

Nitrate reduction by denitrifying bacteria within a porous medium
by Evangeline M Begaye-Ibbotson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University
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Abstract:
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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date July 28, 1998

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TABLE OF CONTENTS

LIST OF TABLES vii

LIST OF FIGURES viii

NOMENCLATURE ix

ABSTRACT x

CHAPTER 1. INTRODUCTION 1
Objectives 2

CHAPTER 2. BIOREMEDIATION—GENERAL 5
Microorganisms 9
Denitrification 14
Controlling Factors 17
Oxygen 17
Other Nutrients 18
Carbon Sources 18
pH 19
Temperature 21
Inhibition 21
Bioremediation as a Water Treatment Process 22

CHAPTER 3. MATERIALS AND METHODS
Batch Cultures 24
Porous Medium Reactor 25
Analytical Methods 31

CHAPTER 4. RESULTS
Nitrate Reduction 35
Nitrate Reduction at Sample Ports 1 and 2 36
Nitrite Reduction 39
Nitrate and Nitrite Reduction Patterns 39
Organic Acid Utilization 40
Biomass 45
Most Probable Number 45
Estimation of Column Biomass 46
Yields 46
Maximum Growth Rate Comparisons 47

TABLE OF CONTENTS - Continued

CHAPTER 5. DISCUSSION 56

CHAPTER 6. GENERAL CONCLUSIONS
Column Experiments 62

REFERENCES 64

APPENDICES 70
Appendix 1: Basal Medium Composition and Preparation 71
Denitrifying Bacteria Medium with Acetate and Lactate 71
Denitrifying Bacteria Medium with Lactate and Sulfate 72
Sulfate-reducing Bacteria Medium for *Desulfovibrio* Species 73
Composition of Standard Medium 74

Appendix 2: Data Tables for Figures 75
Sandstone-Packed Column Data – Influent 75
Effluent 77
Sample Port 1 79
Sample Port 2 80

Appendix 3: Standard Curves
Nitrate IC Standard Curve 81
Nitrite Spectrophotometric Standard Curve 82
Sulfate IC Standard Curve 83
Acetate IC Standard Curve 84
Lactate IC Standard Curve 85

Appendix 4: Calculations 86
Partitioning of Nitrous Oxide (N_2O) 86
Determination of Biomass Volume 87
Biofilm versus Suspended Cells 88
Calculation of Biofilm Thickness 90
Determination of Yields for Entire Column Length 91
Determination of Yields Up to Sample Port 1 92
Determination of Yields Up to Sample Port 2 92

LIST OF TABLES

Table		Page
3.1	Basal Nutrient Composition	30
3.2	Dionex IC Analysis Specifications	32
4.1	Nitrate Reducing Bacteria Yields	50
4.2	Comparison of Maximum Growth Rates	55
A2.1	Influent Column Data	75
A2.2	Effluent Column Data	77
A2.3	Sample Port 1 Effluent Column Data	79
A2.4	Sample Port 2 Effluent Column Data	80

LIST OF FIGURES

Figure		Page
2.1	Bioremediation Requirements	9
3.1	Sandstone-packed Column, Reverse-flow Reactor	27
3.2	Process Flow Diagram of Nitrate-Reduction System	28
4.1	Timeline Comparison between the Nitrate Influent Concentration	37
4.2	Comparison between the Nitrate Influent Concentration	38
4.3	Overall Comparison between the Nitrate Influent	41
4.4	Comparison between the Nitrate Influent Concentration	42
4.5	Overall Comparison between the Acetate Influent Concentration	43
4.6	Comparison between the Acetate Influent	44
4.7	Presentation of the Production of Nitrite and Total Cell Counts	52
4.8	Presentation of Total Cell Counts Found at Sample Port 1	53
4.9	Measurement of Cumulative Biomass by Section	54
A3.1	Nitrate IC Standard Curve	81
A3.2	Nitrite Spectrophotometric Standard Curve	82
A3.3	Sulfate IC Standard Curve	83
A3.4	Acetate IC Standard Curve	84
A3.5	Lactate IC Standard Curve	85

NOMENCLATURE

Latin Characters

Defined as follows:

C_e concentration of electron acceptor

C_s concentration of substrate

K_e Monod half-saturation coefficient for electron acceptor

K_i Monod inhibition constant

K_s Monod half-saturation coefficient for substrate

L_f biofilm thickness

X_a biomass on the surface

X_e cells in suspended phase

Greek Characters

Defined as follows:

μ local specific growth rate

μ_{max} maximum specific growth rate

ABSTRACT

Acid processing of uranium ore resulted in aquifer contamination (nitrate, sulfate, and uranium) of the aquifer associated with the Navajo Sandstone formation at Tuba City, Arizona. The objectives of this study focused on the use of bioprocesses to remediate this aquifer contamination. The bench-scale objective of this study was to evaluate the ability of an indigenous microbial consortium to bioremediate nitrate contamination. The reduction of nitrate typically results in the production of nitrite, which under most conditions is further reduced to dinitrogen gas or ammonia. However, under some conditions inhibitory concentrations of nitrite may accumulate.

Sandstone-packed columns fed with aquifer-relevant concentrations of nitrate were used to assess denitrification rates by indigenous bacteria. To enumerate denitrifying consortia used in the column experiments, most probable number (MPN) techniques were used. Sandstone-packed column influent and effluent data for nitrate, nitrite, carbon substrate and biomass concentrations were collected over time. These data were used to assess nitrate reduction rates within a sandstone column.

This research demonstrates that with indigenous bacteria with stable conditions nitrate is reduced to dinitrogen forming only minimum levels of nitrite which should not inhibit sulfate-reducing bacteria (SRBs). The results of these studies indicate that bacterial denitrification has good potential as a remediation strategy for nitrate-contaminated groundwater to levels below the established regulatory limits of 44 mg/L. Field tests applications are planned for the Department of Energy UMTRA site in Tuba City, Arizona, using an extensive grid of injection and pumping wells.

CHAPTER 1

INTRODUCTION

From the early 1940's through the 1960's widespread uranium ore mining was conducted by private U.S. companies under contract to the federal government. The uranium ore was processed for use in national defense research, weapons development, and the development of a commercial nuclear energy industry. Upon termination of the contracts, piles of exposed uranium tailings remained at the mill sites.

