due to the growth of arsenic-resistant microorganisms. The results of permeability tests on the clogged geotextile from the ETD subdrain revealed that the through-flow capacity was reduced by as much as an order of magnitude when compared to virgin geotextile.

Biopolymers and growth of biofilms are presented herein as a separate group of microbial mechanism in addition to microbial mineral precipitation and transformation to highlight the potential effect of the physical presence of microorganisms on the properties of the bulk soil (e.g., clogging of pore spaces). In addition to altering material properties through their physical presence, microorganisms in a biofilm may also induce mineral precipitation or mineral transformation. For instance, scanning electron microscopy (SEM) and X-ray diffraction analysis on the geotextile samples from the ETD revealed iron oxide precipitates precipitated by microorganisms (Legge et al., 1985).

Biopolymer and biofilm "clogging" of pore spaces may logically be inferred to be beneficial for various physical properties of soil besides permeability, including undrained shear strength, drained shear strength, and shear modulus. Furthermore, a significant reduction in permeability should significantly reduce, if not eliminate, the potential for earthquake-induced liquefaction. One concern with biopolymer and biofilm improvement is that it may not be permanent, i.e. that the soil property changes may be reversible, requiring active maintenance of suitable environmental conditions. However, even if these property changes cannot be relied upon for the long term, there are many situations where "temporary" improvement of soils often is sufficient, e.g., stabilization and groundwater control for excavations and tunneling. In fact, in some situations the "reversibility" of the process may be a desirable trait (e.g., the use of biodegradable biopolymers for construction of permeable reactive barriers). Despite the potential benefits of biofilm and biopolymer accumulation, limited information has been reported on the impact of these phenomena on the shear strength, compressibility, or liquefaction potential of soil and their potential use in corrosion protection.

3.4.2. Application to Groundwater Control

Groundwater control has been a challenging area for geotechnical engineers for many years. Because of the difficulties in identifying all of the geologic features that may cause serious groundwater control problems, high costs and large factors of safety are generally associated with the groundwater control solutions for excavation and tunneling. As described in the previous sections, clogging of the drainage and filter systems in landfills, dams, and water treatment plants has been attributed to the microbial activity and

resulting biofilms and/or microbially precipitated minerals. This observation suggests that one of the microbial mineral precipitation mechanisms or a mechanism that employs microorganisms that develop biopolymers and biofilms can be used to reduce the hydraulic conductivity of soil *in situ*. If groundwater control is achieved through microbial mineral precipitation, then a long-term solution may be achieved. On the other hand, biofilm and biopolymer production may just be suitable for interim (short term) groundwater control because of potential degradation with time. However, there are many situations where a short-term groundwater control solution is desirable, e.g., a temporary excavation below groundwater table. It is possible that in some cases sufficient biofilm or biopolymer production may be stimulated simply through introduction of microorganism with nutrient solution. If the application of this microbial mechanism is feasible, it can also be used as part of barriers for waste containment applications.

3.5. Conclusion

The application of microbiology to geotechnical engineering for soil improvement purposes is largely unexplored. The mechanisms by which microorganisms play a role in geological phenomena, along with observations of certain "adverse" effects of microorganisms on engineered facilities, suggest that in the proper context microorganisms can be used to improve the mechanical properties of soils for engineering purposes. These mechanisms and observed effects have a variety of potential engineering applications, including enhancing soil stability, improving foundation performance, and control of groundwater. Remediation of soil liquefaction

through microbial carbonate precipitation, mitigation of soil swell (expansion) potential through biological mineral transformation, and groundwater control through microbial mineral precipitation or biofilm development are among the potential beneficial applications of microbiology to geotechnical engineering. Once a microbial soil improvement technology is established, an engineered system for application of the technology ideally should have the following properties:

- "Turn-on" and "turn-off" mechanisms so that it can be managed;
- Laboratory testing or alternative method to evaluate the feasibility of the proposed system;
- Environmental and health risks that are predictable and acceptable;
- Predictable performance of the system to facilitate engineering design;
- Lifecycle costs comparable with or superior to existing alternative technologies;
- An improvement rate such that acceptable performance can be achieved over a duration that is manageable within the context of problem; and
- A dependable quality control and assurance system.

In general, the applicability of microbiological processes to soil improvement depends on a number of variables, including the type of microbial metabolism involved in the process, interactions with other microbes present in the environment, soil type, available nutrients, pH, temperature, pressure, concentration of ions, and the availability of oxygen and other oxidants. During development of a microbiological soil improvement technology, permanence/reversability of candidate microbial processes, oxygen availability (i.e., aerobic vs. anaerobic), feasibility of biostimulation or need for

bioaugmentation, delivery techniques of the necessary nutrients to the location of interest, energy consumption, environmental impacts, unanticipated side effects, public and regulatory acceptance should be considered. Thus, extensive laboratory and field study is required to verify the feasibility of candidate mechanisms for real world soil improvement problems.

Among the candidate microbiological soil improvement mechanisms discussed above, calcium carbonate precipitation through microbial denitrification was chosen for further study as part of this dissertation. Calcium carbonate precipitation through microbial denitrification was explored through stoichiometric analyses and bench scale laboratory experiments. Microbial denitrification can offer solutions to geotechnical engineering problems in anoxic zones below groundwater table and may especially applicable in mitigating the liquefaction potential of loose granular deposits. A brief background on the chemistry of calcium carbonate precipitation and the discussion of the results from the analysis and experiments performed as part of this study are presented in the subsequent chapters.

CHAPTER 4

CALCIUM CARBONATE PRECIPITATION THROUGH DENITRIFICATION

4.1. Introduction

Microbially induced precipitation of calcium carbonate for cementation of granular soils involves increasing the total carbonate content, the pH, or both in the pore water of the soil through metabolic activity. In this chapter, the basic principles underlying carbonate equilibrium and the conditions required for calcium carbonate precipitation are presented, along with the affects of denitrification on the geochemistry of the pore fluid medium through a detailed stoichiometric evaluation of energy and growth reactions.

4.2. Carbonate Equilibrium

Understanding aquatic chemistry principles is extremely important in estimating whether or not a change in a geosystem results in calcium carbonate precipitation. The same principles also apply when microorganisms are involved. In this section, the key geochemistry terms, including pH and alkalinity, are defined and followed by a discussion on the change in carbonate content of a liquid medium under various conditions. The discussions presented in this section on carbonate content assumes a closed carbonate system (i.e., no exchange of CO_2 between the aqueous system and the atmosphere or the air within the unsaturated zone overlying the groundwater table). This is a reasonable assumption because denitrification takes place at the anoxic zones below the groundwater table. Thermodynamic and kinetic aspects of calcium carbonate precipitation are also presented in this section.

4.2.1. pH, Alkalinity, Acidity, and Carbonate Content

The terms pH, alkalinity, acidity, and carbonate content are interrelated. Knowing only the pH of a solution may not give a lot of information about the solution, and at least one other parameter needs to measured along with the pH to estimate how that solution is going to behave when an acid is added or precipitation occurs.

The pH of a solution is related to the concentration of the protons (H^+) and defined as:

$$pH = -\log \{H^+\} \tag{4-1}$$

where $\{H^+\}$ = activity of the protons.

Alkalinity refers to acid-neutralizing capacity of a solution and is defined by the sum of the molar concentrations of all ionic species that can neutralize a proton (H^+). In practice, it is a measure of how much acid is needed to bring a solution to a certain pH. Total alkalinity for a solution in which the only basic species are carbonate species can be defined as (Butler, 1991):

$$\text{Alkalinity} = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-] - [H^+] \tag{4-2}$$

where [ ] = mol/L concentration of each species.

Alkalinity is typically reported in "mEq/L" (milliequivalents per liter, mEq/L, equals to millimoles of charge per liter, mM) or in "mg/L as $CaCO_3$." Alkalinity in mEq/L can be converted into mg/L as $CaCO_3$ by multiplying by 50 as $CaCO_3$/mEq. A reaction that produces protons (H^+) results in a decrease in alkalinity, as the produced H^+ binds with carbonate and bicarbonate ions, converting them to bicarbonate and carbonic acid, respectively. The resulting change in pH will depend on the remaining unneutralized H^+.

In parallel, acidity is expressed as the base-neutralizing capacity of a solution or the ability of a solution to donate protons, as follows:

$$\text{Acidity} = 2[H_2CO_3] + [HCO_3^-] + [H^+] - [OH^-] \quad (4\text{-}3)$$

Equations 4-2 and 4-3 can have other species if other components contribute to the acid/base neutralizing capacity. For example, acidity and alkalinity defined here do not account for the acid/base neutralization capacity of a solution due to solid particles.

Total inorganic carbon, C_T, and carbonate equilibria can be defined by the following equations:

$$C_T = [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}] \quad (4\text{-}4)$$

$$K_{a1} = \{HCO_3^-\}\{H^+\} / \{H_2CO_3\} \qquad \text{where } -\log K_{a1} = 6.3 \quad (4\text{-}5)$$

$$K_{a2} = \{CO_3^{2-}\}\{,H^+\} / \{HCO_3^-\} \qquad \text{where } -\log K_{a2} = 10.3 \quad (4\text{-}6)$$

The relative concentrations of each species (carbonic acid, H_2CO_3; bicarbonate, HCO_3^-; and carbonate, CO_3^{2-}) were shown in Figure 3-1, which was developed using the acid dissociation constants, K_a. These equations are important because they quantify the buffering capacity of carbonates. For instance, if protons are consumed through a microbial reaction or bases are added, the alkalinity and pH will increase because more HCO_3^- or CO_3^{2-} are formed from H_2CO_3 and HCO_3^-, respectively.

Microbial reactions often result in production of CO_2, a weak acid (e.g., by oxidation of organic matter with O_2 or NO_3^-). The production of CO_2 results in an increase in the total carbonate content (and, thus, increase in CO_3^{2-} concentration) in the solution and an increase in acidity. The net change in pH of the solution will depend on the buffering capacity of the solution. However, the addition of CO_2 to a solution does

not change the total alkalinity according to Equation 4-2. The removal of CO_2 from a solution (e.g., photosynthesis), on the other hand, will have a small effect on pH if initial pH is lower than 6.3 (i.e., no protons consumed – see Equation 4-4) and a higher impact on pH if the initial pH is greater than 10.3.

If CO_3^{2-} is removed from the solution (e.g., precipitation of a carbonate mineral from a supersaturated solution), total inorganic carbon and alkalinity will decrease, as will the pH. When CO_3^{2-} is added to the solution (e.g., dissolution of a carbonate mineral), the total inorganic carbon and alkalinity increase. When carbonate goes into solution, the carbonate ions in the solution will bond with H^+ ions and the pH will increase.

4.2.2. Calcium Carbonate Precipitation

Calcium carbonate is a common carbonate mineral found in nature and can be precipitated out of a solution abiotically or biologically. Solubility equilibrium for calcium carbonate is presented by the following equation:

$$CaCO_3\ (s) \leftrightarrow Ca^{2+} + CO_3^{2-} \qquad (4\text{-}7)$$

where the ionic species of calcium and carbonate are in equilibrium with the solid phase (calcium carbonate) through an equilibrium constant. The solubility equilibrium can be expressed by using thermodynamic ion activities or by molar concentrations of ionic species (Rittmann et al., 2002). If the ionic activities are assumed to be equal to the molar concentrations, the solubility product, K_{sp}, for calcium carbonate can be defined as:

$$K_{sp} = [Ca^{2+}][CO_3^{2-}] \qquad (4\text{-}8)$$

where $[Ca^{2+}]$ and $[CO_3^{2-}]$ are the molar concentration of calcium and carbonate ions in the solution, respectively.

The solubility product of calcium carbonate is reported to vary from $10^{-8.58}$ to $10^{-7.78}$, depending on temperature, salinity, and type of calcium carbonate polymorph (e.g., calcite, aragonite, vaterite). Theoretically, precipitation of calcium carbonate starts if the product of the concentrations of the calcium and carbonate ions exceed the solubility product. However, energy is required for the formation of new surfaces associated with nucleation of calcium carbonate crystals. If the liquid solution consists of microorganisms or other particles or if the liquid is in contact with a solid surface, the energy required for nucleation is reduced. Once nucleation begins, kinetics control precipitation as long as the Ca^{2+} and CO_3^{2-} concentrations remain super-saturated (Rittmann et al., 2002).

Calcite and aragonite are the most common among the several different polymorphs of calcium carbonate found in nature as part of sedimentary rocks, shells, and corals. Vaterite can also be encountered, especially in gallstones (Bills, 1985), repair tissues of shells of gastropods (Carlson, 1983), and in sediments precipitated by some microorganisms (Rivadeneyra et al., 1991). Among these calcium carbonate polymorphs, calcite is thermodynamically the most stable, and transformation to calcite from less stable forms of calcium carbonate (e.g., aragonite, and vaterite) occurs through dissolution and re-precipitation. The physical properties of the most common polymorphs of calcium carbonate relevant to the discussion presented herein are presented in Table 4-1.

Table 4-1. Polymorphs of calcium carbonate

$CaCO_3$	Density (g/cm^3)	Thermodynamic Solubility Product [1] at 25°C, $-\log K_{sp}$	Hardness[2] (Mohs Scale)
Calcite	2.71	8.33 – 8.58	3
Aragonite	2.93	8.18 – 8.42	3.5 – 4
Vaterite	2.54	7.78	3

(1) Driessens and Verbeeck (1990)
(2) Harrison (2005)

Lowenstam and Weiner (1989) indicate that a precursor phase of the precipitated mineral, which is thermodynamically less stable than the mature form of the mineral, forms first during a sequential precipitation process. Thus, formation of calcite should be expected to take place at a later stage in calcium carbonate development than aragonite and vaterite, and the relative abundance of these polymorphs depends on temperature and the presence of inhibitors. Figure 4-1 indicates that vaterite and calcite are present in almost equal amount at atmospheric pressure when the temperature is around 25°C. However, Morse (1983) suggested that organic acids may decrease the reaction rate and favor the precipitation of calcite over other polymorphs of calcium carbonate. Rivadeneyra et al. (1991) also observed an increase in the relative amount of vaterite to calcite with increasing NaCl concentration. Bills (1985) reported that solutions left unstirred resulted in precipitation in the form of almost all calcite and stirred solutions yielded a mixture of calcite and vaterite.

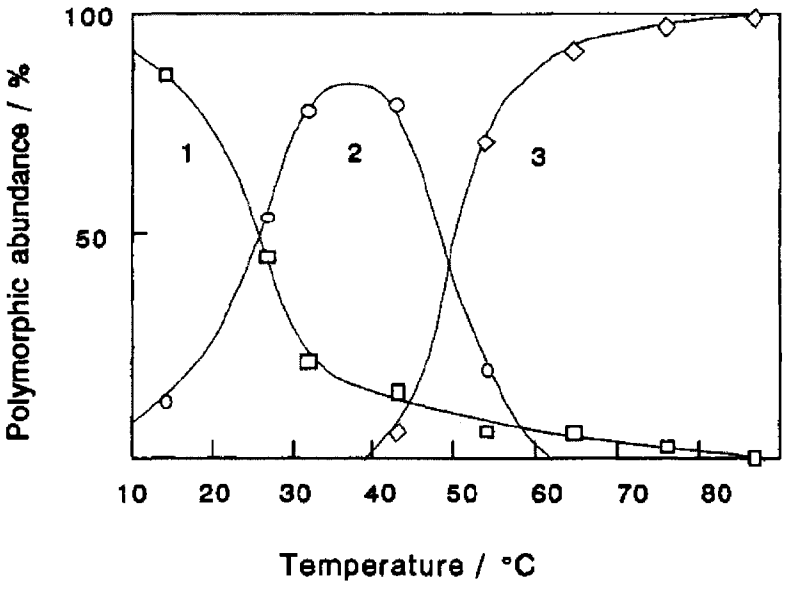


Fig. 4-1. Relative abundance of polymorphs of calcium carbonate based on temperature (Sawada, 1997):(1) calcite; (2) vaterite; and (3) aragonite

In addition to organic acids, NaCl concentration, or mixing of the solution, Mg^{2+} and PO_4^{3-} ions were reported to have inhibitive effects on bacterial precipitation of calcium carbonate (Rivadeneyra et al., 1985a; Rivadeneyra et al., 1985b). Regardless of which form of calcium carbonate is abundant during initial crystallization, the metastable polymorphs eventually transform into calcite in the absence of inhibitors. The presence of Mg^{2+}, organics, and microorganisms affects this transformation and results in occurrences of aragonite and vaterite minerals in the nature.

Although the effect of different surface reactivity of these different polymorphs on mineral growth, dissolution, contaminant uptake, and adsorption has been studied (Sawada, 1997; Warren et al. 2001), research on the effect of relative abundance of these polymorphs on shear strength and stiffness is limited. Harrison (2005) indicates that

calcite and vaterite are relatively softer than aragonite based on the Mohs scale of hardness, a scale commonly used among geologists to describe the relative scratch resistance of minerals (Table 4-1). On the other hand, because the occurrence of vaterite is reported to be assisted by biological activity in nature (Rodriguez-Navaarro et al., 2007), further research is needed to evaluate the effects on the relative abundance of these different polymorphs on engineering properties of a bulk soil cemented with calcium carbonate.

4.3. Denitrification

In addition to promoting nucleation by their physical presence, some microorganisms can directly affect the carbonate concentration of a solution through production of carbon dioxide and alkalinity as a result of their metabolic activity, as discussed previously. A significant amount of recent research regarding the use of microbial calcium carbonate precipitation as a soil improvement mechanism has focused on the use of ureolysis to precipitate calcium carbonate (Whiffin, 2004; DeJong et al., 2006; Whiffin et al., 2007). The research indicates that the drained and undrained shear strength and stiffness of granular soils can significantly be increased by calcium carbonate precipitated through ureolysis. Because the production of urease by soil microorganisms is retarded by lack of oxygen (McCarty and Bremner, 1991), application of ureolysis for soil improvement below groundwater table (i.e., in an anaerobic environment) is suspect. Furthermore, Van Paassen et al. (2008) report that production of large amount of ammonium during ureolysis may pose environmental and health risks, in addition to high costs associated with the treatment of ammonium in groundwater.

As an alternative to employing ureolysis, microbial denitrification can be employed to induce calcium carbonate precipitation within anaerobic zones, as discussed in the previous chapter. Early studies on microbial denitrification date back to late 19th century (e.g., Gayon and Dupetit, 1886), and a significant amount of literature has accumulated since then because of the importance of denitrification for agricultural soils and to the overall nitrogen cycle. Many different bacteria genera are capable of denitrification, including *Alcaligenes, Bacillus, Denitrobacillus, Thiobacillus, Pseuodomonas, Spirillum, Micrococcus,* and *Achromobacter.* Microorganisms capable of denitrification are typically facultative anaerobes (i.e., are capable of switching to nitrate as a terminal electron acceptor when oxygen is limiting). This flexibility in their growth strategy provides them with a competitive advantage over the strictly anaerobic microorganisms. Denitrifiers are widespread and constitute approximately 20 percent of the bacterial population capable of anaerobic growth and 1 to 5 percent of the total heterotrophic bacteria population that can be isolated (Tiedje et al., 1982). In addition, the diversity in the denitrifying bacterial population results in the capability to utilize a wide range of electron donors, including methanol, ethanol, acetone, sugar, neutralized acetic acid, glucose, methane, amino acids, raw domestic wastewater, wastes from food industries, and sludge (McCarty et al., 1969; Kornaros et al., 1996).

The terms denitrification and nitrate reduction have sometimes been used interchangeably in the literature. In this study, the term "denitrification" refers to dissimilatory reduction of nitrate (NO_3^-) to nitrogen gas (N_2) through intermediates, including nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O). Note that some

microorganisms perform dissimilatory nitrate ammonification (reduction of NO_3^- to NH_3) or one-step nitrate reduction (reduction of NO_3^- to NO_2^-); these processes are not included under the term denitrification herein. In addition to denitrification, assimilatory reduction of NO_3^- to cellular nitrogen coupled with denitrification also takes place for cell synthesis in the absence of more reduced nitrogen sources (e.g., NH_4^+).

Each step of denitrification is catalyzed by different enzymes. The enzymes that catalyze these reduction reactions, along with their cofactors, are presented in Table 4-2. Synthesis of the enzymes involved in denitrification depends on various factors, including O_2 content, pH, temperature, and concentration of metal ions. Thus, the rate of heterotrophic denitrification is affected by pH. The optimum pH for most denitrifying strains appears to be between 7 and 8 (Lee and Rittmann, 2003). Lee and Rittmann (2003) also report that heterotrophic denitrification can take place in environments with pH as high as 11, but that intermediates may accumulate outside the optimal pH range. Accumulation of intermediates can be important during denitrification, both from an engineering perspective and an environment/health perspective. From an engineering perspective, only the step that reduces NO_2^- to NO generates alkalinity, and accumulation of intermediates can result in insufficient production of alkalinity and inorganic carbon required for calcium carbonate precipitation. From an environment/health perspective, accumulation of intermediates may become important because increased concentrations of NO_2^- may lead to production of nitrosamines, some of which are carcinogenic (Lijinski, 1977). Thus, the maximum contaminant level (MCL) established by the US

Table 4-2. Steps of denitrification (adapted from Rittmann and McCarty, 2001)

Half Reaction	Catalyzing Enzyme	Cofactors[1]
$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O$	Nitrate reductase	Iron and Molybdenum
$NO_2^- + e^- + 2H^+ \rightarrow NO^- + H_2O$	Nitrite reductase	Iron or Copper
$2NO^- + 2e^- + 2H^+ \rightarrow N_2O + H_2O$	Nitric oxide reductase	Iron
$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O$	Nitrious oxide reductase	Copper

(1) Granger and Ward (2003)

Environmental Protection Agency (EPA) for nitrite (1 mg-N/L) for safe drinking water is much less than the MCL for nitrate (10 mg-N/L) (USEPA, 2001). Also, N_2O gas (g), which may also accumulate due to inhibition of denitrification, is a greenhouse gas and is estimated to contribute to 5 percent of global warming (Bouwman, 1989).

The increase in alkalinity and pH due to denitrification results in increased carbonate (CO_3^-) content of the liquid medium, and the additions of inorganic carbon can enhance this effects. Calcium present in the environment may then precipitate if nucleation sites are available and the solubility product is reached. Researchers have reported precipitation as a result of denitrification in the laboratory and in the field. MacCallum and Guhathakurta (1970) attribute the origins of carbonate sediments on the Bahama Bank to denitrification, though it is not clear whether these carbonate sediments resulted from chemically or microbially induced precipitation. Lee and Rittmann (2003) observed precipitation of calcium carbonate as a result of autohydrogenotrophic denitrification within hollow fiber membrane biofilm reactors. Calcium carbonate precipitation was also reported by Castanier et al. (2000) for denitrification experiments with *Bacillus cereus*. These laboratory findings, as well as the bioclogging and mineral precipitation problems reported for *in situ* permeable reactive barriers that employ denitrification (Israel, 2006), suggest that denitrification is a promising mechanism for calcium carbonate precipitation for soil improvement purposes in anaerobic conditions below the groundwater table. The stoichiometric analysis performed as part of this study to further understand the feasibility of denitrification as a ground improvement technology are discussed in details in the subsequent sections of this chapter.

4.4. Stoichiometric Analysis

Stoichiometric analysis provides insight to the relationship between bacterial growth and the amount of energy released during a microbial oxidation-reduction reaction. Even in the absence of any laboratory test results, a stoichiometric sensitivity analysis can be carried out to quantify bacterial growth, the consumption of electron donor and acceptor, the production of intermediates, and the changes in alkalinity, pH, and inorganic carbon. With the help of actual laboratory data, a complete model predicting time-rate of these changes can also be developed. The stoichiometric analyses presented in this section serve to (a) establish the basis for the batch reactor experiments discussed subsequently; (b) provide an understanding of the reaction kinetics and bacterial growth during dentirification; and (c) evaluate the feasibility of microbial denitrification as a calcium carbonate precipitation for ground improvement. However, development of a detailed biogeochemical model for denitrification accounting for precipitation/dissolution reactions and predicting time rate of changes in geochemistry of the medium is beyond the scope of this study.

Bacteria can utilize reduced organic or inorganic substrates as an electron donor (e.g., glucose, acetate, alanine, ethanol, formate, H_2) and generate energy through transfer of electrons from an electron donor to an electron acceptor (e.g., oxygen, nitrate, nitrite, sulfate, iron). The energy gained through this transfer is used in cellular maintenance, growth, and other microbial functions. Growth, or new cell synthesis, also requires a source of carbon and some of the donor's electrons. The ratio of donor electrons used in reduction and oxidation (redox) reactions that generate energy (catabolic reactions) to

those used for microbial growth (anabolic reactions) depends on the free energy available per one electron transfer from a donor to an acceptor and the energy transfer efficiency. Assuming a constant energy-transfer efficiency, as the available free energy per one electron transfer increases, the portion of electrons transferred to an electron acceptor, f_e^o, required to yield the same amount of new cells for a given electron acceptor goes down. This results in more electrons available for cell synthesis, f_s^o, and a higher microbial yield per one mole of substrate for more reduced electron donors. Microbial yield can be calculated based on stoichiometry, it is expressed as "true yield (Y)," or deduced from laboratory data and expressed as "net yield (Y_n)." The net yield differs from the true yield because some amount of energy is used to support non-growth activities. These non-growth activities are generally called " cell maintenance."

The stoichiometric analysis presented in this section includes evaluation of energy, synthesis, and overall growth reactions for denitrification of different electron donors. Because denitrification is a stepwise process, reduction of nitrate to nitrite, the subsequent reduction of nitrite to nitrogen gas, and the overall reduction of nitrate to nitrogen gas are analyzed independently to provide an insight to the roles of different steps in denitrification affecting the biogeochemistry.

4.4.1. Example Reaction Energetics for Denitrification

Sample stoichiometric calculations are presented in this section for denitrification for one-step reduction from nitrate to nitrogen gas and a two-step reduction using nitrite as an intermediate. For the sample calculations, glucose was employed as the electron donor because it is one of the most common and readily available organic electron

donors. Production of alkalinity, production of CO_2 gas, and microbial yield, all related to microbial calcium carbonate precipitation, are then evaluated based on the results of the stoichiometric analysis of the denitrification of glucose in this section of the dissertation. The results are compared to those from denitrification of other electron donors, including, L-glutamic acid, alanine, acetate, and ethanol in a subsequent section.

Electron donor and acceptor half reactions form the foundation of the energy reaction and are required to estimate the energy generated per one electron transfer (i.e., energy per electron equivalent; kJ/e^- eq). The units (kJ/e^- eq) can be converted to energy per mole using the number of electron equivalents per mole of substrate. The donor half reaction, R_d, for glucose ($C_6H_{12}O_6$) is (Rittmann and McCarty, 2001):

$$R_d:\ 0.25CO_2 + H^+ + e^- \rightarrow 0.042C_6H_{12}O_6 + 0.25H_2O \text{ with } \Delta G^{o'} = 41.35 \text{ kJ/}e^- \text{ eq} \quad (4\text{-}9)$$

where $\Delta G^{o'}$ is Gibb's standard free energy at pH=7.0. The acceptor half reaction, R_a, for nitrate (NO_3^-) to nitrogen gas is (Rittmann and McCarty, 2001):

$$R_a:\quad 0.2NO_3^- + 1.2H^+ + e^- \rightarrow 0.1N_2 + 0.6H_2O \text{ with } \Delta G^{o'} = -72.2 \text{ kJ/}e^- \text{ eq} \quad (4\text{-}10)$$

Then, the energy reaction, R_e, for oxidation of glucose with nitrate reduction to nitrogen gas can be represented by:

$$R_e = R_a - R_d \quad (4\text{-}11)$$

$$R_e:\quad 0.042C_6H_{12}O_6 + 0.2NO_3^- + 0.2H^+ \rightarrow 0.25CO_2 + 0.1N_2 + 0.35\ H_2O \quad (4\text{-}12)$$

with ΔG_r= -113.6 kJ/e^- eq (i.e., energy released per one electron of donor oxidized). Equation (4-12) indicates that, for each mole of NO_3^- reduced to N_2, 1 mole of H^+ is consumed, which is equivalent to the production of alkalinity as 0.05 g as $CaCO_3$ per 1 mmol NO_3^- consumed. In addition, 1.25 mole of CO_2 is produced. At near neutral pH,

only 0.04 percent is present as CO_3^- (Figure 3-1), which means that the CO_2 produced by Equation 4-12 absorbs the base produced to form HCO_3^-.

Assuming that nitrate is being used as a nitrogen source, the cell half reaction can be written as (Rittmann and McCarty, 2001):

$$R_c: \quad 0.036NO_3^- + 0.179CO_2 + 1.036H^+ + 1e^- \rightarrow 0.036C_5H_7O_2N + 0.393H_2O \qquad (4\text{-}13)$$

Then, the synthesis reaction is:

$$R_s = R_c - R_d \qquad (4\text{-}14)$$

$$R_s: \quad 0.042C_6H_{12}O_6 + 0.036NO_3^- + 0.036H^+ \rightarrow 0.036C_5H_7O_2N + 0.071CO_2 + 0.143H_2O \qquad (4\text{-}15)$$

Note that the energy and synthesis reactions are per one electron transferred from the donor to the acceptor. Thus, the portion of donor electrons used in energy reactions, f_e^o, and the portion of donor electrons used in synthesis reactions, f_s^o, should be determined to combine these two reactions to generate overall growth reaction per unit electron transfer.

The values of f_s^o and f_e^o can be estimated by balancing the energy released from the energy reaction to the energy consumed for synthesis. Assuming an energy transfer efficiency, ε, of 0.55, which is within the range of suggested by Rittmann and McCarty (2001) under optimum conditions, the energy required for synthesis of new cells can be estimated as outlined by Rittmann and McCarty (2001) wherein the cell synthesis is simplified into two steps; conversion of the carbon source to pyruvate and conversion of pyruvate to cellular carbon. The energy required to convert the carbon source to

pyruvate, ΔG_p, can be calculated based on free energies available for the carbon source and the pyruvate:

$$\Delta G_p = (35.09 \text{ kJ/e}^- \text{ eq}) - \Delta G^{o'} \quad \text{where } \Delta G^{o'} = 41.35 \text{ kJ/e}^- \text{ eq for glucose} \qquad (4\text{-}16)$$

$$\Delta G_p = -6.26 \text{ kJ/e}^- \text{ eq}$$

When nitrate is used as a source of cellular nitrogen, approximately 0.036 moles of new cells is formed through 1 e^- is transfer from the donor (Equation 4-13), equivalent to 4.036 g cells/e^- eq (i.e., 0.036 moles/e^- eq × 113 g cells/mole = 4.036 g cells/e^- eq). The energy required to convert pyruvate to cellular carbon is 3.33 kJ/g cells (McCarty, 1971). Then, 1 e^- equivalent of the energy required to convert pyruvate to cellular carbon, ΔG_{pc}, is:

$$\Delta G_{pc} = 3.33 \text{ kJ/g cells} \times 4.04 \text{ g cells/e}^- \text{ eq} = 13.44 \text{ kJ/e}^- \text{ eq} \qquad (4\text{-}17)$$

Hence, the total energy needed for cell synthesis, ΔG_s, is:

$$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon) \qquad (4\text{-}18)$$

where $n = +1$ if ΔG_p is positive and -1 if ΔG_p is negative.

$$\Delta G_s = [(-6.26 \text{ kJ/e}^- \text{ eq}) / (0.55^{-1})] + [(13.44 \text{ kJ/e}^- \text{ eq}) / (0.55)]$$

$$\Delta G_s = 20.99 \text{ kJ/e}^- \text{ eq}$$

This amount of energy has to be provided by the oxidation of the electron donor. The amount of energy generated through oxidation of glucose with nitrate, ΔG_r, through one electron transfer is:

$$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'} \qquad (4\text{-}19)$$

where $\Delta G_a^{o'}$ = e^- acceptor Gibb's free energy; -72.2 kJ/e^- eq for $NO_3^- \rightarrow N_2$ reduction;

$\Delta G_d^{o'}$ = e^- donor Gibb's free energy; 41.35 kJ/e^- eq for glucose.

Thus, ΔG_r = -113.55 kJ/e^- eq

The portion of electron donor that needs to be oxidized to supply energy required for cell synthesis, A, is also equal to the ratio of donor electrons used in energy reactions to those used in cell synthesis reactions (f_e^o / f_s^o):

$$A = - \Delta G_s / (\varepsilon \Delta G_r) \quad (4\text{-}20)$$

$$A = - 20.99 / (0.55 \times - 113.55) = 0.34$$

$$A = 0.34 = f_e^o / f_s^o \quad \text{and} \quad (4\text{-}21)$$

$$f_e^o + f_s^o = 1 \quad \text{because the reaction is written per 1 } e^- \text{ equivalent} \quad (4\text{-}22)$$

Solving Equations 4-21 and 4-22 together results in $f_e^o = 0.25$ and $f_s^o = 0.75$, indicating 25 percent of the donor electrons are used in energy production and the rest for new cell synthesis.

The overall growth reaction, R, can be written by combining Equations 4-12 and 4-15 as follows:

$$R = (f_e^o \times R_e) + (f_s^o \times R_s) \quad (4\text{-}23)$$

where,

$$f_e^o \times R_e: \ 0.01C_6H_{12}O_6 + 0.05NO_3^- + 0.05H^+ \rightarrow 0.0625CO_2 + 0.025N_2 + 0.088H_2O \quad (4\text{-}24)$$

$$f_s^o \times R_s: \ 0.032C_6H_{12}O_6 + 0.027NO_3^- + 0.027H^+ \rightarrow 0.027C_5H_7O_2N + 0.053CO_2 + 0.107H_2O \quad (4\text{-}25)$$

Comparison of Equations 4-24 and 4-25 indicate that almost equal amount of CO_2 is produced by both reactions. The ratio of the stoichiometric coefficients for NO_3^- suggest that almost 65 percent of the total nitrate is consumed in the energy reactions and

converted to nitrogen gas. The rest is used as nitrogen for new cell synthesis. In addition, approximately 65 percent of the H^+ consumed during denitrification of glucose is during energy reactions.

By combining Equations 4-24 and 4-25, the overall growth reaction is:

$$0.042C_6H_{12}O_6+0.077NO_3^-+0.077H^+ \rightarrow 0.027C_5H_7O_2N+0.116CO_2+0.025N_2 + 0.195H_2O \quad (4\text{-}26)$$

Equation 4-26 indicates, that for each mole of glucose oxidized, 1.83 moles of nitrate and acidity (H^+) are consumed. The microbial yield, then, is:

$$Y = (0.027/0.042) \times 113 \text{ g cells/mol} = 73 \text{ g cells/mol glucose} \quad (4\text{-}27)$$

So, the true yield, Y, can be expressed as 73 g cells/mol glucose or 0.41 g cells/g glucose.

If denitrification is analyzed in two separate steps, the energy reactions would be:

$NO_3^- \rightarrow NO_2^-$:

$$R_e: \quad 0.042C_6H_{12}O_6 + 0.5NO_3^- \rightarrow 0.5NO_2^- + 0.25CO_2 + 0.25\ H_2O \quad (4\text{-}28)$$

with $\Delta G_r = -83.6$ kJ/e^- eq; and

$NO_2^- \rightarrow N_2$:

$$R_e: \quad 0.042C_6H_{12}O_6 + 0.33NO_2^- + 0.33H^+ \rightarrow 0.167N_2 + 0.25CO_2 + 0.417\ H_2O \quad (4\text{-}29)$$

with $\Delta G_r = -133.91$ kJ/e^- eq.

Although 0.25 moles of weak acid is produced through one electron transfer, alkalinity is produced (i.e., consumption of 0.33 moles of H^+) only during nitrite to nitrogen gas reduction. Also, the energy generated during nitrite to nitrogen gas reduction is almost 60 percent higher than the energy generated during nitrate to nitrite reduction through transfer of one electron.

The synthesis reactions for the two step denitrification process would be as follows:

$NO_3^- \rightarrow NO_2^-$ (NO_3^- as source of nitrogen):

R_s: $0.042C_6H_{12}O_6+0.036NO_3^-+0.036H^+ \rightarrow 0.036C_5H_7O_2N+0.071CO_2+0.143H_2O$ (4-30)

$NO_2^- \rightarrow N_2$ (NO_2^- as source of nitrogen):

R_s: $0.042C_6H_{12}O_6+0.038NO_2^-+0.038H^+ \rightarrow 0.038C_5H_7O_2N+0.058CO_2+0.135H_2O$ (4-31)

Following the same procedure outlined above, [f_e^o, f_s^o] can be estimated as [0.32, 0.68] and [0.24, 0.76] for nitrate to nitrite reduction and for nitrite to nitrogen gas reduction, respectively. The results from these calculations are summarized in Table 4-3. Then, the overall growth reaction for these two steps are:

$NO_3^- \rightarrow NO_2^-$:

$0.042C_6H_{12}O_6+0.184NO_3^-+0.024H^+ \rightarrow 0.024C_5H_7O_2N+0.16NO_2^-+0.128CO_2+0.177H_2O$ (4-32)

$NO_2^- \rightarrow N_2$:

$0.042C_6H_{12}O_6+0.108NO_2^-+0.108H^+ \rightarrow 0.029C_5H_7O_2N+0.04N_2+0.104CO_2+0.203H_2O$ (4-33)

Comparison of the two overall growth reactions presented in Equations 4-32 and 4-33 indicate that relatively less CO_2 is produced and more acidity (H^+) is consumed during nitrite-to-nitrogen reduction than nitrate-to-nitrite reduction. The microbial yield is also greater for the second step of denitrification (Table 4-3).

Table 4-3. Reaction energetics for denitrification of glucose ($\varepsilon = 0.55$)

Electron Donor	Electron Acceptor	ΔG_r (kJ/e^- eq)	ΔH^+ (mol/e^- eq)	ΔCO_2 (mol/e^- eq)	f_e^o	f_s^o	Y (g cells/ g donor)
Glucose	$NO_3^- \rightarrow N_2$	-113.55	-0.077	0.116	0.25	0.75	0.41
	$NO_3^- \rightarrow NO_2^-$	-83.0	-0.024	0.128	0.32	0.68	0.36
	$NO_2^- \rightarrow N_2$	-133.91	-0.108	0.104	0.24	0.76	0.43

ε : Energy transfer efficiency (assumed to be 0.55)

ΔG_r : Equivalent energy generated through oxidation of glucose

ΔH^+ : Acidity consumed/produced (-/+) during overall growth reaction

ΔCO_2 : Carbon dioxide gas consumed/produced (-/+) during overall growth reaction

f_e^o and f_s^o: Portion of donor electrons used in energy and synthesis reactions, respectively ($f_e^o + f_s^o = 1$)

Y : True microbial yield

4.4.2. Stoichiometric Analysis of Denitrification with Different Electron Donors

The procedure outlined in Section 4.2.1 was followed to investigate the effects of utilizing other electron donors, including L-glutamic acid, ethanol, alanine (representing the contents of nutrient broth medium), and acetate, for denitrification. The energy, synthesis, and overall growth reactions for reduction of nitrate to nitrogen gas are given below. The details of the stoichiometric analysis for these reactions and the analysis of two-step denitrification with these different electron donors are presented in Appendix A.

(a) The energy (R_e), synthesis (R_s), and overall growth (R) reactions if Glucose is used as an electron donor are:

$$R_e: 0.042C_6H_{12}O_6 + 0.2NO_3^- + 0.2H^+ \rightarrow 0.25CO_2 + 0.1N_2 + 0.35\ H_2O \quad (4\text{-}34)$$

$$R_s: 0.042C_6H_{12}O_6 + 0.036NO_3^- + 0.036H^+ \rightarrow 0.036C_5H_7O_2N + 0.071CO_2 + 0.143H_2O \quad (4\text{-}35)$$

$$R: 0.042C_6H_{12}O_6 + 0.077NO_3^- + 0.077H^+ \rightarrow 0.027C_5H_7O_2N + 0.116CO_2 + 0.025N_2 + 0.195H_2O \quad (4\text{-}36)$$

(b) The energy (R_e), synthesis (R_s), and overall growth (R) reactions if L-glutamic acid is used as an electron donor are:

$$R_e: 0.056COOHCH_2CH_2CHNH_2COO^- + 0.2NO_3^- + 0.311H^+ \rightarrow 0.278CO_2 + 0.1N_2 + 0.056NH_4^+ + 0.35\ H_2O \quad (4\text{-}37)$$

$$R_s: 0.056COOHCH_2CH_2CHNH_2COO^- + 0.036NO_3^- + 0.147H^+ \rightarrow 0.036C_5H_7O_2N + 0.056NH_4^+ + 0.099CO_2 + 0.06H_2O \quad (4\text{-}38)$$

$$R: 0.056COOHCH_2CH_2CHNH_2COO^- + 0.095NO_3^- + 0.206H^+ \rightarrow 0.023C_5H_7O_2N + 0.056NH_4^+ + 0.036N_2 + 0.164CO_2 + 0.134\ H_2O \quad (4\text{-}39)$$

(c) The energy (R_e), synthesis (R_s), and overall growth (R) reactions if Ethanol is used as an electron donor are:

R_e: $0.083CH_3CH_2OH + 0.2NO_3^- + 0.2H^+ \rightarrow 0.167CO_2 + 0.1N_2 + 0.35\ H_2O$ (4-40)

R_s: $0.083CH_3CH_2OH + 0.036NO_3^- + 0.012CO_2 + 0.036H^+ \rightarrow 0.036C_5H_7O_2N + 0.143H_2O$ (4-41)

R: $0.083CH_3CH_2OH + 0.094NO_3^- + 0.094H^+ \rightarrow 0.023C_5H_7O_2N + 0.036N_2 + 0.052CO_2 +$ $+ 0.217\ H_2O$ (4-42)

(d) The energy (R_e), synthesis (R_s), and overall growth (R) reactions if Alanine is used as an electron donor are:

R_e: $0.083CH_3CHNH_2COO^- + 0.2NO_3^- + 0.367H^+ \rightarrow 0.25CO_2 + 0.1N_2 + 0.083NH_4^+ +$ $0.267H_2O$ (4-43)

R_s: $0.083CH_3CHNH_2COO^- + 0.036NO_3^- + 0.202H^+ \rightarrow 0.036C_5H_7O_2N + 0.083NH_4^+ +$ $0.071CO_2 + 0.06H_2O$ (4-44)

R: $0.083CH_3CHNH_2COO^- + 0.094NO_3^- + 0.261H^+ \rightarrow 0.023C_5H_7O_2N + 0.083NH_4^+ +$ $0.035N_2 + 0.135CO_2 + 0.133\ H_2O$ (4-45)

(e) The energy (R_e), synthesis (R_s), and overall growth (R) reactions if Acetate is used as an electron donor are:

R_e: $0.125CH_3COO^- + 0.2NO_3^- + 0.325H^+ \rightarrow 0.25CO_2 + 0.1N_2 + 0.35H_2O$ (4-46)

R_s: $0.125CH_3COO^- + 0.036NO_3^- + 0.161H^+ \rightarrow 0.036C_5H_7O_2N + 0.071CO_2 + 0.143H_2O$ (4-47)

R: $0.125CH_3COO^- + 0.103NO_3^- + 0.228H^+ \rightarrow 0.021C_5H_7O_2N + 0.041N_2 + 0.145CO_2 +$ $0.228H_2O$ (4-48)

All these reactions, along with the stoichiometric analyses presented in Appendix A are summarized in Table 4-4. The equivalent amount of acidity consumption, ammonium production, carbon dioxide production, and the microbial yield are presented in Table 4-4 for the two generalized steps of denitrification ($NO_3^- \rightarrow NO_2^-$ and $NO_2^- \rightarrow N_2$) and the overall denitrification reaction ($NO_3^- \rightarrow N_2$). The amount of calcium carbonate that can be precipitated is also shown in Table 4-4 for overall denitrification, assuming that sufficient amount of calcium ions are available in the medium and that production of either alkalinity or CO_2 controls the amount of precipitation.