Ore processing for uranium recovery resulted in nitrate, sulfate, and uranium contamination of the aquifer in the Navajo Sandstone formation near Tuba City, Arizona. Rare Metals Corporation of America built the Tuba City mill in 1955. From 1956 to 1966, uranium was extracted at this site using a process that involved initial pulverization of the rock followed by acidic or alkaline leaching. Acidic ore treatment used sulfuric and/or nitric acids to enhance the leaching process. The mill tailings resulting from the processed ore were discharged to unlined tailings and evaporation ponds without any subsurface barrier to groundwater. It is estimated that 38 million cubic feet of water contaminated with dissolved metals seeped into the ground and migrated down to the groundwater. This ore processing water constituted the most significant source of contaminants to the aquifer. Large quantities of sulfate and nitrate from the processing acids are present in the groundwater. Other

contaminants include uranium, selenium, strontium, molybdenum, and cadmium. Nitrate exists at concentrations greater than 1000 mg/L and sulfate concentrations near 3600 mg/L. Uranium is present at concentrations up to 40 times greater than the U.S. Environmental Protection Agency (EPA) maximum contaminant limit (MCL). As the tailings piles became exposed to weathering processes, infiltrating rainwater and melting snow enhanced groundwater contamination.

In 1978, the Uranium Mill Tailings Reclamation Act (UMTRA) was passed due to health concerns over low-level radiation exposure. The Federal Law assigned the Department of Energy (DOE) responsibility for remediating the Tuba City site and 23 other uranium mill tailing sites nationwide. A program was initiated to develop remediation strategies for these contaminated sites. The first phase of the UMTRA project resulted in encapsulation of the tailings piles to minimize future contamination of the groundwater. In 1988, DOE began surface remediation at Tuba City. By April, 1990 approximately 1.4 million cubic yards of tailings and associated contaminated materials had been consolidated and stabilized in an engineered 50-acre disposal cell.

The objectives of this study focused on assessing the ability of indigenous microbial consortia to remediate nitrate groundwater contamination. In this study, an anaerobic system was designed to enrich for any indigenous denitrifying bacteria present in the well waters at the uranium tailings site.

Nitrate removal by dissimilatory bacterial denitrification occurs by the following metabolic pathway:

$$NO_3^- \Rightarrow NO_2^- \Rightarrow NO \Rightarrow N_2O \Rightarrow N_2$$

The process is carried out solely by bacteria, which use nitrogen oxides as terminal electron acceptors in lieu of molecular oxygen. The denitrification pathway between NO_2^- and N_2O has been the subject of much research since the early 1980s. Facultative aerobes in the strict sense are organisms that can grow best aerobically by utilizing the normal complements of a full respiratory cytochrome system in which oxygen serves as the terminal electron acceptor, but which can be grown anaerobically by shifting to a completely different electron transport system in which organic compounds serve as electron acceptors. Much work has focused on dissimilatory nitrite reductase (dNiR) since this is the first step that shunts NO_3^- to a dissimilatory fate instead of an assimilatory fate and, therefore, is a pivotal step in the biogeochemical N cycle.

Dissimilatory nitrate reduction to ammonium (DNRA) has recently become more widely recognized as a process distinct from assimilatory nitrate reduction (Tiedje, 1988). Both of these processes produce NH_4^+, but the former is regulated by O_2 and the latter by NH_4^+ and organic N. DNRA can be stimulated in soils by excessive C addition, but it still does not become the dominant NO_3^- consuming process (Caskey et al., 1979). Assimilatory nitrate reduction process can be carried out by many bacteria but its significance in soil seems to be limited because it is rapidly inhibited by low concentrations

(e.g., 0.1 ppm) of ammonia-N or organic N (Rice et al., 1989). In anaerobic environments, these compounds are typically in high concentration so this process would not be expected to coexist with respiratory denitrification. Dissimilatory processes are distinguished from assimilatory processes in that the amount of N reduced in the dissimilatory process is in excess of that needed for synthesis of new biomass.

From an engineering perspective in order to engineer complex metabolic processes for successful bioremediation in the field, a highly diverse microbial consortium may be necessary. The existence of complex, multiple-step biotransformation pathways makes it unlikely that one specific organism will be sufficient for successful cleanup of mixed contaminants. Although identification of the active microbial species may not be required in order to engineer a specific microbial process, an understanding of the microbial processes and how they influence activity within the general microbial community may be very important. Initially, it was necessary to determine if indigenous denitrifying bacteria could be enriched from the sandstone material. Secondly, sandstone-packed columns were used to assess the denitrification rates by the indigenous bacteria. Finally, the question of nitrite accumulation and how it might affect SRB inhibition issues was important to consider but was not examined experimentally.

CHAPTER 2

BIOREMEDIATION—GENERAL

Contaminant removal processes fall into two categories: those employing physical and/or chemical means and those employing biologically mediated reactions. Examples of physical and/or chemical treatments include ion exchange, reverse osmosis and electrodialysis. Advantages are their simplicity and process automation. A main disadvantage is the separation of the contaminant from one liquid phase to concentrate into another phase.

Bioremediation has demonstrated significant potential as an innovative remedial technology for removal of groundwater contaminants because it is based on microbial-mediated transformation or detoxification of hazardous contaminants. Chemicals are transformed by enrichment for specific microorganisms that supply necessary enzymes in a desired degradation process. As a remediation technology, a major advantage of bioremediation is lower capital cost due to on-site treatment. Bioremediation is especially cost-effective when both impacted soil and groundwater phases can be treated simultaneously. Disadvantages include the inherent uncertainty associated with the design and operation of an engineered biological process in heterogeneous soil environments. With bioremediation, one must manage the system extremely well while maintaining low capital cost of the process. A significant concern of some engineers, scientists, and regulators is that

bioremediation is an unproven technology in the field. In addition, the production of toxic metabolites during the cleanup of hazardous materials is a concern. A final disadvantage is that not all chemicals can be bioremediated.

For successful bioremediation, a suitable energy source, a terminal electron acceptor, other nutrient elements, contaminant-degrading microbes, favorable temperature, favorable pH, and water must all be present to achieve the desired kinetics of any biological process (Hicks et al., 1993). The process design, operation, and management of bioremediation systems must be based on a full understanding of the chemical reactions and the physiological complexities involved. As knowledge about the mechanisms, kinetics, and microbial interactions for the transformation of hazardous materials increases, optimization of design and operation parameters for biological remediation will continue to develop.

Bioremediation engineering results in the controlled manipulation of the environment to generate the proper enzymes for catalyzing the desired reactions. To design the bioremediation process, one must first determine the biologically-mediated reactions which will result in elimination of the target compounds.

Application of bioremediation technology to an environmental problem includes the use of two primary respiratory pathways: aerobic and anaerobic. To date, aerobic systems using naturally occurring microorganisms are most widely implemented. Aerobic systems tend to be more efficient when

degrading petroleum based organic contaminants such as benzene, toluene, ethylbenzene, xylenes, and naphthalene. However, the effectiveness of aerobic bioremediation is often limited by the availability of the terminal electron acceptor, oxygen (Borden et al., 1986). In oxygen limited matrices, bioremediation may proceed anaerobically. Organisms capable of anaerobic degradation use compounds such as nitrate rather than oxygen as terminal electron acceptors. The substrates are oxidized to CO_2 with the successive removal of electrons and H^+ pairs. Other terminal electron acceptors, including sulfate, thiosulfate, and sulfur are used in the same way by another physiological class of prokaryotes generally known as the sulfate- and sulfur-reducing bacteria. Nitrate-based, anaerobic bioremediation of groundwater provides several advantages over aerobic processes. Nitrate is much more soluble in water than molecular oxygen; it is less toxic to microorganisms than hydrogen peroxide, and generally exhibits a lower tendency than hydrogen peroxide to cause unfavorable geochemical reactions and consequent reductions in aquifer permeability (Lee, et al., 1988). Nitrate is a recognized drinking water contaminant, and thus its use in *in situ* groundwater remediation applications must be controlled to avoid exceeding drinking water standards in downgradient sources.

Anaerobic bioremediation approaches have several limitations. For a strict anaerobic system to be effective, no oxygen can be present in the environment because oxygen itself is toxic to strictly anaerobic

microorganisms. This condition is difficult to implement under field conditions, especially when a mechanical pumping system is used to extract groundwater. Secondly, anaerobic degradation of some contaminants can produce intermediate end-products that may be less desirable than the target substance. Thirdly, anaerobic degradation can produce unpleasant and potential dangerous off-gases such as CH_4 and H_2S. For these reasons, full-scale anaerobic bioremediation technologies have lagged behind aerobic approaches (Hicks et al., 1993).

In situ bioremediation is based on the principal of enrichment of indigenous microbial populations so that they become metabolically active and reduce contaminants of concern. *In situ* bioremediation requires environmental conditions in an optimum range for the microorganisms to effectively metabolize the contaminants found at the site. The contaminant must be present in a form that is available to the microorganism. Finally, *in situ* bioremediation may involve the transport of the microorganism from an origin site to the location of the contaminant within the soil (Young et al., 1993).

A basic requirement for *in situ* bioremediation includes attempts to optimize environmental conditions for microorganisms to maximize metabolic transformation of contaminants found at a site. The requirements are presented in the diagram below in descending order of importance (Cookson, 1995).

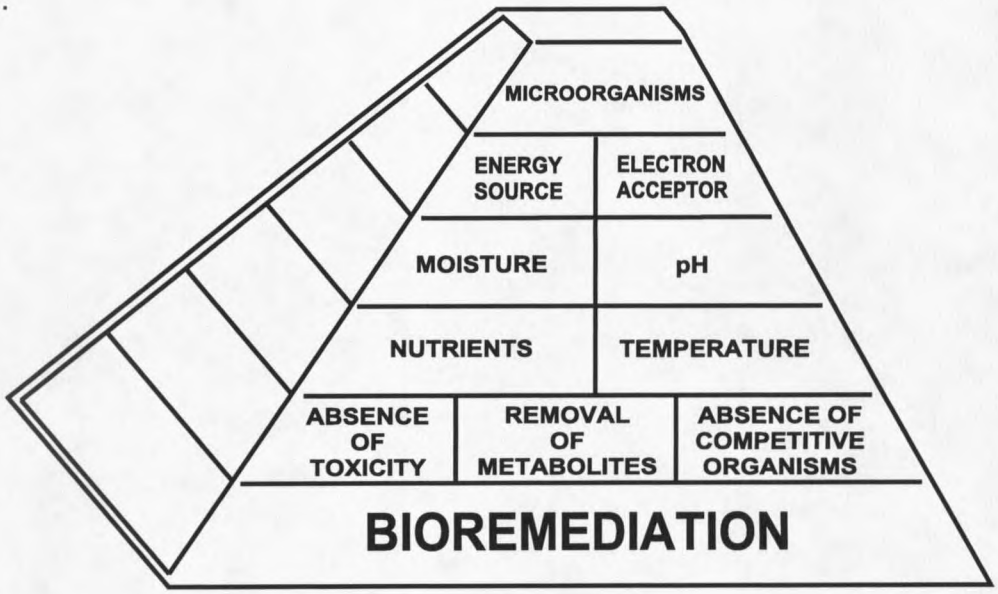

Figure 2.1 Bioremediation requirements

Microorganisms

The first requirement for designing an *in situ* bioremediation system is determination of the presence of pertinent microorganisms that can reduce the target compound. The second requirement is determination of which parameter may be limiting contaminant degradation so that degradation rates may be optimized.

Microbial enrichment is the utilization of an energy and carbon source that is readily available. The production of enzymes is triggered by this energy-yielding source, which may or may not be the target compound. Understanding the microorganisms induced, their nutrient needs, the biochemistry of their mediated reactions, and why they promote the reaction is helpful in designing an effective bioremediation system.

Metabolic steps involved in the reduction of NO_3^- ultimately to N_2 is termed denitrification. Denitrification occurs via the action of denitrifying microorganisms under specific operating conditions. A few aerobic bacteria (principally *Pseudomonas* and *Bacillus* species) can use nitrate as the electron acceptor by reducing it to molecular nitrogen. Denitrification requires specific enzymes and the normal aerobic respiration electron transport chain (Cookson, 1995). It is, therefore, an alternative mode of respiration. The specific enzymes needed for denitrification are not synthesized in the presence of oxygen, even when nitrate is present.

Environmental scientists have used the term denitrification to describe any process in which nitrate (or nitrite) is reduced. However, microbiologists usually restrict the meaning of denitrification to a bacterial respiratory process in which electron transport phosphorylation is coupled to the sequential reduction of nitrogenous oxides. It is the organisms with this respiratory process that rapidly consume soil nitrate when oxygen is limiting. Denitrification occurs naturally in confined aquifers in the absence of oxygen where the reduction of adequate concentrations of nitrate can be coupled to the oxidation of (usually) organic carbon compounds. These conditions, however, are rarely met in a natural environment.

Microorganisms obtain energy by a complex sequence of redox (oxidation-reduction) reactions. Oxidation is the removal of electrons from an atom or molecule. Reduction is the addition of electrons, e.g.

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$$

The reduction half-reaction, that is, the reaction involving the electron acceptor, establishes the metabolism mode. When a site contains more than one potential electron acceptor, the primary electron acceptor is generally determined by the potential energy yield of the reaction.

Oxidation occurs only with reduction and another atom or molecule must accept the electron. This electron flow sequence is known as the electron transport chain to generate energy. This chain consists of molecules that undergo repeated oxidation and reduction and electrons are transferred from one atom to another. The electrons are transported into the microbial cell system by such compounds as nicotinamide adenine dinucleotide phosphate (NADP).