For example, according to Table 4-4, when glucose is used as an electron donor, 0.024 mol of H^+ is consumed and 0.104 mol of CO_2 is produced per 1 e^- transfer during reduction of NO_3^- to NO_2^-. Sixty eight (68) percent of the donor electrons used during this reduction is consumed for cell synthesis and the resulting microbial yield is 0.36 g of cells per 1 g of donor. The consumption of H^+ (0.108 mol/e^- eq) is greater (i.e., more alkalinity is produced) during NO_2^- to N_2 reduction, in comparison to the reduction of NO_3^- to NO_2^-. When adjusted by the amount of electrons transferred in each step (i.e., $NO_3^- \rightarrow NO_2^-$: 2 e; and $NO_2^- \rightarrow N_2$: 3 e^-), the second step of denitrification also results in more CO_2 than the first step:

$NO_3^- \rightarrow NO_2^-$: (0.128 mol CO_2/e^- eq) × (2 e^- eq/$N_{reduced}$) = 0.248 mol $CO_2/N_{reduced}$; and

$NO_2^- \rightarrow N_2$: (0.104 mol CO_2/e^- eq) × (3 e^- eq/$N_{reduced}$) = 0.312 mol $CO_2/N_{reduced}$.

When the overall denitrification of glucose is complete ($NO_3^- \rightarrow N_2$), 0.077 mol/e^- eq of H^+ is consumed and 0.104 mol of CO_2 is produced.

Table 4-4. Reaction energetics and stoichiometric analysis of denitrification ($\varepsilon = 0.55$)

e^- donor	e^- acceptor	ΔH^+ (mol/e^- eq)	ΔNH_4^+ (mol/e^- eq)	ΔCO_2 (mol/e^- eq)	f_e^o	f_s^o	Y g cells/ g donor	g-$CaCO_3$ (s) per g-NO_3^- reduced (1) Alk.is limiting	g-$CaCO_3$ (s) per g-NO_3^- reduced (2) CO_2 is limiting
Glucose	$NO_3^- \rightarrow NO_2^-$	-0.024	--	0.128	0.32	0.68	0.36		
	$NO_2^- \rightarrow N_2$	-0.108	--	0.104	0.24	0.76	0.43		
	$NO_3^- \rightarrow N_2$	-0.077	--	0.116	0.25	0.75	0.41	0.8	2.4
L-Glutamic Acid	$NO_3^- \rightarrow NO_2^-$	-0.131	0.056	0.179	0.45	0.55	0.28		
	$NO_2^- \rightarrow N_2$	-0.248	0.056	0.149	0.33	0.67	0.36		
	$NO_3^- \rightarrow N_2$	-0.206	0.056	0.164	0.36	0.64	0.32	1.7	2.8
Ethanol	$NO_3^- \rightarrow NO_2^-$	-0.020	--	0.067	0.44	0.56	0.59		
	$NO_2^- \rightarrow N_2$	-0.136	--	0.038	0.33	0.67	0.76		
	$NO_3^- \rightarrow N_2$	-0.094	--	0.052	0.36	0.64	0.68	0.8	0.9
Alanine	$NO_3^- \rightarrow NO_2^-$	-0.187	0.083	0.149	0.44	0.56	0.31		
	$NO_2^- \rightarrow N_2$	-0.301	0.083	0.121	0.33	0.67	0.39		
	$NO_3^- \rightarrow N_2$	-0.261	0.083	0.135	0.35	0.65	0.35	2.2	2.3
Acetate	$NO_3^- \rightarrow NO_2^-$	-0.143	--	0.161	0.50	0.50	0.27		
	$NO_2^- \rightarrow N_2$	-0.275	--	0.131	0.38	0.62	0.37		
	$NO_3^- \rightarrow N_2$	-0.228	--	0.145	0.41	0.59	0.32	1.8	2.3

ε : Energy transfer efficiency (assumed to be 0.55)

ΔG_r : Free energy released per equivalent of donor oxidized

ΔH^+ : Acidity consumed/produced (-/+) during overall growth reaction

ΔNH_4^+ : Ammonium consumed/produced (-/+) during overall growth reaction

ΔCO_2 : Carbon dioxide gas consumed/produced (-/+) during overall growth reaction

f_e^o and f_s^o: Portion of donor electrons used in energy and synthesis reactions, respectively ($f_e^o + f_s^o = 1$)

Y : True microbial yield

g-$CaCO_3$ (s) per g-$NO_3^-_{reduced}$: Estimated amount of $CaCO_3$ that can be precipitated per 1 g of NO_3^- reduced during overall denitrification (assuming that sufficient amount of Ca^{2+} is available):

(1) when alkalinity is limiting the precipitation (i.e., all of the produced alkalinity is consumed through $CaCO_3$ precipitation)

(2) when carbon is limiting the precipitation (i.e., all of the produced CO_2 is consumed through $CaCO_3$ precipitation)

Table 4-4 indicates that, with all the electron donors analyzed in this study, production of alkalinity (i.e., consumption of acidity or ΔH^+) and CO_2, needed for carbonate precipitation, takes place when the overall growth reactions are considered. Although the production of CO_2 affects the net change in pH at the end of the reaction, CO_2 is needed as a source of carbonate for calcium carbonate precipitation. Most of the acidity is consumed during the reduction of nitrite for all the electron donors listed in Table 4-4.

If L-glutamic acid is used as an electron donor instead of glucose, 0.056 mol/e^- eq NH_4^+ is produced, in addition to consumption of 0.206 mol/e^- eq of H^+ and production of 0.116 mol/e^- eq of CO_2 for the overall denitrification reaction. The production of ammonium provides additional increase in alkalinity. For instance, Equation 4-45 indicates that through reduction of 0.094 mol of NO_3^-, 0.094 mol of H^+ is consumed through oxidation of 0.083 mol of the donor when ethanol is the electron donor. When alanine is the electron donor, the reduction of 0.094 mol of NO_3^- results in consumption of a higher amount of H^+ (and, thus, higher production of alkalinity) due to production of NH_4^+ (Equation 4-48 and Table 4-4). In general, if the oxidized organic compound contains nitrogen, the released NH_3 is a base that increases the alkalinity of the medium.

Another factor that influences alkalinity is the biomass yield. An organic donor that gives a higher yield puts more of its electrons and carbon into biomass, instead of into reducing nitrate. Therefore, normalized to the organic donor consumed, a higher-yield system should show a smaller impact on alkalinity. For example, Table 4-4 indicates that 75 percent of the donor electrons is spent for cell synthesis when glucose is

used as an electron donor, resulting in a consumption of 0.077 mol of H^+. When acetate is the electron donor, only 59 percent of the electrons is used for cell synthesis, and the amount of H^+ consumed is higher.

The net changes in the pH of the medium depends on relative consumption of acidity, ΔH^+, compared to the production of CO_2, ΔCO_2 (Drtil et al., 1995). Drtil et al. (1995) report that the oxidation state of carbon in the electron donor can be used to assess whether or not the pH increases as a result of the growth of denitrifying microorganism. The ΔH^+ to ΔCO_2 ratio is controlled mainly by the oxidation state of carbon. If the organic donor is more reduced (has a more negative oxidation state), then the ratio of CO_2 released to nitrate reduced goes down, since each mole of carbon (C) can donate more electrons; then, the alkalinity goes up in higher proportion to the CO_2 release, giving a stronger pH impact. For example, using the values presented in Table 4-4, if ethanol (average oxidation state of carbon is -2) is used as an electron donor, $\Delta H^+/\Delta CO_2$ is equal to 0.094/0.052 = 1.8. When acetate (average oxidation state of carbon is 0) is the electron donor, $\Delta H^+/\Delta CO_2$ drops down to 0.228/0.145 = 1.6. Thus, denitrification of ethanol will result in a higher impact on the pH of the medium than denitrification of acetate.

Also shown in Table 4-4 is the amount of calcium carbonate that can be precipitated as a result of the overall denitrification, assuming that the precipitation is not limited by Ca^{2+} concentrations. If the calcium carbonate precipitation is mainly controlled by the production of alkalinity (i.e., all the alkalinity produced during

denitrification is consumed through precipitation of calcium carbonate), the amount of calcium carbonate precipitation for glucose can be estimated as:

$$(\text{g-CaCO}_3/\text{g-NO}_3^-{}_{\text{reduced}}) = [(0.077 \text{ mol H}^+)/(0.077 \text{ mol NO}_3^-)]\times(50 \text{ g CaCO}_3/1 \text{ mol H}^+) \times(1 \text{ mol NO}_3^-/62 \text{ g NO}_3^-{}_{\text{reduced}}) = 0.8 \text{ g-CaCO}_3/\text{g-NO}_3^-{}_{\text{reduced}} \quad (4\text{-}49)$$

Similarly, if we assume that all the CO_2 produced during denitrification of glucose is consumed by $CaCO_3$ precipitation, the amount of $CaCO_3$ precipitation can be calculated as:

$$(\text{g-CaCO}_3/\text{g-NO}_3^-{}_{\text{reduced}}) = [(0.116 \text{ mol CO}_2)/(0.077 \text{ mol NO}_3^-)]\times (100 \text{ g CaCO}_3/1 \text{ mol CO}_2)\times(1 \text{ mol NO}_3^-/62 \text{ g NO}_3^-{}_{\text{reduced}}) = 2.4 \text{ g-CaCO}_3/\text{g-NO}_3^-{}_{\text{reduced}} \quad (4\text{-}50)$$

Table 4-4 indicates that the amount of $CaCO_3$ that can be precipitated by the consumption of produced alkalinity is smaller than the amount of $CaCO_3$ that can be precipitated by the consumption of produced CO_2. Thus, the precipitation of calcium carbonate is mainly controlled by the production of alkalinity during denitrification of glucose as well as during denitrification of the other electron donors included in Table 4-4. Based on Table 4-4, the use of alanine in denitrification results in the largest consumption of acidity (up to 0.261 mol H^+/e^- eq) among all the electron donors studied and results in the highest amount of $CaCO_3$ precipitation, up to 2.2 g-$CaCO_3$/g-$NO_3^-{}_{\text{reduced}}$. Glucose and ethanol yield the lowest amount $CaCO_3$ precipitation, on the basis of NO_3^- reduced, approximately 0.8 g-$CaCO_3$/g-$NO_3^-{}_{\text{reduced}}$.

The $CaCO_3$ precipitation potential can also represented on the basis of the amount of calcium carbonate precipitated per 1 g of donor used, which may be helpful in evaluating the cost of the application when an electron donor needs to be added to the medium. For instance, if glucose is used as an electron donor, the $CaCO_3$ precipitation on the basis of 1 g of donor can be calculated as follows:

$$\begin{aligned}(\text{g-CaCO}_3/\text{g-donor}) &= (0.077 \text{ mol } H^+/0.042 \text{ mol glucose})\times(50 \text{ g } CaCO_3/1 \text{ mol } H^+) \\ &\quad \times(1 \text{ mol glucose}/180.16 \text{ g-donor}) \\ &= 0.5 \text{ g-CaCO}_3/\text{g-donor} \end{aligned} \quad (4\text{-}51)$$

The results from the calculations for the rest of the electron donors considered herein are presented in Table 4-5. Table 4-5 indicates that the highest amount of $CaCO_3$ precipitation takes place when alanine is used as an electron donor, followed by acetate, L-glutamic acid, ethanol, and glucose, in respective order.

Table 4-5. Calcium carbonate precipitation controlled by production of alkalinity

e^- donor	g-$CaCO_3$ (s) /g-$NO_3^-{}_{reduced}$	g-$CaCO_3$ (s)/ g-donor
Alanine	2.2	1.8
Acetate	1.8	1.5
L-glutamic acid	1.7	1.3
Ethanol	0.8	1.2
Glucose	0.8	0.5

4.4.3. Stoichiometric Analysis of Denitrification under Limited Growth Conditions

The values presented in Table 4-4 are based on growth reactions developed under optimum growth conditions. If the growth takes place under conditions that induce stress on the microorganism (e.g., limited substrate and pH, temperature, and salinity out of optimum range), the microorganism may expend more of its energy for maintenance than for synthesizing more cells. Thus, the portion of electrons used for synthesis reactions and the net microbial yield will decrease resulting in different amounts of acidity consumed and CO_2 produced than the values reported in Table 4-4. For instance, if a denitrifying microorganism uses all of the energy produced from denitrification of L-glutamic acid for maintenance (i.e., no new cell synthesis), which represents the stationary growth phase, the portion of electrons used for cell synthesis, f_s, decreases from 0.64 to 0. The overall growth reaction, R, will then be equal to the energy reaction (f_e=1.0):

$$0.056COOHCH_2CH_2CHNH_2COO^- + 0.2NO_3^- + 0.311H^+ \rightarrow 0.278CO_2 + 0.1N_2 + 056NH_4^+ + 0.35\ H_2O \quad (4\text{-}49)$$

Evaluation of Equations 4-39 and 4-49 suggests that, the donor-to-acceptor ratio will decrease as a result of limited growth conditions. The consumption of acidity and production of CO_2 increases as the donor-to-acceptor ratio decreases. Thus, the stoichiometric coefficients in Equations 4-39 (or the coefficients in Table 4-4) and 4-49 represent a range of possible values for consumption of acidity and CO_2 production.

4.4.4. Summary

Denitrification using organic compounds results in production of CO_2 and consumption of acidity (or production of alkalinity), which promote calcium carbonate precipitation. Stoichiometric analysis of denitrification presented in this chapter and the results from laboratory experiments presented by Drtil et al. (1995) indicate that the net changes in pH and alkalinity are influenced not only by dissimilative reactions but also by assimilative reactions. The net changes in the alkalinity and the pH of the denitrifying environment depend on a number of factors, including relative consumption of acidity compared to the production of CO_2 (Drtil et al., 1995) and precipitation of carbonates. The ratio of the acidity (H^+) consumed to the CO_2 produced is controlled mainly by the oxidation state of carbon in the electron donor. The nitrogen content of the electron donor also plays a role in the production of alkalinity through addition of base, NH_3, to the medium. Another factor that affects the production of alkalinity is the biomass yield. An organic donor that gives a higher microbial yield puts more of its electrons and C into biomass, instead of into reducing nitrate. Therefore, normalized to the amount of organic donor consumed, a higher-yield system should show a smaller increase in alkalinity. The stoichiometric analysis performed using five different electron donors (glucose, L-glutamic acid, ethanol, alanine, and acetate) indicates that the precipitation of $CaCO_3$ is mainly controlled by the production of alkalinity and, when alanine is the electron donor, up to 2.2 g $CaCO_3$ precipitation is possible through reduction of 1 g NO_3^- (equivalent to 1.8 g-$CaCO_3$/g-donor).

The precipitation of carbonates affects the acid-base chemistry of the denitrifying

environment by consuming alkalinity and causing the pH to decrease. The effects of calcium carbonate precipitation and accumulation of denitrification intermediates on the geochemistry of the medium are discussed based on the results from batch reactor experiments in the subsequent chapter.

CHAPTER 5

BATCH REACTOR EXPERIMENTS

5.1. Introduction

Bench-scale experiments were conducted to explore the potential for microbially induced calcium carbonate precipitation through denitrification for improvement of engineering properties of granular soils. These laboratory experiments were conducted in two stages. The first stage experiments consisted of bench-scale, closed-system batch reactors inoculated with a pure culture of a bacteria capable of denitrification. These first stage experiments were performed to serve as a proof-of-concept and were used to investigate the effects of various factors on the change in alkalinity and pH in the reactor, including electron donor type, accumulation of intermediates, nutrient limitations, concentration of salts, initial pH, and calcium carbonate precipitation. An overview of batch reactor systems and the microorganism, growth medium, inoculation and liquid medium preparation technique, sampling methods, and analytical techniques employed in the first stage bench scale batch reactor experiments are presented in this chapter, followed by a description of the results of the batch reactor experiments.

Denitrification experiments with liquid medium lacking calcium were performed to provide a basis for assessment of change in geochemistry during microbially induced calcium carbonate precipitation through denitrification. The first-stage bench-scale experiments also included batch reactors with calcium added to the bacteria-growth medium. A summary of the results of the bench scale experiments and potential field application strategies developed based on these results are also presented at the end of this chapter. Second stage bench-scale experiments, consisting of soil-column

experiments, assessing the cementation through microbially induced calcium carbonate precipitation are presented in a subsequent chapter.

5.2. Batch Reactor

A batch reactor is a simple and a very efficient tool in environmental biotechnology to develop a basic understanding of a microbial reaction. A batch reactor is a closed system in which the liquid medium (i.e., bacteria culture, electron donor, electron acceptor, carbon source, and other nutrients required for growth) is stirred to yield suspended growth. The bacterial growth in a batch reactor often is divided into four main phases: lag, exponential, stationary, and death phases. These different phases of bacterial growth are illustrated in Figure 5-1. Colony forming unit (CFU) and the optical density plotted on the vertical axis in Figure 5-1 are quantitative representation of bacteria cells in a medium. The lag phase may include the time in which the growth of the microorganisms is delayed due to adaptation of the inoculum of bacteria to new environmental conditions. In addition, the lag phase includes the duration for the small inoculum to grow enough so that the impact of the reactions carried by the microorganisms is observable. The lag phase can be minimized by using larger inoculum sizes and/or growing the culture where the inoculum is obtained from under similar conditions to the reactor. During the exponential phase, the number of cells increase geometrically until any of the limiting substrates is consumed. Stationary phase begins when the limiting substrate is readily depleted or that the synthesis rate become small and is approximately balanced by the decay rate. When the cell decay exceeds the rate of new cell synthesis, the system enters into the death phase.

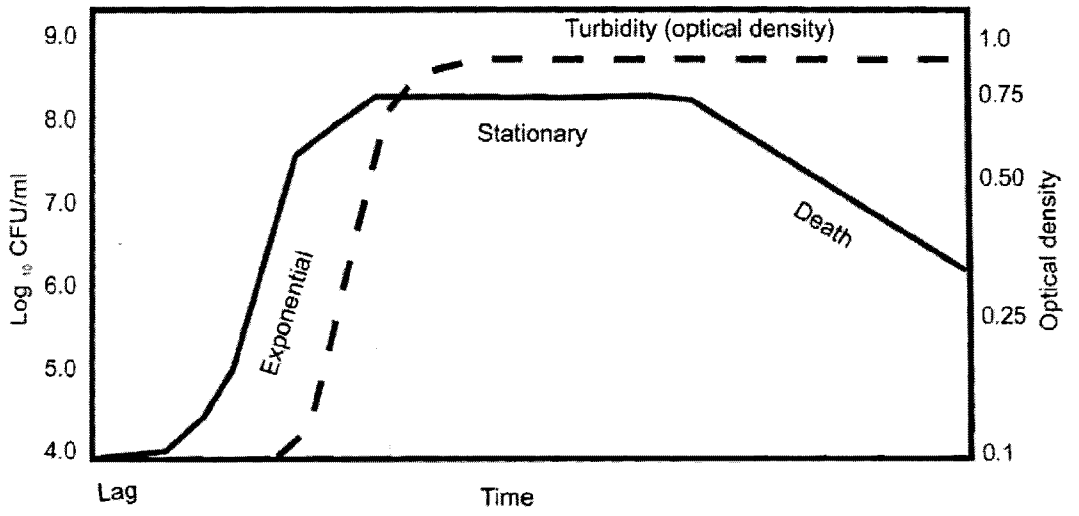


Fig. 5-1. Bacteria growth phases (Maier et al., 2000)

The bacterial growth in a batch reactor can be evaluated by direct and/or indirect measurement techniques. The cells can be counted directly by sampling and using different enumeration techniques (e.g., standard plate count, SPC, or maximum probable number technique, MPN). Indirect methods include monitoring turbidity of the liquid medium using a spectrophotometer and estimating the quantity of cells based on a calibrated sample of cells enumerated by SPC or MPN. In addition, the consumption of electron donor, carbon source, electron acceptor, or production and consumption of intermediates and the production of gases (e.g., CO_2) within the reactor can be monitored to evaluate bacterial growth, as illustrated in Figure 5-2.

5.3. Methods

5.3.1. Microorganism and Growth Medium

The first stage denitrification-precipitation experiments were performed with a strain of

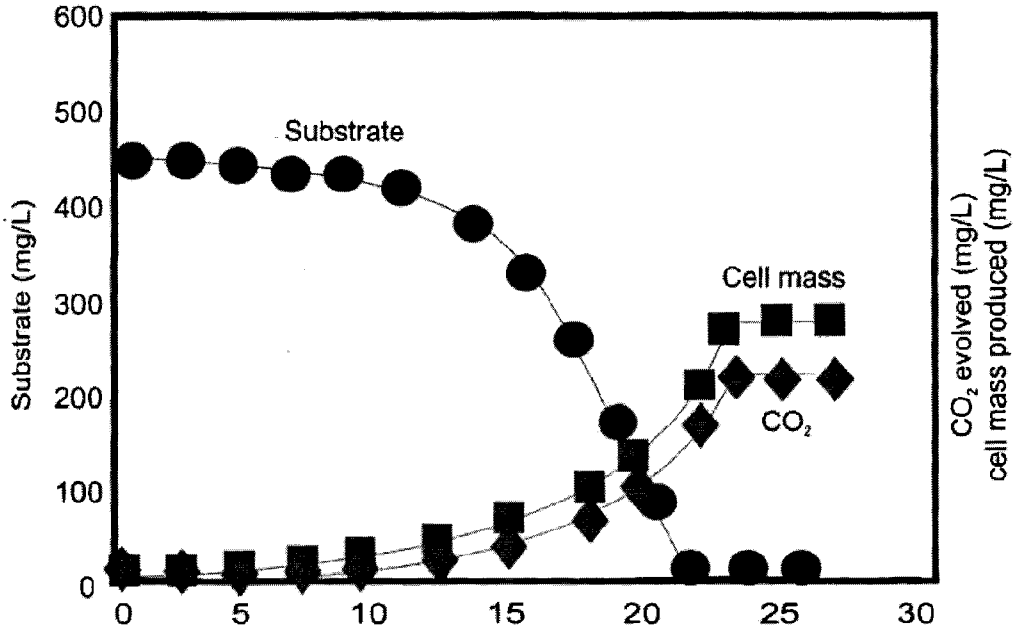


Fig. 5-2. Same bacterial growth represented in three different ways (Maier et al., 2000)

Pseudomonas denitrificans (*P. denitrificans*), American Type Culture Collection (ATCC) designation 13867. *P. denitrificans* is one of the most widely studied microorganisms in denitrification experiments (Radcliffe, 1969; Koike and Hattori, 1975a; Koike and Hattori, 1975b; Wang et al., 1995; Kornaros et al., 1996) and is a gram negative, facultative anaerobe. Kornaros et al. (1996) reported that *P. denitrificans* (ATCC 13867) lacks the capability of fermentation, which eliminates one of the mechanisms that may complicate interpretation of the results. Studies on the effect of pH on the nitrate and nitrite reduction by *P. denitrificans* (ATCC 13867) have indicated that the optimal pH range is approximately 7.4 to 7.6 and 7.2 to 7.3, respectively, at 30°C (Wang et al., 1995).

A freeze-dried pellet of *P. denitrificans* received from the ATCC was revived by aerobically by growing it in a sterilized nutrient broth medium and incubating it

overnight at 30°C. Samples of 10 ml of this nutrient broth-bacteria solution were frozen at -85°C with 5 percent glycerol to store the bacteria for subsequent use in batch reactor experiments. For each batch reactor test, one of the frozen samples was grown anoxically in a 150 ml glass serum bottle containing 2.5 g/L of $NaNO_3$ and 10 g/L of nutrient broth to reduce the lag (i.e., time necessary for a bacteria to synthesize and activate the enzymes necessary when switching between different electron donors or acceptors) and to minimize the possibility of cross-contamination.

5.3.2. Liquid Medium Preparation and Inoculation Technique

The bench scale denitrification-precipitation experiments were performed using reactors of two different sizes: 150-mL glass serum bottles and 2.0-L glass bottles (Figure 5-3). 150-mL glass serum bottles were capped with rubber septum and an aluminum cap. 2.0-L glass bottles were capped with a rubber stopper and placed in between two plywood sheets connected through the corners by threaded steel rods. The steel rods were screwed tightly to prevent the displacement of the rubber stopper upon generation of any positive gas pressure inside the glass bottle. The 2.0-L glass bottles have a cylindrical sampling port at the bottom, which was capped with a rubber septum and an aluminum cap. The rubber septum ports allowed repetitive sampling of the contents of the reactor, both liquid and gas, with minimal disturbance to the microbial reactions inside the reactors. The reactors were periodically injected with an electron donor solution upon sampling to minimize the effect of sample size on the results.

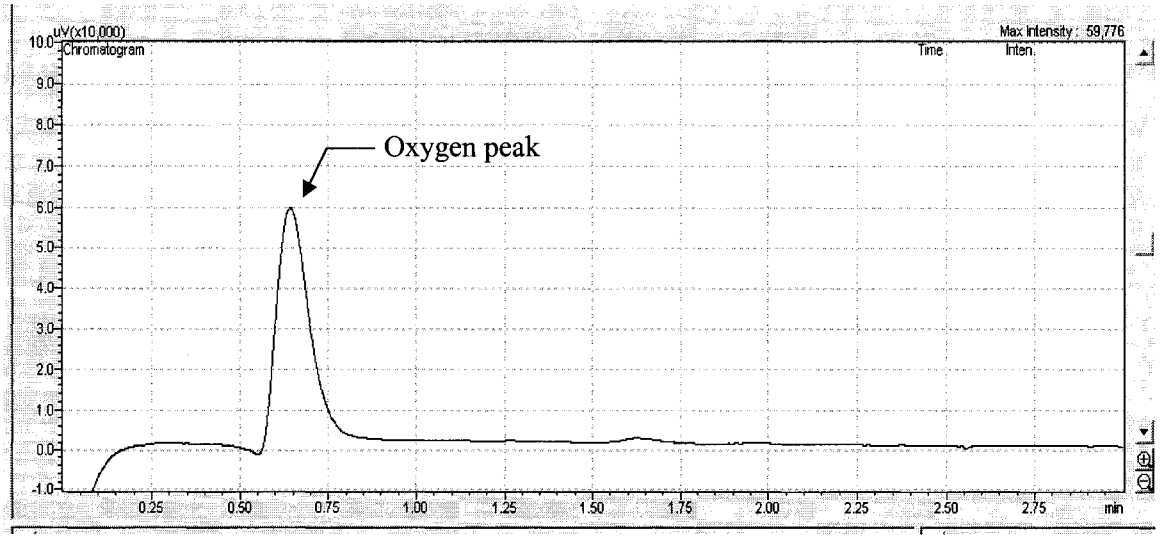
The components of the liquid medium for each experiment (i.e., electron donor, electron acceptor, phosphates, and inorganic salts) were autoclaved separately to prevent

Fig. 5-3. Batch reactors – 150-mL glass serum bottles and 2.0-L glass bottle

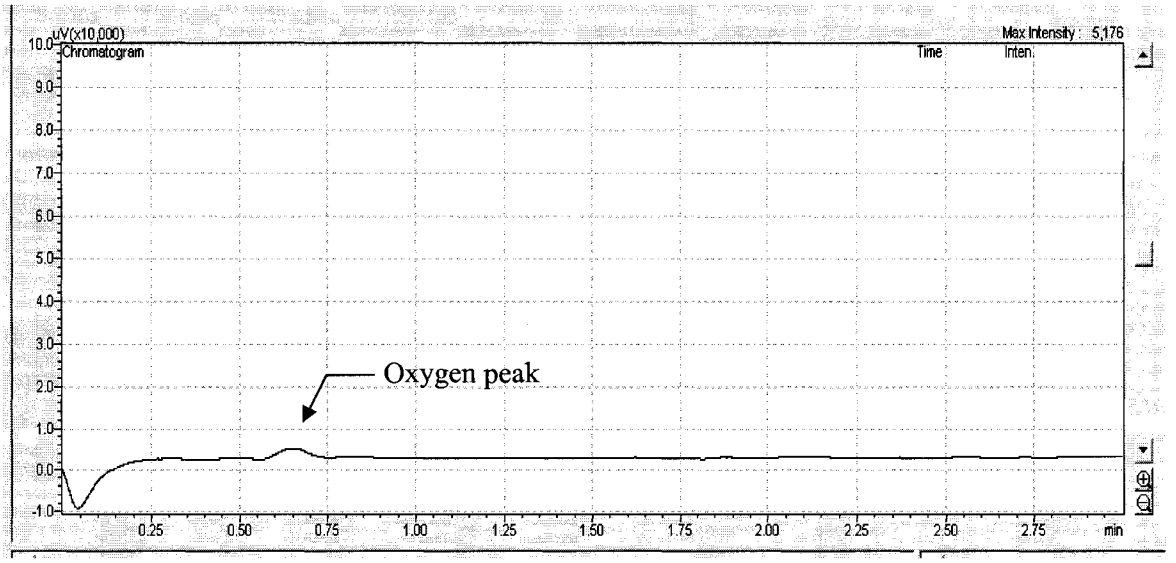
any chemical reaction during sterilization and poured into sterilized glass bottles in a biological safety cabinet. A liquid medium volume corresponding to approximately 75 to 80 percent of the total reactor size was prepared to minimize the pressures generated due to the formation of gases during denitrification.

The pH of the initial medium was modified to approximately 7.0 using 1M NaOH or 1M HCl solution, and the glass bottles were capped tightly. The reactors were then removed from the biological safety cabinet and purged with filtered N_2 (g) for approximately 1 hr using sterilized needles through the sampling port to minimize the O_2 (g) within the reactor. At the end of each N_2-purging episode, the gas in the headspace of the reactors were analyzed using a gas chromatography (GC) device (Shimadzu GC-2010 with thermal conductivity detector) to verify that O_2 levels within the reactor were reduced to minimal levels. A comparison of chromatography of samples from the headspace of a reactor before and after it was purged with N_2 is shown in Figure 5-4.

After the O_2 (g) levels within the reactor were confirmed to be minimal, inoculums of anoxically grown *P. denitrificans* (ATCC 13867) of 3 mL and 12 mL were injected into 120-mL and 1.5-L liquid mediums, respectively. Insufficient inoculum size typically results in prolonged lag phases at the beginning of the experiment but is not likely to affect the substrate utilization rate during the exponential phase of the growth. Because the lag phase lengths in our experiments were modest (a few hours to a day), no adjustments were made to the inoculum size in later experiments. The inoculated batch reactors were then placed on a shaker at 120 rpm in a dark room at constant temperature.



(a)



(b)

Fig. 5-4. Sample gas chromatograms (a) before and (b) after purging reactors with N_2 plotted on same scale: x-axis is the retention time (e.g., for the device used in this study, the retention time for oxygen is 0.65 min) and y-axis is the thermal conductivity detector signal

Unless otherwise noted, the temperature was kept at 30°C throughout each denitrification and precipitation experiments.

5.3.3. Analytical Methods

Samples 4 and 6 mL in volume were collected periodically from the 150-mL and 2.0-L batch reactors, respectively, through the sampling ports using sterilized needles and syringes. These samples were first analyzed for pH and then filtered through a 0.2-μm membrane filter to remove all the biomass from the sample after the pH measurement. Although the pH of the sample exposed to the atmosphere may not be equal to the pH of the medium within the closed batch reactor due to the difference in the partial pressure of CO_2 (g), N_2O (g), and NH_3 (g), if present, the measured pH is an indicator of the relative changes in the chemistry of the liquid medium. The filtered samples were used in the analysis of alkalinity, calcium hardness, and nitrate and nitrite concentration. The alkalinity measurements were performed using a digital titrator and Method 8203 proposed by HACH® (2006). The calcium hardness was evaluated by titration of diluted samples using ethylenediaminetetraacetic acid (EDTA), a chemical buffer, and HACH® indicator powders. The nitrate and nitrite concentrations were analyzed using HACH® DR/4000 Spectrophotometer. The chromotropic acid method (Method 10020) and the ferrous sulfate method (Method 8153) proposed by HACH® (2003) were followed for evaluation of nitrate and nitrite concentrations, respectively. Based on the evaluated nitrite concentrations, if necessary, the samples were treated with urea before assessment of nitrate concentrations to prevent interference of nitrite, as suggested by HACH® (2003).

Gas chromatography analyses were periodically performed on samples from the headspace during each experiment to monitor the CO_2 (g), O_2 (g), and N_2O (g) levels within the reactor. CO_2 (g) levels were measured to assess the rate of bacterial growth along with consumption of nitrate and nitrite. O_2 (g) levels were measured to evaluate whether or not there was any leakage through the sampling ports of the reactors during the experiment. Even though most of the O_2 (g) was removed at the start of the experiment through purging the reactors with N_2 (g), rubber septum stoppers may become susceptible to leakage upon degradation of the rubber due to continuous sampling with syringe needles. Because the accumulation of N_2O (g) may indicate inhibition of denitrification, N_2O (g) levels were also monitored during testing.

In addition to direct measurements, the concentration of nitrous acid (HNO_2), which may inhibit bacterial denitrification, was estimated based on the acid dissociation constant (i.e., $K_a = 6.76\times10^{-4}$ at 25°C; Lange's Handbook of Chemistry, 1987), the measured pH (i.e., H^+ concentration), and the measured nitrite concentration, as shown in Equations 5-1 through 5-3.

$$HNO_2 \leftrightarrow H^+ + NO_2^- \tag{5-1}$$

$$[HNO_2] = [H^+]\,[NO_2^-] / K_a \tag{5-2}$$

$$\text{where} \quad [H^+] \approx 10^{-pH} \text{ and } [NO_2^-] = \text{measured directly} \tag{5-3}$$

Control reactors were also prepared with the liquid medium used for each experiment (no bacteria added) and kept under the same conditions as the corresponding denitrification experiment. Samples from the control reactors were periodically assayed

for pH, alkalinity, calcium hardness, and nitrate and nitrite concentration, and the reactors were periodically monitored for CO_2 (g), N_2O (g), and O_2 (g) levels.

5.4. Results and Discussion

A complete set of tables and figures summarizing the results from the batch reactor experiments is presented in Appendix B. The results are categorized and discussed below to provide insight to the denitrification-precipitation system. The initial set of batch reactor experiments, which did not include calcium in the liquid medium, were performed to confirm the aspects of the denitrification process favorable for calcium carbonate precipitation. These experiments are designated as "denitrification experiments." Another set of batch reactor experiments, designated as "precipitation experiments," was conducted to quantitatively assess calcium carbonate precipitation through denitrification and to investigate the effect of various factors on the accumulation of intermediates. Throughout the experiments discussed herein, excess amounts of electron donor and carbon source were added to ensure that the growth was mainly controlled by electron acceptor concentration.

5.4.1. Denitrification Experiments

Available data on the effect of microbial denitrification on the pH and alkalinity of the medium is limited because most of the experiments to study the kinetics of denitrification were performed with chemical buffers. Drtil et al. (1995) performed a series of experiments using different electron donors (glycerine, glucose, acetate, and ethanol) to investigate the pH decrease reported in some denitrification experiments by previous researchers. The stoichiometric analysis presented in the previous chapter and

the experiments performed by Drtil et al. (1995) with various electron donors confirmed that alkalinity increased as a result of denitrification. As discussed in the previous chapter, the net changes in alkalinity and pH depended on dissimilative and assimilative reactions, oxidation state of carbon in the electron donor/carbon source, and biomass yield. In addition to these factors, accumulation of denitrification intermediates and precipitation-dissolution reactions also affects the change in the geochemistry of the medium. The denitrification experiments presented herein were performed to provide a basis for evaluation of the effects of calcium carbonate precipitation on the geochemistry of the medium. The effects of the accumulation of denitrification intermediates are also discussed as part of the precipitation experiments.

Electron donors used in experiments with *P. denitrificans* by others included L-glutamic acid, methanol, aspartate, alanine formate, and peptone-yeast extract (Koike and Hattori, 1975a; Koike and Hattori, 1975b; Nishimura et al., 1980; Wang et al., 1995, Kornaros et al., 1996). As part of the study presented herein, a simple set of experiments were prepared using nutrient broth and L-glutamic acid as electron donors.

The experiment designated as DN-N3 (denitrification experiment N3) was performed in a 2.0-L reactor and included 1.5 L of 20.7 g/L nutrient broth (manufacturer/supplier: BD Diagnostic Systems) and 1.25 g/L (15 mM) $NaNO_3$. Nutrient broth is one of the most common media used to grow microorganisms and is rich in proteins. 8 g of a powder form of nutrient broth typically consists of 5 g of peptone and 3 g of beef extract, and the composition may slightly differ from one manufacturer to another.

The assays of the samples collected from DN-N3 indicated that the pH of the medium increased from 6.8 to 7.7 over 24 hrs through reduction of all of the nitrate and nitrite (Figure 5-5). During the same period, the alkalinity rose from 0.55 g/L to approximately 1.9 g/L as $CaCO_3$. Temporary accumulation of nitrite was also observed until all the nitrate was depleted (Figure 5-5). Temporary accumulation of nitrite is generally expected due to the inhibitory effect of nitrate on nitrite reduction (Kornaros et al., 1996). The nitrous acid concentration was estimated to be less than 0.01 mg HNO_2-N/L during this period. No change in pH, alkalinity, and nitrate and nitrite concentrations was observed after 24 hrs.

Approximately 64 hrs (2.7 days) and 105 hrs (4.4 days) from the beginning of experiment DN-N3, solutions of $NaNO_3$ with similar concentrations to the initial amount were injected to the reactor. The initial nitrate reduction and these two nitrate injections are referred to as the 1st, 2nd, and 3rd denitrification episodes, respectively, to facilitate the discussion presented herein (Figure 5-6). The comparison of the results from these three denitrification episodes indicates the following:

- As a result of 3-episode denitrification, the pH in the reactor increased from 6.8 to 8.3 over 7.2 days through reduction of a total of approximately 36 mM of nitrate. An increase in pH from 6.8 to 8.3 typically results in a significant change in carbonate speciation, approximately from 0.02 percent to 1.0 percent CO_3^{2-} based on Figure 3-1, for a given inorganic carbon concentration.
- Total alkalinity also increased from 0.5 g/L to over 4.0 g/L as $CaCO_3$ over 7.2 days. When normalized to the amount of NO_3^- reduced during each

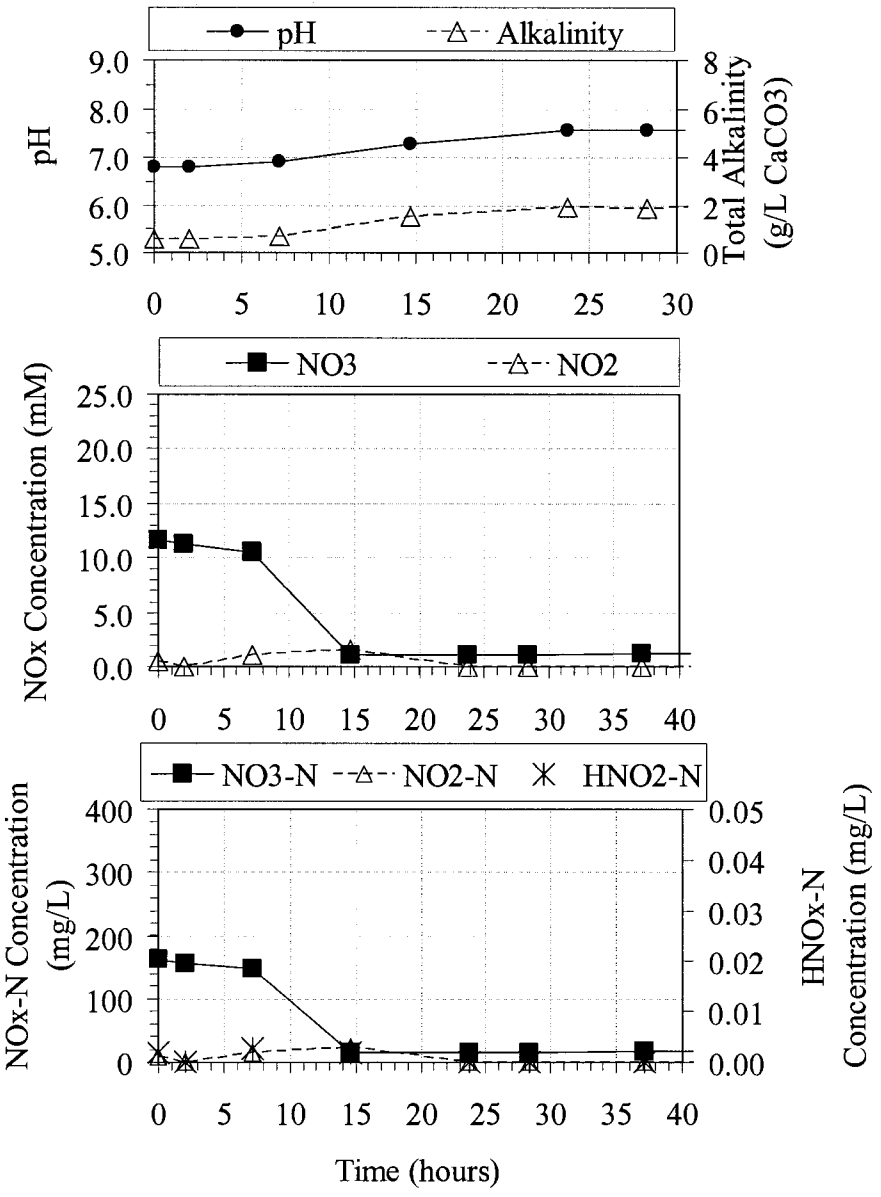


Fig. 5-5. Effect of denitrification on pH and alkalinity (DN-N3: nutrient broth)

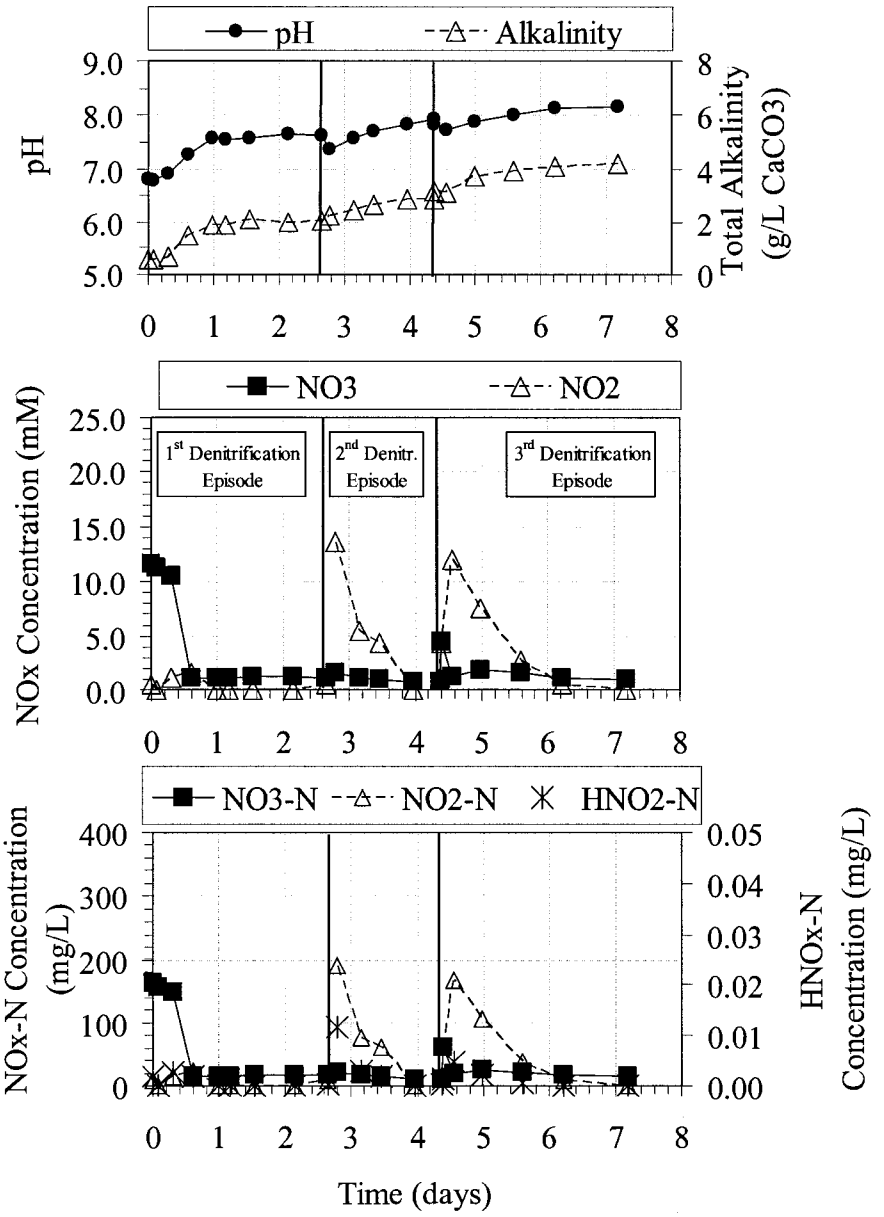


Fig. 5-6. Effect of continuous denitrification on pH and alkalinity (DN-N3: nutrient broth)

denitrification episode, the increase in alkalinity is within 1.4 to 2.1 g as $CaCO_3/g\text{-}NO_3^-{}_{reduced}$, which is consistent with the results of the stoichiometric analysis for alanine (2.2 $g\text{-}CaCO_3/g\text{-}NO_3^-{}_{reduced}$; Table 4-4).