Enzymes enable the microbial redox reactions. Enzymes are complex organic protein catalysts generated by the microbial cell. A catalyst increases the velocity of the overall reaction and is both a reactant and a product of the reaction. Catalysts are effective in extremely small amounts, remain intact in the reaction, do not change the equilibrium of the chemical reaction, and are usually very specific in their ability to accelerate a given chemical reaction.

The amount of free energy from a reaction depends on the Gibbs free energies for the substrates and products. The relationship is given by

$$\Delta G^\circ = \Delta G_f^\circ \text{ (products)} - \Delta G_f^\circ \text{ (substrates)}$$

where ΔG° is the increment in free energy of the products and reactants relative to the elements for the reaction under standard conditions (25°C and 1 atm). A useful concept for solution-based redox reactions is electron activity (pE). Electrical activity, pE, is a measure of the availability of electrons in much the same manner that pH measures the availability of protons. Electron activity is related to Gibbs free energy by

$$pE = \frac{-\Delta G^\circ}{2.3nRT}$$

where R = gas constant
T = absolute temperature
n = number of electrons involved in the reaction

The amount of free energy that can be obtained by microorganisms from redox reactions is directly proportional to the electrical activity (pE) of the redox system. For denitrification of NO_3^- to N_2, the pE value is given as +21.0 mV and the pE value of +14.9 mV for nitrate reduction to nitrite (Snoeyink et al., 1980).

It is unlikely that just one specific organism will be important to successful cleanup of contaminated sites. For most projects a highly diverse population of microorganisms must be present for successful bioremediation of a contaminant which generally requires multiple biochemical steps, and an organism often can only perform one of the steps. Specific identification of individual organisms may not be important. A highly diverse population also results in complicated and extraordinary diverse nutrient and water chemistry needs. Microorganisms apply oxidation and reduction reactions in a variety of

specialized mechanisms that are frequently specific to an organism or consortium. This fact leads to the need for diverse microbial communities when performing bioremediation at a site with mixed contaminants.

In many cases, a consortium of microorganisms can accomplish more than the individual sums of each microorganism. Synergism is where the total activity is greater than that which can occur with just a single organism. It can occur when one member of a microbial community may be unable to synthesize a particular reactant, but will reduce that compound when a second organism synthesizes the needed component. Microorganisms also produce metabolites that may be self-inhibitory or inhibit the growth of other organisms. Maintaining the enzyme reaction synergistically depends on the activity of a second organism to remove compounds excreted by the first organism.

In situ soil bioremediation may require transport of the microorganisms from an origin to the location of contaminant within the soil (Young et al., 1993). In many cases the contaminant is located far below the soil surface and significant vertical transport within the soil is necessary. The main processes, which govern microbial transport, excluding survival, are sorption to soil components and physical filtering in which cell size inhibits transport through small soil pores. Laboratory columns packed with larger sand-sized particles as compared to smaller-pore columns resulted in greater bacterial transport. Increased flow rates may also result in increased cell transport (Trevors et al., 1990).

Denitrification

Since the early 1970's, high nitrate concentrations in groundwater sources have become a national problem, limiting the direct use of groundwater resources. In response, the U.S. Environmental Protection Agency (EPA) has established the maximum contaminant level (MCL) for nitrate-nitrogen (NO_3^--N) in drinking water at 10 mg/L (714 µmol/L). Groundwater is a significant source of water for both domestic and agricultural use; it is the source of domestic water for 50% of the total U.S. population and for nearly 90% of the rural population (Bouchard et al. 1992).

Denitrification and nitrate reduction are used synonymously when nitrate (as a terminal electron acceptor) reduction is coupled to energy-yielding oxidation of an organic compound. The nitrogen oxides that serve as terminal electron acceptors are formed in discrete steps within the pathway of denitrification and this pathway is presumed to be (Payne, 1973)

$$\text{nitrate } (NO_3^-) \xrightarrow[\text{reductase}]{\text{nitrate}} \text{nitrite } (NO_2^-) \xrightarrow[\text{reductase}]{\text{nitrite}} \text{nitric oxide}$$

$$(NO\uparrow) \xrightarrow[\text{reductase}]{\text{nitric oxide}} \text{nitrous oxide } (N_2O\uparrow) \xrightarrow[\text{reductase}]{\text{nitrous oxide}} \text{dinitrogen } (N_2\uparrow)$$

A distinct enzyme, a nitrogen oxide reductase that transfers electron from the chain to the particular intermediate of the denitrification pathway, catalyzes each step of the pathway.

Denitrification also refers to the dissimilatory reduction of one or both of the ionic nitrogen oxides (nitrate, NO_3^-, and nitrite, NO_2^-) to the gaseous oxides

(nitric oxide, NO, and nitrous oxide, N_2O), which may themselves be further reduced to dinitrogen (N_2). The nitrogen oxides act as terminal electron acceptors in the absence of oxygen. The gaseous nitrogen species are major products of these reductive processes (Knowles, 1982).

Denitrifying bacteria able to use nitrogen oxides as electron acceptors in place of oxygen with the evolution of major gaseous products are biochemically and taxonomically very diverse. Most bacteria possess all of the reductases necessary to reduce NO_3^- to N_2, some lack NO_3^- reductase and are termed NO_2^--dependent, and others lack N_2O reductase and therefore yield N_2O as the terminal product. Still other organisms possess N_2O reductase but cannot produce N_2O from NO_3^- or NO_2^- (Knowles, 1982).

Not all denitrifiers can use nitrate as a terminal electron acceptor. These organisms apparently possess all of the other enzymes required. However, because they can use NO_2^-, and N_2 is evolved (Vangai et al., 1974; Pichinoty et al., 1976a) most denitrifiers can grow with NO_2^- as sole electron acceptor.

There are two distinct metabolic pathways that bacteria utilize to effect nitrate metabolism. By the assimilatory pathway, nitrate is reduced to ammonia; or under certain conditions nitrate is used in place of oxygen as terminal electron acceptor in respiration forming the dissimilatory pathway. The assimilatory process occurs both aerobically and anaerobically, is not an energy-yielding process and the product, ammonia, is not excreted into the medium. In contrast, dissimilatory process mostly occurs anaerobically, is not

affected by ammonia, and is energy yielding; the intermediates, nitrite, nitric oxide, nitrous oxide and final product nitrogen gas are excreted into the medium.

In anaerobic conditions, nitrate can serve as an electron acceptor for the oxidation of organic (and sometimes inorganic) compounds, with the yield of energy and the release of gaseous N_2 or N_2O. Knowledge of the energy yield in a particular reaction does not necessarily assure an accurate prediction of what will happen. Denitrification to the product N_2 yields more energy than does the production of N_2O, but the cell may not be able to take advantage of the difference. When nitrate is abundant and organic substrate is limiting, the energy yield to N_2 per unit carbohydrate would appear determinant. This difference is small but still favors the production of N_2. Yet N_2O is formed in the denitrification reaction; under field conditions the yield of N_2O relative to N_2 ranges from negligible to 20% (Stefanson, 1972; Rolston et al., 1976). This suggests that other factors are involved. When the concentration of nitrate ion is high compared with available organic substrate, N_2O is usually a larger fraction of the total denitrified gas. Nitrous oxide production is also a function of pH. As pH increases, the ratio of N_2O:N_2 also increases.

Nitric oxide, NO, is also a possible product of the denitrification process. Its production is enhanced at low pH values. Its detection in the gas phase is uncommon, possibly due to its high solubility and high reactivity. In contrast, N_2O, is relatively stable and when produced, is generally detected in the gas

phase. The assessment for nitrate reduction is analysis for nitrite in the medium.

Controlling Factors

Denitrifiers share a common reaction to oxygen availability and have similar growth requirements.

Oxygen

Nitrate reduction begins when oxygen is depleted and ceases immediately when oxygen is reintroduced. Oxygen does not seem to affect the stability of the denitrifying enzymes already present (Ruiz-Herrera et al., 1976; John, 1977). In oxygen limited matrices, bioremediation can proceed anaerobically. Compounds other than oxygen, such as nitrate and sulfate, perform as terminal electron acceptors. These metabolic pathways may give rise to off-gases such as hydrogen sulfide (H_2S), depending on the initial organic compound present.

Oxygen affects both the activity and synthesis of the denitrifying enzymes. Oxygen affects the redox potential and is more readily utilized by a consortium with obligate aerobes. It acts as an inhibitor to denitrifying bacteria. The gradual depletion of oxygen or provision of semi-anaerobic conditions appears to favor denitrification. Knowles (1982) explains that in soils there is frequently an inter-aggregate air-filled porosity surrounding intra-aggregate water filled pores which become virtually anaerobic permitting denitrification to occur. The anaerobic zones are about 200 μm in diameter (Hiscock et al., 1991).

Anaerobic incubation near 30°C generally favors denitrification. Many *in situ* bioremediation systems use anaerobic processes (Hooker et al., 1994), but no one has reported microbial transport data under anoxic conditions.

Other Nutrients

The availability of nutrients is an important requirement in sustaining biological cell growth. For biosynthesis to occur, nutrients consist of those elements required in large amounts (C, H, O, N, P, and S), various minerals required in minor amounts (K, Na, Mg, Ca and Fe), and trace amounts of certain metals (Mn, Zn, Cu, Co and Mo) (Champ et al., 1979). Additional organic carbon is required as an electron donor for heterotrophic denitrification. Most groundwaters contain adequate concentrations of the necessary minerals and trace metals to support biosynthesis (Champ et al., 1979).

Carbon Sources

In many cases, the choice of a specific carbon source is generally based upon its availability and economic considerations. Denitrifiers can use a wide variety of carbohydrates and organic acids, as well as many other organic compounds, as carbon and energy sources during aerobic growth but may be more restricted under denitrifying conditions.

The stoichiometric relationship for the overall denitrification reaction with sodium acetate as the carbon source is

$$8NO_3^- + 2CH_3COO^- + 2H^+ \rightarrow 8NO_2^- + 4H_2O + 4CO_2$$

$$8NO_2^- + 3CH_3COO^- + 11H^+ \rightarrow 4N_2 + 10H_2O + 6CO_2$$

The minimal amount of organic compound required for complete removal of nitrogen and the ratio of organic carbon utilized to nitrogen denitrified (C:N), depends on both the type of organic compound and the form of the terminal electron acceptor (McCarty et al., 1969). The C:N ratio for the sodium acetate/nitrate redox couple was estimated at 1.7 (Dodd et al., 1975). Other authors using sodium acetate for denitrification of nitrite found the optimum C:N ratio to be 0.83 (Blaszczyk et al, 1979) and a C:N ratio of 1 was necessary for 80-90% denitrification (Bitton et al., 1984).

The use of acetic acid for denitrification of nitrate is inconvenient for two reasons: (1) it is expensive compared to other energy sources such as methanol (Francis et al., 1975; Francis et al., 1977), (2) the requirement of denitrifying bacteria for acetic is 3.30 g compared to only 2.47 g methanol in the denitrification of 1 g NO_3^--N (McCarty et al., 1969).

pH

Optimum pH for denitrification varies with the organism. Denitrification is generally optimal in the range of 7.0-8.0. Temperature is also a significant controlling factor. At low temperatures, denitrification decreases markedly but is

measurable between 0-5°C. Synergistic effect of temperature and oxygen upon denitrification is observed at a high temperature where oxygen solubility is less, thus increasing the biological rate process, and vice versa. In 1938, Karlsen (Delwiche, 1956) reported that *Pseudomonas aeruginosa* denitrified in a pH range of 5.8 to 9.2 with an optimum range of 7.0 to 8.2. When the enzyme system from *Pseudomonas aeruginosa* was tested in three different buffer systems, denitrification was optimum at pH 7.4, 30% at pH 6 and 50% at pH 10 (Fewson et al., 1961). Optimal aerobic microbial growth occurs at about the same pH value that defines optimal denitrification conditions. At low pH values, the nitrogen oxide reductases, especially nitrous oxide reductase, is progressively inhibited such that the overall rate of denitrification decreases. However, the mole fraction of nitrous oxide produced increases, and at pH 4.0 nitrous oxide may be the major product. Therefore, it appears that both decreasing pH and increasing O_2 concentration tend to decrease the overall denitrification rate, at the same time increasing the proportion of nitrous oxide in the products evolved.

Most common environmental microbes metabolize contaminants most efficiently in pH environments between 6 and 8. In conjunction with microbial growth, pH may decline in response to the generation of organic acids from metabolic activity. The effect of this phenomenon is site specific and is related to the composition and buffering capacity of the treated matrices.

Wijler (1954) reported on the effect of pH on the distribution of gases from denitrification with mixed flora. At pH 7.0 and below, N_2O is the major gaseous product; above pH 7.0 it is produced but is subsequently reabsorbed and further denitrified to N_2. At pH 4.9, N_2O and NO are evolved at almost equal volumes; at higher pH values, little NO is produced.

Temperature

Most environmental microbes are effective at reducing contaminants when matrix temperatures range between 8°C and 30°C. When all other parameters are maintained at an optimum condition within this operating temperature range, soil microbial activity will double every 10°C rise in temperature.