- 15 mM of NO_3^- was reduced within approximately 4 hrs after it was injected during the 2nd and 3rd denitrification episodes, compared to the approximately 15 hrs it took for the 1st denitrification episode to reduce the same amount of NO_3^-, due to small inoculum size at the beginning of the experiment. Once that lag-time factor was eliminated, the rates of nitrate reduction were comparable in the 2nd and 3rd denitrification episodes.
- The pH increased during the entire 1st denitrification episode. On the other hand, during the 2nd and 3rd denitrification episodes, the pH first decreased until all the nitrate was consumed and, then, increased while nitrite was being consumed as primary electron acceptor. This difference in the trend in pH between the 1st and the other denitrification episodes is possibly related to the rate of nitrite reduction relative to nitrate reduction rate. Note that only the step that involves reduction of NO_2^- produces alkalinity. When CO_2 is released without production of alkalinity (i.e., during reduction of nitrate to nitrite), the pH decreases. Because nitrite reduction was not as fast as nitrate reduction during the 2nd and 3rd denitrification episodes, accumulation of higher concentrations of nitrite in the 2nd and 3rd denitrification episodes compared to the 1st denitrification episode was observed. On the other hand, during the 1st denitrification episode, the nitrite and nitrate reduction rates

were comparable and nitrite accumulation was not as pronounced as in the other two denitrification episodes.

- The alkalinity stayed almost constant while nitrate was being consumed as the primary electron donor during the 2nd and 3rd denitrification episodes. On the other hand, during the same denitrification episodes, when nitrite was being used as primary electron donor, the alkalinity increased.
- The rate of increase in pH and alkalinity decreased in the 2nd and 3rd denitrification episodes. This decrease in the rate of increase in pH and alkalinity may be attributed to a decrease in the rate of enzyme activities.
- The maximum nitrous acid concentration was estimated to be approximately 0.012 mg HNO_2-N/L, which is less than the inhibitive concentration of 0.04 mg HNO_2-N/L reported by Abeling and Seyfried (1992). The concentration of 0.012 mg HNO_2-N/L also corresponds to an inhibition coefficient, I, of 0.42 reported by Almeida et al. (1995), where I is the ratio of specific growth rate under different levels of nitrous acid to maximum specific of growth rate in the absence of inhibition for denitrifying *Pseuodomonas fluorescens*. Because the nitrite reduction rates for the 2nd and 3rd denitrification episodes are similar, the estimated nitrous acid concentrations are more comparable with the inhibitive concentrations reported by Abeling and Seyfried (1992).

Similar results were observed with denitrification experiment with L-glutamic acid. The liquid medium for the experiment performed with L-glutamic acid, designated by DN-N19, consisted of 11 g/L (75 mM) L-glutamic acid, 2.08 g/L (25 mM) $NaNO_3$, 1

g/L (17 mM) NaCl, 0.28 g/L (2 mM) KH_2PO_4, 0.2 g/L (1 mM) $MgSO_4.7H_2O$, and a drop of trace metal solution, as described in Table 5-1. The liquid medium volume at the beginning of the experiment was 120 mL, and a 150-mL glass serum bottle was used as a reactor. The results from DN-N19 illustrated in Figure 5-7 indicate that the pH increased from 7.3 to 8.3 through denitrification of approximately 25 mM nitrate over 1.6 days. The total alkalinity also rose from 1.4 g/L to 3.4 g/L as $CaCO_3$ (equivalent to 1.3 g-$CaCO_3$/g-$NO_3^-{}_{reduced}$), slightly lower than 1.7 g-$CaCO_3$/g-$NO_3^-{}_{reduced}$ as indicated by the stoichiometric evaluation of L-glutamic acid in the previous chapter (Table 4-4). The estimated nitrous acid concentrations stayed less than 0.002 mg HNO_2-N/L.

The assays on samples from the control reactors indicated that the pH, alkalinity, and nitrate concentrations stayed the same for the duration of the experiment. The increase in pH and alkalinity observed in these experiments should promote precipitation

Table 5.1. Nutrient Composition for DN-N19 Liquid medium

	Chemical	Concentration, g/L	Concentration, mM
Inorganic Salts	NaCl	1.0	17
	$MgSO_4.7H_2O$	0.2	1
	Trace metals	(a)	(a)
Phosphates	KH_2PO_4	0.28	2
Electron Donor / Carbon Source	L-glutamic acid	11.0	75
Electron Acceptor / Nitrogen Source	$NaNO_3$	2.08	25

(a) A drop of trace metal solution of 0.5% (w/v) of $CuSO_4$, F_eCl_3, $MnCl_2$, $Na_2MoO_4.2H_2O$.

of carbonates when the amount of CO_3^- added to the medium and the concentration of Ca^{2+} exceed the solubility product of calcium carbonate. The results from these denitrification experiments are used as a reference when the results from the calcium carbonate precipitation experiments are discussed in the subsequent section.

5.4.2. Precipitation Experiments

Additional experiments with calcium added to the liquid media used in the "denitrification experiments" were performed to evaluate calcium carbonate precipitation potential. All of the precipitation experiments performed in this stage (L-glutamic acid or nutrient broth were used as carbon source/electron donor) resulted in calcium carbonate precipitation, unless denitrification was inhibited. Precipitation of 0.5 to 2.4 g $CaCO_3$ per denitrification of 1.0 g NO_3^- was observed, depending on the initial calcium concentration and accumulation of intermediates. In addition to the potential environmental and health risks associated with the accumulation of nitrite and nitrous oxide during denitrification, the accumulation of intermediates can pose technical challenges during the application of this technology in the field. For instance, the production of alkalinity and CO_2 lower than the desired levels for precipitation of carbonates due to incomplete denitrification or resulting in much slower denitrification rates would make calcium carbonate precipitation through denitrification impractical. Thus, this section provides an overview of potential factors that may result in accumulation of nitrite and nitrous oxide and presents a discussion on the relevance of these factors to the precipitation experiments performed as part of this study through a summary of the results from the precipitation experiments.

5.4.2.1. Effect of Phosphorus

Even though each component used in the liquid medium in an experiment was either autoclaved separately and mixed after it cooled down or filter sterilized, precipitation of mineral complexes always occurred before the start of the experiment. Individual chemical precipitation experiments with liquid media of different compositions and comparison of the solubility products of possible mineral complexes suggest that it was calcium phosphate that was precipitating during the preparation of the liquid media (i.e., the relatively low solubility product of $Ca_3(PO_4)_2$; $K_{sp}=2.07\times10^{-33}$). Two experiments were performed with liquid media with no phosphates, designated by DN-N9 and DN-N10. The components of these two media are listed in Table 5-2.

Experiments DN-N9 and DN-N10 are illustrated in Figures 5-8 and 5-9. The assays on the samples collected from DN-N9 and DN-N10 indicated relatively very low nitrate reduction rates as compared to the denitrification experiments presented in the previous section. For instance, in experiment DN-N19 approximately 25 mM of nitrate was completely reduced to nitrite within 24 hrs, as shown in Figure 5-7. However, during experiment DN-N9, it took approximately 38 days for the reduction of 30 mM nitrate, as illustrated in Figure 5-8, using the same type of electron donor/carbon source (i.e., L-glutamic acid). Although alkalinity increased slightly or stayed almost constant during these two experiments, the pH decreased to approximately 6.6 and stayed constant after 5 days from the beginning of the experiments.

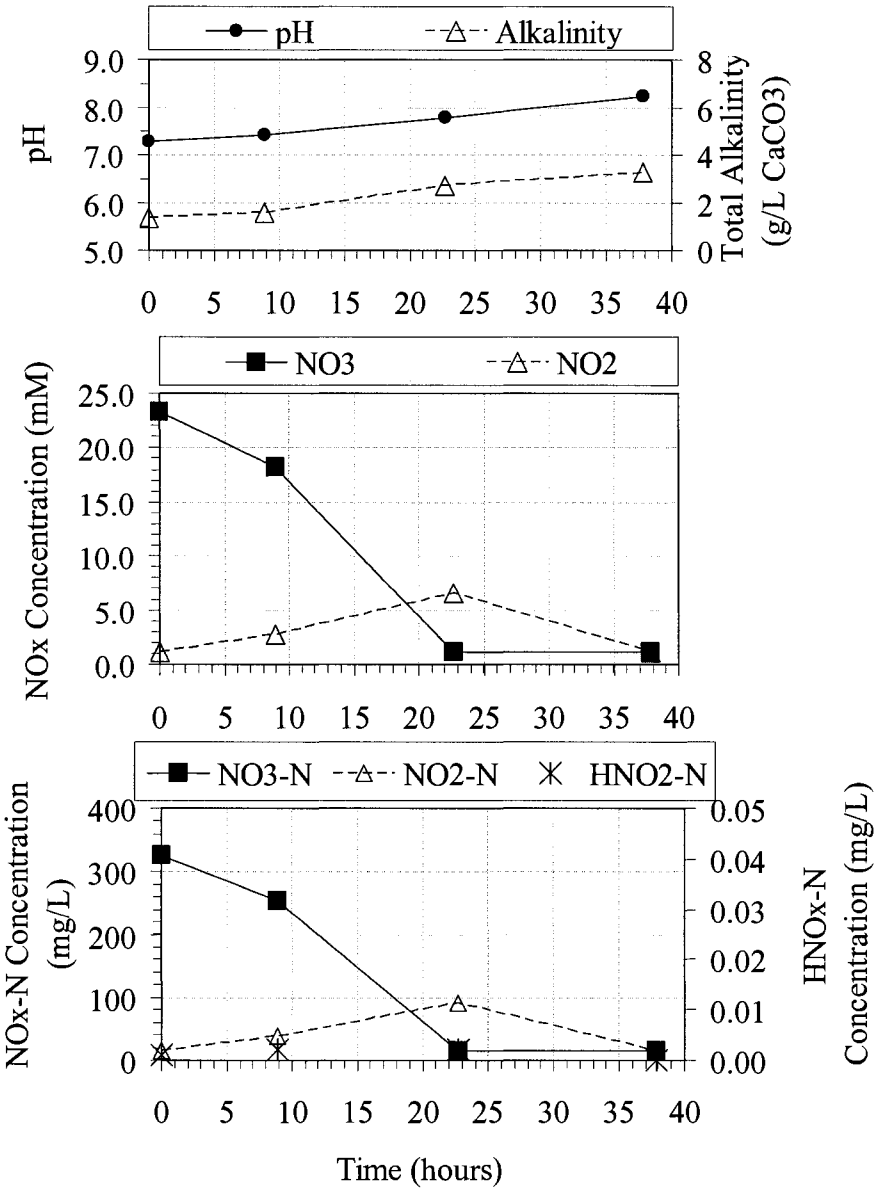


Fig. 5-7. Denitrification experiment with L-glutamic acid (DN-N19)

Table 5.2. Composition of DN-N9, DN-N10, and DN-N13 Liquid medium

	DN-N9 and DN-N13		DN-N10	
	Chemical	Concentration, mM	Chemical	Concentration, mM
Inorganic Salts	NaCl	17	NaCl	17
	$MgSO_4.7H_2O$	1	$MgSO_4.7H_2O$	1
	$CaCl_2.2H_2O$	100	$CaCl_2.2H_2O$	100
	Trace metals	(a)	Trace metals	(a)
Phosphates	KH_2PO_4	(b)	KH_2PO_4	--
Electron Donor / Carbon Source	L-glutamic acid	75	$CH_3COONa.3H_2O$	75
Electron Acceptor / Nitrogen Source	$NaNO_3$	25	$NaNO_3$	25

(a) A drop of trace metal solution of 0.5% (w/v) of $CuSO_4$, F_eCl_3, $MnCl_2$, $Na_2MoO_4.2H_2O$.
(b) DN-N9 and DN-N10 did not include any phosphates. DN-N13, with the same composition as DN-N9, were performed with 2 mM KH_2PO_4.

The estimated maximum HNO_2 concentrations were 0.07 and 0.12 mg-N/L for DN-N9 and DN-N10, respectively. DN-N9 and DN-N10 resulted in incomplete denitrification due to permanent accumulation of NO_2^- and N_2O (g). The N_2O (g) in the headspace reached maximum when HNO_2 concentrations reached 0.05 mg-N/L and 0.12 mg-N/L during DN-N9 and DN-N21, respectively, at 14 days from the beginning of the experiments, followed by a stationary microbial growth phase.

To investigate the reason(s) for the very low nitrate reduction rates and accumulation of nitrite in DN-N9 and DN-N10, first, additional electron donor/carbon source was injected to each reactor at Day 15. The results, presented in Figures 5-8 and 5-9, showing no increase in nitrate reduction rates and no change in NO_2^- and N_2O (g) accumulation, indicate that the electron donor/carbon source was not limiting the

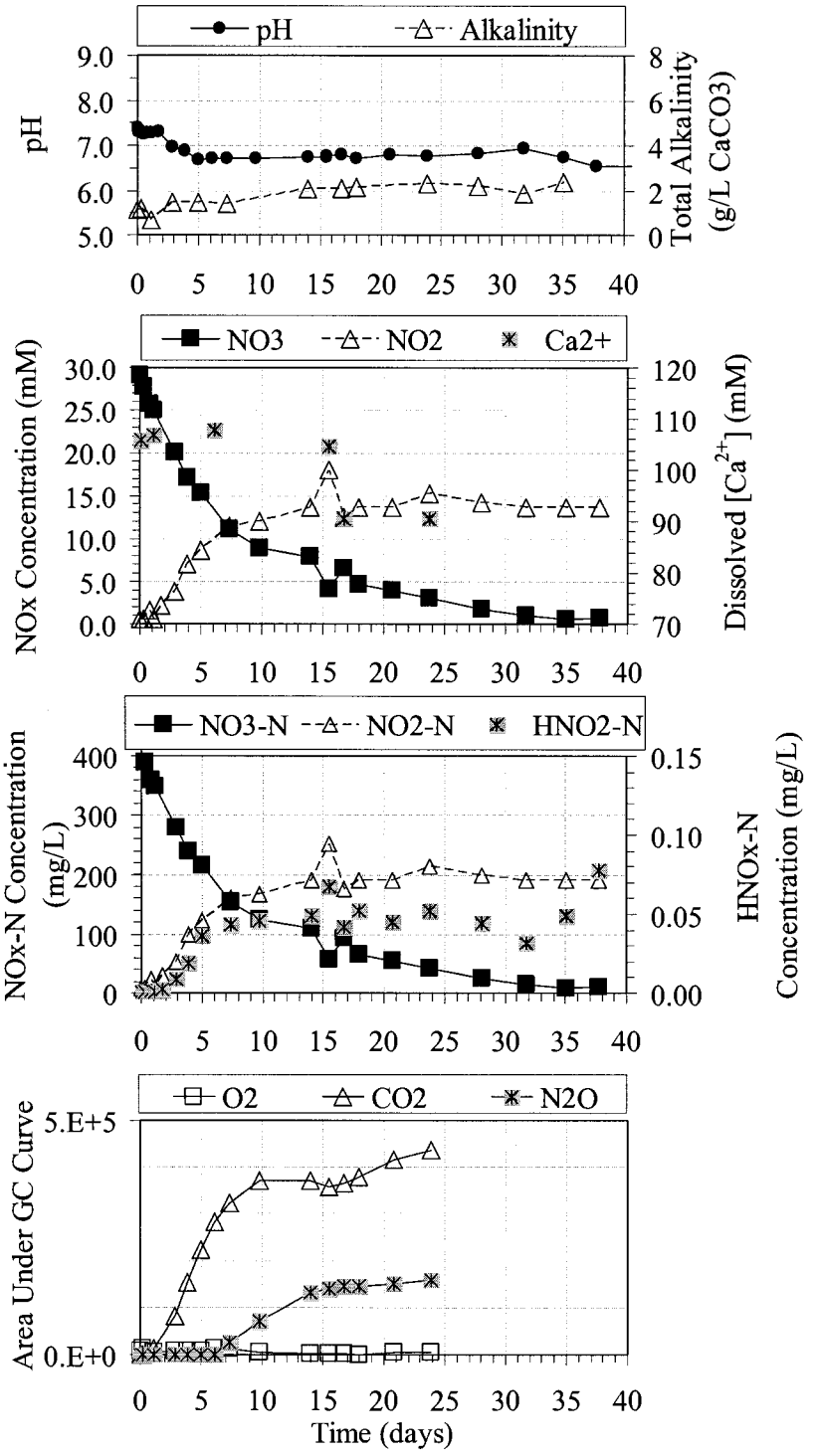


Fig. 5-8. Incomplete denitrification-precipitation experiment with L-glutamic acid (DN-N9)

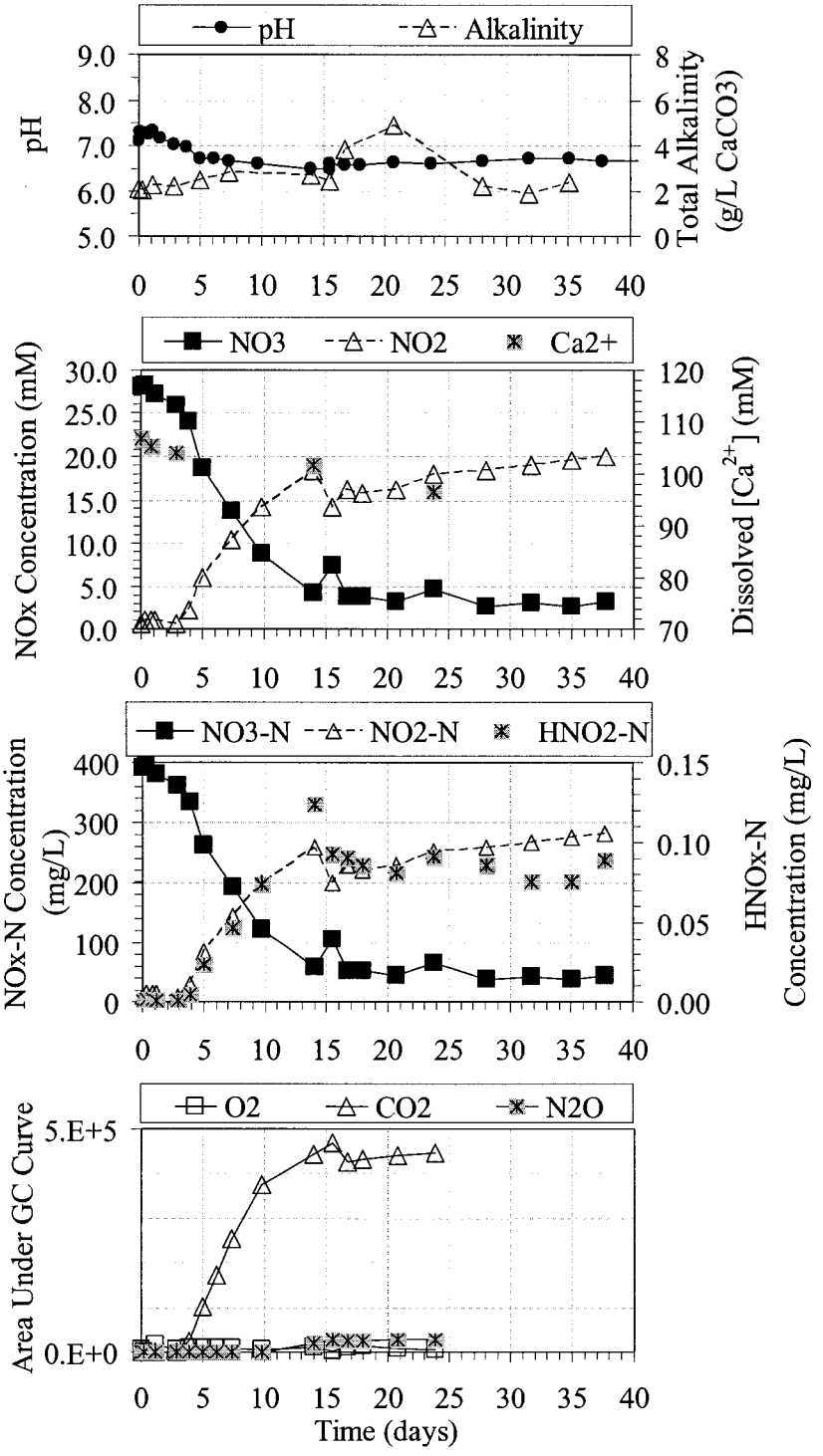


Fig. 5-9. Incomplete denitrification-precipitation experiment with L-glutamic acid (DN-N10)

growth in these reactors. Another experiment, designated by DN-N13, was designed with phosphorus (P), in the form of 0.47 g/L (2mM) KH_2PO_4, added to the liquid medium composition used for DN-N9 (Table 5-2). The addition of P did not prevent nitrite accumulation (Figure 5-10), but stopped N_2O (g) accumulation. On the other hand, DN-N13 showed a much faster nitrate reduction rates compared to the results from DN-N9 (Figure 5-8).

The effect of P concentration on the nitrate reduction rate is consistent with the findings by Hunter (2003). Phosphorus is one of the essential nutrients required for microbial growth and is used in the synthesis of adenosine triphosphate (ATP) and of cellular components, including nucleic acids, phospholipids, and proteins. A series of sand column tests performed by Hunter (2003) to investigate the ability of denitrifying barriers to function under low-phosphate conditions revealed that nitrate reduction rates increased with increasing phosphate concentration for the phosphate levels employed in his experiments. Hunter (2003) concluded that a nitrogen-to-phosphorus (N/P) ratio of 100 or less is required to mitigate nitrite accumulations in denitrifying subsurface permeable reactive barriers. Although the N/P ratio for DN-N13, of approximately 15, was much less than 100, the addition of P did not mitigate the nitrite accumulation but increased the microbial growth rate (i.e., increased the nitrate reduction rate). The cause of the nitrite accumulation was further investigated using a few other experiments discussed in detail in subsequent sections. However, subsequent experiments included liquid media consisting of some form of P, to avoid P limitation to microbial growth.

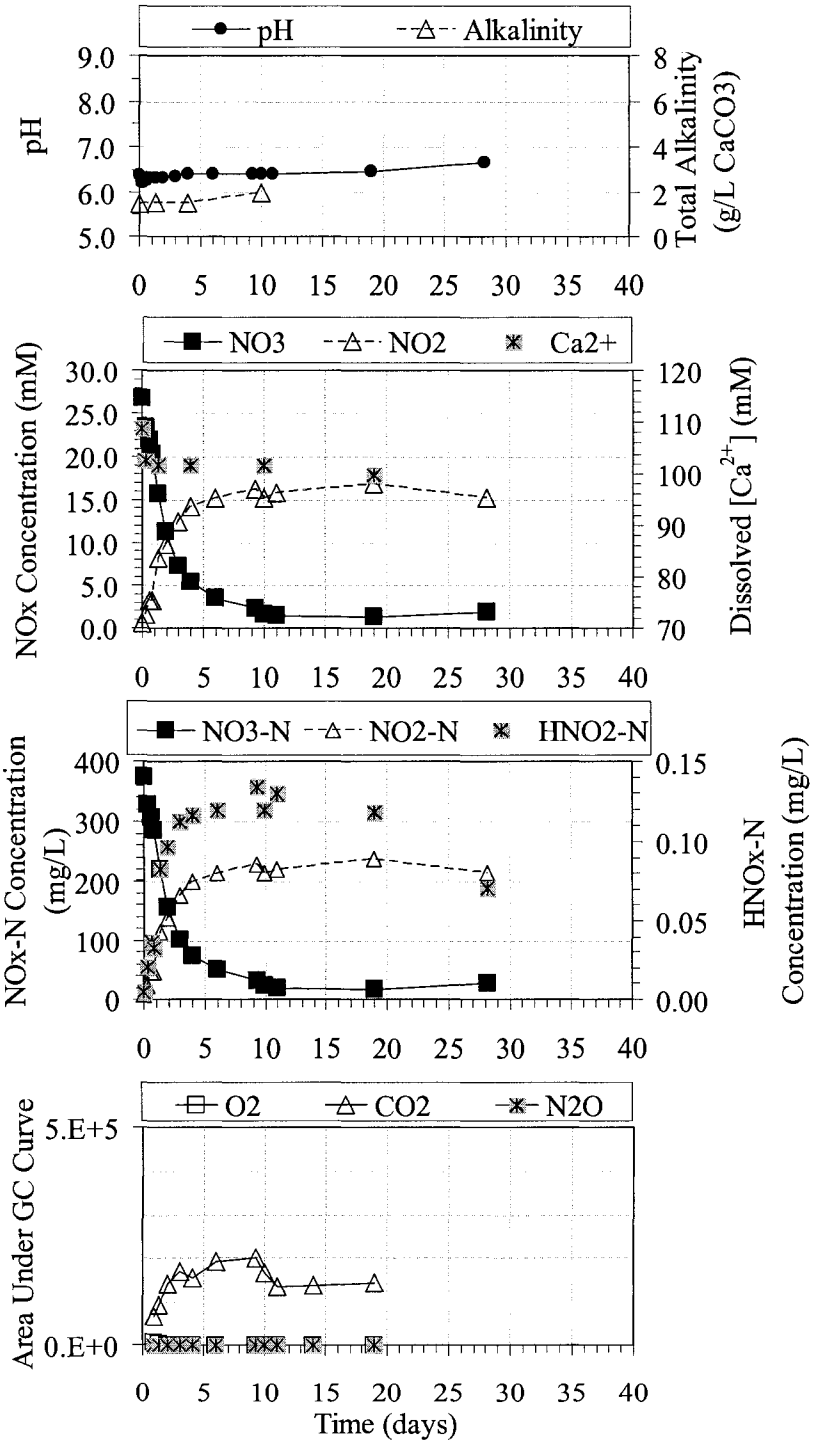


Fig. 5-10. Incomplete denitrification-precipitation experiment with L-glutamic acid and phosphates (DN-N13)

5.4.2.2. Nitrite Accumulation

Temporary accumulation of nitrite (i.e., reduction of nitrite after all or most of the nitrate is consumed) during denitrification has been reported by several investigators due to differences in the maximum rates of reduction by nitrate and nitrite reductases (Betlach and Tiedje, 1981) or due to inhibitory effect of nitrate on each one of the reduction steps from nitrite to nitrogen gas (Kornaros et al., 1996). Temporary accumulation of nitrite, which was observed during denitrification experiments performed as part of this study, is not of a great concern because the nitrite concentrations can be controlled by the initial nitrate concentrations in order not to affect the microbial growth or pose environmental or health risks to humans.

In addition to temporary accumulation of nitrite, permanent accumulation of nitrite (i.e., minimal or no consumption of nitrite even after all the nitrate is consumed) was also observed during precipitation experiments DN-N9, DN-N10, and DN-N13. Review of the literature on denitrification revealed several factors, listed in Table 5-3, that may result in accumulation of nitrite. Only a few studies listed on Table 5-3 made an attempt to propose a mechanism for inhibition of denitrification and permanent nitrite accumulation. Batch reactor experiments performed to investigate the nitrite accumulation during DN-N9, DN-N10, and DN-N13 indicated that HNO_2 concentrations and salinity were the two main factors for inhibition of denitrification. Results from these batch reactor experiments and the relevance of the various factors listed in Table 5-3 along with possible inhibitory mechanisms are discussed in this section.

Table 5.3. Factors that may cause nitrite accumulation during denitrification

Factor	Reference	Notes
Differences in the maximum rates of reduction by nitrate and nitrite reductases	Betlach and Tiedje (1981)	
Electron donor selective of nitrate-reducers	Wilderer et al. (1987); Turk and Mavinic (1987); Veydovec et al. (1994)	Accumulation of extracellular nitrite as a result of imbalance between nitrate-respiring and true denitrifiers. Cell yield for nitrate reduction can reach 3 times as high as the denitrifiying bacteria using nitrite as the electron donor.
Different response of different strains to nutrient-limited environment	Blaszczyk (1992); Blaszcyzyk (1993)	Based on experiments with *Paracoccus denitrificans, P. stutzeri, P. aeruginosa.*
Differential repression of nitrate and nitrite reductase synthesis activity	Korner and Zumft (1989); Coyne and Tiedje (1990)	
Limited carbon source/electron donor concentration relative to electron acceptor	Oh and Silverstein (1999); van Rijn et al. (1996); Thomsen et al. (1994); Rittmann and McCarty (2001)	Nitrate is typically the preferred electron acceptor , rather than nitrite, when substrate electrons are limited, based on experiments with *P. fluorescens, P.stutzeri, and Pa. denitrificans.*
Presence of oxygen	Skerman and MacRae (1957); Tiedje (1988); Sacks and Barker (1949); Rittmann and McCarty (2001)	Oxygen can control denitrification by repression of the production and/or the activity of denitrification enzymes.
Carbon source/electron donor type	Blaszczyk (1993); van Rijn et al. (1996)	
Ammonia toxicity	Francis and Mankin (1977)	At higher pH, ammonia toxicity may inhibit denitrification if high concentrations of ammonium is supplied to the medium in the form of a nitrate source (NH_4NO_3) or in another form.
Nitrate Source	Francis and Mankin (1977)	Francis and Mankin (1977) report that KNO_3 and $NaNO_3$ resulted in two orders of magnitude higher nitrite concentrations in the effluent than NH_4NO_3 with similar initial nitrate concentrations.

Table 5.3. (cont'd)

Factor	Reference	Notes
Presence of heavy metals, pesticides, or pesticide derivatives	Mitsui et al. (1964); Bollag and Henneringer (1976); Bollag and Barabasz (1979); Bollag and Kurek (1980)	
Exposure to light	Barak et al. (1998)	At low light intensities, revesible photooxydation of cytochrome c is possible, which plays the role of the electron donor during reduction of nitrite to nitric oxide.
Temperature	Glass et al. (1997); Napier and Bustamante (1988); Radcliffe (1969)	Temperature affects the activity of denitrification enzymes.
Phosphate limitation	Hunter et al. (2003)	Nitrite accumulates when N/P (nitrogen-to-phosphorus ratio) > 100.
Copper limitation	Catalan-Sakari et al. (1997)	2,000 μg Cu and 285 μg Cu per 1 g NO_3-N required to avoid nitrite accumulation in seawater and freshwater, respectively.
pH	Francis and Mankin (1977); Silverstein et al. (1993); Timmerhaus and van Haute (1983); van der Hoek et al. (1987); Glass et al. (1997); Chen et al. (1991); Almeida et al. (1995); Napier and Bustamante (1988)	pH can inhibit denitrification in two ways; affecting the activity of enzymes and/or affecting the speciation of different chemicals toxic tomicroorganisms.
High nitrate concentration	Francis and Mankin (1977); Glass and Silverstein (1998); Glass and Silverstein (1999)	
Nitrite toxicity	Radcliffe (1969); Veydovec et al. (1994); van Verselfeld et al. (1977); Tiedje (1988); Beccari et al. (1983); Horseley et al. (1982); Chen et al. (1991); Meijer et al. (1979)	
Nitrous acid toxicity	Abeling and Seyfried (1992); Glass et al. (1997); Glass and Silverstein (1998); Almeida et al. (1995); Meijer et al. (1979)	
Salinity/electrical conductivity of medium/total dissolved solids	Denton (1995); Smith and Goran (1996); Glass and Silverstein (1999); Ucisik and Henze (2004)	

Nitrite accumulation has been observed in experiments with pure bacteria cultures as well as in experiments with activated sludge, where mixed cultures are present (Glass and Silverstein, 1998). Because only a pure culture of *P. denitrificans*, rather than a mixed culture of bacteria, was used in our batch reactor experiments, "electron donor selective of nitrate-reducers" and "different response of different strains to nutrient-limited environment" can be eliminated as a factor inhibiting denitrification from the list presented in Table 5-3.

As discussed earlier in this chapter, all of the denitrification and precipitation experiments were performed with carbon source/electron donor concentrations well above the required levels indicated by stoichiometric analysis. In addition, the concentration of the remaining oxygen, within the reactors used for DN-N9, DN-N10, DN-N13 (reactors where nitrite accumulation was observed) after purging with N_2 (g), was comparable with DN-N3, where nitrite accumulation was not observed. Oxygen can affect denitrification by repression of the synthesis or the activity of denitrification enzymes. Dissolved oxygen concentrations greater than 2.5 to 5.0 mg/L have been reported to repress the synthesis of denitrification enzymes in *Pseudomonas stutzeri* (Korner and Zumft, 1989). Skerman and MacRae (1957), on the other hand, report that dissolved oxygen levels above 0.2 mg/L completely suppresses nitrate reduction (i.e., the activity of enzyme) in *P. denitrificans*. Thus, "limited carbon source/electron donor concentration" and "presence of O_2" cannot explain the nitrite accumulation within the reactors used for DN-N9, DN-N10, and DN-N13.

Blaszczyk (1993) reported that denitrification experiments with either methanol, acetate, or ethanol as a sole carbon source, resulted in substantial amounts of nitrite accumulation, whereas no nitrite accumulation was observed when nutrient broth was used as sole carbon source because of parallel and balanced activities of nitrate and nitrite reductases. On the other hand, experiments DN-N19 and DN-N9 included similar amounts of the same type of electron donor/carbon source" (i.e., L-glutamic acid), but yielded different responses in terms of nitrite accumulation (Figure 5-7 vs. Figure 5-8). Thus, type of electron donor/carbon source is not the reason for nitrite accumulation in DN-N9

In addition to the carbon source/electron donor, Francis and Mankin (1977) observed varying amount of nitrite accumulation with different nitrate sources. Because the precipitation experiments where nitrite accumulation was observed (DN-N9, DN-N10, and DN-N13) were performed with the same nitrate source as DN-N3 and DN-N19 (where nitrite accumulation was not observed), "nitrate source" is not relevant to the discussion presented herein. In addition, the liquid media used in all the batch reactor experiments did not include any "heavy metals, pesticides, or pesticide derivatives."

Ammonia toxicity has been known to inhibit anaerobic systems (Rittmann and McCarty, 2001). Francis and Mankin (1977) observed a decrease in specific nitrate removal rates with increasing pH in continuous flow stirred reactors when NH_4NO_3 was used as a nitrate source and suggested that inhibition was due to ammonia toxicity. Typically, ammonium is released to an environment when a carbon source/electron donor containing nitrogen (e.g., glutamate), or an ammonium based electron acceptor (e.g.,

NH_4NO_3), or salts containing ammonium (e.g., NH_4Cl) are present within an denitrifying system. If the concentrations of electron donors containing organic nitrogen and the pH are high, ammonia concentrations may reach inhibitory limits (i.e., 100 mg/L; McCarty and McKinney, 1961) through the following equilibrium:

$$NH_4^+ \leftrightarrow H^+ + NH_3 \quad \text{with} \quad K_a = 5.56\times10^{-10} \text{ (at } 35^oC) \qquad (5\text{-}4)$$

As presented in Figure 5-9, Experiment DN-N10, which included acetate as a carbon source/electron donor instead of L-glutamic acid and nutrient broth (i.e., electron donors containing nitrogen), resulted in nitrite accumulation. In addition, because DN-N9 and DN-N19, both of which included L-glutamic acid, yielded different response in terms of nitrite accumulation, "ammonia toxicity" cannot be used to explain the nitrite accumulation observed during the precipitation experiments.

The batch reactors were kept in a dark room and were only exposed to light during sampling. Barak et al. (1998) report that exposure of *Pseudomonas sp.* Strain JR12 to light caused photo-oxidation of cytochrome c, an intermediate electron carrier in the denitrification pathway, and resulted in reversible nitrite accumulation. Temperature also plays an important role in growth and Wang et al. (1995) report that the maximum temperature that *P. denitrificans* are able to grow is 30 ^oC. Because the same protocol in terms of light exposure and temperature was followed for all the experiments, "exposure to light" and "temperature" are not the possible causes of nitrite accumulation during DN-N9, DN-N10, and DN-N13.

As discussed in the previous section, addition of "phosphorus" at concentrations well above the required levels reported in the literature did not mitigate the accumulation

of nitrite in DN-N13 , although it increased the rate of nitrate reduction.

The nitrite reductase in *P. denitrificans* is a copper-containing protein (Wang et al., 1995). Thus, if copper is limited, expression of the enzyme can be inhibited. Catalan-Sakari et al. (1997) suggest that 2,000 μg Cu and 285 μg Cu per 1 g of NO_3-N are required to avoid nitrite accumulation in denitrifying seawater and freshwater systems, respectively. Not only copper but also iron and molybdenum are required for the synthesis of denitrification enzymes (Granger and Ward, 2003; Rittmann and McCarty, 2001). "Copper limitation" also cannot be a valid reason for the nitrite accumulation observed in DN-N13, because DN-N19, performed with similar amounts and same type of electron donor and acceptor and supplied with the same amount of trace metal solution, did not yield any permanent nitrite accumulation.

The remaining factors in Table 5-3, salinity, pH, high nitrate concentration, nitrite toxicity, and nitrous acid toxicity, are all relevant to the batch reactor experiments conducted as part of this study. The pH and the concentrations of nitrate, nitrite, and nitrous acid are interdependent, and these factors were investigated together. Nitrite concentrations are related to initial nitrate concentration through the stoichiometry of denitrification, and nitrous acid is in equilibrium with nitrite through the equilibrium equations presented in Equations 5-1 through 5-3 (i.e., the effect of pH). Salinity is discussed separately at the end of this section.

The levels of nitrate observed in domestic and agricultural wastewater treated with denitrification range from 10 to 200 mg-N/L (Glass and Silverstein, 1998), which are comparable with the nitrate concentrations employed in the experiments performed as

part of this study (i.e., less than 400 mg-N/L). Francis and Mankin (1977) reported inhibition of denitrification at nitrate concentrations over 6,000 mg/L as NO_3^- (1,355 mg/L as NO_3-N). Napier and Bustamante (1988), on the other hand, observed a decrease in nitrate concentrations from 40,000 mg/L (9,030 mg/L as NO_3-N) to 10 mg/L (2.2 mg/L as NO_3-N) over a year through microbial denitrification but reported that calcium hydroxide addition was necessary to keep the pH above 6.8 and sustain denitrification. Radcliffe (1969) indicates that $NaNO_2$ added to the medium with anaerobically grown cultures of *P. denitrificans* had no inhibitory effect up to 4 g/L (approximately 800 mg/L as NO_2-N) on the microbial growth, which is much higher than the maximum nitrite observed during DN-N9, DN-N10, and DN-N13 (280 mg/L as NO_2-N). On the other hand, along the same lines with Napier and Bustamante (1988), several investigators have also observed the pH dependency of inhibitive nitrite concentrations (Horseley et al., 1982; Beccari et al., 1983; Chen et al., 1991; Almeida et al., 1995; Glass et al., 1997; Glass and Silverstein, 1998). For instance, Glass et al. (1997) indicated that high concentrations of nitrite, e.g., 2,000 mg-N/L, can be denitrified with relatively little inhibition if the pH is greater than 8.0. The inhibitive nitrite concentrations that Glass et al. (1997) reported for pH 6.0 and 7.0 were 30 mg-N/L and 250 mg-N/L, respectively.

Because of the pH dependency observed in inhibitive levels of nitrate and nitrite, some researchers have suggested that the inhibition was actually due to undissociated nitrous acid (HNO_2), rather than high nitrate or nitrite concentrations. For example, the 30 mg/L NO_2-N for pH 6.0 and 250 mg/L NO_2-N for pH 7.0 inhibitive nitrite concentrations reported by Glass et al. (1997) correspond to 0.04 mg/L of HNO_2-N

(Figure 5-11). Similarly, Abeling and Seyfriend (1992) also reported that the inhibition of denitrification occurred when HNO_2 concentrations reached 0.13 mg/L (0.04 mg-N/L).

Figure 5-11 was developed using Equations 5-1 through 5-3 describing the nitrite-nitrous acid equilibrium. Figure 5-11 indicates that the nitrous acid concentrations stays below the inhibitive level of 0.04 mg/L if pH is above 7.5, unless the nitrite concentrations exceed 1,400 mg-N/L (100 mM). Almeida et al. (1995) also observed pH dependency of inhibitive nitrite concentrations and performed analysis to quantify the level of inhibition due to different nitrous acid concentrations (Figure 5-12). The inhibitive nitrous acid concentration of 0.04 mg-N/L indicated by Abeling and Seyfried (1992) and Glass et al. (1997) corresponds to an 80 percent inhibition (i.e., inhibition coefficient of 0.2) based on the denitrification experiments performed by Almeida et al. (1995).

Only a few researchers have made an attempt to propose a mechanism for inhibition of denitrification by nitrous acid. Glass et al. (1997) suggest that the proton donated by HNO_2 (Equation 5-1) directly interferes with the pH gradient across the cell membrane required for adenosine triphosphate (ATP) synthesis, which is termed as "decoupling" by Rittmann and McCarty (2001). On the other hand, Meijer et al. (1979) propose that, based on the results from their experiments, although the uncoupling action of nitrite is dependent on the pH of the outer medium, the irreversibility of the proton

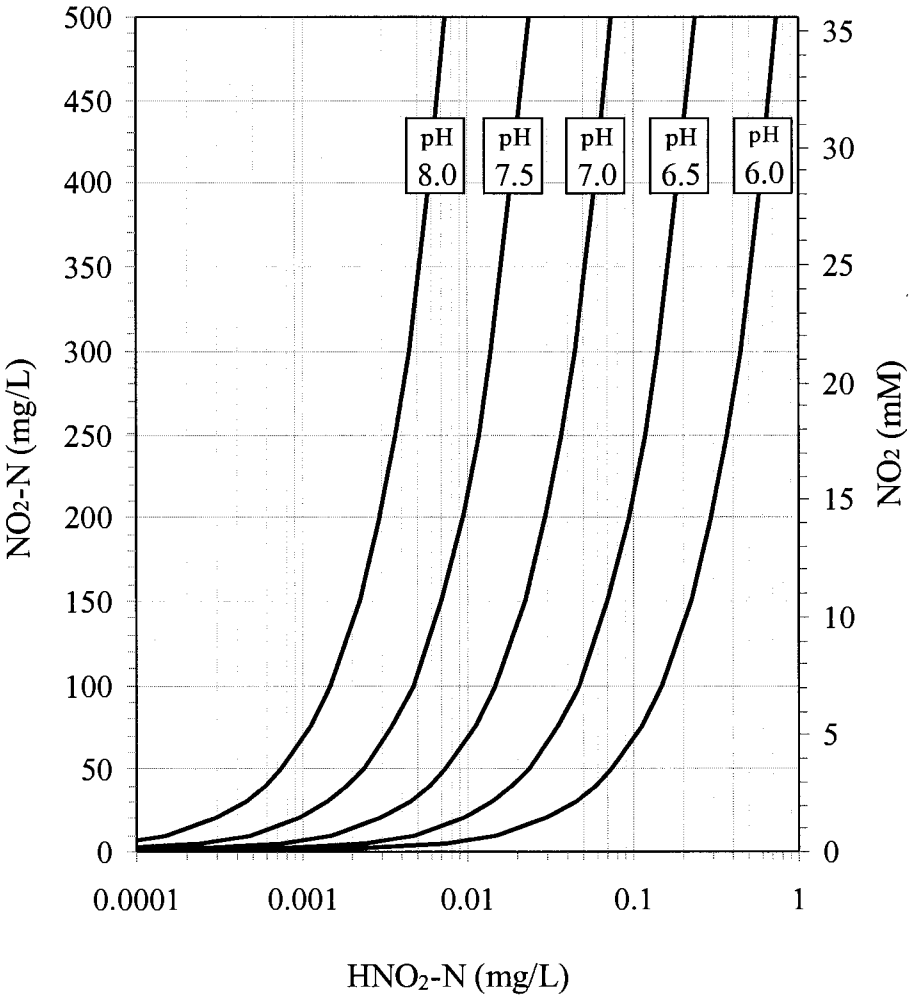


Fig. 5-11. Nitrite – Nitrous Acid equilibrium

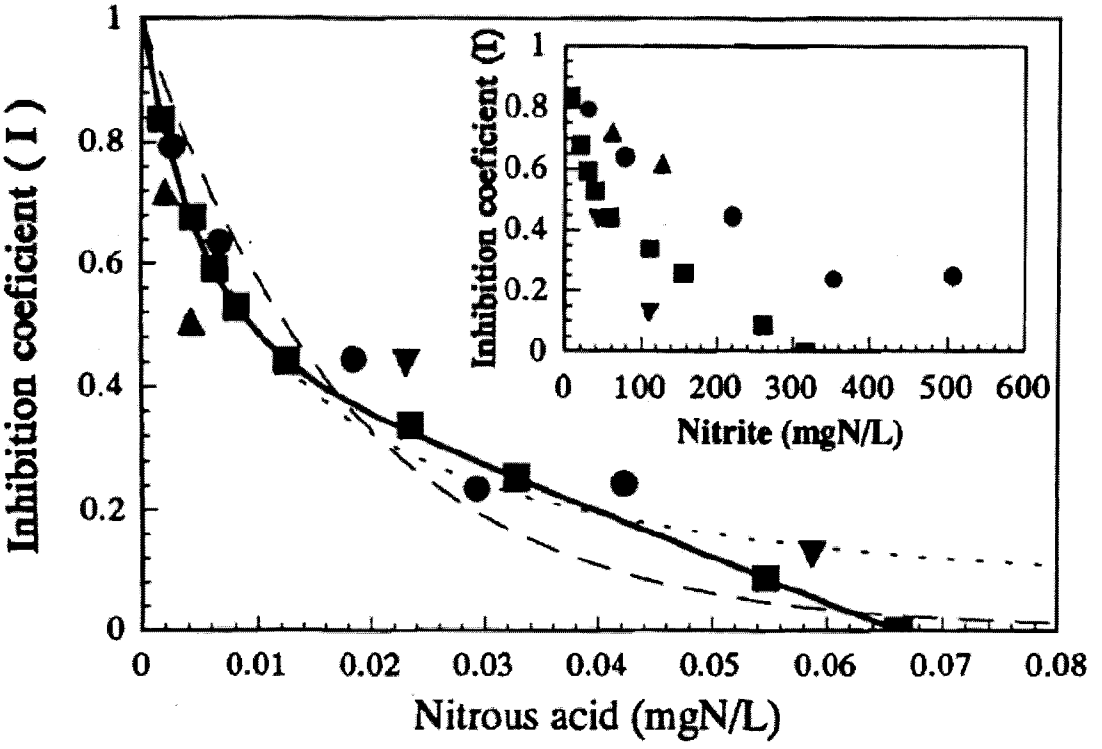


Fig. 5-12. Growth Inhibition due to nitrous acid concentration for *P. fluorescens* (Almeida et al. 1995): ▼pH=6.6; ■ pH=7.0; ● pH=7.4;▲ pH= 7.8; and dashed/continuous lines represent different equations used to fit laboratory data

permeability of the membrane even when all of the nitrite has been consumed points to a direct effect of nitrite on the cytoplasmic membrane. Because of the interdependency among "high nitrate concentration", "nitrite toxicity", and "nitrous acid toxicity", these factors will be termed as the "nitrite/nitrous acid toxicity" throughout the remainder of this study.

Chromatography analysis of the CO_2 concentration in gas samples collected from the headspace during DN-N9 and DN-N10 indicate that the rate of microbial growth decreased after 6 days and10 days from the beginning of the experiments, respectively (A decrease in the slope of the CO_2 production curve indicates a decrease in growth rate unless there is calcium carbonate precipitation and change in pH). Thus, Figures 5-8 and 5-9 indicate that the decrease in growth rate corresponds to estimated nitrous acid concentrations of approximately 0.04 mg/L HNO_2-N for DN-N9 and 0.07 mg-N/L for DN-N10, which are comparable with the inhibitive nitrous acid levels reported by previous researchers. On the other hand, the microbial growth rate started to decrease when the estimated nitrous acid concentrations exceeded 0.10 mg-N/L for DN-N13 (Figure 5-13).

An additional experiment, designated by DN-N17, was performed with liquid medium composition similar to DN-N13, except for a lower $NaNO_3$ concentration, to investigate the inhibition of denitrification by nitrous acid concentrations. Instead of the 2.1 g/L (25 mM) of $NaNO_3$ used for DN-N13, the DN-N17 liquid medium included 0.67 g/L (8 mM) of $NaNO_3$. The resulting estimated nitrous acid concentrations at DN-N17 did not exceed 0.02 mg HNO_2-N/L (much lower than the concentrations observed during DN-N9, DN-N10, and DN-N13), and nitrite or nitrous oxide (N_2O) accumulation was not observed (Figure 5-13). Thus, nitrous acid was a factor resulting in nitrite accumulation.

Another experiment was performed to investigate the possibility of the effect of salinity on accumulation of denitrification intermediates. Salinity is a measure of the dissolved salt content of the liquid medium, including Cl, Na, Mg, S, Ca, K, expressed in

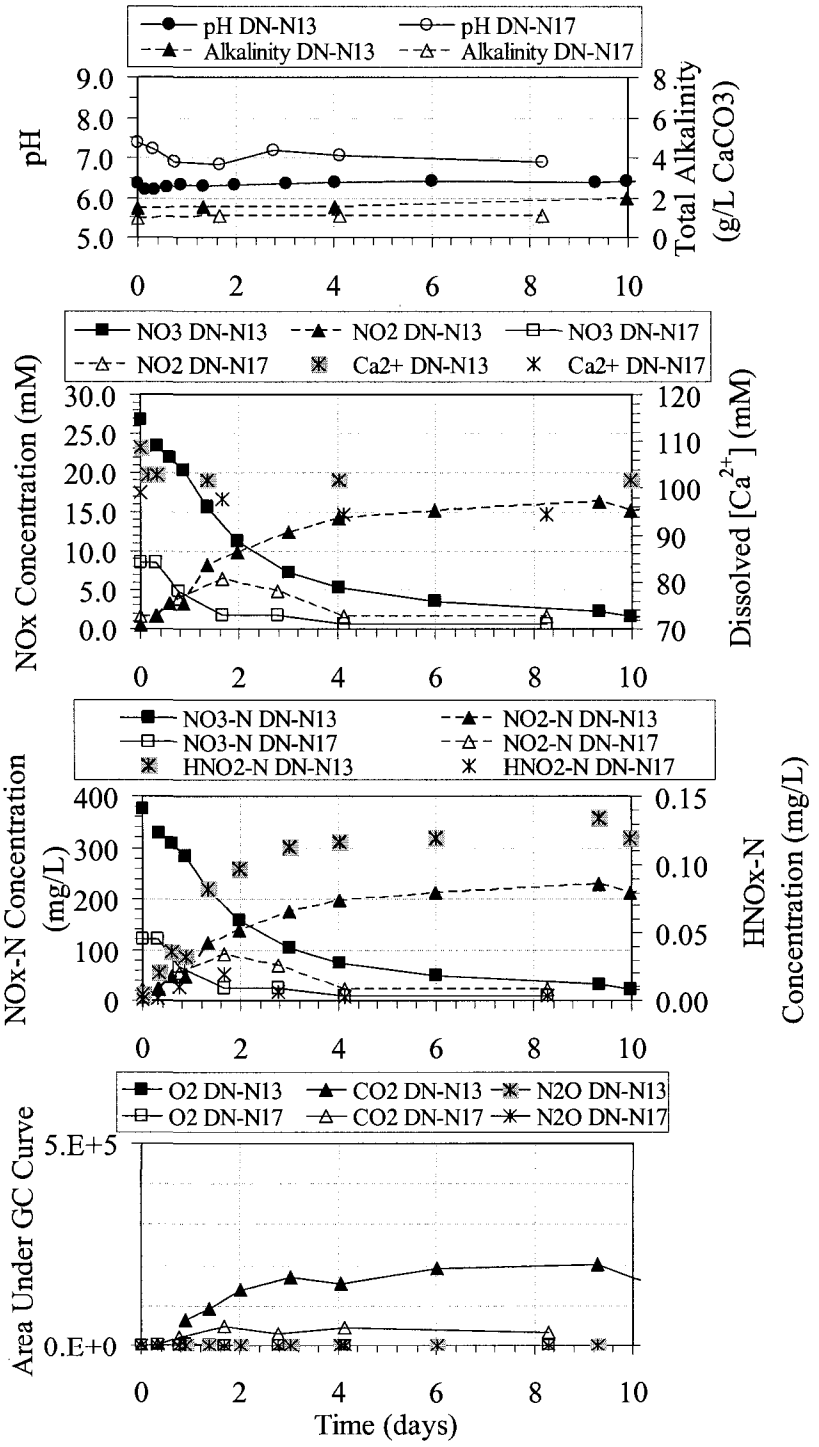


Fig. 5-13. Effect of initial nitrate concentrations on denitrification with L-glutamic acid: Initial NO_3^- concentrations for DN-N13: 25 mM and DN-17: 8 mM.

mg of dissolved solids in 1.0 L of water. Electrical conductivity, total dissolved solids (TDS), or ionic strength are all related to salinity and increase as salt concentration within a liquid medium increases. Rittmann and McCarty (2001) indicate that inhibition of the anaerobic process in wastewater with high salt concentration is related to the cation part of the salt, rather than the anion. The ionic strength of the liquid media used in DN-N9, DN-N10, and DN-N13 is approximately 0.4 with a TDS of 14.5 g/L. Glass and Silverstein (1999) were able to achieve denitrification using a synthetic waste water with ionic strength up to 3.0 (TDS up to 180 g/L) at a pH of 9.0, but nitrate reduction was inhibited at pH 7.5. Ucisik and Henze (2004) report decreased denitrification rates due to presence of chloride (Cl^-) between 4.8 g/L and 96.7 g/L as Cl, with maximum reduction up to an order of magnitude. The chloride concentrations within the liquid media used for DN-N9, DN-N10, and DN-N13 were less than 4.0 g/L as Cl, another experiment, designated by DN-N21, was conducted to examine the effects of salinity on denitrification.

The liquid medium used in DN-N21 included 2.92 g/L (20 mM) $CaCl_2.2H_2O$, instead of 14.7 g/L (100 mM) in DN-N13, with all other components staying the same as DN-N13 (Table 5-4). During DN-N21, the pH increased from 7.05 to 7.35 through reduction of 25 mM NO_3^- (Figure 5-14). The nitrate reduction rate was also higher during DN-N21, where reduction of approximately 25 mM of NO_3^- took approximately 2.5 days, compared to 10 days needed for the reduction of 30 mM of NO_3^- during DN-N13. On the other hand, the pH was also higher during DN-N21 (7.05 to 7.37) compared to DN-N13 (6.37 to 6.65). Figure 5-15, which was developed by Radcliffe (1969) based

Table 5.4. Composition of DN-13 and DN-N21 Liquid medium

	DN-N13		DN-N21	
	Chemical	Concentration, mM	Chemical	Concentration, mM
Inorganic Salts	NaCl	17	NaCl	17
	$MgSO_4.7H_2O$	1	$MgSO_4.7H_2O$	1
	$CaCl_2.2H_2O$	100	$CaCl_2.2H_2O$	20
	Trace metals	(a)	Trace metals	(a)
Phosphates	KH_2PO_4	2	KH_2PO_4	2
Electron Donor / Carbon Source	L-glutamic acid	75	L-glutamic acid	75
Electron Acceptor / Nitrogen Source	$NaNO_3$	25	$NaNO_3$	25

(a) A drop of trace metal solution of 0.5% (w/v) of $CuSO_4$, F_eCl_3, $MnCl_2$, $Na_2MoO_4.2H_2O$.

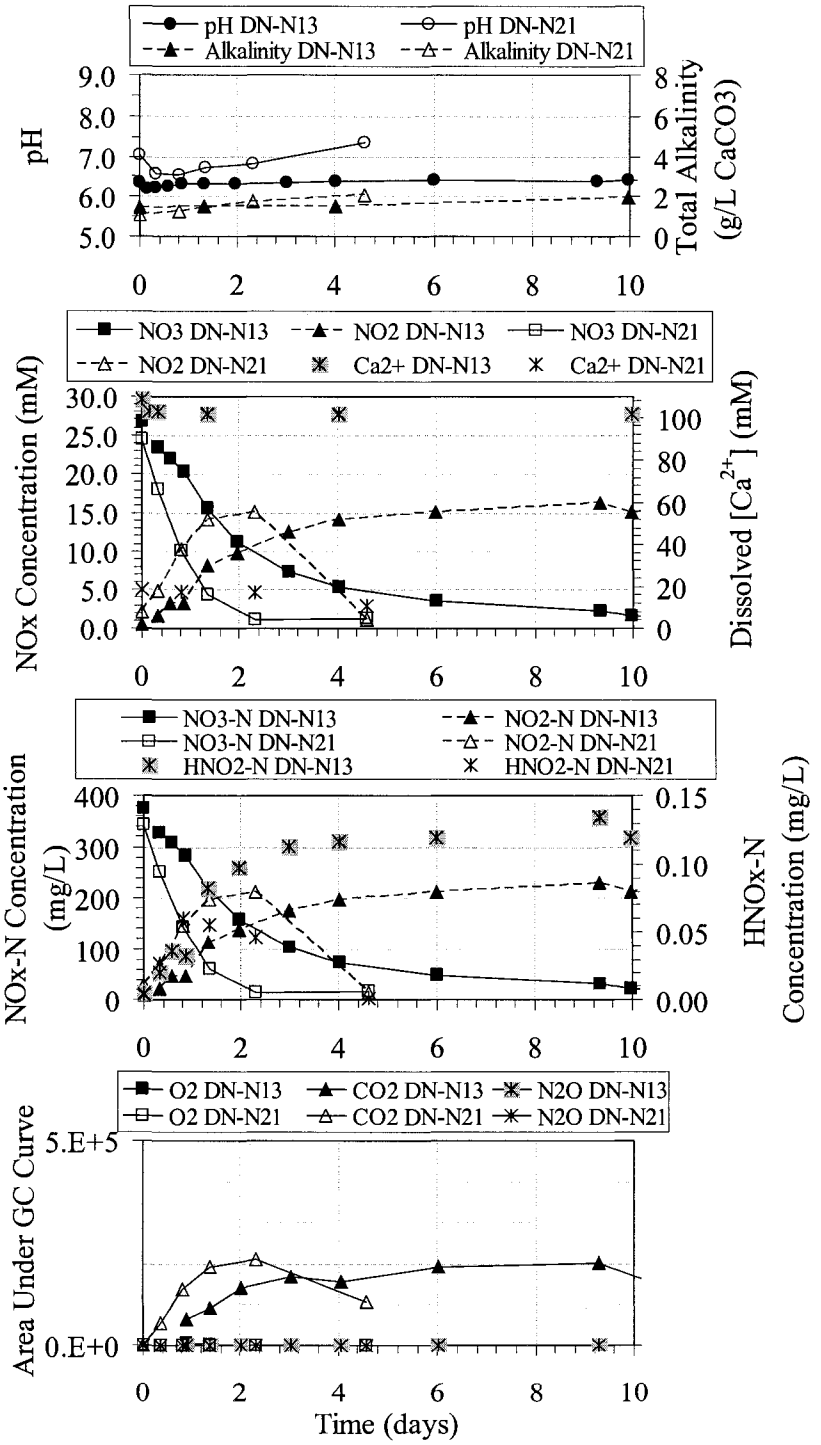


Fig. 5-14. Effect of total dissolved solids on denitrification with L-glutamic acid: Initial Ca^{2+} concentrations for DN-N13 and DN-21 are 100 mM and 208 mM.

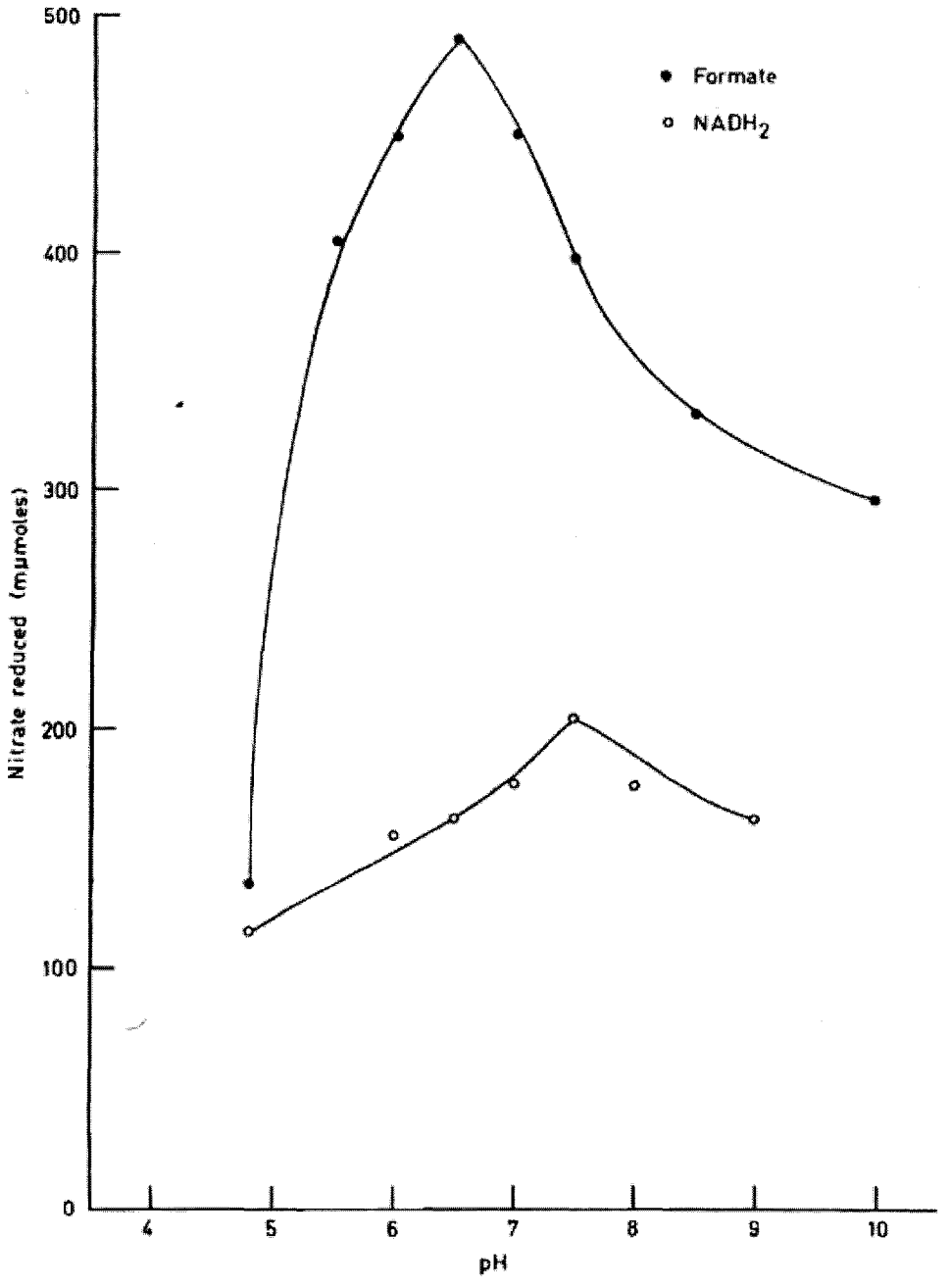


Fig. 5-15. Nitrate reductase activity for *P. denitrificans* (Radcliffe, 1969)

on a series of experiments performed by using *P. denitrificans*, shows that the activity of nitrate reductase at pH levels observed during DN-N13 and DN-N21 are comparable. Thus, the increase in nitrate reduction rate can be attributed to a decrease in salinity. Figure 5-14 also indicates that, although the NO_2^- concentrations reached 15 mM and the estimated nitrous acid concentrations reached 0.06 mg-N/L, all of the nitrate and nitrite was consumed within 5 days after the beginning of Experiment DN-N21. Therefore, the results from DN-N21 suggest that lowering TDS of the liquid media from 14.5 g/L to 4.8 g/L mitigated nitrite accumulation for *P. denitrificans*.

In conclusion, nitrite/nitrous acid concentration, which is controlled by initial nitrate concentration and pH, and salinity were the two main factors resulting in nitrite accumulation during DN-N9, DN-N10, and DN-N13.

5.4.2.3. Nitrous Oxide Accumulation

Nitrous oxide accumulation was also observed in precipitation experiments DN-N9 (Figure 5-8), DN-N10 (Figure 5-9), DN-N18 (Figure 5-16), and DN-N21 (Figure 5-17). Permanent accumulation of nitrous oxide (i.e., minimal or no consumption of nitrous oxide even after all the nitrate or nitrite consumed) was observed in DN-N9 and DN-N10. The liquid media used for DN-N9 and DN-N10 were discussed in the previous section and the results are summarized in Figures 5-8 and 5-9. DN-N18 and DN-N21 yielded temporary accumulation of nitrous oxide. Experiment DN-N18 was performed with a liquid medium consisting of 16.7 g/L nutrient broth as carbon source/electron donor and 1.0 g/L (12 mM) of $NaNO_3$ as electron acceptor and with the remaining components of the liquid medium the same as used in DN-N18. DN-N18 included

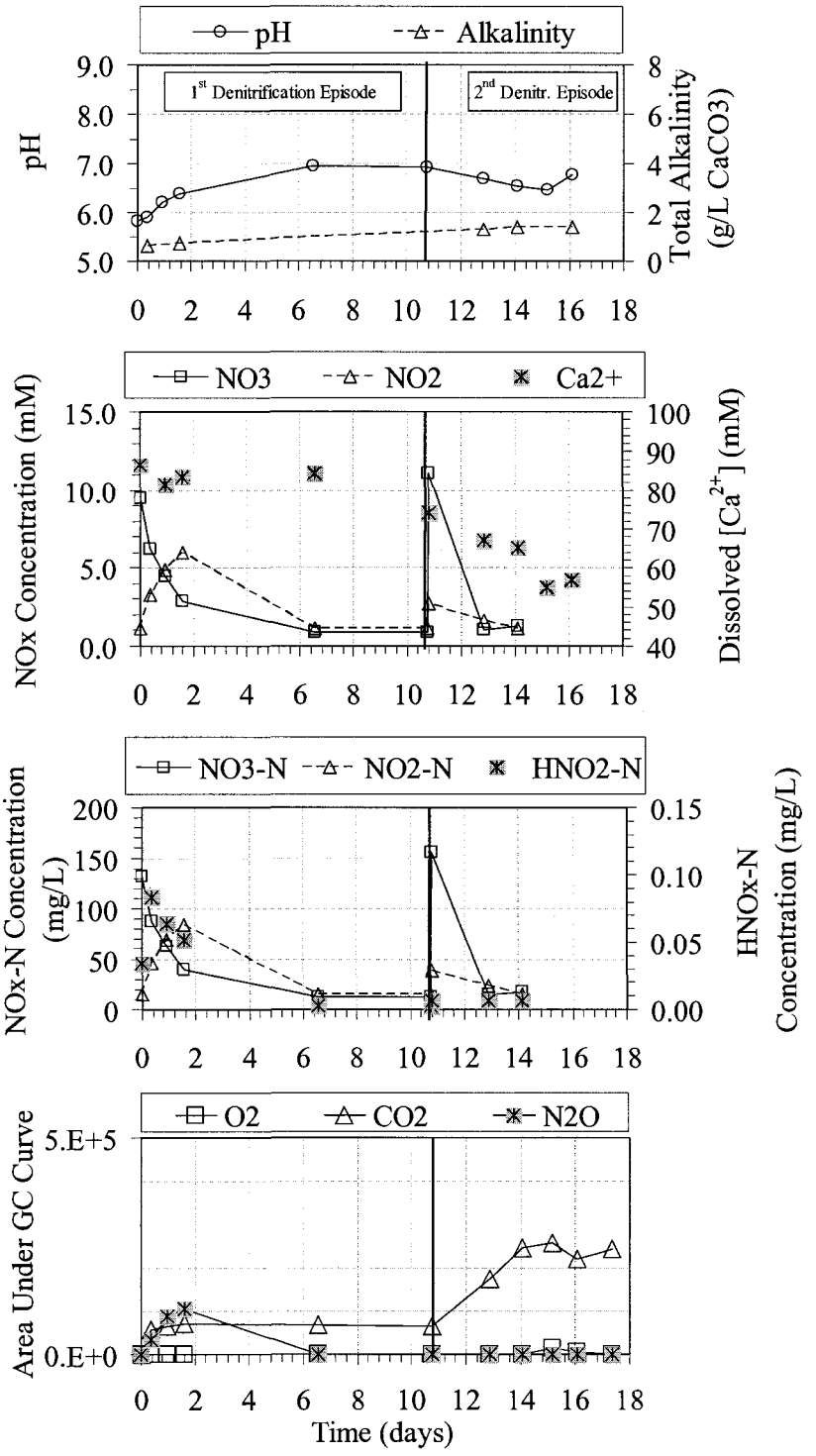


Fig. 5-16. Nitrous oxide accumulation during denitrification-precipitation experiment with nutrient broth (DN-N18)

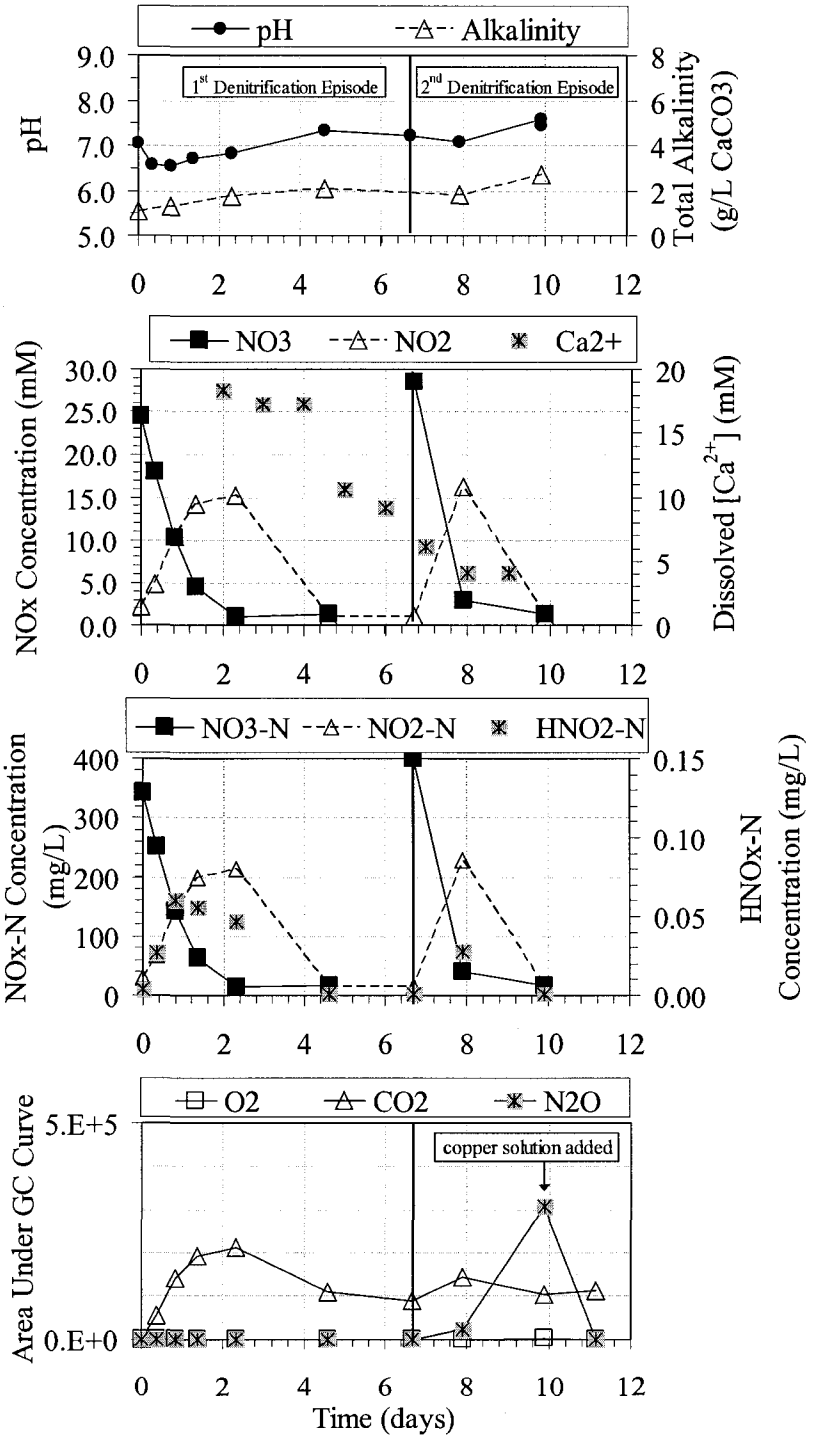


Fig. 5-17. Nitrous oxide accumulation during denitrification-precipitation experiment with L-glutamic acid (DN-N21)

Table 5.5. Factors that may cause nitrous oxide accumulation during denitrification

Factor	Reference
Presence of oxygen	Shulthess and Gujer (1996)
Low temperature	Keeney et al. (1979); Knowles (1981)
High Nitrite Concentration	Shulthess and Gujer (1996)
Limited carbon source	Tiedje (1988); Hunt et al. (2007)
Acetylene	Yoshinari and Knowles (1976)
Presence of pesticide and derivatives	Bollag and Kurek (1980)
Copper limitation	Cervantes et al. (1998); Granger and Ward (2003)

two episodes of denitrification of 12 mM of nitrate and nitrous oxide accumulation was observed only during the 1st denitrification episode. Similarly, DN-21 also included two episodes of denitrification of L-glutamic acid (i.e., electron donor) with 25 mM of nitrate, but nitrous oxide accumulation was only observed during the 2^{nd} denitrification episode. The results from DN-N18 and DN-N21 are presented in Figures 5-16 and 5-17.

The factors reported in the literature that may result in nitrous oxide accumulation during denitrification are listed in Table 5-5. Nitrous oxide can accumulate theoretically as a result of any inhibitory mechanism affecting the early stages of denitrification and cell synthesis. Thus, accumulation of nitrous oxide that takes place concurrently with nitrite accumulation can also be a result of most of the factors listed in Table 5-3, such as limited carbon source, presence of oxygen, ammonia toxicity, presence of heavy metals, pesticides, or pesticide derivatives, temperature, phosphate limitation, pH, nitrite/nitrous acid toxicity, and salinity. DN-N9 and DN-N10 lacked phosphorus in their liquid media

to prevent any precipitation prior to inoculation and resulted in nitrous oxide accumulation. DN-N13, which was performed with the liquid media used for DN-N9 amended with phosphorus, did not yield any nitrous oxide accumulation (Figure 5-10). Although the author has not encountered a study reporting nitrous oxide accumulation due to phosphorus limitation in the literature, the comparison of the results from DN-N9 and DN-N13 suggests that the nitrious oxide accumulation observed during DN-N9 and DN-N10 can be explained by phosphorus limitation.

On the other hand, DN-N18, performed with a nutrient broth rich in phosphorus, showed nitrous oxide accumulation only during 1st denitrification episode when nitrous acid concentrations reached 0.08 mg HNO_2-N/L (Figure 5-16). The accumulated nitrous oxide diminished with decreasing nitrous acid concentrations and was not observed during the 2nd denitrification episode. Thus, the nitrous oxide accumulation observed at DN-N18 probably was caused by the decoupling effect of nitrous acid and was reversible.

Nitrous oxide accumulation observed during the 2nd denitrification episode of DN-N21 was due to nutrient limitation. As presented in Figure 5-17, nitrous oxide in the headspace started to decrease after 1.0 mL of the trace metal solution (Table 5-1) was injected into the reactor. Because nitrous oxide-reductase is a copper containing enzyme (Granger and Ward, 2003) and the nitrous oxide levels diminished immediately after the trace metal solution containing copper was injected into the reactor, temporary nitrous oxide accumulation observed during DN-N21 may be attributable to copper limitation, which is in agreement with the observations of Cervantes et al. (1998) and Granger and Ward (2003).

In conclusion, investigation of the results from DN-N9, DN-N10, DN-N13, DN-N18, and DN-N21 indicate that phosphorus limitation, nitrous acid toxicity, and copper limitation may result in accumulation of nitrous oxide.

5.4.2.4. Calcium Carbonate Precipitation

All of the precipitation experiments performed with L-glutamic acid or nutrient broth as carbon source/electron donor resulted in calcium carbonate precipitation, unless denitrification was inhibited. Although some researchers use the ratio of carbonate mineral deposited to the organic carbon source (i.e., carbonatogenic yield) as a measure of the degree of carbonate precipitation, the ratio of carbonate precipitate to the amount of nitrate reduced was used in this study to interpret the results from the precipitation experiments because (1) nitrate was limiting during the experiments presented herein and (2) organic carbon content as chemical oxygen demand (COD) or biochemical oxygen demand (BOD) was not measured in these experiments. Thus, the ratio of calcium carbonate precipitated to the amount of nitrate reduced was considered to be the appropriate measure of precipitation for the denitrifying environments employed herein, where nitrate is limited.

Precipitation of 0.3 to 2.4 g $CaCO_3$ (s) per denitrification of 1.0 g NO_3^- was observed throughout the precipitation experiments described herein. The results from the precipitation experiments are summarized in Table 5-6. Note that DN-N9, DN-N10, DN-N13, and DN-N14 included in Table 5-6 are the experiments where denitrification was inhibited (i.e., incomplete denitrification) and correspond to the lower levels of $CaCO_3$ precipitation due to insufficient production of alkalinity and CO_2.

Table 5.6. Summary of calcium carbonate precipitation experiments

	e-donor Conc.	NO_3^- Consumed	Initial Ca^{2+} Conc.	Initial pH	Final pH	Duration $CaCO_3$[(b)]	$CaCO_3$ (s) ($g/g\text{-}NO_3^-$)
DN-N9	LGA[(a)] 75 mM	25 mM	100 mM	7.40	6.54	37 days 1.9 g/L	1.2
DN-N10	Acetate 75 mM	25 mM	100 mM	7.10	6.64	40 days 1.1 g/L	0.7
DN-N13	LGA[(a)] 75 mM	25 mM	100 mM	6.37	6.65	28 days 1.0 g/L	0.7
DN-N14	LGA[(a)] 50 mM	16 mM	100 mM	6.64	6.40	10 days 0.4 g/L	0.4
DN-N17 1st Episode	LGA[(a)] 50 mM	8 mM	100 mM	7.39	6.96	4 days 0.4 g/L	1.0
DN-N17 2nd Episode		8 mM		6.96	6.63	3 days 0.9 g/L	1.8
DN-N17 3rd Episode		8 mM		6.63	6.53	2 days 0.7 g/L	1.4
DN-N18 1st Episode	NB[(c)] 16.7 g/L	12 mM	100 mM	5.82	6.92	6 days 0.2 g/L	0.3[(f)]
DN-N18 2nd Episode		12 mM		6.92	6.78	3.5 days 1.8 g/L	2.4
DN-N21 1st Episode	LGA[(a)] 75 mM	25 mM	20 mM	7.05	7.35	4.6 days 0.8 g/L	0.5[(f)]
DN-N21 2nd Episode		25 mM		7.35	7.46	3.3 days 0.6 g/L	0.4

(a) LGA : L-glutamic acid.
(b) Normalized to liquid medium volume of 1.0 L
(c) NB: Nutrient Broth
(d) $CaCO_3$ (s) quantity estimated based on change in measured dissolved calcium ion concentrations
(e) DN-N9, DN-N10, DN-N13, and DN-N14 incomplete denitrification.
(f) Does not include the calcium carbonate precipitation after all the nitrite is consumed within an episode until the beginning of the next episode.

The effect of calcium carbonate precipitation on the geochemistry of the medium and quantitative evaluation of the $CaCO_3$ precipitation is discussed below based on the results from the experiments where complete denitrification was observed (DN-N17, DN-18, and DN-N21). The remainder of this section is divided into two parts with respect to the carbon source/electron donor used in the experiment to facilitate the discussion. DN-N18, where nutrient broth was the electron donor, is presented first, followed by a discussion of the precipitation experiments with L-glutamic acid (DN-N17 and DN-N21).

The results from DN-N18, a precipitation experiment performed with nutrient broth and calcium chloride, are presented in Figure 5-18. The results from DN-N3 and DN-N4 , denitrification experiments performed with nutrient broth as the carbon source and electron donor, are collectively shown in Figure 5-18, as well, for comparison. DN-N3 and DN-N4 are designated as "denitrification only" experiments while DN-N18 is designated as a "denitrification+precipitation" in Figure 5-18. Although the "denitrification only" experiments were not performed at the same time as DN-N18, the time scale for DN-N4 and DN-N3 were shifted to provide a better comparison among these experiments and to match the beginning of the 1^{st} and 2^{nd} denitrification episodes, respectively. DN-N18 was performed with 100 mM of calcium chloride dihydrate ($CaCl_2.2H_2O$) and 22.05 g of $CaCl_2.2H_2O$ was added to the 1.5 L liquid medium to reach a 100 mM initial Ca^{2+} concentration. The assay of the sample taken from the liquid medium in DN-N18 prior to inoculation of the medium with *P. denitrificans* indicated dissolved Ca^{2+} concentrations approximately 90 mM. This suggests that some of the

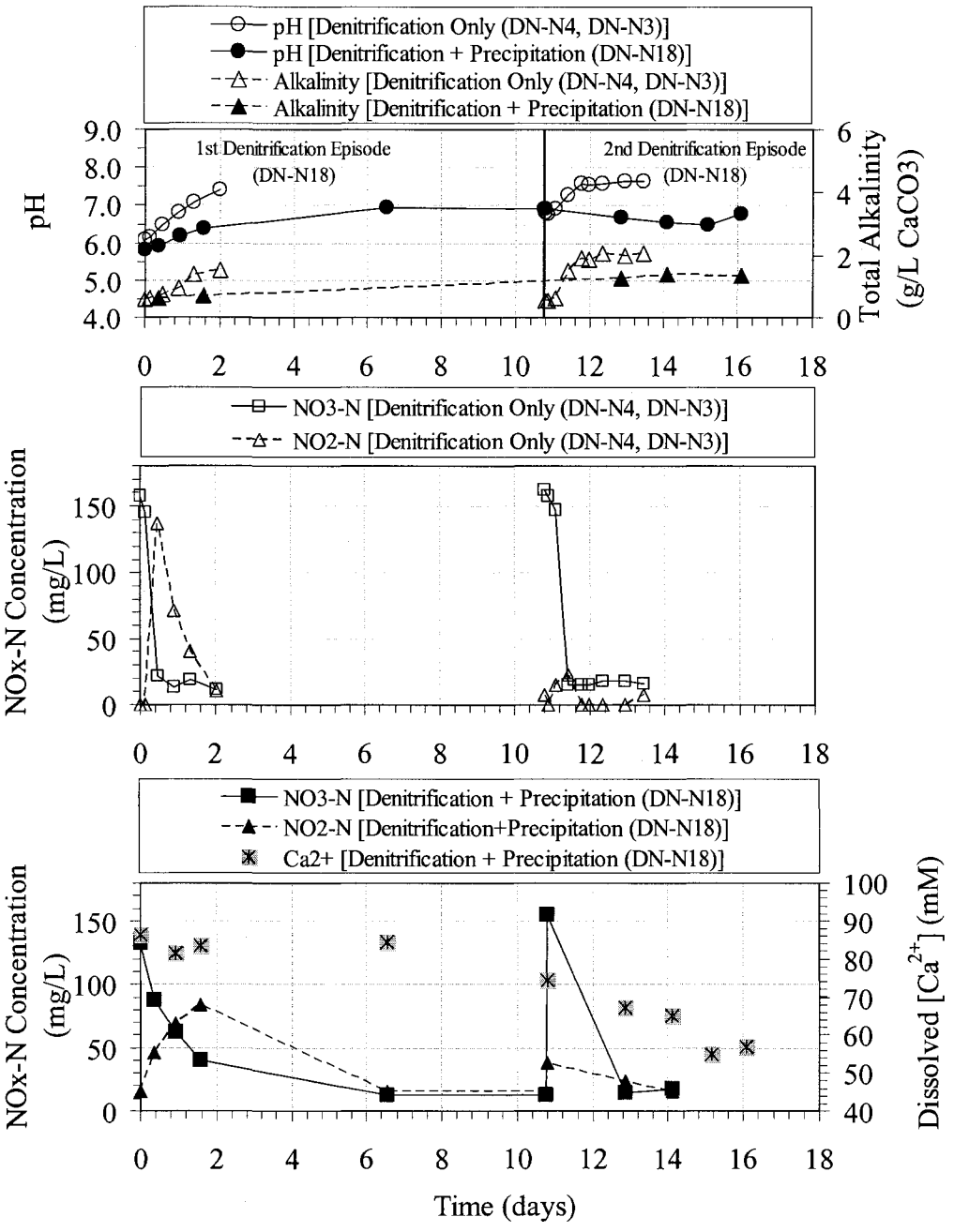


Fig. 5-18. Effect of calcium carbonate precipitation on geochemistry (DN-N18: initial Ca^{2+} concentration:90 mM electron donor: nutrient broth)

initial calcium precipitated out of the solution (Figure 5-18), most probably as calcium phosphate, as discussed in the section on "Effect of Phosphorus."

The pH during the 1st denitrification episode of DN-N18 rose from 5.8 to approximately 7.0, whereas the corresponding "denitrification only" experiment yielded a greater increase in the pH to over 7.4 within a shorter time frame (Figure 5-18). Also, the "denitrification only" experiments resulted in a greater increase in total alkalinity. During the 1st denitrification episode of DN-N18, the dissolved calcium concentrations did not change significantly (i.e., only 2 mM decrease in calcium concentrations, equivalent to approximately 0.3 g $CaCO_3/g\text{-}NO_3^-{}_{reduced;}$ Table 5-6) due to insufficient production of alkalinity (much lower than the increase in alkalinity in "denitrification only" experiment). Note that calcium carbonate precipitation that took place after all the nitrite was consumed within the 1st denitrification episode is not included in 0.3 g $CaCO_3/g\text{-}NO_3^-{}_{reduced}$ in Table 5-6. The difference in the alkalinity response can be explained by the development of nitrous acid concentrations above the inhibitive concentrations (Figure 5-16). The effect of nitrous acid toxicity on nitrate reduction rates and accumulation of intermediates were discussed in detail in the previous section.

The pH continuously decreased during the 2nd denitrification episode of DN-N18 as a result of the decrease in dissolved calcium concentration (i.e., calcium carbonate precipitation), whereas the pH increased during "denitrification only" experiment (Figure 5-18). The total alkalinity almost stayed constant during the 2nd episode of denitrification, indicating that almost all of the produced alkalinity during denitrification was consumed through calcium carbonate precipitation during the 2nd denitrification

episode. Figure 5-18 indicates that the dissolved calcium concentrations decreased by 18 mM during the 2nd denitrification episode of DN-N18, suggesting a total of 2.4 g of $CaCO_3$ (s) precipitation throughout DN-N18 per denitrification of 1.0 g NO_3^-, slightly higher than 2.2 g-$CaCO_3$/g-$NO_3^-_{reduced}$ as suggested by the stoichiometric analysis for alanine presented in the previous chapter (Table 4-4).