Inhibition

A few compounds inhibit denitrification. The most important are the sulfur compounds. For example, sodium sulfide added to soil that is incubated anaerobically depresses N_2 production, but stimulated the reduction of nitrate to ammonium and to nitrite (Myers, 1972). Acetylene is a well-known inhibitor of N_2O reduction to N_2 (Knowles, 1982). Also, nitrate, even in small quantities, can suppress NO reduction to N_2O (Goering, 1972). The suppression of NO reductase may explain the observed accumulation of nitrite in some localized anaerobic aquatic environments.

Tiedje (1988) suggested that carbon substrate limitations to the denitrification process in anaerobic habitats rarely occur. On the other hand, organic carbon had an indirect effect on denitrification, enhancing oxygen consumption by aerobic bacteria leading to anaerobiosis. Addition of water to dry soil often causes large increases in denitrification (Robertson et al., 1984). However, if the soil is flooded, oxygen regulation becomes less important and nitrate availability regulates the rate. There is a long-term effect of accumulation of organic matter. Organic matter can have a great impact on the total heterotrophic population sizes, including denitrifiers, by providing both energy and attachment surfaces. The size of the denitrifier population in the active zones in the soils is an important parameter for the nitrate removal efficiency (Davidson et al., 1996).

Bioremediation as a Water Treatment Process

Natural denitrification of nitrate-contaminated groundwater is not extensive and is limited by the availability of NO_3^--N and organic carbon. As an alternative, artificial stimulation of denitrification as a water treatment process is more effective since the nutrients necessary for biosynthesis can be supplied.

In situ denitrification has the advantage of performing both denitrification and secondary treatment, for example, degradation of organic residuals, within the aquifer. Furthermore, underground processes are independent of any seasonal temperature variations, thus maintaining efficiency of any such

system. A possible disadvantage is the clogging of aquifer pore spaces with the gaseous products of denitrification and dead biological matter.

The choice of biological denitrification as a treatment technology will need to be assessed on an individual site basis. *In situ* treatment is customary for treating small, remote groundwater supplies obtained from shallow, intergranular aquifers.

CHAPTER 3

MATERIALS AND METHODS

Batch Cultures

Monitoring-well water samples from the Tuba City site were used as inoculum for establishing batch enrichment cultures of indigenous denitrifying and sulfate-reducing bacteria. Aquifer nitrate concentrations had been periodically measured for several years and averaged about 1300 mg/L. Average groundwater sulfate concentrations from periodic sampling and analyses was 3640 mg/L. (CeRAM, 1996). The presence of denitrifying bacteria in batch enrichment cultures was confirmed by significant, measured nitrate reduction and nitrite production (See Analytical Methods for procedures). In addition, some enrichment consortia produced hydrogen sulfide (H_2S) in anaerobic serum bottles incubated at 35°C. Black precipitate formation and H_2S odor occurred, characteristic of sulfate reduction to sulfide (Lovely, 1992).

Batch cultures used to inoculate the sandstone column reactor were prepared using 30 mL of well water samples as inoculum per 1L of sterile, anaerobic enrichment medium. The inoculated medium (1L) was incubated at a room temperature of 20°C for 40 days. The anaerobic enrichment medium (Table 3.1) used for the batch cultures contained a short-chain fatty acid, acetate, as the carbon and energy source at a concentration of approximately

400 mg/L (Bryant, 1972). Two pore volumes of the anaerobic batch culture were used to inoculate the sandstone-packed reactor. After inoculation with the second pore volume, the column was allowed to remain in "batch mode" for 24 hours to encourage attachment of bacterial cells to the sandstone medium. A continuous flow of new influent nutrient medium containing 400 mg/L acetate and 500 mg/L nitrate was resumed at a flow rate of 0.13 mL/min. Samples were collected daily for nitrate, nitrite, and acetate analyses.

Porous Medium Reactor

Navajo Sandstone cores were obtained from the drilling operations at the Tuba City site and transported under cold storage to Montana State University. Sections of the core were pulverized using a mortar and pestle. Previous analysis of similarly crushed sandstone identified a homogenous matrix of fine to coarse sand grains in the range of 70-600 microns (Sandia National Laboratory, 1995). A plexiglass column was sterilized by washing with ethanol followed by a 30-minute exposure to ultraviolet (UV) light in a biological laminar flow hood. Stainless steel gauge mesh #60 (used on both column ends to contain the packed sand) and tubing were sterilized at 120°C for 20 minutes prior to column assembly. The stainless steel meshes were seated with rubber o-rings. Moist sand was packed into each cylindrical column by tapping and intermittent vigorous shaking to an approximate bulk density of 2.07 g/cm^3. The anaerobic, reverse-flow porous media reactor used for this

study was a sandstone-packed polycarbonate column (5-cm ID x 30-cm) equipped with three sampling ports (Figure 3.1). The linear sampling ports were located at 5-, 15- (middle port), and 25-cm intervals measured from either column end. Oxygen-impermeable butyl rubber stoppers (as septa) were inserted in the three ports for sample extraction directly from inside the column. Liquid samples were extracted with sterile syringes from the first two ports on the influent side of the column. In addition, column influent and effluent were sampled. Samples were collected daily and analyzed for nitrate, nitrite, and acetate concentrations. Sterile water was immediately pumped through the column at a flow rate of 0.13 mL/min to remove any very fine matrix material and to condition the column. Approximately two pore volumes of sterile, deionized water was passed in reverse-flow through the column. The porosity of the sandstone-packed column was approximately 22% with a permeability of about 300 mD at an operating pressure of 0.01 atm gauge (data from Sandia National Lab study).