Similar behavior was observed in the precipitation experiment performed with L-glutamic acid as carbon source/electron donor, designated by DN-N17. DN-N17 was conducted with three episodes of denitrification, each one with approximately 8 mM of NO_3^- as electron donor, to mitigate nitrous acid accumulation. The results from DN-N17 were compared with DN-N19, a "denitrification only" experiment. The components of the liquid media used in DN-N17 and DN-N19 are listed in Table 5-7. The results from the assays of the samples from DN-N19 indicate that denitrification of approximately 25 mM of NO_3^- resulted in a pH increase from 7.3 to 8.3 over 1.5 days. When calcium ions were present, the reduction of the same amount of nitrate in three episodes resulted in a pH decrease from 7.4 to 6.5 over 16 days (Figure 5-19). The nitrate reduction rates were also slower during precipitation experiments, and the total alkalinity almost stayed constant as a result of calcium carbonate precipitation. Table 5-6 indicates up to 1.8 g $CaCO_3$ precipitation per denitrification of 1.0 g of NO_3^- during the different denitrification episodes of DN-N17, consistent with the $CaCO_3$ precipitation potential of L-glutamic acid estimated based on the stoichiometric analysis presented in Table 4-4 (i.e., 1.7 g-$CaCO_3$/g-$NO_3^-_{reduced}$).

Table 5.7. Composition of DN-17 and DN-N19 Liquid medium

	DN-N17		DN-N19	
	Chemical	Concentration, mM	Chemical	Concentration, mM
Inorganic Salts	NaCl $MgSO_4.7H_2O$ $CaCl_2.2H_2O$ Trace metals	17 1 100 (a)	NaCl $MgSO_4.7H_2O$ Trace metals	17 1 (a)
Phosphates	KH_2PO_4	2	KH_2PO_4	2
Electron Donor / Carbon Source	L-glutamic acid	75	L-glutamic acid	75
Electron Acceptor / Nitrogen Source	$NaNO_3$	25	$NaNO_3$	25

(a) A drop of trace metal solution of 0.5% (w/v) of $CuSO_4$, F_eCl_3, $MnCl_2$, $Na_2MoO_4.2H_2O$.

A different geochemical response was observed during precipitation experiment DN-N21, performed with a liquid medium consisting of 20 mM calcium, instead of the 100 mM of calcium used in DN-N18 and DN-N17. The results from DN-N21 are shown in Figure 5-20 along with "denitrification only" experiment DN-N19 for comparison. The decrease in dissolved calcium concentrations during DN-N21 suggests only 0.4 to 0.5 g of calcium carbonate precipitation per 1.0 g of NO_3^-.

The pH decreased initially during DN-21, but this decrease was not due to calcium carbonate precipitation as the dissolved calcium concentration almost stayed constant until almost all the nitrate was reduced. The pH decrease during this period was probably due to lower nitrite reduction rates as alkalinity was produced from conversion

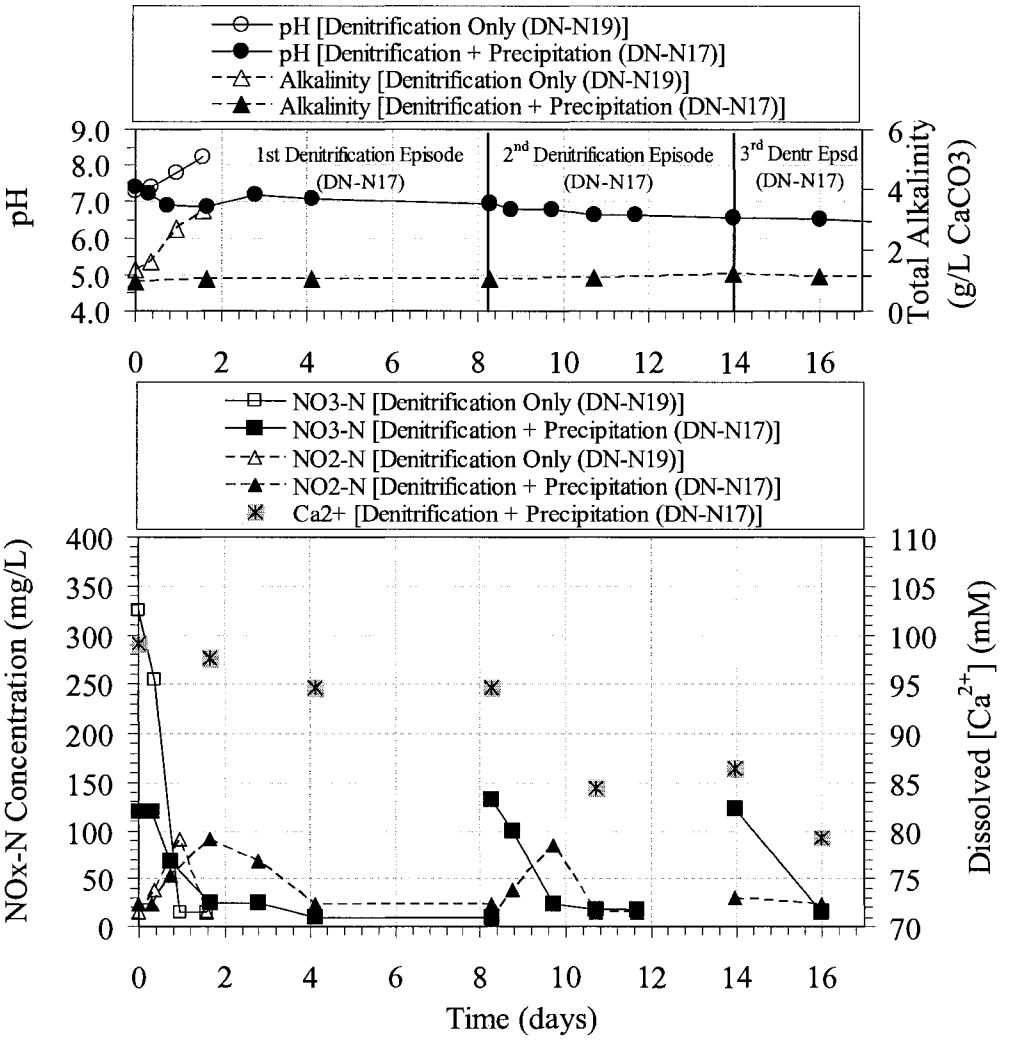


Fig. 5-19. Effect of calcium carbonate precipitation on geochemistry (DN-N17: initial Ca^{2+} concentration:100 mM electron donor: L-glutamic acid)

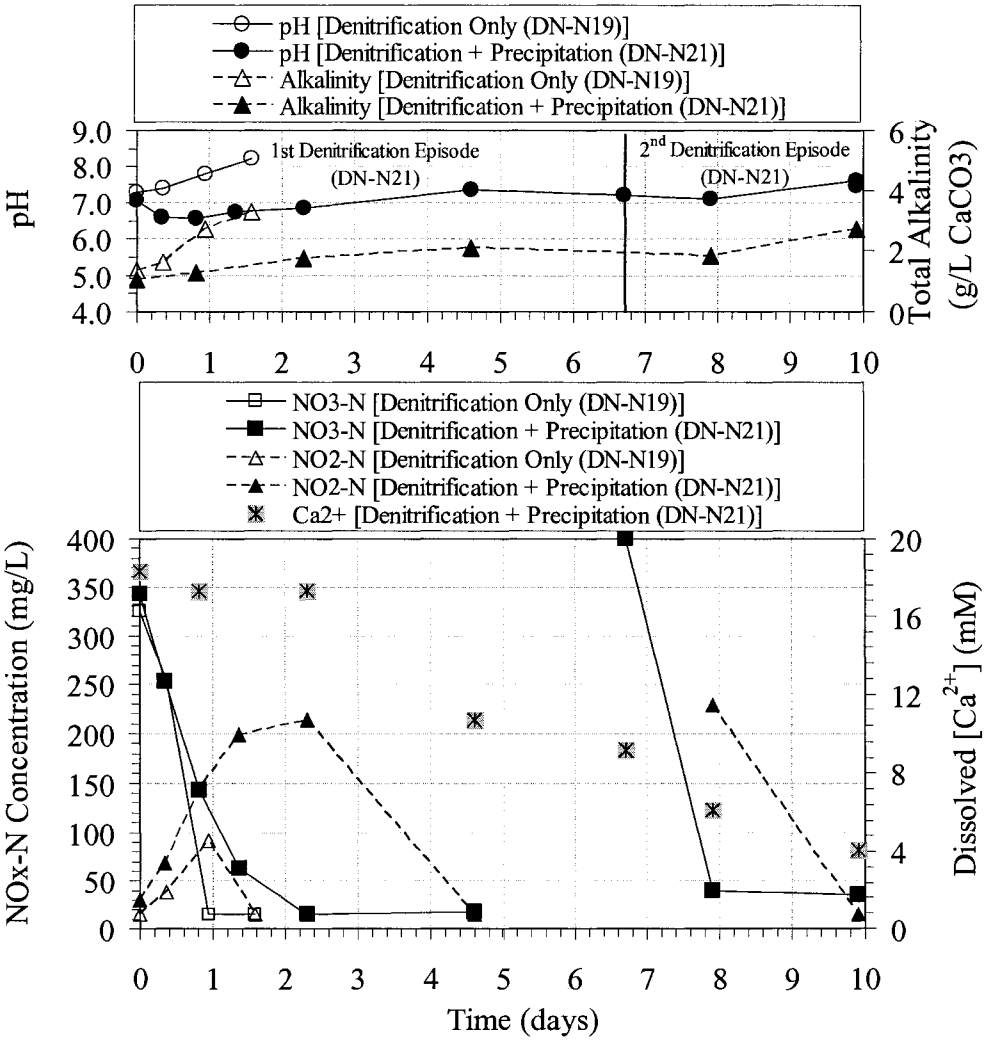


Fig. 5-20. Effect of calcium carbonate precipitation on geochemistry (DN-N21: initial Ca^{2+} concentration:20 mM; electron donor: L-glutamic acid)

of nitrite. On the other hand, during DN-N19, a smaller peak of nitrite was observed compared to DN-N21, indicating a higher nitrite conversion rate concurrent with nitrate reduction, resulting in a pH increase. After all of the nitrate was consumed in DN-N21, the pH and alkalinity started to increase even though the dissolved calcium concentration was decreasing.

Because the initial Ca^{2+} concentrations were lower than the previous experiment with the same electron donor (DN-N17), only a portion of the alkalinity was consumed through calcium carbonate precipitation. Contrary to the pH decrease observed in DN-N17 during calcium carbonate precipitation (Figure 5-19), partial consumption of the alkalinity produced through denitrification (shown by increase in alkalinity in Figure 5-20) resulted in a net increase in alkalinity and pH during DN-N21.

In addition, salinity, mainly caused by the calcium salt, resulted in higher nitrite peaks (i.e., lower nitrite reduction rates until all the nitrate is consumed as electron donor), which determines if the pH will increase or decrease at the beginning of denitrification and may become important when developing field application strategies to minimize nitrous acid concentrations.

The calcium carbonate precipitation calculated based on the measured change in dissolved calcium concentrations are compared with the results from the stoichiometric analysis in Table 5-8. The results from the incomplete denitrification experiments (DN-N9,DN-N10, DN-N13, DN-N14) and experiments with insufficient calcium source (DN-N21) are not included in Table 5-8. The comparison of the results indicates that the calcium carbonate precipitation in DN-N17 was mainly controlled by the production of

Table 5-8. Comparison of results from stoichiometric analysis and batch experiments

Experiment	e-donor	Calculated based on measured change in dissolved $[Ca^{2+}]$ *(See Table 5-6)*	Estimated based on Stoichiometry *(See Table 4-4)*	
			(a) Alkalinity is limiting;	(b) CO_2 is limiting;
		$CaCO_3$ (s) ($g/g\text{-}NO_3^-$)	$CaCO_3$ (s) ($g/g\text{-}NO_3^-$)	$CaCO_3$ (s) ($g/g\text{-}NO_3^-$)
DN-N17 1st Episode	L-glutamic Acid	1.0	1.7	2.8
DN-N17 2nd Episode		1.8		
DN-N17 3rd Episode		1.4		
DN-N18 1st Episode	Nutrient Broth	0.3[c]	2.2[d]	2.3[d]
DN-N18 2nd Episode		2.4		

(a) All of the produced alkalinity is consumed through $CaCO_3$ precipitation.
(b) All of the produced CO_2 is consumed through $CaCO_3$ precipitaton.
(c) Does not include the calcium carbonate precipitation after all the nitrite is consumed within an episode until the beginning of the next episode.
(d) Based on stoichiometric analysis of alanine as electron donor
(e) Table does not include results from incomplete denitrification experiments (DN-N9,DN-N10, DN-N13, DN-N14) or when calcium source was not sufficient (DN-N21)

alkalinity. During the first denitrification episode of DN-N18, the produced alkalinity less than the alkalinity production estimated by the stoichiometric analysis, resulting in a much lower calcium carbonate precipitation. During the second denitrification episode of DN-N18, the batch reactor experiment resulted in a slightly higher amount of calcium carbonate precipitation, which may indicate a slightly higher production of alkalinity and CO_2 in the reactor than the quantities estimated based on stoichiometric analysis.

5.4.3. X-ray Diffraction Analysis

The sediment deposited by microorganisms within the reactors were filtered out using a vacuum-filter system. The sediments became hard and brittle after being air-dried over night. A sample of sediment deposited during DN-N17 is shown in Figure 5-21. A few drops of hydrochloric acid (HCl) added to the sediments collected on filter papers resulted in gas production in all cases, which indicates there is calcium carbonate in the sediment deposited by the microorganisms. Samples of these sediments were subsequently analyzed by X-ray diffraction to identify the mineralogical content of these microbial deposits. The results from X-ray diffraction analysis on sediments from the DN-N9 and DN-N17 are shown in Figures 5-22 and 5-23, respectively. The results from

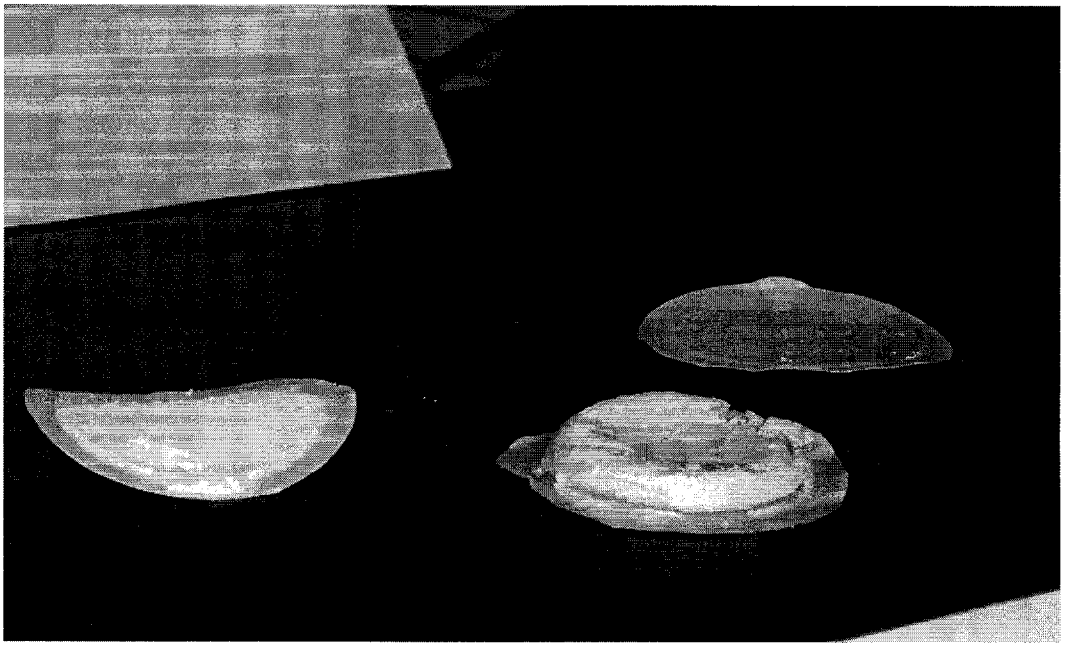


Fig. 5-21. Sediment collected on filter paper - DN-N17 (Filter paper dia.=1.75 in. or 4.45 cm)

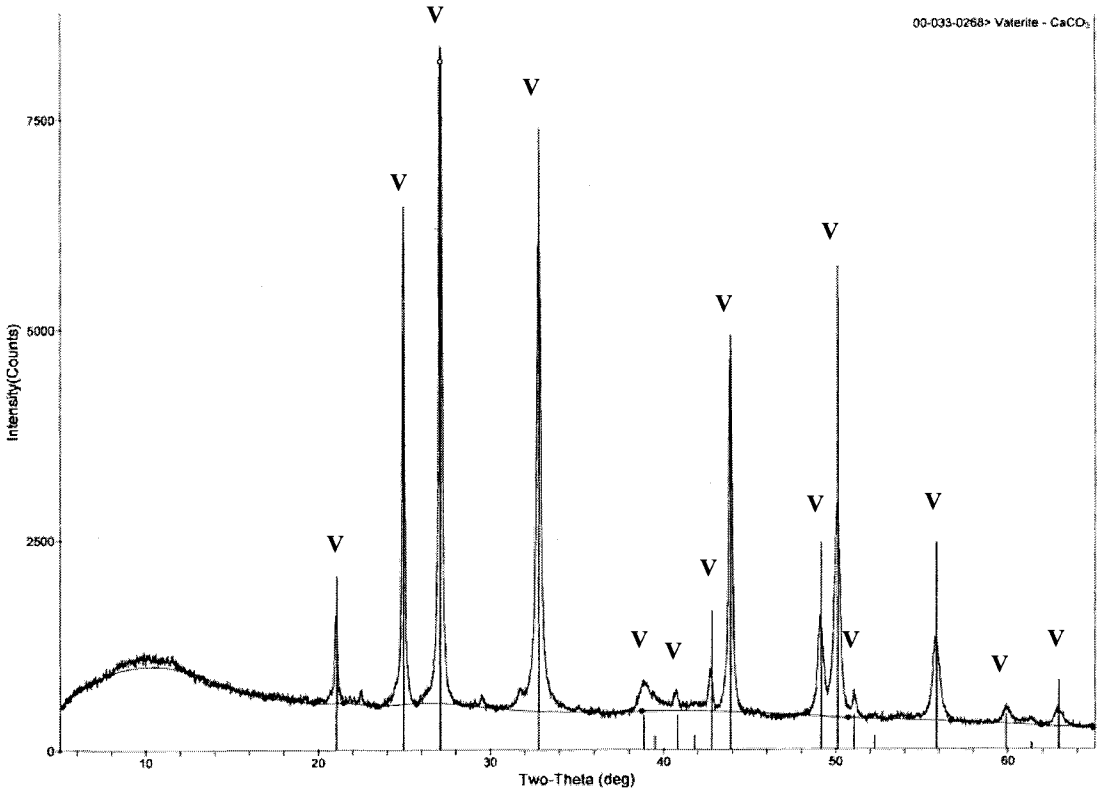


Fig. 5-22. X-ray diffraction test results – DN-N9 sediment (V:Vaterite)

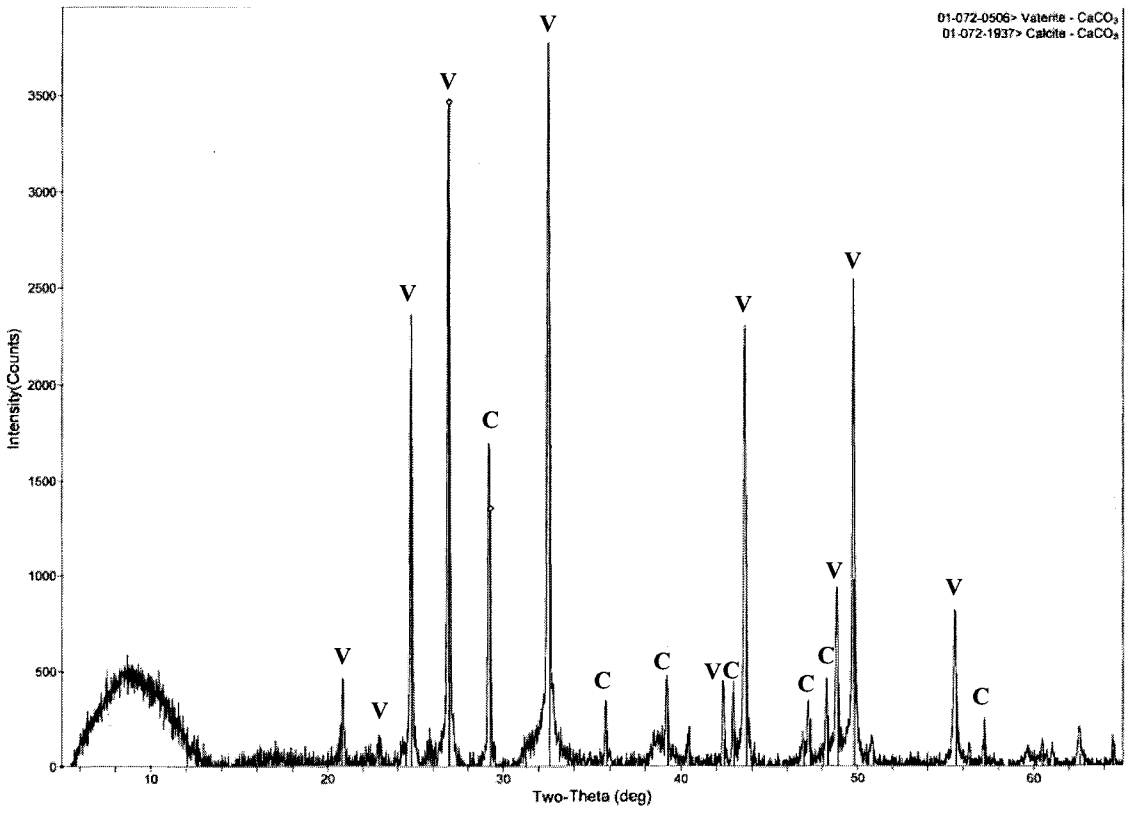


Fig. 5-23. X-ray diffraction test results – DN-N17 sediment (V:Vaterite; C:Calcite)

the X-ray diffraction analysis indicate that vaterite and calcite minerals make up the bulk of these microbial calcium carbonate deposits.

5.5. Summary of Test Results and Field Application Strategies

The results from the batch reactor experiments were used to evaluate the calcium carbonate precipitation potential through denitrification, to investigate the effects of the accumulation of denitrification intermediates, and to verify the findings from the stoichiometric analysis. Batch reactor experiments performed as part of this study included a suspended growth system whereas in the soil column experiments and for future *in situ* applications, a composite microbial growth consisting of biofilm and suspended growth can be expected due to attachment of the microorganisms on soil particle surfaces.

Although the quantitative results from the batch reactor experiments are specific to *P. denitrificans* (ATCC 13867), these results are consistent with the results from the stoichiometric analysis of denitrification and can be generalized conceptually for application of denitrification for calcium carbonate precipitation in soil. The following is a summary of the results from the denitrification and precipitation experiments and review of the literature, followed by a field application strategy based on these results:

- Denitrification typically results in an increase in alkalinity and pH, which is favorable for calcium carbonate precipitation.
- The pH may increase or decrease initially during reduction of nitrate to nitrite, based on the relative rate of nitrate reduction to nitrite reduction. The production of alkalinity is generally associated with the reduction of nitrite.

When CO_2 is released without production of alkalinity (e.g., during the reduction of nitrate to nitrite), the pH will decrease unless concurrent nitrite reduction with comparable rates takes place while nitrate is being reduced to nitrite. If nitrite reduction is inhibited during reduction of nitrate to nitrite, the decrease in pH will be observed.

- Accumulation of denitrification intermediates (i.e., nitrite and nitrous oxide) can inhibit the production of alkalinity and inorganic carbon required for carbonate precipitation.
- Removal of carbonate from a solution (e.g., precipitation of calcium carbonate) typically results in a decrease in alkalinity and pH. If the medium has low calcium concentration, $[Ca^{2+}]<20$ mM, pH may increase or stay constant due to limited consumption of alkalinity through $CaCO_3$ precipitation.
- If sufficient amount of Ca^{2+} is present in the medium, alkalinity stays constant or increases only slightly when liquid medium with calcium is used, due to precipitation of calcium carbonate (i.e., consumption of all the produced alkalinity) during denitrification.
- Phosphorus limitation affects the rate of denitrification through its affects on cell synthesis and can result in accumulation of intermediates.
- High salinity results in lower denitrification rates and accumulation of intermediates, if the microorganisms are not tolerant to salinity.

- Synthesis of denitrification enzymes requires iron, molybdenum, and copper. Although copper was the only limiting metal in the experiments presented herein, limitation of other inorganic nutrients necessary for the synthesis of denitrification enzymes can result in accumulation of denitrification intermediates.
- Concentrations of nitrous acid, the undissociated form of nitrite, above 0.04 mg HNO_2-N/L resulted in accumulation of denitrification intermediates, probably through affecting the proton gradient across cell membrane.

Nitrite/nitrous acid toxicity can be controlled by initial nitrate concentrations and/or the pH. Initial low nitrate concentrations (<8 mM), or using a buffering agent to keep pH above 7.0 (based on Figure 5-11), or both can mitigate nitrite/nitrous acid toxicity. Consistent with the results of the stoichiometric analysis presented in the previous chapter (Table 4-4), the denitrification-precipitation experiments resulted in precipitation of $CaCO_3$ up to 2.4 g per 1 g of NO_3^- reduced, which suggests that denitrification is a potentially feasible mechanism for calcium carbonate precipitation for soil improvement. All the factors that can potentially affect the success of the application of denitrification as a calcium carbonate precipitation mechanism suggest that the geochemistry of the groundwater should be analyzed for nitrate, nitrite, metal, and phosphorus concentrations prior to field application to evaluate the limiting conditions. Dissolved oxygen content, pH, alkalinity, salinity, COD or BOD, and total and calcium hardness should also be measured to develop the "recipe" for the nutrient solution to be amended to the ground to foster the growth of denitrifiers.

The nutrient solution prepared based on the analysis of the geochemistry of the groundwater may include an electron donor or electron acceptor along with other inorganic nutrients. If the electron donor is the limiting factor for denitrification at a particular location, an appropriate organic electron donor can be selected considering relevant factors including solubility, cost, and ammonium content. If the electron acceptor is the limiting factor, nitrate salts or nitrite salts can be included in the nutrient solution to be injected into the ground. Utilization of small quantities of nitrate (i.e., successive short denitrification episodes, instead of one long denitrification episode) may provide a better implementation strategy during field applications because remedial measures can be taken easily to mitigate the accumulation of hazardous intermediates and minimize the environmental and health risks that may rise as a result of this application. Alternatively, although not shown in the bench scale experiments discussed in this chapter, nitrite salts can be used instead nitrate salts as the electron acceptor because most of the alkalinity produced during denitrification is produced during reduction of nitrite. This would also provide direct control over the nitrite concentrations in the groundwater. However, injection of nitrite, a chemical agent that may be carcinogenic, directly into the ground may entail challenges associated with regulatory and public acceptance. Thus, whether or not small quantities of nitrate or nitrite is being used, a comprehensive quality control/assurance plan detailing monitoring and sampling is required for the management of the environmental and health risks and to increase the efficiency of the application.

The precipitation experiments performed as part of this study all included liquid medium containing calcium ions at the beginning of the experiments. As an alternative

to injecting a solution containing calcium salts at the beginning of implementation of this technology, an initial nutrient mix designed to stimulate the growth of indigenous denitrifiers *in situ* can be amended to the ground to minimize the effect of pH decrease associated with calcium carbonate precipitation on the activity of denitrification enzymes and microbial growth. Calcium salts can be added at a later stage based upon the change in pH and alkalinity of the medium.

The batch reactor experiments discussed in this chapter were performed at a constant temperature of approximately 30°C. Soil temperatures may vary significantly from 30°C. Because lower temperatures will slow the microbial kinetics and may alter the equilibria, further experiments to study the temperature effects are recommended.

A biogeochemical model developed based on the results from these batch experiments, soil column tests, and pilot scale field trials (recommended as part of future work following this study) can be helpful to engineers in analyzing different scenarios prior to a field application and in performing a cost-benefit analysis.

CHAPTER 6

EFFECTS OF MICROBIAL CEMENTATION ON THE SHEARING BEHAVIOR OF GRANULAR SOILS AND SOIL-COLUMN EXPERIMENTS

6.1. Experience with Ex-Situ Artificial Cementation Experiments

The effects of cementation on the shearing behavior of granular soils have been studied by many researchers using artificially cemented soils (Sitar et al., 1980; Bacus et al., 1981; Rad, 1983; Abdulla and Kiousis, 1997; Fernandez and Santamarina, 2001; Asghari et al. 2003; Haeri et al., 2005; Kasama et al., 2006). Most of these experiments were performed using Portland cement (the most common type of cement used in engineering, prepared by heating and grinding a mixture of limestone containing a minimum of 80 percent calcium carbonate and clay) or gypsum cement (another common type of cement that consists primarily of gypsum mineral, which is composed of calcium sulfate dihydrate). Tests on artificially cemented granular soils indicate shear strength increases primarily due to an increase in cohesion among the particles, with only a slight increase in peak and residual internal friction angles for the cemented soil (Sitar et al., 1980). Rad (1983) performed a series of tests on sands with varying degrees of relative density and observed increase in unconfined compressive strength with as little as 1 percent Portland cement by weight. The experiments conducted by Rad (1983), shown in Figure 6-1 and 6-2, also indicated that the strength gain due to cementation was primarily due to increased cohesion and that there was only a slight increase in the friction angle. Sitar et al. (1980) reported that cementation with 2 percent Portland cement by weight induced cohesion on the order of 45kPa in one sand (45 kPa cohesion is enough to allow a vertical cut over 10 m-high to stand unsupported).

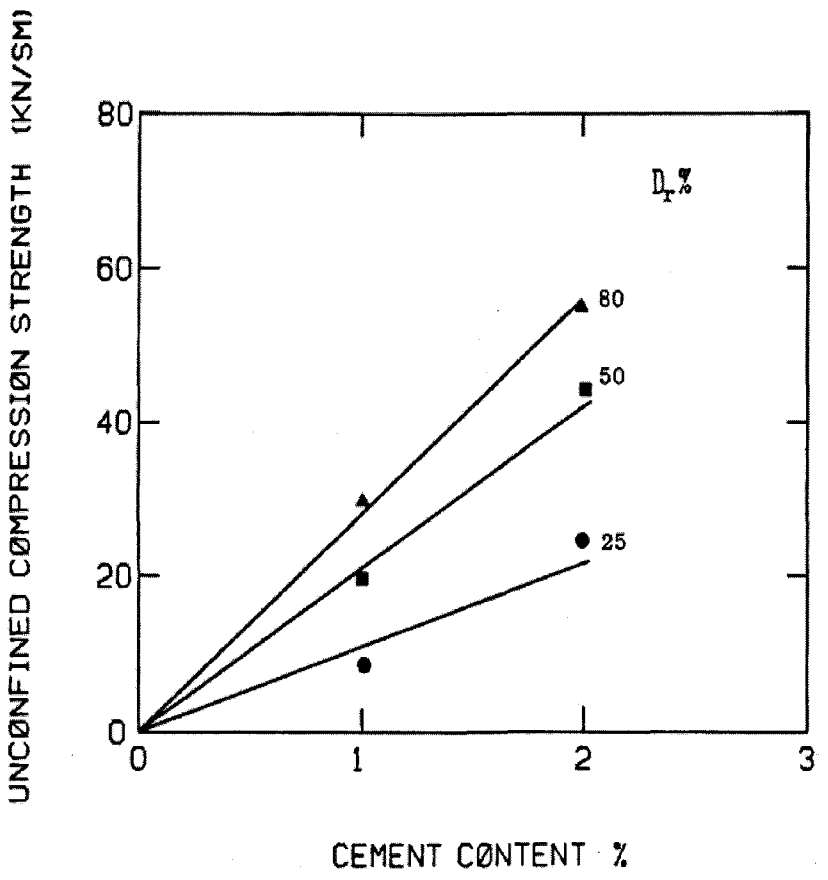


Fig. 6-1. Change in unconfined compressive strength with cement content: samples with 25, 50, and 80 percent relative density, D_r (Rad, 1983)

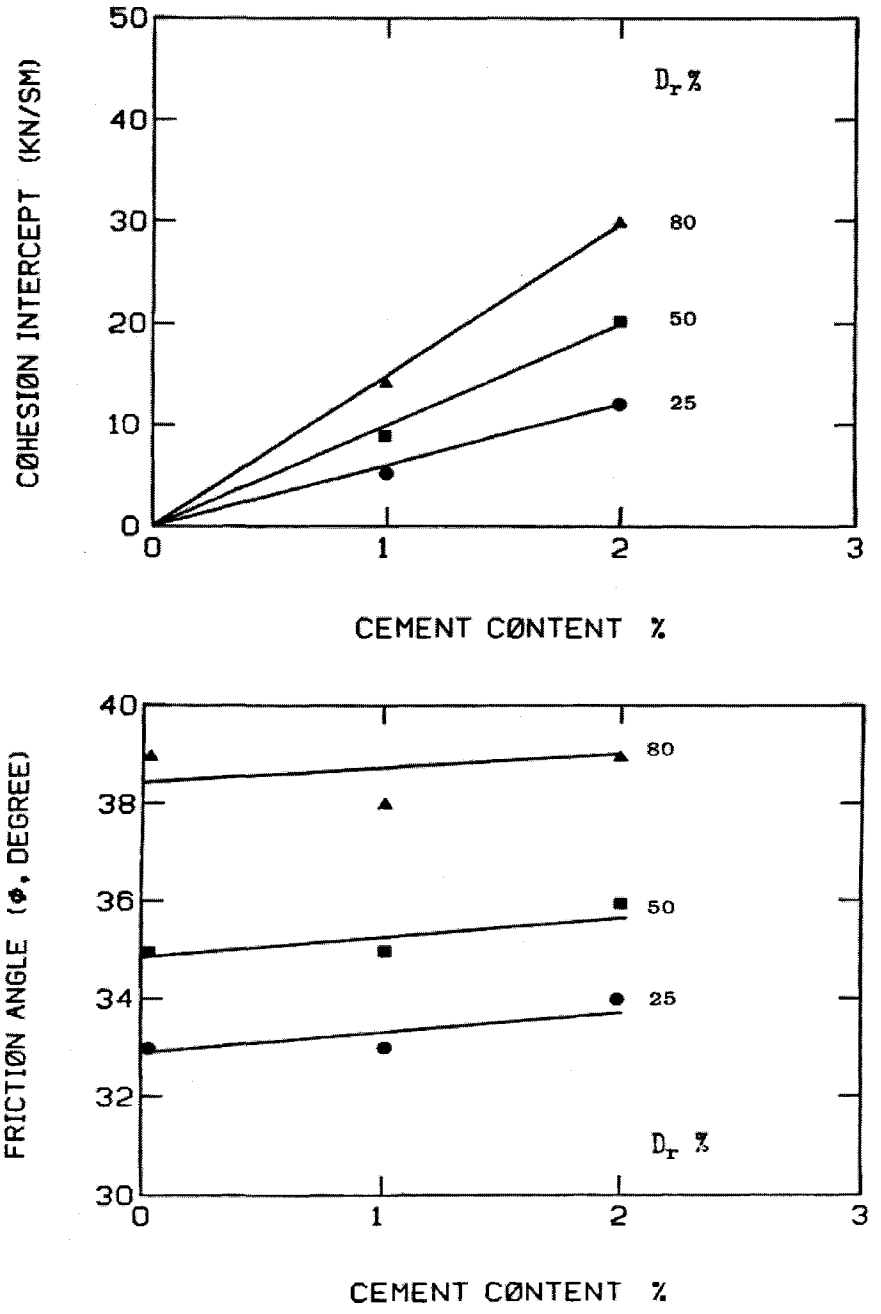


Fig. 6-2. Effect of cementation on cohesion and friction angle: samples with 25, 50, and 80 percent relative density, D_r (Rad, 1983)

Tests conducted by Bachus et al. (1981) suggest that cementation can also increase the initial tangent modulus (i.e., increase the small strain stiffness) of a soil by up to an order of magnitude at low confining pressures, though the effect was much smaller at higher confining pressures (where the confining pressure was applied after the artificially cemented soil was prepared). Based on the results of experiments with gypsum-cemented gravelly sand, Haeri et al. (2005) reported that the friction angle of sand increases slightly due to cementation but that the increase in cohesion is more noticeable and increases as the cement content increases. Fernandez and Santamarina (2001) reported that the small strain stiffness of sands can increase by an order of magnitude or more due to cementation with 2 percent Portland cement by weight.. The results published by Fernandez and Santamarina (2001) indicate an increase in shear wave velocity (which is proportional to the square root of the small strain stiffness) of fine subangular sand from 230 m/s to 620 m/s at 100 kPa confining pressure when mixed with 2 percent Portland cement by weight and cured before loading. These investigators also noted that cemented soils exhibit very limited changes in shear wave velocity (and thus in small-strain stiffness) due to stress change until de-cementation (breaking of the cementation bonds) begins (Fernandez and Santamarina, 2001).

However, all of these results are for abiotic cementation. Due to a difference in mineral content, structure, particle size, and organic content of the cement, soils improved by microbially induced cementation may display a different shearing and volume change response than soils improved with abiotic cementation.

6.2. Previous Research on Microbial Cementation

Whiffin (2004) studied the effects of microbial precipitation of calcium carbonate through hydrolysis of urea on the physical properties of sands. An aerated solution of urea, calcium, and urea-hydrolyzing bacteria was injected into sand specimens to induce calcium carbonate precipitation. Whiffin (2004) reported up to 1.5 M calcium carbonate precipitation within 18 hours of incubation period. The change in physical properties due to the induced calcium carbonate precipitation was initially evaluated using compressional wave (P-wave) velocity measurements that were assumed to be correlated with uniaxial compressive strength. The P-wave velocity measurements indicated an increase in cementation (and thus shear strength) with increasing concentration of hydrolyzed urea (Whiffin, 2004). However, the degree of saturation of the soil specimens, which may affect the interpretation of P-wave velocities measured during these experiments, was not reported.

Whiffin (2004) subsequently performed triaxial shear strength tests on Dutch Koolschhijn sand injected with urea, calcium, and urea-hydrolyzing bacteria and reported that shear strength increased by a factor of 8 and stiffness (secant modulus at 50 percent of peak shear stress) increased by a factor of 3, without a significant change in pore volume. However, important details of the triaxial tests, e.g., the confining pressures, and drainage conditions, were not reported.

DeJong et al. (2006) evaluated the effects of calcium carbonate precipitation induced microbially through urea hydrolysis on the shearing properties of loose sands. Shear wave velocity measurements were employed by DeJong et al. (2006) to monitor

the development of cementation during microbial treatment (a period of approximately 24 hours) and consolidated undrained triaxial shear tests were performed at the end of the treatment period. DeJong et al. (2006) observed an increase in shear wave velocity from approximately 200 m/s to 540 m/s due to microbial treatment and reported that the microbially cemented soils displayed a similar shearing response to gypsum cemented soils under undrained conditions (Fig. 6-3). DeJong et al. (2006) concluded that pH, oxygen supply, metabolic status and concentration of microorganisms, and calcium concentration are critical factors for the success of this application.

Recently, Whiffin et al. (2007) performed a series of drained triaxial tests on samples (25 cm high, 6.6 cm dia.) from a sand column cemented with calcium carbonate precipitated through ureolysis under a confining pressure of 50 kPa. These investigators observed improvement in the peak compressive strength for samples with calcium carbonate concentrations greater than 60 kg/m^3 (Fig. 6-4). Based on the dry unit weight reported for the sand used in these experiments (1.65 g/cm^3), this "threshold" calcium carbonate content of 60 kg/m^3 corresponds to 3.6 percent cement by weight. This reported threshold for strength improvement of microbially cemented soil is in contrast with the reported increase in shear strength with as low as 1 percent cement by weight for Portland cement mixed with sand ex-situ (Rad, 1983). Whiffin et al. (2007) reported that the drained peak compressive strength of the sand samples increased from 167 kPa to 570 kPa for a calcium carbonate content of approximately 110 kg/m^3 (6.7 percent cement by weight). Whiffin et al. (2007) also reported that microbially induced calcium carbonate

cementation did not seem to affect either the residual compressive strength or the hydraulic conductivity of their samples.

Recently, Harkes et al. (2008) performed a series of unconfined compression tests on sand samples cemented with microbially induced calcium carbonate through ureolysis and observed a similar increasing trend as noted by Whiffin et al. (2007). The unconfined compressive strengths increase ranged from 0.1 MPa to 100 MPa depending on level of cementation. Because important details about the experiments were not reported, the tests results from Harkes et al (2008) are not presented here.

Because most of the experiments concentrated on the shear strength, further research on stress-strain behavior and volume change response under static and dynamic loading, and the effects of confining pressure, fines content, cement properties should be evaluated.

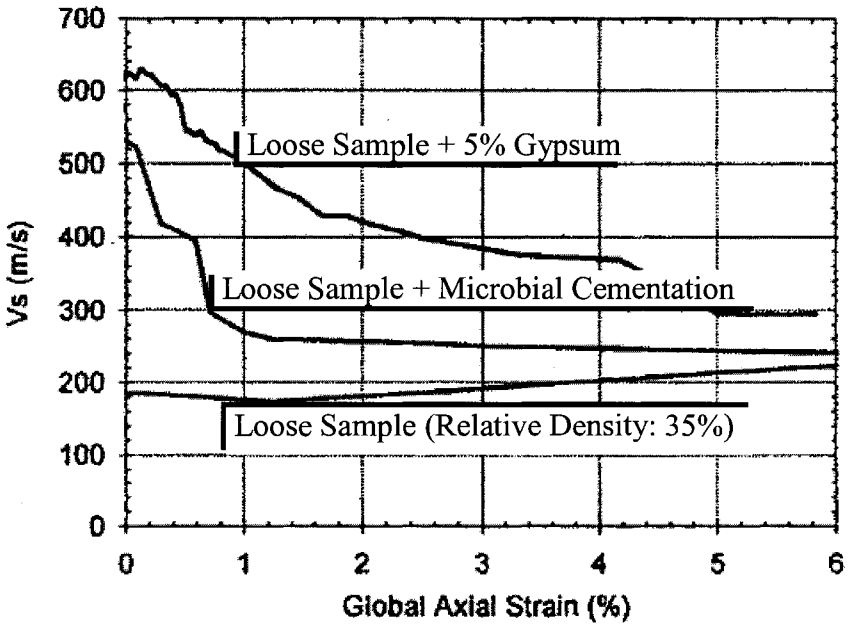


Fig. 6-3. Shear wave velocity measured during monotonic triaxial tests (DeJong, 2006)

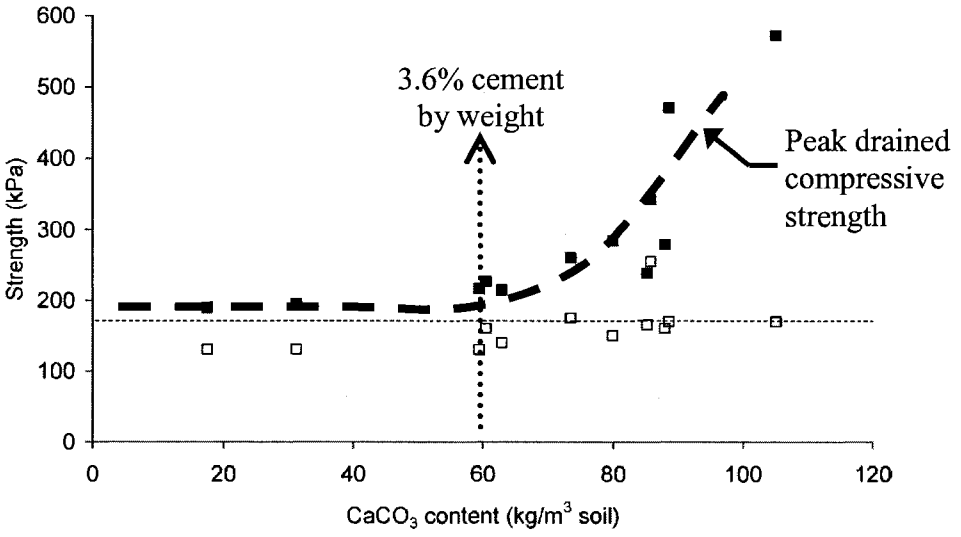


Fig. 6-4. Change in unconfined compressive strength with microbially induced calcium carbonate precipitation: ■ peak strength; □ residual strength (adapted from Whiffin et al., 2007)

6.3. Soil-Column Experiments

As a follow up to the batch reactor tests described in the previous chapter, soil-column experiments were performed to evaluate requirements for inducing cementation in cohesinless soils through precipitation of calcium carbonate during microbial denitrification. Closed-system soil-column experiments were conducted using acrylic cylinders filled with cohesionless soil and liquid nutrient solution. The liquid nutrient solution was sampled frequently to monitor denitrification. The soil samples recovered from the "closed-system soil-columns" at the end of the experiments were analyzed with the help of scanning electron microscopy (SEM) to produce an image of the soil particle surfaces and grain contacts. In addition to the physical SEM evaluation, energy dispersive spectrometer (EDS) analysis performed concurrently with the SEM to provide information on the elemental composition of the specimens, including substances accumulated on grain surfaces and at the particle contacts.

The methodology employed in the closed-system soil-column experiments and the results from these tests are discussed in detail in the remainder of this chapter.

6.3.1. Closed System Soil Column Experiments – Methods

Soil column experiments were performed using the American Society of Testing and Materials (ASTM) 20/30 Ottawa Sand furnished by US Silica Company. The 20/30 Ottawa sand is commercially available "standard" silica sand with known properties and approximately 99 percent of the grains are between 0.60 and 0.85 mm in diameter. The product data sheet listing the properties of the 20/30 Ottawa sand is included in Appendix C.

The sand was placed in the acrylic cylinders with an inside diameter of 7.3 cm and a height of 15.5 cm, shown in Figure 6-5. The sand was placed n a controlled manner to achieve a target dry density of 1.6 g/cm^3 (100 lbs/ft^3). The acrylic cylinders used in the soil column experiments are made up of three pieces, a base platen, a hollow cylinder, and a top cap, that fit together easily and were manufactured at the ASU Engineering Technical Services Machine Shop. The hollow cylinder fits into the base platen and the top cap fits into the hollow cylinder. O-ring seals were used to provide a seal between the cylinder, the base platen, and the top cap. A total of 4 sampling ports controlled by stainless steel valves, two along the side of the hollow cylinder and one each at the top cap and base platen, were employed to provide liquid delivery into and out of the column.

Prior to placing the sand in the acrylic cylinders, the cylinders were first filled with liquid nutrient solution. The content of the liquid nutrient solution was same as the DN-N17 liquid medium, are listed in Table 5-7. The initial nitrate and calcium concentrations were approximately 8 mM and 100 mM, respectively. The initial pH of the liquid nutrient solution was adjusted to approximately 7.0 using sodium hydroxide (NaOH). Once the liquid nutrient solution was filled up to approximately 2/3 of the total height of the cylinder, the sand was poured through a funnel placed at a few centimeters from the surface of the liquid in order to minimize the spatial variability in sand density and inclusion of air pockets within the soil profile. The excess liquid was allowed to flow over the sides of the cylinder. Once the sand was filled up to the target height, the top cap of the acrylic cylinder was placed carefully. The top valve was left open and

Fig. 6-5. Acrylic cylinders used in closed system soil column tests

the extra liquid between the top cap and the top of the soil column was removed using a vacuum applied by a syringe and a thin hose as the top cap was pushed down to the top of the soil surface. Once the top cap was in contact with the top of the soil surface, the top valve was closed. The top cap, and base platen connections were then covered with a thin epoxy to provide extra protection against leakage out of the cylinder. The liquid nutrient solution, the acrylic cylinders, the soil, and the tools used in constructing the soil columns were not sterilized.

The acrylic cylinders were kept in a dark room with temperature set to 30°C throughout the soil column experiments. Approximately 6-ml samples were periodically

collected from the cylinders with a syringe and analyzed for pH, nitrate, nitrite concentrations, alkalinity, and calcium concentrations. The liquid samples were first analyzed for pH and filtered through 0.2-μm membrane filter prior to storing in a refrigerator for further analysis.

6.3.2. Closed-System Soil-Column Experiments – Results

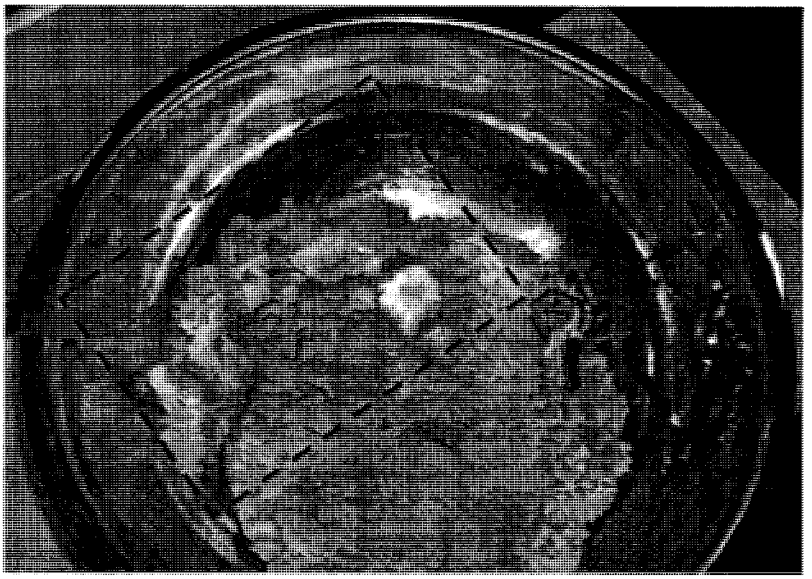
The first closed-system soil-column experiments was performed with two successive denitrification episodes using an initial nitrate concentrations of 8 mM using one acrylic cylinder. Another acrylic cylinder was filled with sand and distilled water as a control specimen. The assays on the three liquid samples collected from the initial soil column indicated that all of the initial 8 mM NO_3^- was reduced within approximately 3.5 days, consistent with the nitrate reduction times observed in DN-N17. The pH of the liquid samples decreased from 7.0 to 6.82 at the end of the first denitrification episode. Following the first denitrification episode, a liquid solution containing nitrate was injected to restore the NO_3^- concentration to 8 mM within the soil column. The liquid samples collected during the second denitrification episode were not assayed for nitrate and nitrite concentrations due to contamination observed after they were placed in the refrigerator, along with the other samples from the first denitrification episode waiting for alkalinity and calcium concentration analyses.

Four days following the beginning of the second denitrification episode, the two acrylic cylinders (one containing the sand and the liquid nutrient solution and another with just sand and distilled water) were opened, and the contents were placed in two separate aluminum bowls. These samples were left to air drying for approximately 2 days. At the

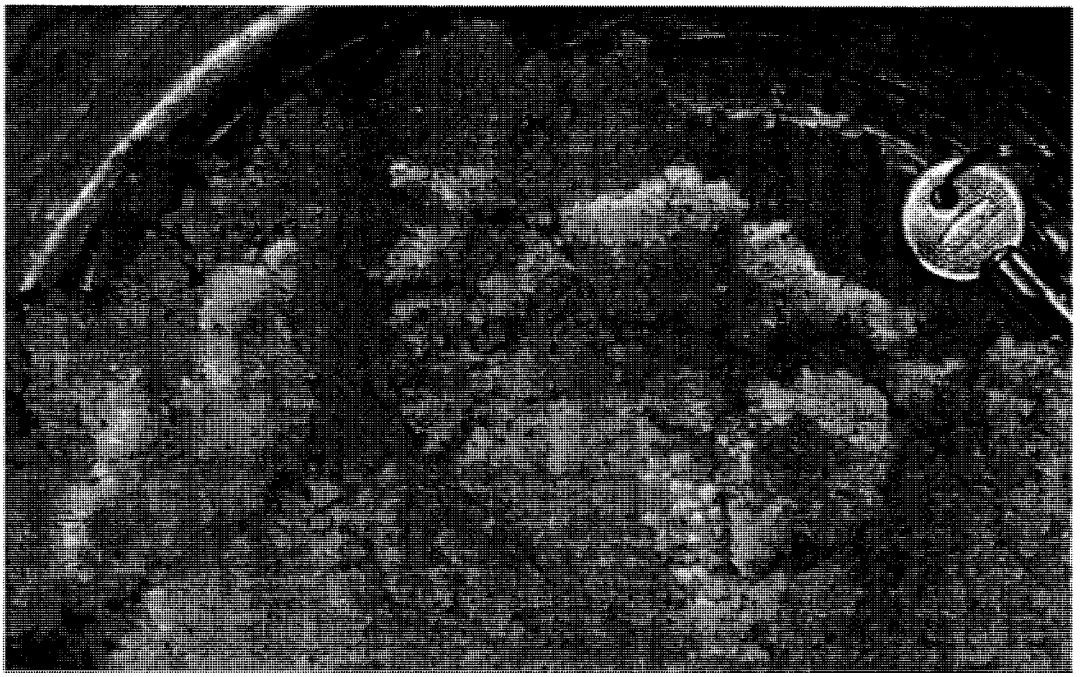
end of the 2-day drying period, the soil from the column that was treated with nitrate resulted in lumps of sand particles. These lumps of soil particles, , shown in Figure 6-6, were easily breakable by hand. A few ml of HCL dropped on some of the lumps that were broken up by hand resulted in gas generation. These lumps were further analyzed by SEM and EDS to verify calcium carbonate precipitation. The air dried soil samples from the control experiment did not result in gas generation upon treatment with HCl.

Sample scanning electron micrographs are presented in Figures 6-7 and 6-8. Figure 6-7 shows two grains of the Ottawa sand attached to each other through an intermediate material shown in close-up in Figure 6-8. The section shown in Figure 6-8 (b) was further analyzed by EDS for elemental composition. The results from the EDS analysis are shown in Figure 6-9. The EDS results indicate that calcium and carbon elements are present in the material attaching the two sand particles, which suggests that $CaCO_3$ may be present within the material cementing the soil from the column treated with nitrate.

Assuming the cementation was caused by $CaCO_3$ precipitation, the amount of $CaCO_3$ precipitation can be increased by employing additional denitrification episodes and/or replacing the L-glutamic acid used in the soil column experiment with alanine or another electron donor/carbon source that yield more $CaCO_3$ precipitation.



(a)



(b)

Fig. 6-6. Air dried contents of the soil column test: Picture (b) is a closer view of the sand in the aluminum bowl shown in Picture (a), marked with a box

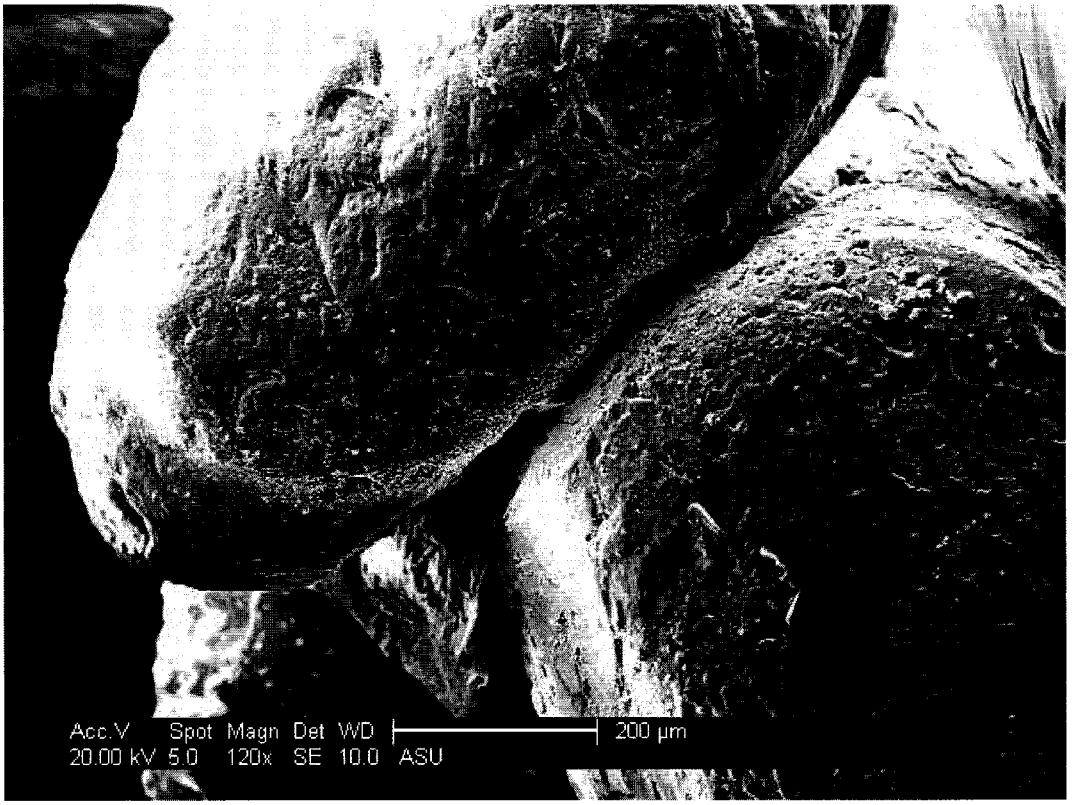
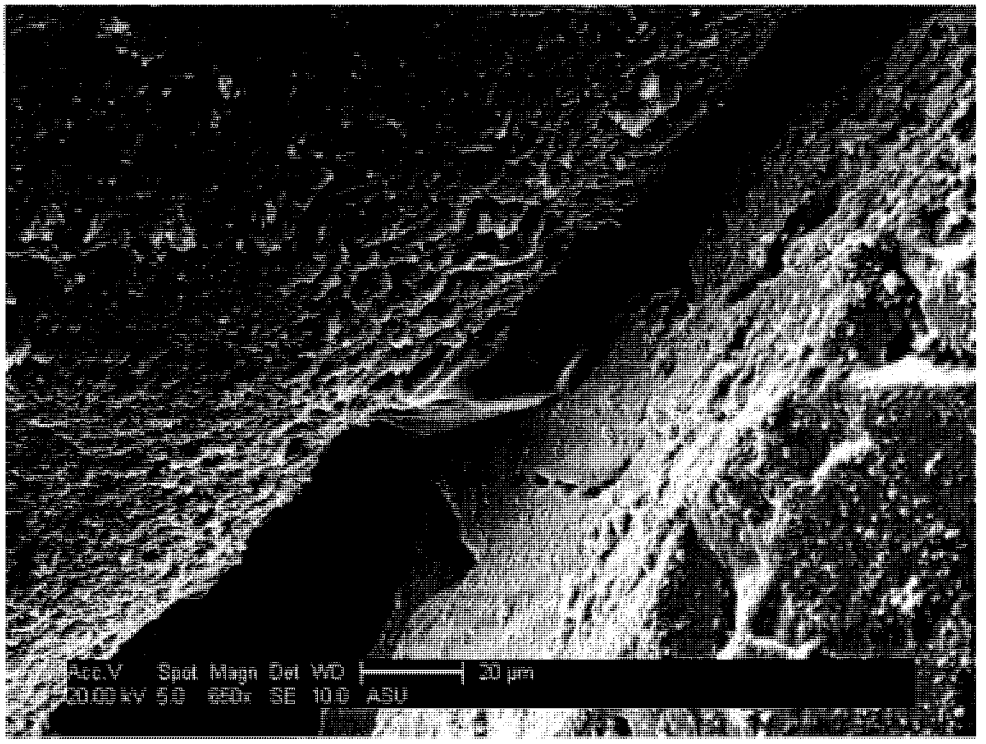
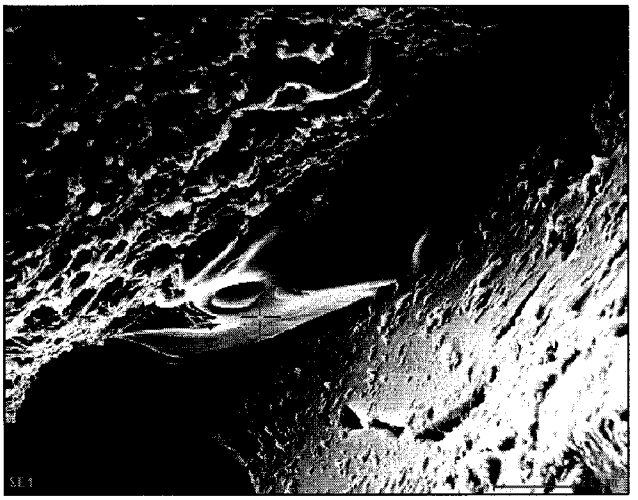


Fig. 6-7. Scanning electron micrograph of weakly cemented Ottawa sand

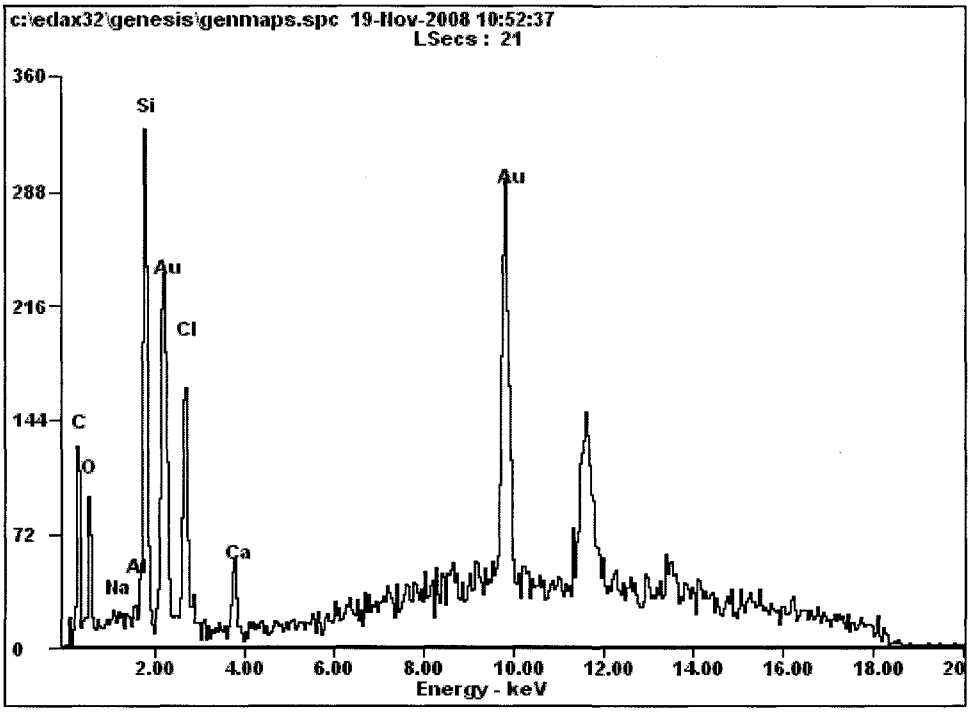


(a)



(b)

Fig. 6-8. (a) Closer view of the intergranular contact shown in Figure 6-7 and (b) the section used in EDS analysis



Element	Weight (%)
C	55.98
O	18.41
Na	00.72
Si	12.07
Cl	09.54
Ca	03.27

Fig. 6-9. Elemental analysis of the section shown in Figure 6-8 (b) by EDS

6.3.3. Experiments with Bender Elements

In addition to the acrylic cylinders used in the closed-system soil-column experiments described above, additional soil columns made up of 7.6-cm (3.0-in) inner diameter Schedule 40 polyvinyl chloride (PVC) were manufactured to accommodate piezo-ceramic plates, referred to as bender elements. Bender elements are commonly used in geotechnical engineering research to measure the shear wave velocity of soil specimens. The PVC columns were also designed to accommodate continuous flow of a liquid nutrient solution.

The piezo-ceramic bender elements vibrate when a voltage is applied to them and generate a voltage when they are mechanically excited, e.g., when they vibrate. To measure shear wave velocity using bender elements, elements are placed at either end of the specimen and waves are generated at one end of a soil sample by applying a voltage to the "input" bender element. These waves travel through the soil and are received by the other bender element at the other end of the soil specimen. The waves generated by these bender elements can be both compression waves (P-waves) and shear waves (S-waves). The speed of P-waves are affected by the degree of saturation of the soil. Therefore, S-waves, which are not affected by the presence of water, are typically used to quantify the stiffness of the soils. Interpretation of the results of a bender element tests (i.e., estimation of the shear wave velocity) requires evaluation of the signal from the receiver element by an oscilloscope for either the S-wave first arrival time or the natural frequency of the soil sample through analysis of the amplitude of the output wave at different frequencies.

The shear wave velocity measurements performed using the PVC column were inconclusive due to difficulties in interpreting the signals from the elements. The main problem with interpretation was attributed to the stiff boundaries of the soil column (i.e., the sides of the PVC tube) located in close proximity to the input wave source (i.e., the piezo-ceramic plate either at the bottom or the top of the soil column). Although PVC columns with the height to diameter ratio consistent with the ratio proposed by Sawangsuriya et al. (2006) for bender element tests were employed (i.e., diameter to height ratio greater than 1.0), insufficient electrical grounding of the soil samples contributed to the difficulty in interpreting output signal amplitudes.

To overcome the difficulties in evaluating the shear wave velocity (or small strain stiffness) of a microbially cemented sample, a special type of triaxial set-up accommodating bender elements attached to the top and base platen may be required. Special attention should also be paid to providing sufficient electrical grounding for the soil sample.

6.4. Conclusion

The soil column experiments performed as part of this study resulted in weak cementation of sand particles through microbial denitrification. Research with artificially cemented soils and experiments on microbially cemented soils indicate that sufficient cementation will result in an increase in the peak undrained and drained shear strength of the specimen. Further experiments are needed to evaluate the relationship between the amount of microbially induced cementation and the shear strength, the stress-strain behavior and volume change response of the cemented specimen under static and

dynamic loading. These experiments should include evaluation of the effects of confining pressure, fines content, and cement properties on microbial cementation. Ultimately, a relationship between a target $CaCO_3$ precipitation amount and the induced change in engineering properties is required for engineering design purposes.

CHAPTER 7

CONCLUSION

7.1. Summary of Results

Microbiological improvement of physical properties of soils has the potential to provide a sustainable, cost-effective, and non-disruptive alternative to current ground improvement technologies for a variety of problems. Potential microbiological improvement techniques include mineral precipitation, mineral transformation, *in situ* and *ex situ* enhancement of soils with biopolymers, and growth of biofilms. The applicability of microbiological processes to soil improvement will likely depend on a variety of factors, including the type of microbial metabolism desired, interactions with other microbes present in the environment, soil type, available nutrients, depth below ground surface, pH, temperature, pressure, concentration of ions, and the availability of oxygen and other oxidants.

Successful development and implementation of microbiological processes for ground improvement would have wide application to a variety of important geotechnical problems, including enhancing foundation bearing capacity and reducing associated settlements, mitigating the susceptibility to earthquake-induced liquefaction, reducing the swell (expansion) potential beneath foundations and roadways, enhancing slope stability, facilitating excavation and tunneling, and reducing permeability for controlling soil erosion and scour, reducing under-seepage of levees and cut-off walls. Applications of microbiological processes may be especially useful near or beneath existing structures, where the application of traditional soil improvement techniques is limited because of the ground deformations and/or high cost associated with current technologies. Many of the

interaction processes between minerals and microorganisms are known to improve the engineering properties of soil on a geological time scale, and some of these processes are known to induce undesired effects in shorter time frames (e.g., clogging of treatment plant filters, drainage facilities at dams, mines, and landfills). The engineering challenge is to induce the desired process over a time frame of engineering interest in the location of interest.

The bench-scale experiments on mineral precipitation, including batch reactors and soil column tests, performed as part of this study suggest that inducement of calcium carbonate precipitation through microbial denitrification has the potential to be developed as a ground improvement technology for granular soils below groundwater table. Stoichiometric analysis and the results from batch reactor experiments indicate denitrification typically results in an increase in pH and alkalinity, which is favorable for calcium carbonate precipitation. Stoichiometric analysis also suggests that the calcium carbonate precipitation is mainly controlled by the production of alkalinity during denitrification. Subsequent precipitation experiments resulted in precipitation of $CaCO_3$ up to 2.4 g per 1 g of NO_3^- reduced (consistent with the results of the stoichiometric analysis), which suggests that denitrification is potentially a feasible mechanism for calcium carbonate precipitation in granular soils.

Anaerobic and aerobic oxidation of an organic compound results in production of CO_2. If the medium is a well-buffered neutral or alkaline environment, CO_2 produced as a result of oxidation of an organic compound transforms into carbonate and then precipitates if there is an adequate amount of appropriate cations, such as Ca^{2+}.

Precipitation is enhanced if the pH increases due to microbial production of alkalinity, which can occur through several microbial mechanisms (e.g., ureolysis, denitrification, sulfate reduction). Batch reactor experiments performed as part of this study indicate that during denitrification, the pH may increase or decrease initially during reduction of nitrate to nitrite, based on the relative rate of nitrate reduction to nitrite reduction, in the absence of cations to form solid complexes. On the other hand, the overall denitrification process (i.e., reduction of nitrate to nitrogen gas) results in an increase in alkalinity and pH, with a major contribution from the step of denitrification that involves reduction of nitrite to nitrogen gas. The alkalinity is then consumed when the carbonate ions precipitate with the available cations.

Bacterial denitrification offers advantages over ureolysis, another calcium carbonate precipitation mechanism currently under investigation (by others), for mineral precipitation applications below groundwater table. Because the production of urease by soil microorganisms is retarded by lack of oxygen, application of ureolysis below the groundwater table is complicated and may require air sparging and/or bioaugmentation. On the other hand, calcium carbonate precipitation through microbial denitrification can be applied through biostimulation (i.e., use of indigenous bacteria), as denitrifying microorganisms are widespread in the nature. Furthermore, production of ammonium during ureolysis may pose environmental and health risks, and a relatively high costs is generally associated with the treatment of ammonium in groundwater. The nitrogen gas produced by bacterial denitrification (i.e., assuming that all CO_2 will be dissolved in the groundwater) does not pose any known risks to the human health or the environment.

Denitrification also provides advantages over other anaerobic mechanisms because of high microbial yield and the diversity of denitrifying microorganisms. Diversity in the denitrifying bacterial population results in capability to utilize a wide range of electron donors and provides a competitive advantage over other groups of microorganisms.

Although accumulation of denitrification intermediates (i.e., nitrite and nitrous oxide) can inhibit the production of alkalinity and inorganic carbon required for carbonate precipitation, the denitrification process can be managed in a manner to reduce any associated risks to the environment or health and to facilitate the success of calcium carbonate precipitation as a soil improvement technique. The geochemistry of the groundwater should be analyzed for nitrate, nitrite, metal, and phosphorus concentrations prior to a field application along with other parameters typically measured for any environmental application (e.g., dissolved oxygen content, pH, alkalinity, salinity, COD or BOD, and total and calcium hardness) to evaluate limiting conditions and to develop the "recipe" for the nutrient solution to be amended to the ground. The nutrient solution prepared based on the analysis of the geochemistry of the groundwater may include an electron donor or electron acceptor along with other inorganic nutrients. If the electron donor is the limiting factor for denitrification at a particular location, an appropriate organic electron donor can be selected considering relevant factors, including solubility, cost, microbial yield, and potential $CaCO_3$ precipitation quantity. If the electron acceptor is the limiting factor, nitrate salts or nitrite salts can be included in the nutrient solution to be injected into the ground. Utilization of small quantities of nitrate (i.e., successive short denitrification episodes, instead of one long denitrification episode) may provide a

better management strategy during field applications because remedial measures can be taken easily to mitigate the accumulation of hazardous intermediates and to minimize the environmental and health risks that may arise as a result of application of this process.

Although not included in the experiments presented in this study, as an alternative to injecting a solution containing calcium salts at the beginning of application, an initial nutrient mix designed just to stimulate the growth of indigenous denitrifiers *in situ* can be amended to the ground to minimize the effect of pH decrease associated with calcium carbonate precipitation on the activity of denitrification enzymes and microbial growth. Calcium salts can be added at a later stage based upon the change in pH and alkalinity of the medium.

Geotechnical laboratory experiments conducted previously indicate that microbially induced calcium carbonate precipitation results in increase in shear strength of granular soils. However, due to a difference in mineral content, structure, particle size, and organic content and distribution of the cement, the net effect on shear strength, stress-strain behavior, and volume change response may differ from artificially cemented soils. Therefore, relationship between microbially induced precipitation and the change in engineering properties of the soil requires additional study.

Regardless of whether denitrification or another microbial mechanism is employed for soil improvement through calcium carbonate precipitation, a comprehensive quality control/assurance plan detailing monitoring and sampling is required for the management of the environmental and health risks and to increase the efficiency of the application.

7.2. Future Research

Additional interdisciplinary research by microbiologists, chemists, geologists, and geotechnical engineers, collaboratively, is required to realize the potential of microbially induced carbonate precipitation using denitrification and other microbiological soil improvement technologies. Almost all of the research in the literature on the interactions of minerals and microorganisms has been carried out by microbiologists, geologist, and environmental engineers and involvement of geotechnical engineers in these research efforts has been limited. Involvement of geotechnical engineers is an absolute necessity for development of ground improvement technologies based on microbial mechanisms.

Subsequent to completion of the ASU soil column experiments, further geotechnical laboratory testing is planned to investigate the behavior of granular soils cemented with microbially induced calcium carbonate precipitation, followed by pilot scale testing of microbial denitrification as a ground improvement mechanism. Because most of the previous experiments on the properties of artificially cemented soils has concentrated on the shear strength, further research on stress-strain behavior and volume change response under static and dynamic loading, and on the effects of confining pressure, fines content, and cement properties on soil behavior is recommended.

A biogeochemical model for calcium carbonate precipitation through microbial denitrification should be developed based on the results of the ASU batch experiments and subsequent soil column tests to estimate time-rate of changes during microbial growth, which can be helpful to engineers in analyzing different scenarios prior to a field application and performing a cost-benefit analysis.

Laboratory experiments on microbial calcium carbonate precipitation indicate a potential challenge with respect to homogeneous distribution of the cementation within the soil matrix. Thus, special efforts may be required to develop methods to induce a uniform distribution of microorganisms and microbial cements within the soil.

In addition to calcium carbonate precipitation, research on precipitation of other minerals (e.g., silicates, iron oxides) that can potentially be used for ground improvement is also recommended. For example, silica cements are the strongest and the most resistant to weathering among all the cements found in sedimentary rocks.

In addition to mineral precipitation, other microbial mechanisms (i.e., mineral transformation, biopolymers and growth of biofilms also have a potential to be employed for ground improvement. Thus, further research is required to fully explore the potential of microbial mechanisms to be applied to several geotechnical engineering problems.

References

ABAG (2003). *The REAL dirt on liquefaction*. Association of Bay Area Governments. http://www.abag.ca.gov/bayarea/eqmaps/liquefac/buildings.html

Abeling, U., Seyfried, C. (1992). "Anaerobic-aerobic treatment of high strength ammonium wastewater-nitrogen removal via nitrite." *Wat. Sci. Technol.*, 26(56), 1007-1015.

Abd-el-Malik, Y. and Rizk, S.G. (1963a). "Bacterial sulfate reduction and the development of alkalinity. II. laboratory experiments with soils." *Journal of Applied Bacteriology*, 26, 14-19.

Abd-el-Malik, Y. and Rizk, S.G. (1963b). "Bacterial sulfate reduction and the development of alkalinity. II. experiments under natural conditions in the Wadi Natrun." *Journal of Applied Bacteriology*, 26, 20-26.

Abdulla, A.A. and Kiousis, P.D. (1997). "Behavior of cemented sands – I. Testing." *International Journal for Numerical and Analytical Methods in Geomechanics*, Vol. 21, 533-547.

AIR (2006). *35th Anniversary of the 1971 San Fernando Earthquake*. AIR Worldwide Corporation, http://www.air-worldwide.com/_public/html/air_currentsitem.asp?ID=882

Almeida, J.S., Julio, S.M., Reis, M.A.M., Carrondo, M.J.T. (1995). "Nitrite inhibition of denitrification by *Pseudomonas fluorescens*." *Biotechnology and Bioengineering*, Vol.46, 194-201.

Asghari, E., Toll, D.G., and Haeri, S.M. (2003). "Triaxial behavior of a cemented gravelly sand, Tehran Alluvium." *Geotechnical and Geological Engineering*, Vol. 21, 1-28.

Bachus, R.C., Clough, G.W., Sitar, N., Shafii-Rad, N., Crosby, J., and Kaboli, P. (1981). *behavior of weakly cemented soil slopes under static and seismic loading conditions – Volume II.* Prepared for the US Geological Survey Dept. of Interior Office of Earthquake Studies, Final Report, July.

Barak, Y., Tal, Y., and van Rijn, J. (1998). "Light-Mediated Nitrite Accumulation during Denitrification by Pseudomonas sp. Strain JR12." *Applied and Environmental Microbiology*, American Society of Microbiology, 64(3):813-817.

Bavendamm, W. (1932). "Die mikrobiologische kalkfällung in der tropischen see." *Arch Mikrobiol.*, 3, 205-276.

Beccari, M., Passion, R., Ramadori, R., and Tandovi, V. (1983). "Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture." *Journal of Wat. Pollut. Control Fed.*, 55, 58.

Betlach, M.R. and Tiedje, J.M. (1981). "Kinetic explanation for accumulation of nitrite, nitric oxide and nitrous oxide during bacterial denitrification." *Appl. Environ. Microbiol.*, Vol. 42, 1074– 1084.

Bills, P.M. (1985). "The precipitation of calcium carbonate polymorphs in vitro at 37°C." *Calcified Tissue International*, Vol. 37, 174-177.

Birnbaum, S.J., and Wireman, J.W. (1984). "Bacterial sulfate reduction and pH: implications for early diagenesis." *Chemical Geology*, 43: 143–149.

Birnbaum, S.J., Wireman, J.W., and Borowski, R. (1989). "Silica precipitation induced by the anaerobic sulfate reducing bacterium *Desulfovibrio desulfuricans*: effects upon the cell morphology and implications for preservation." In *Origin, evolution, and modern aspects of biomineralization in plants and animals*. Edited by R.E. Crick. Plenum Press, New York, 289–298.

Blaszczyk, M., (1992). "Comparison of denitrification by *Paracoccus denitrificans*, *Pseudomonas stutzeri* and *Pseudomonas aeruginosa*." *Acta Microbiol.* Pol. 41, 203– 210.

Blaszczyk, M., (1993). "Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*." *Appl. Environ. Microbiol.* 59, 3951–3953.

Bollag, J.M and Barabasz, W., (1979). "Effect of heavy metals on the denitrification process in soil." *J. Environ. Qual.* 8, 196–201.

Bollag, J.M. and Henneringer, H.M. (1976). "Influence of pesticides on denitrification in soil and with an isolated bacterium." *J. Environ. Qual.* 5, 15– 18.

Bollag, J.M. and Kurek, E.J., (1980). "Nitrite and nitrous oxide accumulation during denitrification in the presence of pesticide derivatives." *Appl. Environ. Microbiol.* 29 (4), 845– 849.

Boquet, E., Boronat, A., Ramos-Cormenzana, A. (1973). "Production of calcite (Calcium Carbonate) crystals by soil bacteria is a general phenomenon." *Nature*, Vol. 246, December 21/28, 527-529.

Bouwman, A.F. (1989). "The role of soils and land-use in the green house effect." *Netherlands Journal of Agricultural Science*, 37(1), 13-19.

Brune, M., Ramke, H.G., Collins, H., and Hanert, H.H. (1991). "Incrustations process in drainage systems of sanitary landfills." *Proceedings, 3rd International Landfill Symposium*, Cagliari, Italy, 999-1035.

Bryner, L.C., Beck, J.V., Davis, D.B., and Wilson, D.G. (1954). "Microorganisms in leaching sulfide minerals." *Ind Eng Chem*, 46, 2587-2592.

Buchanan, R. E., Gibbons, N. E. (ed.). (1974). *Bergey's manual of determination bacteriology*. 8th ed.

Butler, J.M. (1991). *Carbon dioxide equilibria and their applications.* CRC Press.

Cabalar, A.F. and Canakci, H. (2005). "Ground improvement by bacteria." *Proceedings of the Third Biot Conference on Poromechanics*, Norman, Oklahoma, Editors: Abousleiman, Y.N., Cheng, A.H.-D, and Ulm, F.-J..

Carlson, W.D. (1983). "The polymorphs of $CaCO_3$ and the aragonite-calcite transformation." *Reviews in Mineralogy – Vol. 11 Carbonates: Mineralogy & Chemistry*, R.J. Reeder (Ed.), P.H. Ribbe (Series Ed.), Mineralogical Society of America.

Castanier, S., Le Metayer-Levrel, G., and Perthuisot, J.-P. (2000). "Bacterial roles in the precipitation of carbonate minerals", *Microbial Sediments*, R.E. Riding and S.M. Awramik (Ed.s), Springer-Verlag Berlin Heidelberg.

Catalan-Sakairi, M, Wang, P and Masatoshi, M (1997). "High-rate seawater denitrification utilizing a macro-porous cellulose carrier." *Journal of Fermentation and Bioengineering,* 83, 102–108.

Cecil, J. (2001). *The destruction of Sodom and Gomorrah, Ancient History – Other Cultures*. http://www.bbc.co.uk/history/ancient/cultures/sodom_gomorrah_01.shtml

Cervantes, F., Monroy, O., and Gomez, J. (1998). "Accumulation of intermediates in a denitrifying process at different copper and high nitrate concentrations." *Biotechnology Letters,* Vol. 20, No. 10, October 1998, 959–961.

Chen, S., Juaw, C., and Cheng, S. (1991). "Nitrification and denitrification of hugh-strength ammonium and nitrite wastewater with biofilm reactors." *Water Sci. Tech.*, 23, 1417.

Cooke, A.J., Rowe, R.K., Rittmann, B.E., VanGulck, J.F., and Millward, S. (2001). "Biofilm growth and mineral precipitation in synthetic leachate columns." *Journal of Geotech. and Geoenviron. Engineering*, ASCE, 127(10), 849-856.

Coyne, M.S. and Tiedje, J.M., (1990). "Induction of denitrifying enzymes in oxygen limited *Ascromobacter cycloclastes* continuous culture." *FEMS Microbiol. Ecol.* 73, 263– 270.

Cunningham, A.B., Characklis, W.G., Abdeen, F., and Crawford, D. (1991). "Influence of biofilm accumulation on porous media hydrodynamics." *Environ. Sci. Tech.*, 25:1305-1311.

Denton L. (1995). *Microbiological Diversity of Denitrifying Bacteria in High Nitrate Brines of Varying Salinity*. MS Thesis, Univ. of Colorado, Boulder.

DeJong, J.T., Fritzges, M.B., and Nusslein, K. (2006). "Microbially induced cementation to control sand response to undrained shear." *Journal of Geotechnical and Geoenvironmental Engineering*, ASCE, Vol. 132, No. 11, pp. 1381-1392.

Donald, R. (1998). "Sulfide mineral diagenesis: A bacterially mediated, rapid, low temperature, anaerobic process." In: *Abstracts of 98th General Meeting of ASM*, Atlanta, May 17–21, p. 15.

Dong, H., Kostka, J.E., and Kim, J. (2003). "Microscopic evidence for microbial dissolution of smectite." *Clays and Clay Minerals*, Vol. 51, No. 5, 502-512.

Donlan R.M. (2002). "Biofilms: Microbial life on surfaces." *Emerg. Infect. Dis. 8 (9),* Center for Disease Control and Prevention (CDC), www.altcorp.com/AffinityLaboratory/biofilms.html

Drew G.H. (1911). "The action of some denitrifying bacteria in tropical and temperate seas, and the bacterial precipitation of calcium carbonate in the sea." *J. of Mar. Bio. Assoc, UK*, 9:142-155.

Drew G.H. (1914). "On the precipitation of calcium carbonate in the sea by marine bacteria, and on the action of denitrifying bacteria in tropical and temperate seas." *Carnegie Inst. Publ.*, Washington, 182, 5:7-45.

Driessens, F.C.M. and Verbeeck, R. M. H. (1990). *Biominerals*. CRC Press.

Drtil, M., Nemeth, P., Kucman, K., Bodik, I., and Kasperek, V. (1995). "Acidobasic balances in the course of heterotrophic denitrification." *Water Research*, 29:1353-1360.

Dutta, L. Nuttall, H.E., Cunningham, A.I., James, G., and Hiebert, R. (2005). "In-situ biofilm barriers: case study of a nitrate groundwater plume, Albuquerque, New Mexico." *Remediation Journal*, Vol. 15, Issue 4, pp 101-111.

Ehrlich, H.L. (2002). *Geomicrobiology.* Fourth Edition, Revised and Expanded, Marcel Dekker, Inc., New York.

Fernandez, A.L. and Santamarina, J.C. (2001). "Effect of cementation on the small strain parameters of sands." *Canadian Geotech. J.*, Vol. 38, 191-199.

Fleming, I.R., Rowe, R.K., and Cullimore, D.R. (1999). "Field observations of clogging in a landfill leachate collection system." *Canadian Geotechnical Journal*, 36(4), 289-296.

Fortin, D., Ferris, F.G., and Beveridge, T.J. (1997). "Surface-mediated mineral development by bacteria." *Geomicrobiology: Interactions between microbes and minerals (Rev Mineral, Vol.35)*, edited by J.F. Banfield and K.H. Nealson, Mineralogical Society of America, Washington D.C., 161-180.

Fortin, D., Rioux, J.-P., and Roy, M. (2002). "Geochemistry of iron and sulfur in the zone of microbial sulfate reduction in mine tailings." *Water, Air, and Soil Pollution: Focus*, 2:37-56.

Francis C. and Mankin J. (1977). "High nitrate denitrification in continuous flow-stirred reactors." *Water Research*, 11, 289-294.

Fujita, Y., Ferris, F.G., Lawson, R.D., Colwell, F.S., and Smith, R.W. (2000). "Calcium carbonate precipitation by ureolytic subsurface bacteria." *Geomicrobiology Journal*, 17, 305-318.

Gayon, E. and Dupetit, G. (1886). "Recherches sur la réduction des la nitrates par les infiniments petits." *Soc. Sci. Phys. Nat. Bordeaux*, Sér. 3, 2, 201-307.

GeoDelft (2006). *SmartSoils®.* http://www.smartsoils.nl//EN/page12.asp.

Giroud, L.P. (1996). "Granular filters and geotextile filters," *Proceedings of Geo-filters '96*, Editors: J. Lafleur and A.L. Rollins, Montreal, Canada, 565-580.

Glass, C., Silverstein, J., and Oh, J. (1997). "Inhibition of denitrification in activated sludge by nitrite." *Water Environment Research*, Vol. 69, No.6, 1086-1093.

Glass, C. and Silverstein, J. (1998). "Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation." *Water Resources*, 32 (3), 831– 839.

Glass, C. and Silverstein, J. (1999). "Denitrification of high nitrate, high-salinity wastewater," Water Resources, 33 (1), 223-229.

Granger, J. and Ward, B.B. (2003). "Accumulation of Nitrogen Oxides in copper-limited cultures of denitrifying bacteria." *Limnology and Oceanography*, Vol. 48, No. 1, Part 1 (Jan., 2003), 313-318.

Guidry, S.A. and Chafetz, H.S. (2003). "Siliceous shrubs in hot springs from Yellowstone National Park, Wyoming, U.S.A." *Can. J. Earth Sci.*, 40: 1571–1583.

HACH (2003). *DR/4000 Spectrophotometer Procedures Manual.*

HACH (2006). *Digital Titrator User Manual*, June, Ed. 24.

Haeri, S.M., Hamidi, A., and Tabatabee, N. (2005). "The effect of gypsum cementation on the mechanical behavior of gravelly sands." *Geotechnical Testing Journal*, ASTM, Vol. 28, No. 4, 380-390.

Haigh, S.K. and Madabhushi, S.P.G. (2002). "Dynamic centrifuge modeling of the destruction of Sodom and Gomorrah." *International Conference on Physical Modeling in Geotechnics*, St John's, Newfoundland, Canada, July.

Hammes, F., Boon, N., de Villiers, J., Vestraete, W., and Siciliano, S.D. (2003). "Strain-specific ureolytic microbial calcium carbonate precipitation." *Applied and Environmental Microbiology*, August, 4901-4909.

Harkes, M.P., van Paassen, L.A., van Loosdrecht, M.C.M., and Whiffin, V.S. (2008). "Co-relation of unconfined compressive strength and carbonate content in the BioGrout process." *1st International Conference on BioGeoCivil Engineering*, 23 - 25 June, The Netherlands.