The sandstone-packed column was maintained at a room temperature of 20°C. This temperature value is only slightly greater than the average site groundwater temperature of 16°C. Flow rate of the nutrient influent was maintained at about 187 mL/day, which represents a pore water velocity of 29 cm/day (Figure 3.2). Nutrient medium was transported through Tygon tubing

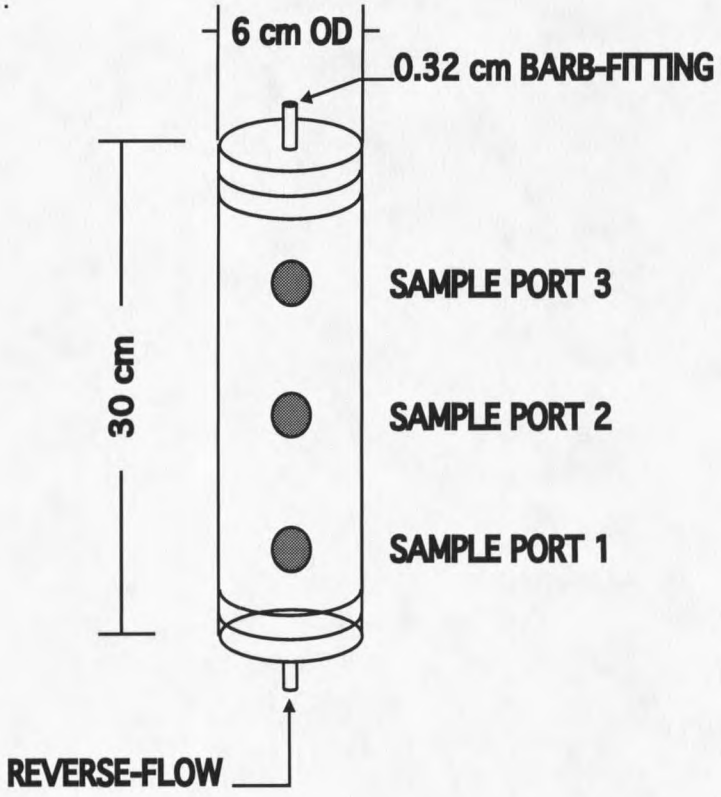

Figure 3.1 Sandstone-packed column; reverse-flow reactor.

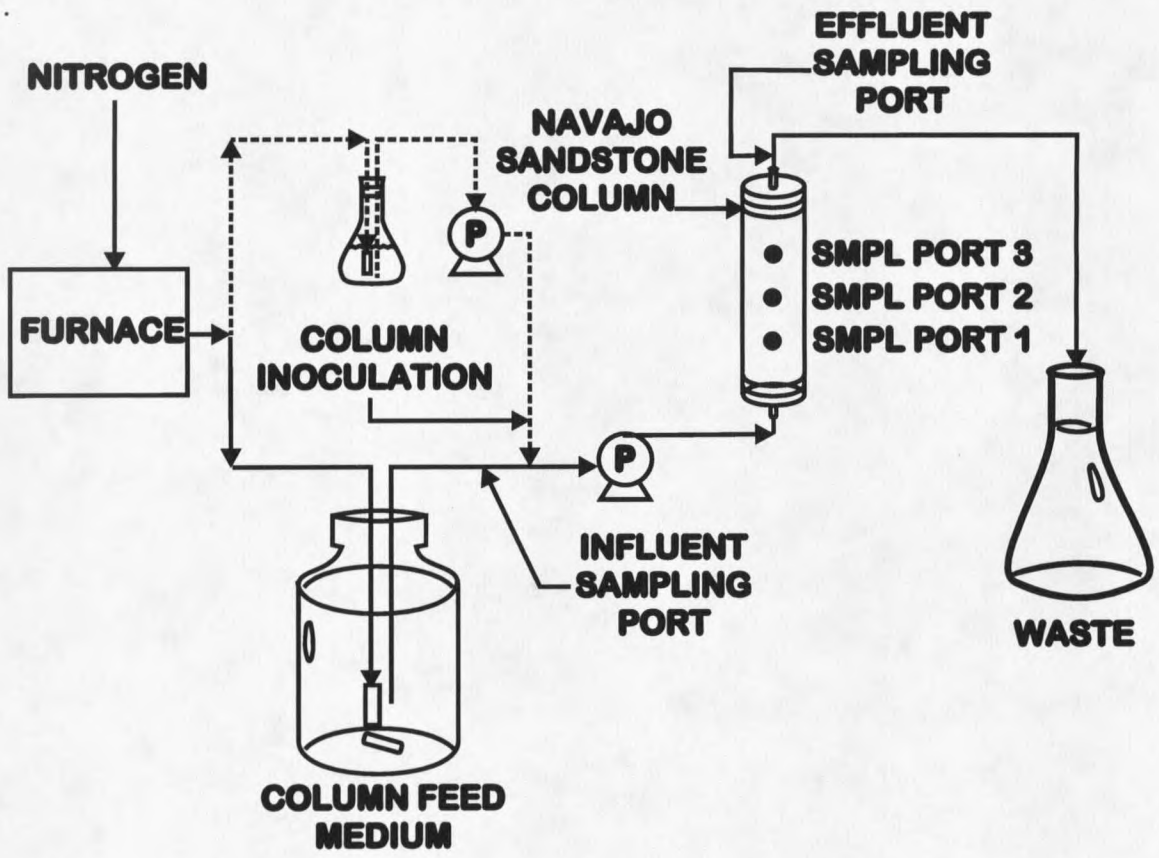

Figure 3.2 Process flow diagram of nitrate-reduction system.

(Masterflex Tygon® 6419-13) to minimize oxygen diffusion into the system. Slow, continuous nitrogen purging of the influent nutrient medium maintained anaerobic conditions inside the column. Dissolved oxygen (DO) levels less than 2 mg/L in the column influent were detected using a colorimetric kit (CHEMets, Dissolved Oxygen, K-7512). Sterile, oxygen-free nitrogen used to continuously purge the column influent was ensured by using 0.22 μm bacterial air vents (Gelman) at the inlet and outlet sides of a heated copper-filing-containing oxygen trap (Lindberg (General Signal) Model 847) furnace at 400°C (Hungate, 1969). Nitrogen purge gas was set at a flow rate of 15 cm^3/min. Cylinder gases contain small amounts of oxygen (O_2). The gases react with the heated copper to yield black copper oxide (CuO). Introducing hydrogen gas (H_2) through the line periodically reduced the oxidized copper filings.

In a preliminary sandstone-packed column experiment, a pressure gauge (0 - 5000 psi) was used to determine the backpressure at the inlet side of the column. Backpressure was expected to increase as a result of the combined effects of precipitate accumulation, biomass plugging, and gas accumulation. Although column pressure at the effluent end was not measured, it was assumed to be ambient.

Similar to the batch culture enrichment medium, the anaerobic influent medium used for the sandstone-packed columns contained 400 mg/L acetate

and 500 mg/L nitrate. The basal nutrient composition is listed below in Table 3.1. Ammonium chloride, phosphorous and potassium dihydrogen phosphate were added as the respective nitrogen sources. Vitamin and trace minerals were added to enhance the growth of the inoculum (Balch et al., 1979) (See Appendix for composition). A 20 mM phosphate buffer was added to help maintain a solution pH above 7.0. Microbial activity usually decreases as pH decreases much below 6.0, thereby possibly inhibiting denitrification in the column.