Harrison, J. (2005). "TecEco cement concretes; abatement, sequestration utilization in the built environment." *Concrete 05*, Concrete Institute of Australia, 17-19 October, Melbourne.

Horseley, R., Roscoe, J., and Talling I. (1982). "Nitrate reduction by Pseuodomonas spp: Antagonism by Fermentative Bacteria." *Applied and Environmental Microbiology*, 53:57.

Hunter, W.J. (2003). "Accumulation of nitrite in denitrifying barriers when phosphate is limiting." *Journal of Contaminant Hydrology*, 66, 79-91.

Hunt, P.G., Matheny, T.A., and Ro, K.S. (2007). "Nitrous Oxide accumulation in soils from riparian buffers of a coastal plain watershed Carbon/Nitrogen ratio control." *J. Environ. Qual.*, July 17, 36(5): 1368 - 1376.

Israel S. (2006) *In situ Denitrification of Nitrate Rich Groundwater in Marydale, Northern Cape*, M.S. Thesis, Department of Soil Science, Stellenbosch University, South Africa, 157 p.

Ivanov, V. and Chu, J. (2008). "Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ." *Rev. Environ. Sci. Biotechnology*, Vol. 7., No. 2, 139-153.

Jones, D.E. and Jones, K.A. (1987). "Treating expansive soils." *Civil Engineering*, ASCE, Vol. 57, No. 8, August.

Karimi, S. (1998). *A study of geotechnical applications of biopolymer treated soils with an emphasis on silt*. Ph.D. Dissertation, University of Southern California, 167 p.

Kasama, K., Zen, K., and Iwataki, K. (2006). "Undrained shear strength of cement treated soils." *Soils and Foundations*, Vol. 46, No. 2, 221-232.

Keeney, D.R., Fillery, I.R., and Marx, G.P. (1979). "Effect of temperature on the gaseous nitrogen products of denitrification in a silt loam soil." *Soil Sci. Soc. Am. J.*, 43, 1124.

Khachatoorian, R., Petrisor, I.G., Kwan, C-C., and Yen, T.F. (2003). "Biopolymer plugging effect: laboratory-pressurized pumping flow studies." *Journal of Petroleum Science and Engineering*, v. 38, 13-21.

Kim, J., Dong, H., Seabaugh, J., Newell, S., and Eberl, D. (2004). "Role of microbes in the smectite-to-illite reaction." *Science*, v. 303, 830-832.

Kim, J., Furukawa, Y., Dong, H., and Newell, S.W. (2005). "The effect of microbial fe (iii) reduction on smectite flocculation." *Clays and Clay Minerals*, Vol. 53, No. 6, pp 572-579.

Knowles, R. (1981). *Denitrification*. In Terrestial Nitrogen Cycles, F.E. Clark and T. Roswall (Eds.), Swedish National Research Council, Stockholm.

Koike, I. and Hattori, A. (1975a). "Growth yield of denitrifying bacterium, *Pseudomonas denitrificans*, under aerobic and denitrifying conditions." *J. Gen. Microbiol.*, 88:1-10.

Koike, I. and Hattori, A. (1975b). "Energy yield of denitrification: an estimate from growth yield in continuous cultures of *Pseudomonas denitrificans* under nitrate-, nitrite-, and nitorus oxide-limited conditions", *J. Gen. Microbiol.*, 88:11-19

Komlos, J., Cunningham, A.B., Warwood, B., and Lames, G. (1998). "Biofilm barrier formation and persistence in variable saturated zones." *Proceedings of the 1998 Conference on Hazardous Waste Research.*

Kornaros, M., Zafiri, C., Lyberatos, G. (1996). "Kinetic denitrification by *Pseudomonas denitrificans* under growth conditions limited by carbon and/or nitrate or nitrite", *Water Environment Research.*, 68(5):934-945.

Korner, H. and Zumft, W.G., (1989). "Expression of denitrification enzymes in response to the dissolved oxygen and respiratory substrate in continuous culture of *Pseudomonas stutzeri.*" *Appl. Environ. Microbiol.* 55, 1670– 1676.

Kostka, J.E., Dalton, D.D., Skelton, H., Dollhopf, S., and Stucki, J.W. (2002). "Growth of iron (III)-reducing bacteria on clay minerals as the sole electron acceptor and comparison of growth yields on a variety of oxidized iron forms." *Applied and Environmental Microbiology,* Dec., 6256-6262.

Krumbein, W.E. (1974). "On the precipitation of aragonite on the surface of marine bacteria." *Naturwissenschaften*, 61-167.

Krumbein, W.E. (1979). "Photolithotrophic and chemoorganotrophic activity of bacteria and algae as related to beachrock formation and degradation (Gulf of Aqaba, Sinai)." *Geomicrobiology Journal*, 1, 139-203.

Krynine, D.P. and Judd, W.R. (1957). *Principles of Engineering Geology and Geotechnics*. McGraw-Hill, New York, 1957.

Lange's Handbook of Chemistry (1987). 13th Ed., John A. Dean (Ed.), McGraw-Hill, Inc., New York, N.Y.

Lee, K.C. and Rittmann, B.E. (2003). "Effects of pH and precipitation on autohydrogenotrophic denitrification using the hollow fiber membrane biofilm reactor", *Water Research*, 37:1551-1556

Legge, K.R., Scheurenburg, R., Clever, C., James, G., and Claus, R. (1985). *Investigation into apparent clogging of a geotextile recovered from ergo tailings dam wall drain.* Preliminary Report, Department of Water Affairs and Forestry, Republic of South Africa, 22 October, 19 p.

Lewin, J. (1965). *Calcification.* Physiology and Biochemistry of Algae, Ed. R.A. Lewin, Academic Press, 457-465.

Lijinski, W. (1977). "How nitrosamines cause cancer." *New Science*, 27, 216-217.

Lowenstam, H.A. and Weiner, S. (1989). *On biomineralization*. Oxford University Press US, 1989, 324 p.

MacCallum, M.F., Guhathakurta, K., (1970). "The precipitation of calcium carbonate from seawater by bacteria isolated from bahama bank sediments." *J. Appl. Bact.*, 33:649-655.

MacLeod, F.A., Lappin-Sott, H.M., and Costerton, J.W. (1988). "Plugging of a model rock system by using starved bacteria." *Applied and Environmental Microbiology*, June, 1365-1372.

Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000). *Environmental Microbiology*. Academic Press.

Marshall, J., Orange, D., and Hochstaedter, A. (1989). *Earthquakes*. http://www.es.ucsc.edu/~es10/fieldtripEarthQ/Damage1.html

Martin, G. R., Yen, T. F., and Karimi, S. (1996). "Application of biopolymer technology in silty soil matrices to form impervious barriers." *Proc., 7th Australia-New Zealand Geomechanics Conference*, Adelaide, Australia.

McCarty, P.L. and McKinney, R.E. (1961). "Salt toxicity in anaerobic digestion." *Journal of Water Pollution Control Federation*, Vol. 33, 399-414.

McCarty, P.L. (1971). "Energetics and bacterial growth." *Organic Compounds in Aquatic Environments*, eds. S.D. Fraust and J.V. Hunter, New York: Marcel Dekker.

McCarty, G.W. and Bremner, J.M. (1991). "Production of urease by microbial activity in soils under aerobic and anaerobic conditions." *Biology and Fertility of Soils*, 11, 228-230.

McCarty, P. L., Beck, L., and St. Amant, P. (1969). „Biological denitrification of wastewater by addition of organic materials." p. 1271. *In Proceedings of the 24th Indiana Waste Conference*, no. 135. Purdue University, Lafayette, Ind.

Meijer, E.M., Van der Zwaan, J., Wever, R., and Stouthamer, A. (1979). "Anaerobic respiration and energy conservation in *Paracoccus denitrificans*." *European Journal of Biochemistry*, Vol. 96(1), 69-76.

Mitchell, J.K. and Santamarina, J.C. (2005). "Biological considerations in geotechnical engineering." *Journal of Geotech. and Geoenvr. Engineering*, Vol. 131, No. 10, 1222-1233.

Mitsui, S., Watanabe, I., Honma, M., and Honda, S. (1964). "The effect of pesticides on denitrification in paddy soil." *Soil Sci. Plant Nutr.* 10, 15–23.

Mobley, H.L. and Hausinger, R.P. (1989). "Microbial ureases: significance, regulation, and molecular characterization." *Microbiology Rev.,* 53:85-108.

Montgomery, D.R. (1990). "Effects of the Loma Prieta earthquake, October 17, 1989, San Francisco Bay Area." *California Geology*, January, Vol. 43, No.1.

Moore, J.N. (1983). "The origin of calcium carbonate nodules forming on Flathead Delta Lake, Northwest Montana." *Limnol. Oceanogr.*, 28, 646-654.

Morse, J.W. (1983). "The kinetics of calcium carbonate dissolution and precipitation." *Reviews in Mineralogy – Vol. 11 Carbonates: Mineralogy & Chemistry*, R.J. Reeder (Ed.), P.H. Ribbe (Series Ed.), Mineralogical Society of America.

NOAA (2005). *Earthquakes.* National Geophysical Data Center. http://www.ngdc.noaa.gov/seg/hazard/slideset/earthquakes/

Napier, J. and Bustamante, R., (1988). "In-situ biodenitrification of the S-3 ponds." *Environ. Prog.,* 7(1), 13-16.

Nadson, G.A. (1903). *Microorganisms as geologic agents.* I. Tr Komisii Isslect Min Vodg St, Petersburg: Slavyanska.

Nishimura, Y., Kamihara, T. and Fukui, S. (1980). "Diverse effects of formate on the dissimilatory metabolism of nitrate in *Pseudomonas denitrificans* ATCC 13867: growth, nitrite accumulation in culture, cellular activities of nitrate and nitrite reductases."*Arch. Microbiol.*, 124:191-195.

Oh, J. and Silverstein, J. (1999). "Acetate limitation and nitrite accumulation during denitrification." *Journal of Environmental Engineering*, Vol. 125, No. 3, 234-242.

Perkins, S.W., Gyr, P., and James, G. (2000). "The influence of biofilm on the mechanical behavior of sand." *Geotechnical Testing Journal*, ASTM, Vol. 23, No. 3, 300-312.

Popa, R. and Kinkle, B.K. (2000). "Discrimination among iron sulfide species formed in microbial cultures." *Journal of Microbiological Methods*, 42:167-174.

Rad, N. S. (1983). *Static and Dynamic Behavior of Cemented Sands.* PhD Dissertation, Stanford University, 320 pp.

Radcliffe, B.C. (1969). *Biochemical studies on denitrification in Pseudomonas denitrificans*. Ph.D. Thesis, University of Adelaide, South Australia.

Rickard, D.T. (1969). "The microbiological formation of iron sulfides." *Stockh. Contrib. Geol.*, 20, 49–66.

Rittmann, B.E. and McCarty, P.L. (2001). E*nvironmental biotechnology: principles and applications*. McGraw-Hill.

Rittmann, B.E., Banaszak, J.E., VanBriesen, J.M., and Reed, D.T. (2002). "Mathematical modeling of precipitation and dissolution reactions in microbiological systems." *Biodegradation*, 13, 239-250.

Rivadeneyra, M.A., Perez-Garcia, I., Salmeron, V., and Ramos-Cormenzana, A. (1985a). "Bacterial precipitation of calcium carbonate in the presence of phosphate." *Soil Bio. Biochem.*, 17:171-172.

Rivadeneyra, M.A., Ramos-Cormenzana, A., and Garcia-Cervigon, A. (1985b). "Etude de l'influence du rapport Mg/Ca sur la formation de carbonate par des bacteria telluriques." *Can. J. Microbiol.*, 31, 229-231.

Rivadeneyra, M.A., Delgado, R., Quesada, E., and Ramos-Cormenzana (1991). "Precipitation of calcium carbonate by *Deleya halophila* in media containing NaCl as sole salt," *Current Microbiology*, Vol. 22, 185-190.

Rodriguez-Navarro, C., Jimenez-Lopez, C., Rodriguez-Navarro, A., Gonzalez-Muñoz, M.T., and Rodriguez-Gallego, M. (2007). "Bacterially mediated mineralization of vaterite." *Geochimica et Cosmochimica Acta.*, Vol.71, Issue 5, 1197-1213.

Rowe, K.R., VanGulck, J.F., and Millward, S.C. (2002). "Biologically induced clogging of a granular medium permeated with synthetic leachate." *Journal of Environ. Eng. Sci.*, 1, 135-156.

Sacks, L.E. and Barker, H.A. (1949). "The influence of oxygen on nitrate and nitrite reduction." *Journal of Water Pollut. Contr. Fed.*, Vol. 58, 11-22.

Sawada, K. (1997). "The mechanisms of crystallization and transformation of calcium carbonates." *Pure and Applied Chemistry*, Vol. 69, No. 5, 921-928

Sawangsuriya, A., Biringen, E., Fratta, D., Bosscher, P. J. and Edil, T. B. (2006). "Dimensionless limits for the collection and interpretation of wave propagation data in soils". *ASCE Geotechnical Special Publication (GSP) No. 149*: "Site and Geomaterial Characterization," pp. 160-166.

Schulthess, R.V. and Gujer, W. (1996). "Release of Nitrous Oxide (N_2O) from denitrifying activated sludge: verification and application of a mathematical model." *Water Research*, 30, 521–530.

Seed, H.B., R.J. Woodward, and R. Lundgren (1962). "Prediction of swelling potential of compacted clays." *Journal of SMFE Div.,* ASCE, June, 53-87.

Silverstein, J.; Cook, N.E.; Figueroa, L.A.; Cutter, K.; Hund, R.; and Deters, J. (1993). "pH control of biological denitrification of high strength wastewater." *Rep. Dep. Energy*, Contract ASC 102824CL, 1.

Sitar, N, Clough, G.W., and Bachus, R.G. (1980). *Behavior of weakly cemented soil slopes under static and seismic loading conditions*. Prepared for the US Geological Survey Dept. of Interior Office of Earthquake Studies, Final Report, June.

Skerman, V.D.B. and MacRae, I.C. (1957). "The influence of oxygen availability on the degree of nitrate reduction by *Pseudomonas denitrificans*." *Canadian Journal of Microbiology*, 3, 505-530.

Smith, J.L. and Doran, J.W. (1996). "Measurement and use of pH and electrical conductivity for soil quality analysis." In: Doran, J.W., Jones, A.J. (Eds.), *Methods for Assessing Soil Quality.* SSSA Special Publication No. 49, Soil Science Society of America, Madison, WI, pp. 169– 185.

Stocks-Fischer, S., Galinat, J.K., and Bang, S.S. (1999). "Microbial precipitation of CaCO3." *Soil Biology and Biochemistry*, Vol 31,1563-1571.

Thomsen, J.K., Geest, T., and Cox, R.P. (1994). "Mass Spectrometric Studies of the Effect of Intermediates in Denitrification by *Paracoccus denitrificans*." *Appl. Environ. Microbiol.*, 60, 2, 536.

Tiedje, J.M., A.J. Sexstone, D.D. Myrold, and J.A. Robinson. (1982). "Denitrification: Ecological niches, competition and survival." *Ant. van Leeuwenhoek J. Microbiol.* 48:569-583.

Timmerhaus, P., and Van Haute, A. (1983). "Denitrification with methanol." *Water Res.* (G.B.), 17, 10, 1249.

Todar, K. (2006). *Todar's online textbook of bacteriology*. University of Wisconsin www.textbookofbacteriology.net

Turk, O. and Mavinic, S. (1987). "Benefits of using selective inhibition to remove nitrogen from highly nitrogenous wastes," *Environ. Tech. Lett.*, 8:419-426.

Ucisik, A.S. and Henze, M. (2004). "Biological denitrification of fertilizer wastewater at high chloride concentration." *Water SA*, 30: 191, 2004..

USEPA (2001). *National primary drinking water regulations consumer fact sheet on Nitrates/Nitrites.* United States Environmental Protection Agency www.epa.gov/safewater/dwh/c-ioc/nitrates.html

van der Hoek, P., Latour J., and Klapwijk A. (1987). "Denitrification with methanol in the presence of high nitrate waste solutions." *Appl. Micro. Biotech.* 27, 199-205.

van Paassen, L.A., Daza, C.M., and Van Loosdrecht, M.C.M. (2008). "In situ sand consolidation by microbial denitrification." *1st International Conference on BioGeoCivil Engineering*, 23 - 25 June, The Netherlands.

van Rijn, J., Tal, Y., and Barak, Y., (1996). "Influence of volatile fatty acids on nitrite accumulation by a *Pseudomonas stutzeri* strain isolated from a denitrifying fluidized bed reactor." *Appl. Environ. Microbiol.* 62, 2615– 2630.

van Versefeld, H., Meijer, E., and Stouthamer, A. (1977). "Energy conservation during nitrate respiration in *Paracoccus denitrificans*." *Arch. Microbiol.,* 112, 17.

Veydovec, W., Silverstein, J., Cook, N., Jr., Figueroa, L., Hund R., and Lehmukhl G. (1994). "Denitrification inhibition by high nitrate wastes." *Proc ASCE Nat. EE Conf.*, ASCE, New York, NY, pp. 415-422.

Wang, J.-H., Baltzis, B.C., and Lewandowski, G.A. (1995). "Fundamental denitrification kinetic studies with *Pseudomonas denitrificans*", *Biology and Biotechnology*, 47:26-41.

Warren, L.A., Maurice, P.A., Parmar, N., and Ferris, F.G. (2001). "Microbially mediated calcium carbonate precipitation: implications for interpreting calcite precipitation for solid-phase capture of inorganic contaminants." *Geomicrobiology Journal*, Jan., Vol.18, Issue 1, pp 93-115.

Whiffin, V. (2004) *Microbial $CaCO_3$ precipitation for the production of biocement*,. Ph.D. Dissertation, School of Biological Sciences and Biotechnology, Murdoch University, Australia, September, 154 p.

Whiffin, V.S., van Paassen, L.A., and M.P. Harkes (2007). "Microbial carbonate precipitation a soil improvement technique." *Geomicrobiology Journal*, 24:1-7.

Wilderer, P., Jones, W., and Dau, U., (1987). "Competition in denitrification systems affecting reduction rate and accumulation." *Water Res.,* 21, 239– 245.

Yoshinari, T and Knowles, R. (1976). "Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria." *Biochem Biophys Res Commun.*, Apr 5; 69(3), 705–710.

APPENDIX A

STOICHIOMETRIC ANALYSIS OF DENITRIFICATION

The stoichiometric analyses presented in this appendix were performed based on the procedure outlined by Rittmann and McCarty (2001). Please refer to Section 4.4.1 of this dissertation for the details of the computation procedure. For all of the analysis presented herein, NO_3^- was assumed to be the nitrogen source during the reduction of nitrate to nitrogen gas and the reduction of nitrate to nitrite. For nitrite reduction to nitrogen gas, NO_2^- was assumed to be the source of nitrogen.

L-glutamic Acid – Reduction of Nitrate, NO_3^-, to Nitrogen, N_2:

R_d: $0.167CO_2+0.111HCO_3^-+0.056NH4^++1e^- \rightarrow 0.056COOHCH_2CH_2CHNH_2COO^- +0.444H_2O$ ($\Delta G^o = 30.93$ kJ/e^- eq)

R_a: $0.2NO_3^-+1.2H^++1e^- \rightarrow 0.1N_2+0.6H_2O$ ($\Delta G^o = -72.2$ kJ/e^- eq)

R_c: $0.036NO_3^-+0.179CO_2+1.036H^++1e^- \rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.056COOHCH_2CH_2CHNH_2COO^- + 0.2NO_3^- +0.311H^+ \rightarrow 0.1N_2 + 0.278CO_2 + 0.056NH_4^+ +0.267H_2O$

R_s: $0.056COOHCH_2CH_2CHNH_2COO^- +0.036NO_3^- +0.147H^+ \rightarrow 0.036\ C_5H_7O_2N + 0.056NH_4^+ + 0.099CO_2+0.06H_2O$

$\Delta G_p = (35.09\ kJ/e^-\ eq) - \Delta G^{o'}$ where $\Delta G^{o'} = 30.93$ kJ/e^- eq for glutamate

$\Delta G_p = 4.16$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells × 4.04 g cells/e^- eq = 13.44 kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 31.998$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -72.2$ kJ/e^- eq and $\Delta G_d^{o'} = 30.93$ kJ/e^- eq

$\Delta G_r = -103.13$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.564$

$A = 0.564 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.361$ and $f_s^o = 0.639$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.056COOHCH_2CH_2CHNH_2COO^-+0.095NO_3^-+0.206H^+ \rightarrow 0.023C_5H_7O_2N+0.056NH_4^+ +0.164CO_2 +0.036N_2 + 0.134H_2O$

$Y = (0.023/0.056) \times 113$ g cells/mol = 46.4 g cells/mol glutamate

L-glutamic Acid – Reduction of Nitrate, NO_3^-, to Nitrite, NO_2^-

R_d: $0.167CO_2+0.111HCO_3^-+0.056NH_4^++1H^++1e^- \rightarrow 0.056COOHCH_2CH_2CHNH_2COO^- +0.444H_2O$ ($\Delta G^o = 30.93$ kJ/e^- eq)

R_a: $0.5NO_3^-+1.2H^++1e^- \rightarrow 0.5NO_2^-+0.5H_2O$ ($\Delta G^o = -41.65$ kJ/e^- eq)

R_c: $0.036NO_3^-+0.179CO_2+1.036H^++1e^- \rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.056COOHCH_2CH_2CHNH_2COO^- + 0.5NO_3^- +0.111H^+ \rightarrow 0.5NO_2^- + 0.278CO_2 + 0.056NH_4^++0.167H_2O$

R_s: $0.056COOHCH_2CH_2CHNH_2COO^- +0.036NO_3^-+0.147H^+ \rightarrow 0.036\ C_5H_7O_2N + 0.056NH_4^+ + 0.099CO_2+0.06H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 30.93$ kJ/e^- eq for glutamate

$\Delta G_p = 4.16$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.04 g cells/e^- eq $= 13.44$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 31.998$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -41.65$ kJ/e^- eq and $\Delta G_d^{o'} = 30.93$ kJ/e^- eq

$\Delta G_r = -72.58$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.802$

$A = 0.802 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.445$ and $f_s^o = 0.555$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.056COOHCH_2CH_2CHNH_2COO^-+0.242NO_3^-+0.131H^+ \rightarrow 0.020C_5H_7O_2N+0.056NH_4^+ +0.179CO_2 +0.222NO_2^- + 0.107H_2O$

$Y = (0.020/0.056) \times 113$ g cells/mol $= 40.4$ g cells/mol glutamate

L-glutamic Acid – Reduction of Nitrite, NO_2^-, to Nitrogen, N_2

R_d: $0.167CO_2+0.111HCO_3^-+0.056NH_4^++1H^++1e^- \rightarrow 0.056COOHCH_2CH_2CHNH_2COO^- +0.444H_2O$ (ΔG^o= 30.93 kJ/e^- eq)

R_a: $0.333NO_2^-+1.333H^++1e^- \rightarrow 0.167N_2+0.667H_2O$ (ΔG^o= -92.56 kJ/e^- eq)

R_c: $0.038NO_2^-+0.192CO_2+1.038H^++1e^- \rightarrow 0.038C_5H_7O_2N+0.385H_2O$

R_e: $0.056COOHCH_2CH_2CHNH_2COO^- + 0.333NO_2^- +0.444H^+ \rightarrow 0.167N_2 + 0.278CO_2 + 0.056NH_4^+ +0.333H_2O$

R_s: $0.056COOHCH_2CH_2CHNH_2COO^- +0.038NO_2^-+0.15H^+ \rightarrow 0.038\ C_5H_7O_2N + 0.056NH_4^+ +0.085CO_2+0.051H_2O$

$\Delta G_p = (35.09\ kJ/e^-\ eq) - \Delta G^{o'}$ where $\Delta G^{o'}$ = 30.93 kJ/e^- eq for glutamate

ΔG_p = 4.16 kJ/e^- eq and ΔG_{pc} = 3.33 kJ/g cells × 4.346 g cells/e^- eq = 14.473 kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and ε = 0.55 (assumed)

ΔG_s = 33.878 kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'}$ = -92.56 kJ/e^- eq and $\Delta G_d^{o'}$ = 30.93 kJ/e^- eq

ΔG_r = -123.49 kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.499$

$A = 0.499 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.333$ and $f_s^o = 0.667$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.056COOHCH_2CH_2CHNH_2COO^-+0.137NO_2^-+0.248H^+ \rightarrow 0.026C_5H_7O_2N+0.056NH_4^+ +0.149CO_2 +0.055N_2 + 0.145H_2O$

Y = (0.026/0.056) ×113 g cells/mol = 52.5 g cells/mol glutamate

Ethanol – Reduction of Nitrate, NO_3^-, to Nitrogen, N_2:

R_d: $0.167CO_2+1H^++1e^- \rightarrow 0.083CH_3CH_2OH+0.25H_2O$ ($\Delta G^o = 31.18$ kJ/e^- eq)

R_a: $0.2NO_3^-+1.2H^++1e^- \rightarrow 0.1N_2+0.6H_2O$ ($\Delta G^o = -72.2$ kJ/e^- eq)

R_c: $0.036NO_3^-+0.179CO_2+1.036H^++1e^- \rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.083\ CH_3CH_2OH + 0.2NO_3^- + 0.2H^+ \rightarrow 0.1N_2 + 0.167CO_2 + 0.35H_2O$

R_s: $0.083\ CH_3CH_2OH + 0.036NO_3^- + 0.036H^+ + 0.012CO_2 \rightarrow 0.036\ C_5H_7O_2N + 0.143H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 31.18$ kJ/e^- eq for ethanol

$\Delta G_p = 3.91$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.04 g cells/e^- eq $= 13.44$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^n) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 31.544$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -72.2$ kJ/e^- eq and $\Delta G_d^{o'} = 31.18$ kJ/e^- eq

$\Delta G_r = -103.38$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.555$

$A = 0.555 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.357$ and $f_s^o = 0.643$

$R = (f_e^o \times R_e) + (f_s^o \times R_s)$

R: $0.083CH_3CH_2OH + 0.094NO_3^- + 0.094H^+ \rightarrow 0.023C_5H_7O_2N +$

$+0.052CO_2 + 0.036N_2 + 0.217H_2O$

Y = (0.023/0.083) $\times$113 g cells/mol = 31.3 g cells/mol ethanol

Ethanol – Reduction of Nitrate, NO_3^-, to Nitrite, NO_2^-:

R_d: $0.167CO_2+1H^++1e^- \rightarrow 0.083CH_3CH_2OH+0.25H_2O$ ($\Delta G^o = 31.18$ kJ/e^- eq)

R_a: $0.5NO_3^-+1.2H^++1e^- \rightarrow 0.5NO_2^-+0.5H_2O$ ($\Delta G^o = -41.65$ kJ/e^- eq)

R_c: $0.036NO_3^-+0.179CO_2+1.036H^++1e^- \rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.083\ CH_3CH_2OH + 0.5NO_3^- \rightarrow 0.5NO_2^- + 0.167CO_2 + 0.25H_2O$

R_s: $0.083\ CH_3CH_2OH +0.036NO_3^-+0.036H^++0.012CO_2 \rightarrow 0.036\ C_5H_7O_2N +0.143H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 31.18$ kJ/e^- eq for ethanol

$\Delta G_p = 3.91$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.04 g cells/e^- eq $= 13.44$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 31.544$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -41.65$ kJ/e^- eq and $\Delta G_d^{o'} = 31.18$ kJ/e^- eq

$\Delta G_r = -72.83$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.787$

$A = 0.787 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.441$ and $f_s^o = 0.559$

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.083CH_3CH_2OH +0.240NO_3^-+0.02H^+ \rightarrow 0.020C_5H_7O_2N+$

$+0.067CO_2 +0.22NO_2^- + 0.190H_2O$

Y = (0.020/0.083) $\times$113 g cells/mol = 27.2 g cells/mol ethanol

Ethanol – Reduction of Nitrite, NO_2^-, to Nitrogen, N_2:

R_d: $0.167CO_2+1H^++1e^- \rightarrow 0.083CH_3CH_2OH+0.25H_2O$ ($\Delta G^o = 31.18$ kJ/e^- eq)

R_a: $0.333NO_2^-+1.333H^++1e^- \rightarrow 0.167N_2+0.667H_2O$ ($\Delta G^o = -92.56$ kJ/e^- eq)

R_c: $0.038NO_2^-+0.192CO_2+1.038H^++1e^- \rightarrow 0.038C_5H_7O_2N+0.385H_2O$

R_e: $0.083\ CH_3CH_2OH + 0.333NO_3^- +0.333H^+ \rightarrow 0.167N_2 + 0.167CO_2 + 0.417H_2O$

R_s: $0.083\ CH_3CH_2OH +0.038NO_3^-+0.038H^++0.026CO_2 \rightarrow 0.038\ C_5H_7O_2N +0.135H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 31.18$ kJ/e^- eq for ethanol

$\Delta G_p = 3.91$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.346 g cells/e^- eq $= 14.473$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^n) + (\Delta G_{pc} / \varepsilon)$ where $n=1$ and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 33.423$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -92.56$ kJ/e^- eq and $\Delta G_d^{o'} = 31.18$ kJ/e^- eq

$\Delta G_r = -123.74$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.491$

$A = 0.491 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.329$ and $f_s^o = 0.671$

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.083CH_3CH_2OH +0.136NO_2^-+0.136H^+ \rightarrow 0.026C_5H_7O_2N+$

$+0.038CO_2 +0.055NO_2^- + 0.228H_2O$

$Y = (0.026/0.083) \times 113$ g cells/mol $= 35.4$ g cells/mol ethanol

Alanine – Reduction of Nitrate, NO_3^-, to Nitrogen, N_2:

R_d: $0.167CO_2+0.083HCO_3^-+0.083NH4^+ +1e^- \rightarrow 0.056CH_3CHNH_2COO^- +0.417H_2O$ ($\Delta G^o = 31.37$ kJ/e^- eq)

R_a: $0.2NO_3^- +1.2H^+ +1e^- \rightarrow 0.1N_2 +0.6H_2O$ ($\Delta G^o = -72.2$ kJ/e^- eq)

R_c: $0.036NO_3^- +0.179CO_2 +1.036H^+ +1e^- \rightarrow 0.036C_5H_7O_2N +0.393H_2O$

R_e: $0.083CH_3CHNH_2COO^- + 0.2NO_3^- +0.367H^+ \rightarrow 0.1N_2 + 0.250CO_2 + 0.083NH_4^+ +0.267H_2O$

R_s: $0.083CH_3CHNH_2COO^- +0.036NO_3^- +0.202H^+ \rightarrow 0.036\ C_5H_7O_2N + 0.083NH_4^+ + 0.071CO_2 +0.06H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 31.37$ kJ/e^- eq for alanine

$\Delta G_p = 3.72$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.04 g cells/e^- eq $= 13.44$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 31.198$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -72.2$ kJ/e^- eq and $\Delta G_d^{o'} = 31.37$ kJ/e^- eq

$\Delta G_r = -103.57$kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.548$

$A = 0.548 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.354$ and $f_s^o = 0.649$.

$R = (f_e^o \times R_e) + (f_s^o \times R_s)$

R: $0.083\ CH_3CHNH_2COO^- +0.094NO_3^- +0.261H^+ \rightarrow 0.023C_5H_7O_2N +0.083NH_4^+ +0.135CO_2 +0.035N_2 + 0.133H_2O$

Y = (0.023/0.083) $\times$113 g cells/mol = 31.3 g cells/mol alanine

Alanine – Reduction of Nitrate, NO_3^-, to Nitrite, NO_2^-:

R_d: $0.167CO_2+0.083HCO_3^-+0.083NH4^++1e^-\rightarrow 0.056CH_3CHNH_2COO^-$

$+0.417H_2O$ ($\Delta G^o= 31.37$ kJ/e^- eq)

R_a: $0.5NO_3^-+1.2H^++1e^-\rightarrow 0.5NO_2^-+0.5H_2O$ ($\Delta G^o= -41.65$ kJ/e^- eq)

R_c:$0.036NO_3^-+0.179CO_2+1.036H^++1e^-\rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.083CH_3CHNH_2COO^- + 0.5NO_3^- +0.167H^+\rightarrow 0.5NO_2^- + 0.250CO_2 +$

$0.083NH_4^++0.167H_2O$

R_s: $0.083CH_3CHNH_2COO^- +0.036NO_3^-+0.202H^+ \rightarrow 0.036\ C_5H_7O_2N +$

$0.083NH_4^+ + 0.071CO_2+0.06H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq) – $\Delta G^{o'}$ where $\Delta G^{o'} = 31.37$ kJ/e^- eq for alanine

$\Delta G_p = 3.72$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells × 4.04 g cells/e^- eq = 13.44 kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 31.198$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -41.65$ kJ/e^- eq and $\Delta G_d^{o'} = 31.7$ kJ/e^- eq

$\Delta G_r = -73.02$ kJ/e^- eq

$A = - \Delta G_s / (\varepsilon \Delta G_r) = 0.777$

$A = 0.777 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.437$ and $f_s^o = 0.563$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R:$0.083\ CH_3CHNH_2COO^- +0.239NO_3^-+0.187H^+\rightarrow 0.02C_5H_7O_2N+0.083NH_4^+$

$+0.149CO_2 +0.219N_2 + 0.106H_2O$

$Y = (0.02/0.083) \times 113$ g cells/mol = 27.2 g cells/mol alanine

Alanine – Reduction of Nitrite, NO_2^-, to Nitrogen, N_2:

R_d: $0.167CO_2+0.083HCO_3^-+0.083NH4^++1e^- \rightarrow 0.056CH_3CHNH_2COO^- +0.417H_2O$ ($\Delta G^o = 31.37$ kJ/e^- eq)

R_a: $0.333NO_2^-+1.333H^++1e^- \rightarrow 0.167N_2+0.667H_2O$ ($\Delta G^o = -92.56$ kJ/e^- eq)

R_c: $0.038NO_2^-+0.192CO_2+1.038H^++1e^- \rightarrow 0.038C_5H_7O_2N+0.385H_2O$

R_e: $0.083CH_3CHNH_2COO^- + 0.333NO_3^- +0.5H^+ \rightarrow 0.167N_2 + 0.250CO_2 + 0.083NH_4^+ +0.333H_2O$

R_s: $0.083CH_3CHNH_2COO^- +0.038NO_3^- +0.205H^+ \rightarrow 0.038\ C_5H_7O_2N + 0.083NH_4^+ + 0.058CO_2+0.051H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 31.37$ kJ/e^- eq for alanine

$\Delta G_p = 3.72$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.346 g cells/e^- eq $= 14.47$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 33.078$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -92.56$ kJ/e^- eq and $\Delta G_d^{o'} = 31.7$ kJ/e^- eq

$\Delta G_r = -123.93$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.485$

$A = 0.777 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.327$ and $f_s^o = 0.673$

$R = (f_e^o \times R_e) + (f_s^o \times R_s)$

R: $0.083\ CH_3CHNH_2COO^- +0.135NO_3^- +0.301H^+ \rightarrow 0.026C_5H_7O_2N+0.083NH_4^+ +0.121CO_2 +0.054N_2 + 0.143H_2O$

$Y = (0.026/0.083) \times 113$ g cells/mol $= 35.4$ g cells/mol alanine

Acetate – Reduction of Nitrate, NO_3^-, to Nitrogen, N_2:

R_d: $0.125CO_2+0.125HCO_3^-+1H^++1e^- \rightarrow 0.125CH_3COO^-$

$+0.375H_2O$ ($\Delta G^o = 27.40$ kJ/e^- eq)

R_a: $0.2NO_3^-+1.2H^++1e^- \rightarrow 0.1N_2+0.6H_2O$ ($\Delta G^o = -72.2$ kJ/e^- eq)

R_c: $0.036NO_3^-+0.179CO_2+1.036H^++1e^- \rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.125CH_3COO^- + 0.2NO_3^- +0..2H^+ \rightarrow 0.1N_2 + 0.250CO_2 + 0.350H_2O$

R_s: $0.125CH_3COO^- +0.036NO_3^-+0.161H^+ \rightarrow 0.036\ C_5H_7O_2N + 0.071CO_2+0.143H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq) $- \Delta G^{o'}$ where $\Delta G^{o'} = 27.4$ kJ/e^- eq for acetate

$\Delta G_p = 7.69$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.04 g cells/e^- eq $= 13.44$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 38.416$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -72.2$ kJ/e^- eq and $\Delta G_d^{o'} = 27.4$ kJ/e^- eq

$\Delta G_r = -99.6$ kJ/e^- eq

$A = - \Delta G_s / (\varepsilon \Delta G_r) = 0.701$

$A = 0.701 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.412$ and $f_s^o = 0.588$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.125CH_3COO^- +0.103NO_3^-+0.228H^+ \rightarrow 0.021C_5H_7O_2N$

$+0.145CO_2 +0.041N_2 + 0.228H_2O$

$Y = (0.021/0.125) \times 113$ g cells/mol $= 19$ g cells/mol acetate

Acetate – Reduction of Nitrate, NO_3^-, to Nitrite, NO_2^-:

R_d: $0.125CO_2+0.125HCO_3^-+1H^++1e^- \rightarrow 0.125CH_3COO^-$

$+0.375H_2O$ ($\Delta G^o = 27.40$ kJ/e^- eq)

R_a: $0.5NO_3^-+1.2H^++1e^- \rightarrow 0.5NO_2^-+0.5H_2O$ ($\Delta G^o = -41.65$ kJ/e^- eq)

R_c: $0.036NO_3^-+0.179CO_2+1.036H^++1e^- \rightarrow 0.036C_5H_7O_2N+0.393H_2O$

R_e: $0.125CH_3COO^- + 0.5NO_3^- +0.125H^+ \rightarrow 0.5NO_2^- + 0.250CO_2 + 0.25H_2O$

R_s: $0.125CH_3COO^- +0.036NO_3^-+0.161H^+ \rightarrow 0.036\ C_5H_7O_2N + 0.071CO_2+0.143H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 27.4$ kJ/e^- eq for acetate

$\Delta G_p = 7.69$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.04 g cells/e^- eq $= 13.44$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 38.42$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -41.65$ kJ/e^- eq and $\Delta G_d^{o'} = 27.4$ kJ/e^- eq

$\Delta G_r = -69.1$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 1.012$

$A = 1.012 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.503$ and $f_s^o = 0.497$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

R: $0.125CH_3COO^- +0.269NO_3^-+0.143H^+ \rightarrow 0.018C_5H_7O_2N$

$+0.161CO_2 +0.251NO_2^- + 0.197H_2O$

$Y = (0.018/0.125) \times 113$ g cells/mol $= 16.3$ g cells/mol acetate

Acetate – Reduction of Nitrite, NO_2^-, to Nitrogen, N_2:

R_d: $0.125CO_2+0.125HCO_3^-+1H^++1e^- \rightarrow 0.125CH_3COO^- +0.375H_2O$ ($\Delta G^o = 27.40$ kJ/e^- eq)

R_a: $0.333NO_2^-+1.333H^++1e^- \rightarrow 0.167N_2+0.667H_2O$ ($\Delta G^o = -92.56$ kJ/e^- eq)

R_c: $0.038NO_2^-+0.192CO_2+1.038H^++1e^- \rightarrow 0.038C_5H_7O_2N+0.385H_2O$

R_e: $0.125CH_3COO^- + 0.333NO_3^- +0.458H^+ \rightarrow 0.167NO_2 + 0.250CO_2 + 0.417H_2O$

R_s: $0.125CH_3COO^- +0.036NO_2^-+0.163H^+ \rightarrow 0.038\ C_5H_7O_2N + 0.058CO_2+0.135H_2O$

$\Delta G_p = (35.09$ kJ/e^- eq$) - \Delta G^{o'}$ where $\Delta G^{o'} = 27.4$ kJ/e^- eq for acetate

$\Delta G_p = 7.69$ kJ/e^- eq and $\Delta G_{pc} = 3.33$ kJ/g cells $\times$ 4.346 g cells/e^- eq $= 14.473$ kJ/e^- eq

$\Delta G_s = (\Delta G_p / \varepsilon^{n}) + (\Delta G_{pc} / \varepsilon)$ where n=1 and $\varepsilon = 0.55$ (assumed)

$\Delta G_s = 40.27$ kJ/e^- eq

$\Delta G_r = \Delta G_a^{o'} - \Delta G_d^{o'}$ where $\Delta G_a^{o'} = -92.56$ kJ/e^- eq and $\Delta G_d^{o'} = 27.4$ kJ/e^- eq

$\Delta G_r = -119.96$ kJ/e^- eq

$A = -\Delta G_s / (\varepsilon \Delta G_r) = 0.611$

$A = 1.012 = f_e^o / f_s^o$ and $f_e^o + f_s^o = 1 \rightarrow f_e^o = 0.379$ and $f_s^o = 0.621$.

$R = (f_e^o \times R_e)+(f_s^o \times R_s)$

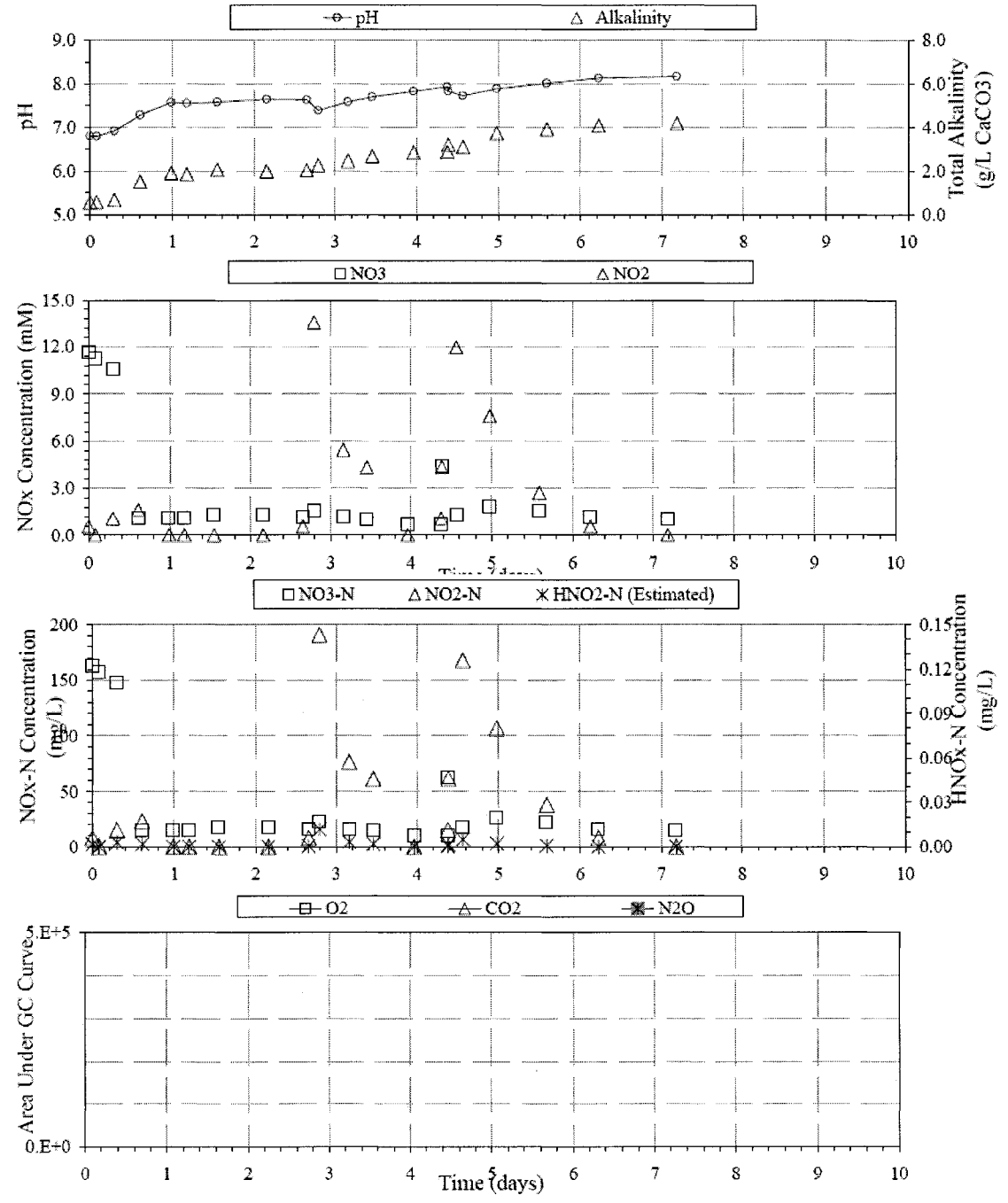
R: $0.125CH_3COO^- +0.15NO_2^-+0.275H^+ \rightarrow 0.024C_5H_7O_2N +0.131CO_2 +0.063N_2 + 0.242H_2O$

$Y = (0.024/0.125) \times 113$ g cells/mol $= 21.7$ g cells/mol acetate

APPENDIX B

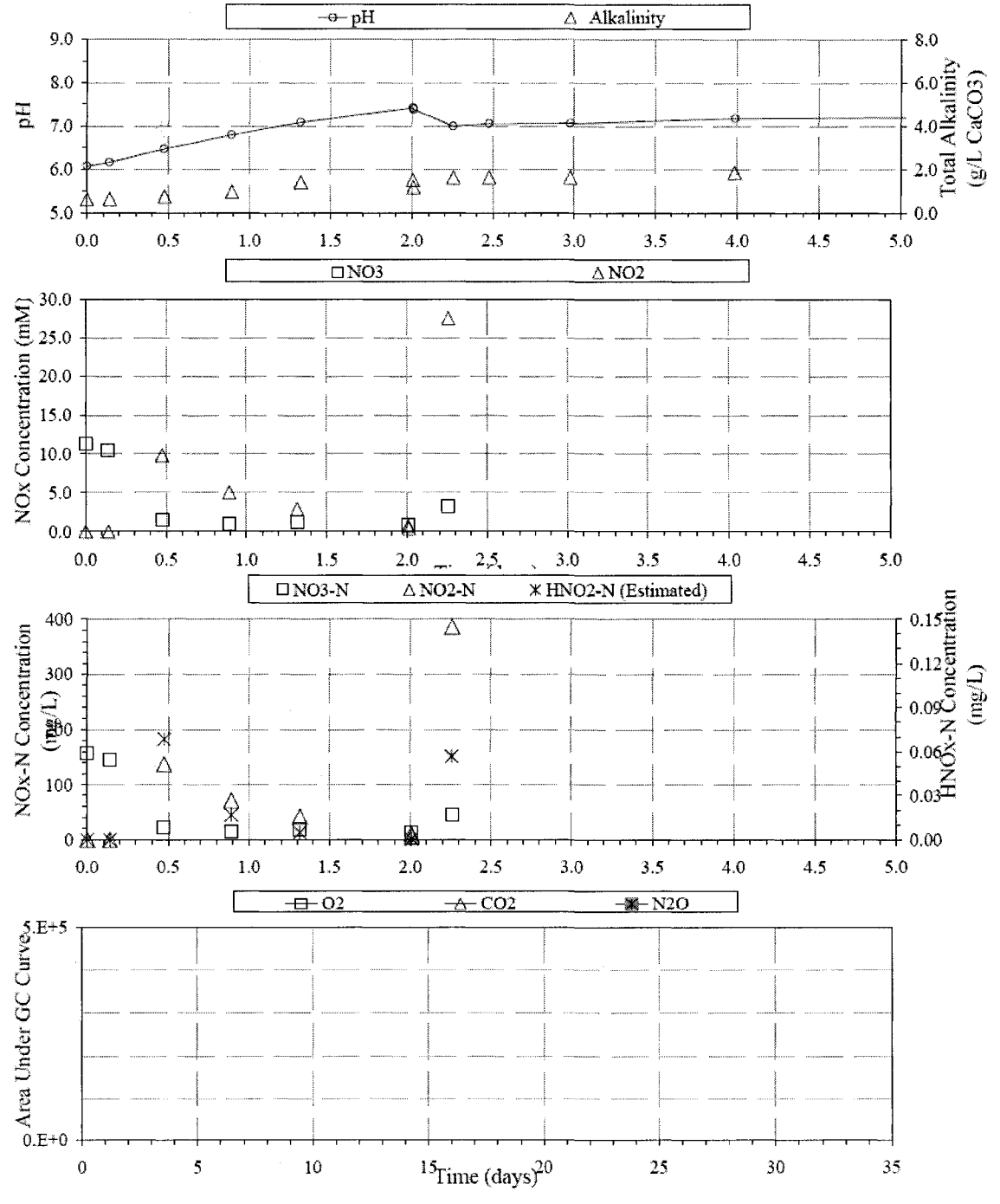
BATCH REACTOR TEST RESULTS

DN-N3

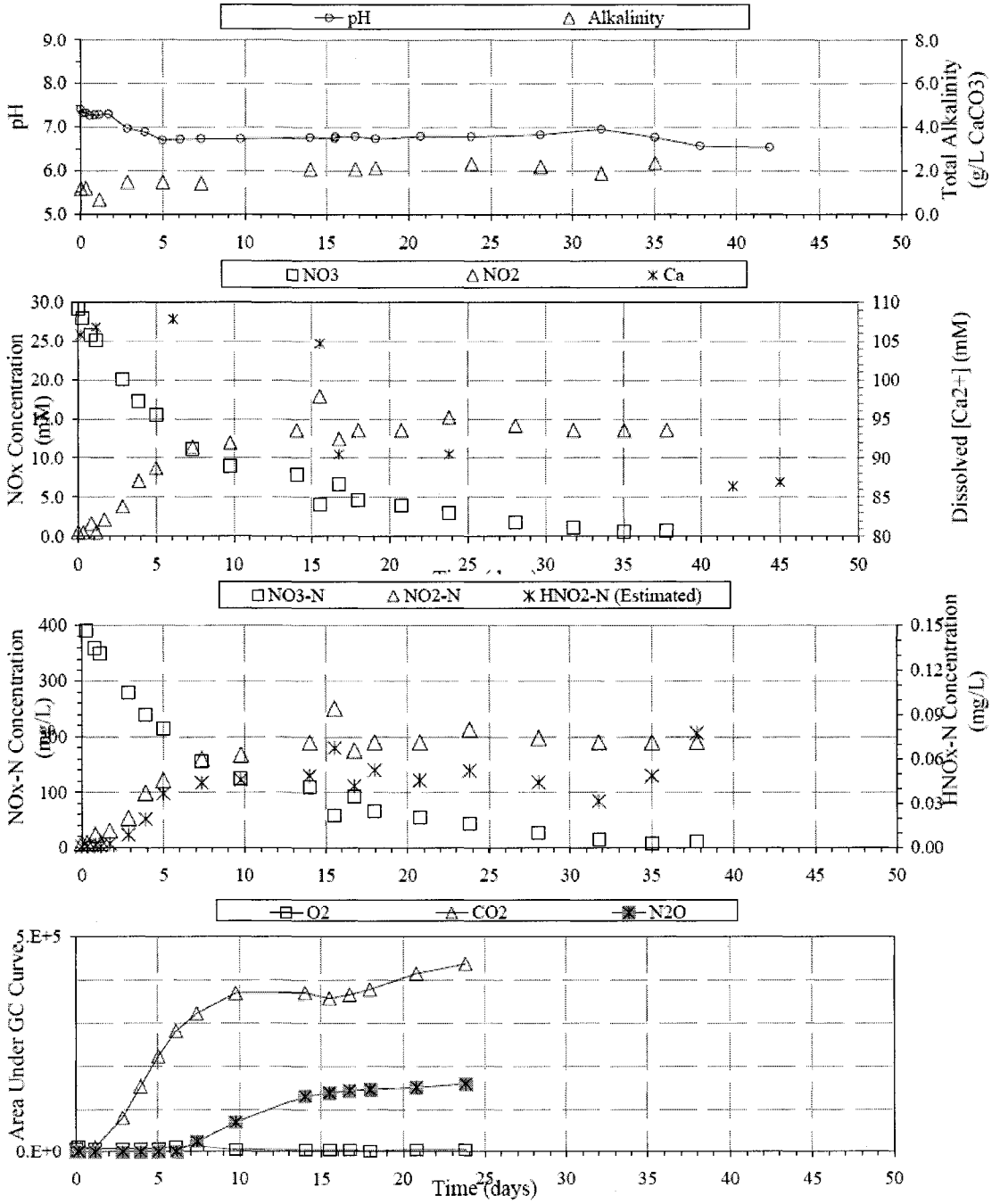


Inorganic Salts	mM	Phosphate	mM	e-donor	mM	e-acceptor	mM
				Nutrient Broth 31 g/1500 ml		NaNO3	15

DN-N4


pH
Alkalinity
pH
Total Alkalinity (g/L CaCO3)
NO3
NO2
NOx Concentration (mM)
NO3-N
NO2-N
HNO2-N (Estimated)
NOx-N Concentration (mg/L)
HNOX-N Concentration (mg/L)
O2
CO2
N2O
Area Under GC Curve
Time (days)
Inorganic Salts mM
Phosphate mM
e-donor mM
Nutrient Broth 2 g/120 ml
e-acceptor mM
NaNO3 10

DN-N9



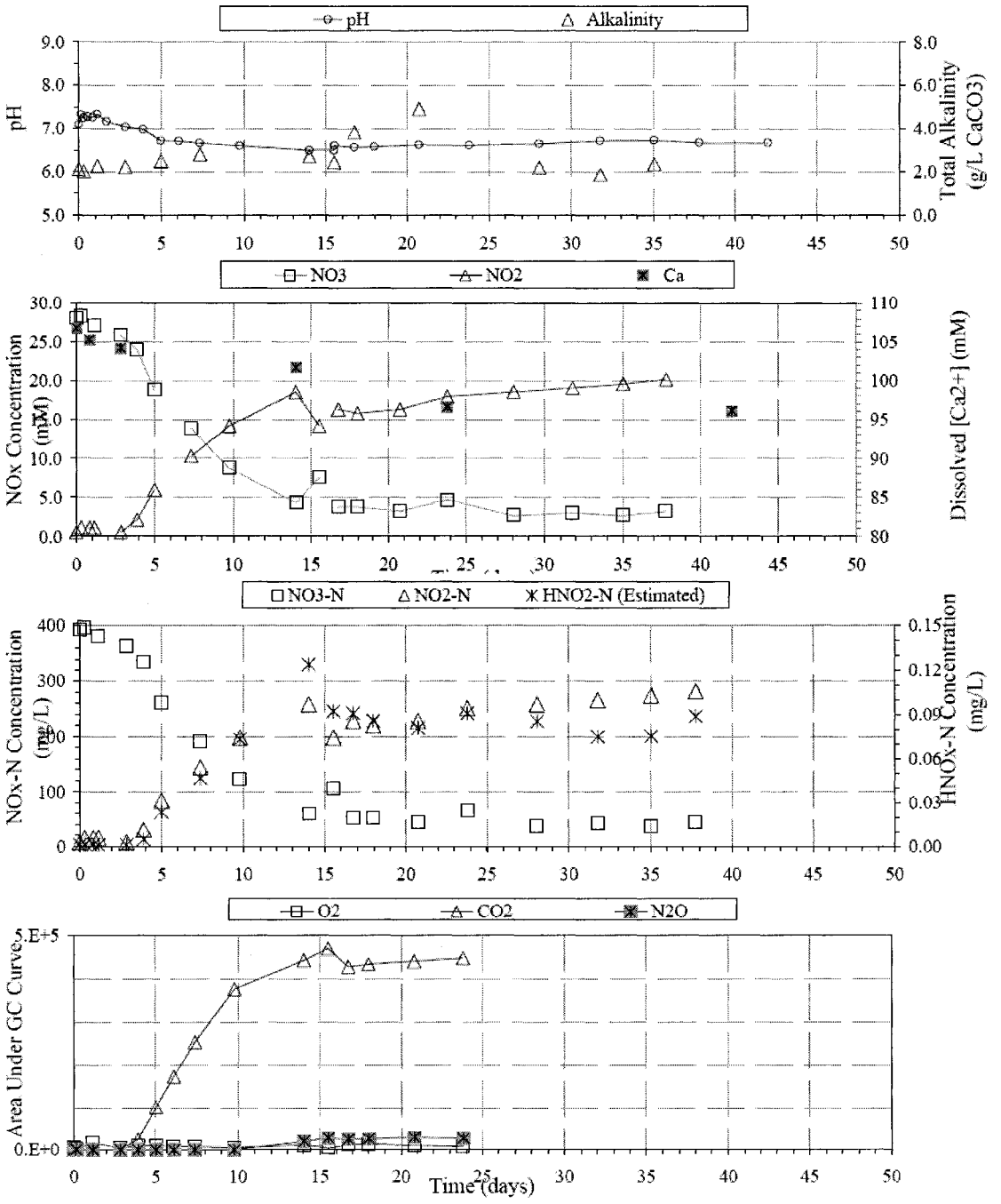
Inorganic Salts	mM
NaCl	17
NH4Cl	0
MgSO4.7H2O	1
CaCl2.2H2O	100
Trace Metal Solution	

Phosphate	mM

e-donor	mM
L-Glutamic Acid	75

e-acceptor	mM
NaNO3	25

DN-N10



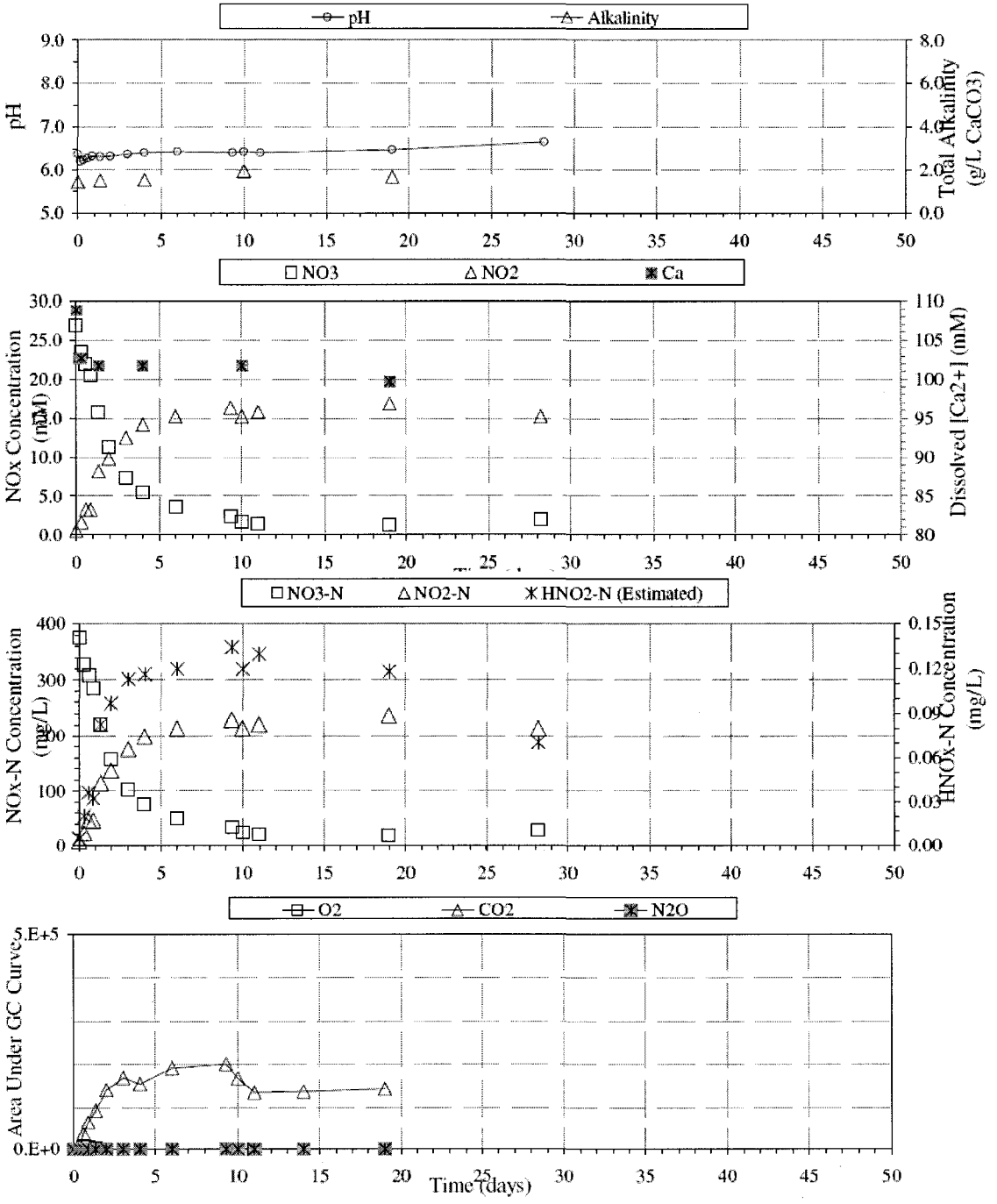
Inorganic Salts	mM
NaCl	17
NH4Cl	0
MgSO4.7H2O	1
CaCl2.2H2O	100
Trace Metal Solution	

Phosphate	mM

e-donor	mM
CH3COONa.3H2O	75

e-acceptor	mM
NaNO3	25

DN-N13



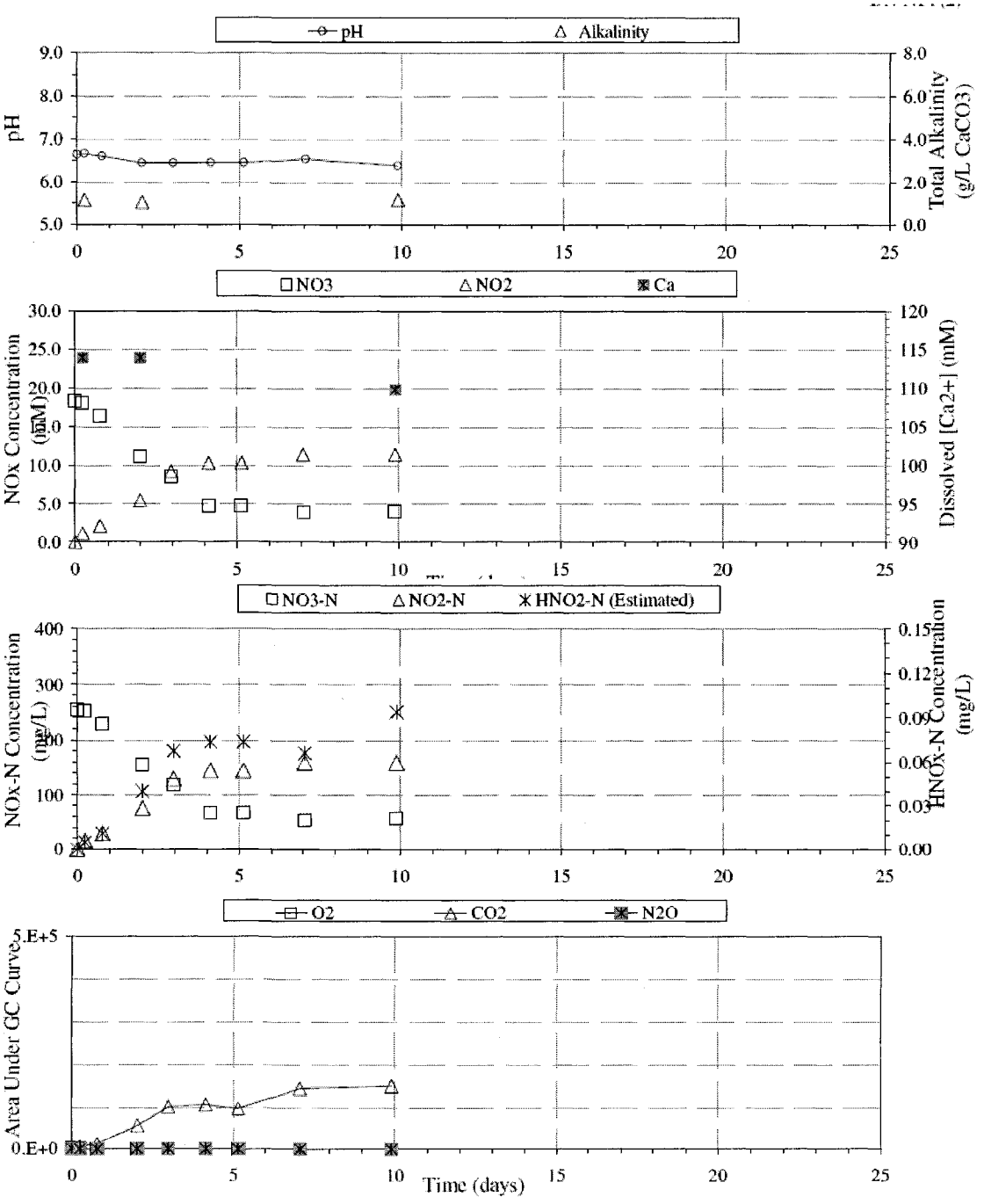
Inorganic Salts	mM
NaCl	17
NH4Cl	0
MgSO4.7H2O	1
CaCl2.2H2O	100
Trace Metal Solution	

Phosphate	mM
KH2PO4	2

e-donor	mM
L-Glutamic Acid	75

e-acceptor	mM
NaNO3	25

DN-N14



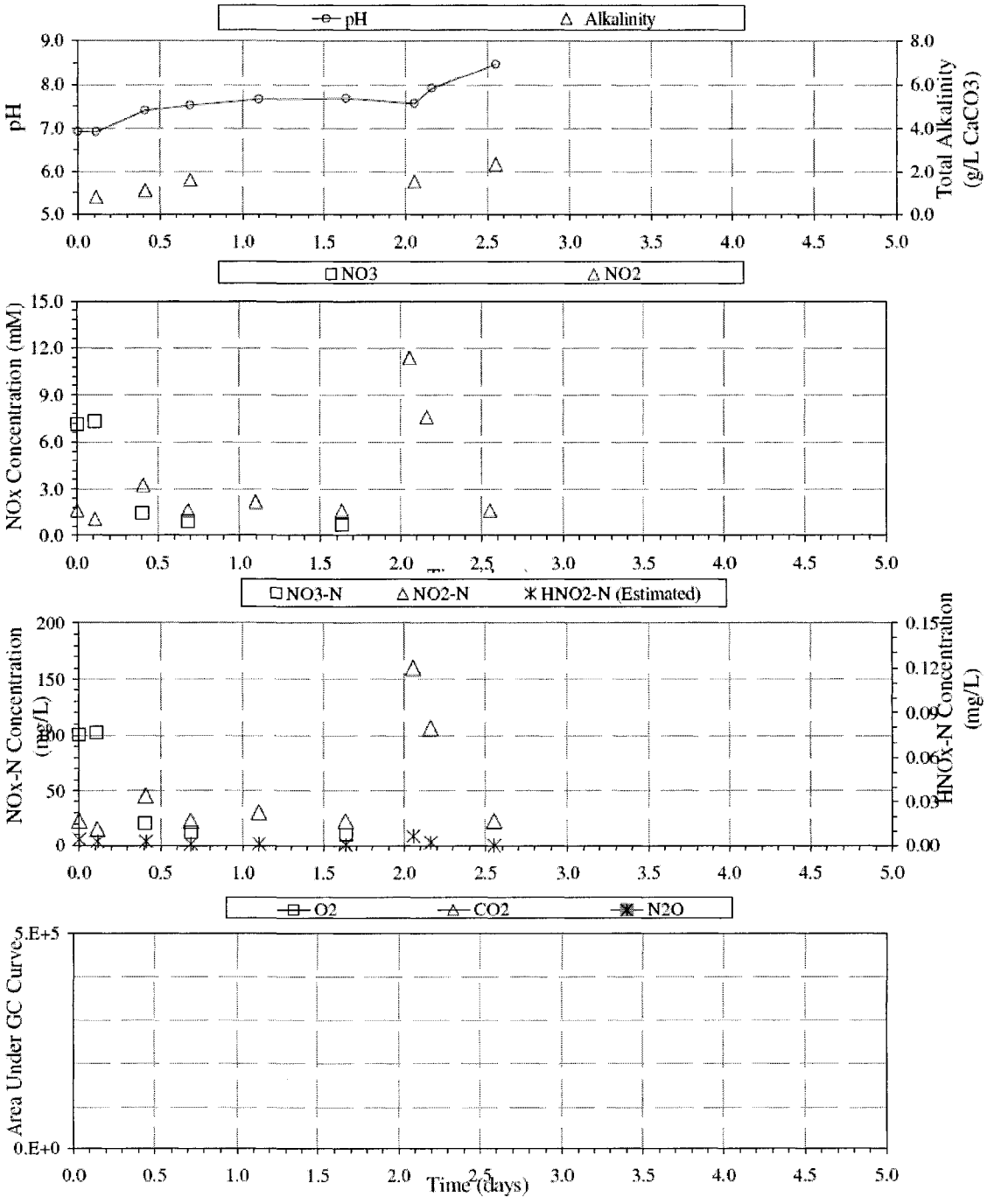
Inorganic Salts	mM
NaCl	17
NH4Cl	12
MgSO4.7H2O	1
CaCl2.2H2O	100
Trace Metal Solution	

Phosphate	mM
KH2PO4	1

e-donor	mM
L-Glutamic Acid	50

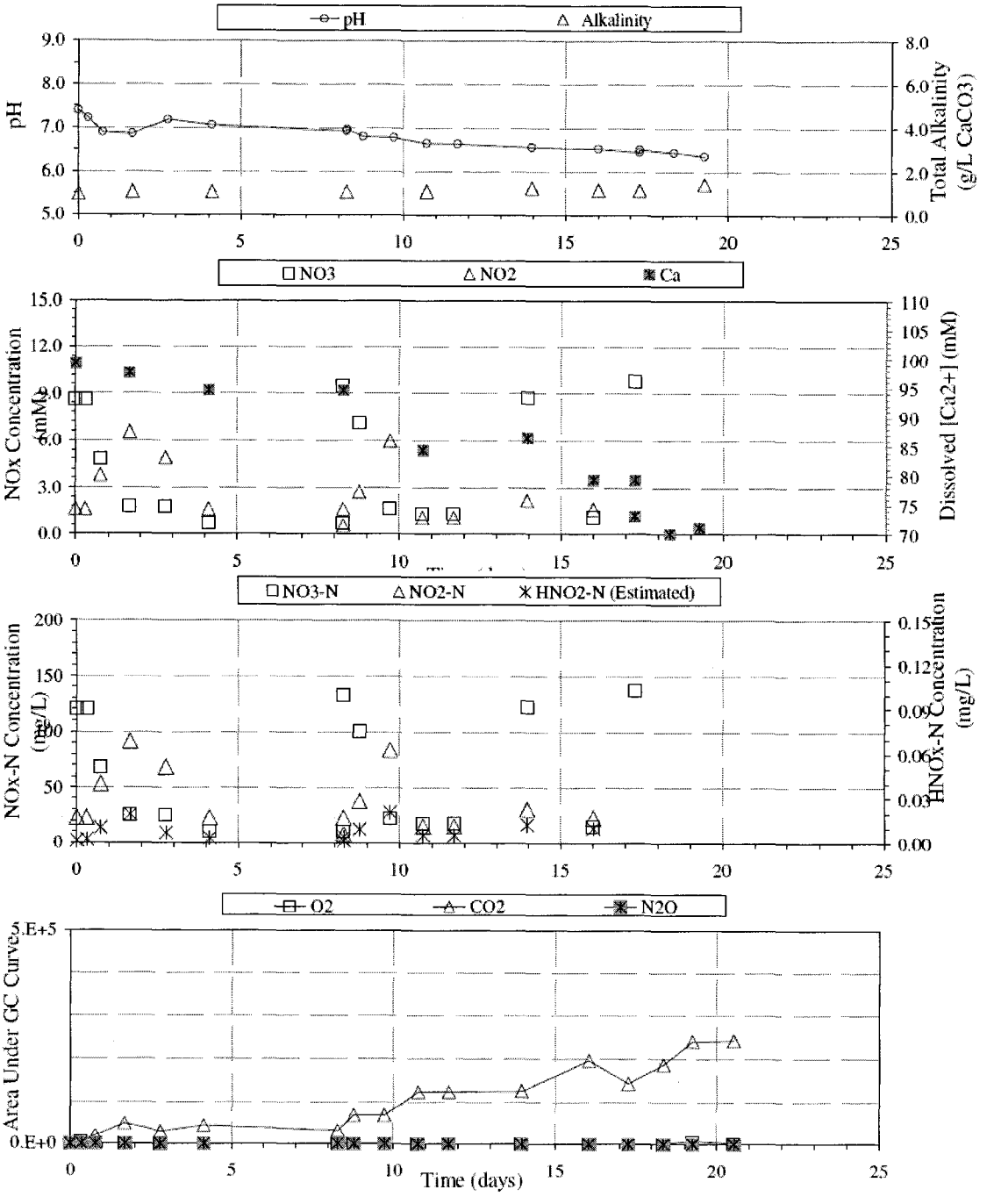
e-acceptor	mM
NaNO3	16

DN-N15



Inorganic Salts	mM	Phosphate	mM	e-donor	mM	e-acceptor	mM
				Nutrient Broth 2 g/120 ml		NaNO3	10

DN-N17



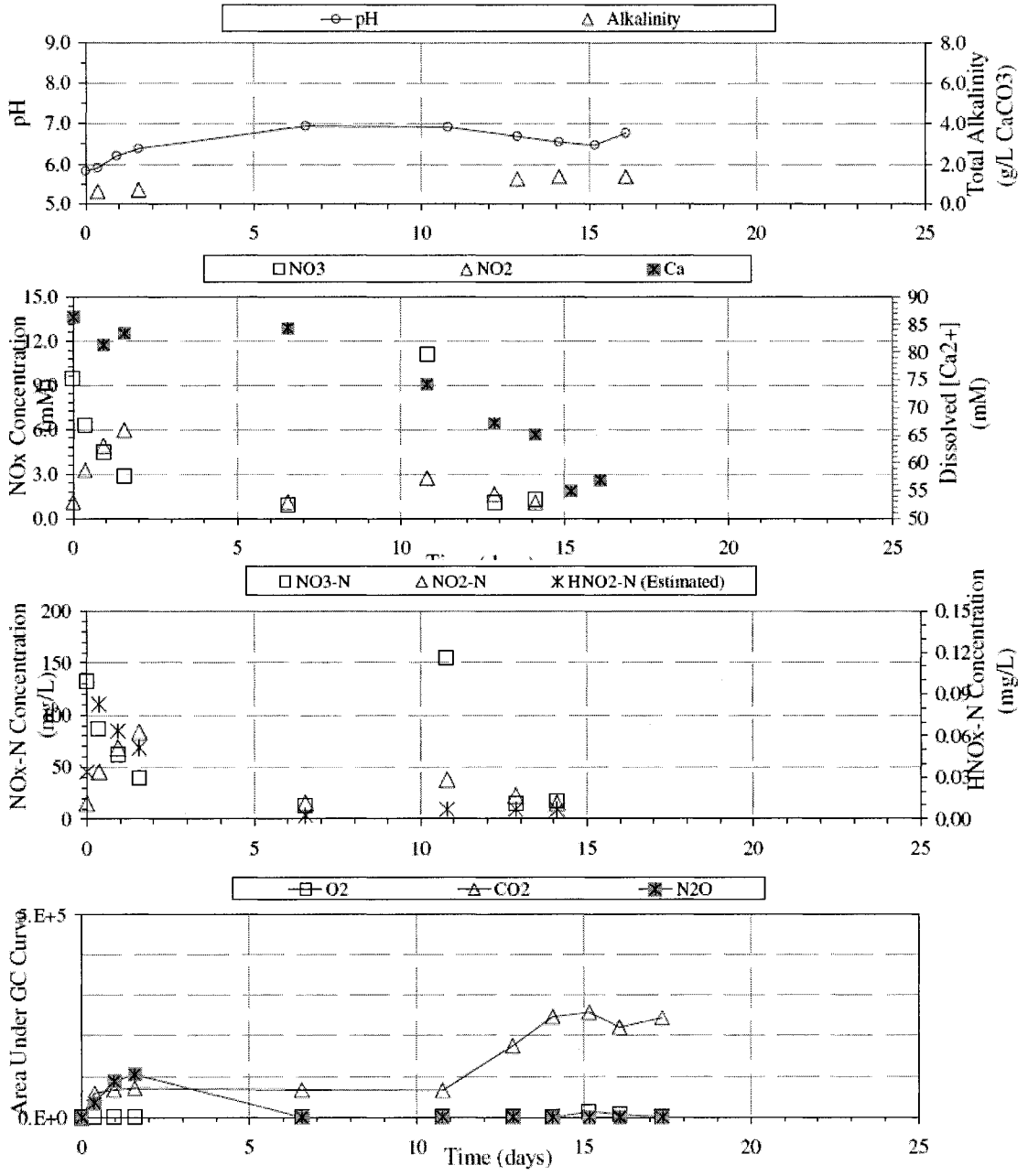
Inorganic Salts	mM
NaCl	17
NH4Cl	0
MgSO4.7H2O	1
CaCl2.2H2O	100
Trace Metal Solution	

Phosphate	mM
KH2PO4	2

e-donor	mM
L-Glutamic Acid	50

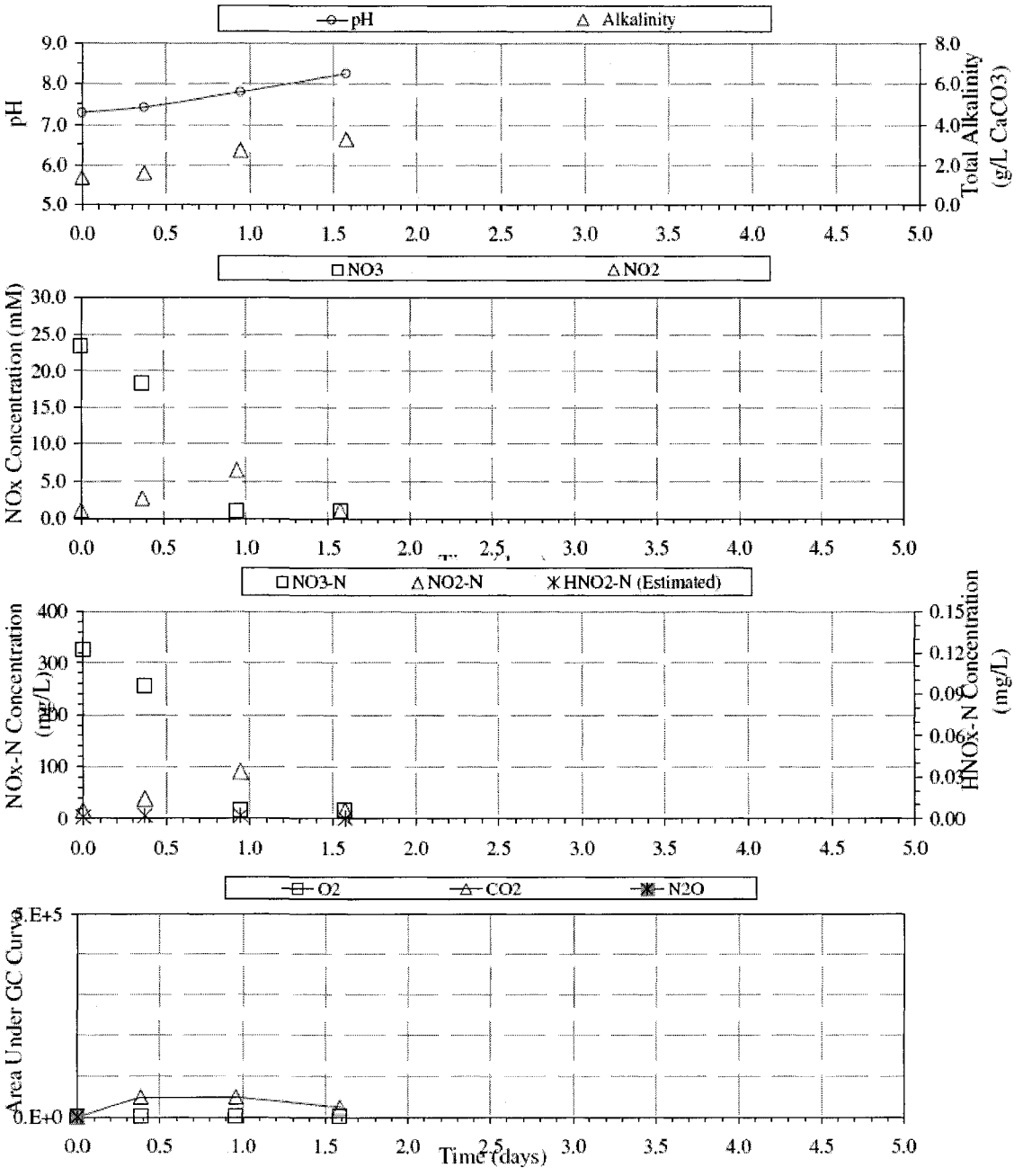
e-acceptor	mM
NaNO3	8

DN-N18



Inorganic Salts	mM	Phosphate	mM	e-donor	mM	e-acceptor	mM
CaCl2.2H2O	100			Nutrient Broth 2 g /120 ml		NaNO3	12

DN-N19



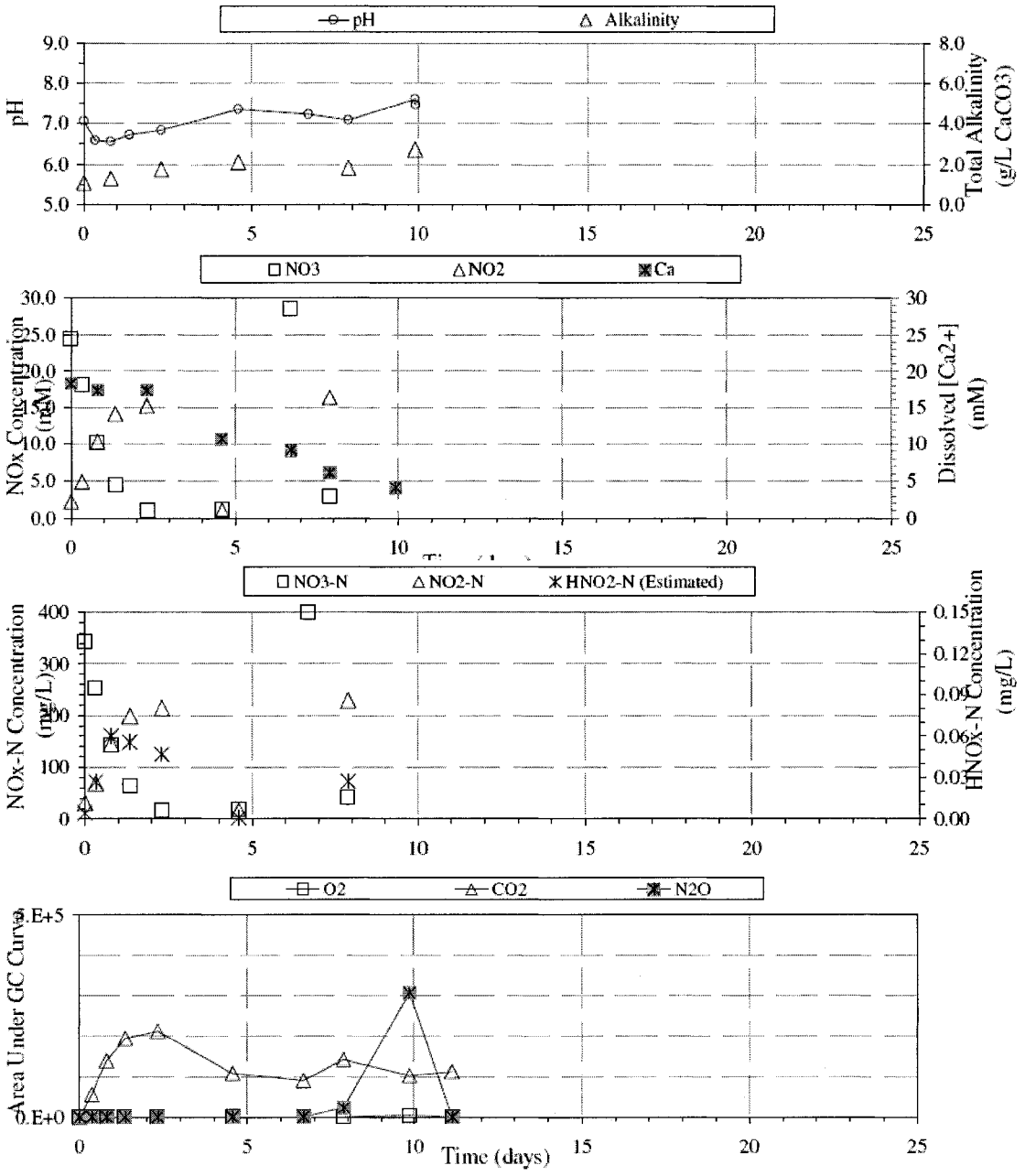
Inorganic Salts	mM
NaCl	17
NH4Cl	0
MgSO4.7H2O	1
CaCl2.2H2O	0
Trace Metal Solution	

Phosphate	mM
KH2PO4	2
Yeast E.	0.006g/120ml
Peptone	0.006g/120ml

e-donor	mM
L-Glutamic Acid	75

e-acceptor	mM
NaNO3	25

DN-N21



Inorganic Salts	mM
NaCl	9
NH4Cl	0
MgSO4.7H2O	1
CaCl2.2H2O	20
Trace Metal Solution	

Phosphate	mM
KH2PO4	2
Yeast E.	0.006g/120ml
Peptone	0.006g/120ml

e-donor	mM
L-Glutamic Acid	75

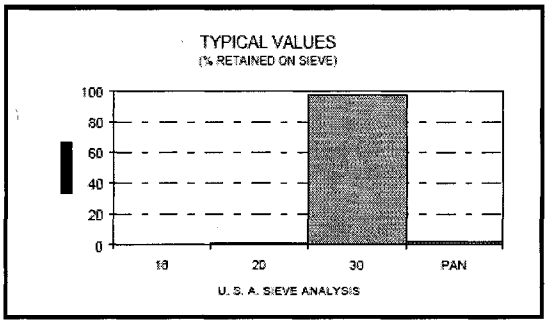
e-acceptor	mM
NaNO3	25

APPENDIX C

ASTM 20/30 OTTAWA SAND PRODUCT DATA SHEET

U.S. SILICA®
Sands of Time

PRODUCT DATA



ASTM[(1)] 20/30

UNGROUND SILICA

PLANT: OTTAWA, ILLINOIS

(1) AMERICAN SOCIETY FOR TESTING AND MATERIALS

USA STD SIEVE SIZE		TYPICAL VALUES		
		% RETAINED		% PASSING
MESH	MILLIMETERS	INDIVIDUAL	CUMULATIVE	CUMULATIVE
16	1.180	0.0	0.0	100.0
20	0.850	1.0	1.0	99.0
30	0.600	97.0	98.0	2.0
PAN		2.0	100.0	0.0

TYPICAL PROPERTIES

COLOR ..... WHITE
GRAIN SHAPE ..... ROUND
HARDNESS (Mohs) 7
MELTING POINT (Degrees F) 3100
MINERAL ..... QUARTZ
pH 7
SPECIFIC GRAVITY 2.65

TYPICAL CHEMICAL ANALYSIS, %

SiO_2 (Silicon Dioxide) 99.8
Fe_2O_3 (Iron Oxide) 0.020
Al_2O_3 (Aluminum Oxide) 0.06
TiO_2 (Titanium Dioxide) 0.01
CaO (Calcium Oxide) ..... <0.01
MgO (Magnesium Oxide) ..... <0.01
Na_2O (Sodium Oxide) ..... <0.01
K_2O (Potassium Oxide) ..... <0.01
LOI (Loss On Ignition) 0.1

CONFORMS TO ASTM C778

December 15, 1997

WARNING: The product contains crystalline silica - quartz, which can cause silicosis (an occupational lung disease) and lung cancer. **For detailed information on the potential health effect of crystalline silica - quartz, see the U.S. Silica Company Material Safety Data Sheet.**

U.S. Silica Company P.O. Box 187, Berkeley Springs, WV 25411-0187 (304) 258-2500