Table 3.1 Basal Nutrient Composition
(Lovely et al., 1988)
(To 1 L of deionized H_2O)

Components	(g/L)
Basal solution	
Sodium bicarbonate	2.5
Ammonium chloride	1.5
Potassium dihydrogen phosphate	0.6
Potassium chloride	0.1
Trace vitamin solution	10 mL
Trace mineral solution	10 mL
Carbon sources	
Potassium acetate	400 mg
Electron acceptors	
Nitrate as N	500 mg

Analytical Methods

The batch cultures were monitored for acetate (sole carbon source) and nitrate (terminal electron acceptor) disappearance, and nitrite production. The analyses were performed using a Waters' high pressure liquid chromatography (HPLC) system that included a Model 510 HPLC pump and Model 486 UV and Model 432 Conductivity detectors. Bacterial cells were also enumerated concurrently from subsamples of samples removed for acetate and nitrate/nitrite analyses. The subsample was enumerated using the acridine orange total direct count (AODC) method (Kepner et al., 1994). In addition, the batch culture headspace was monitored for nitrous oxide (N_2O) production as an indication of true dissimilatory nitrate reduction. Headspace samples were obtained using a gas-tight syringe and analyzed with a Shimadzu Gas Chromatography (GC) with a 60/80 Carboxen-1000 (15'x1/8") column with a Ni^{63} electron capture detector (ECD).

Sample nitrate and nitrite concentrations were measured using standard HPLC techniques and the Dionex Ion Chromatograph (IC) (Model AI-450; Dionex Co., San Francisco, CA) with a pulse electrochemical detector (Model DX 300) and Dionex IonPac AS4A-SC (See Table 3.2) anion separation column (2 mm).

Nitrite concentrations were analyzed using a spectrophotometric method (Garrett et al., 1969) with the spectrophotometer (Milton Roy, Spectronic 601) set at a wavelength of 546 nm. High chloride background concentrations

interfered with the nitrite quantification using chromatographic analytical method. A major dissolved inorganic constituent in groundwater, chloride, has been found to exceed levels greater than 5 mg/L (Freeze et al., 1979). OnGuard-Ag pretreatment cartridges were not able to remove the chloride interference at higher concentration levels. Therefore more accurate results were obtained using the colorimetric method

Carbon substrate concentrations were measured using the Dionex IC. The type of chromatographic column and eluent mixture depended on the analyte of interest (Table 3.2).

Table 3.2 Dionex IC Analysis Specifications

Electron Acceptors		
SPECIFICATION	**NITRATE**	**SULFATE**
Column Type Pore Size	AS4A-SC 2 mm	AS4A-SC 2 mm
Detector	Conductivity	Conductivity
Eluent Concentration	Na_2CO_3+$NaHCO_3$ 1.8 mM/1.7 mM	Na_2CO_3+$NaHCO_3$ 1.8 mM/1.7 mM
Regenerant Concentration	none	none
Flow Rate	0.5 mL/min	0.5 mL/min
Electron Donors		
SPECIFICATION	**ACETATE**	**LACTATE**
Column Type Pore Size	AS10 4 mm	AS11 4 mm
Detector	Conductivity	Conductivity
Eluent Concentration	$K_2B_4O_7$ 3.5 mM	NaOH 1.0 mM
Regenerant Concentration	$K_2B_4O_7$ 100 mM	NaOH 100 mM
Flow Rate	1.0 mL/min	1.0 mL/min

The measurement of acetate alone required the use of an AS10 IonPac (4 mm) column. Determination of acetate and lactate in the same analysis required the use of an AS11 IonPac (4 mm) column for resolution of quantifiable acetate and lactate peaks.

Hydrogen sulfide in the aqueous phase was measured using a methylene blue colorimetric test (Cline, 1969). Using a sample dilution of 1:50, an aliquot of 0.1 mL was added to 4.9 mL of 1% zinc acetate. To this solution 0.4 mL of a diamine reagent was added. After 30 minutes, the absorbance was measured using the Milroy spectrophotometer set at a wavelength of 670 nm.

Total cell enumerations were determined using the acridine orange direct count (AODC) method (Kepner et al., 1994). The cells were first fixed in a 50% buffered gluteraldehyde solution. A vortexed 2 mL sample of the fixed cell solution was transferred to a vacuum filter assembly, and cells were immobilized on a 0.2 μm polycarbonate membrane. A 0.5 mL aliquot of 0.02% acridine orange solution was added to the immobilized, membrane-bound cells for a minimum of two minutes prior to vacuum filtration. The stained membrane filter was then placed on top of an immersion oiled glass slide. Another drop of immersion oil was placed onto the membrane. A cover slip was placed on the top of the oil-soaked membrane for cell enumeration with an Olympus BH-2 UV microscope.

In order to enumerate denitrifying bacteria in the column effluent most probable number (MPN) tests were performed. A phosphate buffer solution was anaerobically prepared for six-fold serial dilution using the effluent sample as inoculum. A function of the buffer is to adjust the tonicity of the suspending medium to that of the cell to avoid possible osmotic shock (Zuberer, 1994). Difco nitrate broth, which has the distinct advantages of being easy to prepare and of providing better nutritional conditions for the growth of more fastidious bacteria than that permitted in other media, was selected for enumerating nitrate reducing and denitrifying bacteria (Valera et al., 1961; Alexander, 1965). Suspended inocula (0.1 mL) were added to sterile media in screw-capped tubes, replicated 5 times per each six-fold dilution, for enumerating nitrate reducers and denitrifiers. Tubes contained 6-mL aliquots of Difco nutrient broth (8 g/L) to which 9.9 mM of nitrate was added and autoclaved together at 121°C for 15 minutes. Nitrate was supplied as a potassium salt. After inoculation of the sterile anaerobic tubes with the appropriate dilutions, the caps were tightened to exclude the diffusion of atmospheric oxygen during incubation for 4 weeks at room temperature (Volz, 1977).

The disappearance of nitrate and nitrite in the MPN tubes constituted a positive test for nitrate reduction (Volz, 1977). An additional confirmatory test for denitrification involved assessment of nitrous oxide (N_2O) gas production by sampling the MPN tube headspace.

CHAPTER 4

RESULTS

Nitrate-reduction results in the sandstone-column will first be introduced. The results show observable levels of nitrite occurring when nitrate reducing bacteria, nitrate, and organic acids are present. The remaining part of this section will focus on nitrite reduction patterns in the sandstone-packed column, utilization of substrates, and biomass yields. Further results, such as the comparison of maximum growth rate (μ_{max}) numbers to literature values for nitrate reducing bacteria, will be presented.

Nitrate Reduction

After inoculation with an enrichment culture in a batch-mode for 24 hours, flow of influent medium through the sandstone-packed column was resumed at an average pore velocity of 29 cm/day. As the medium contained a nitrate concentration of 500 mg/L and an acetate level of 400 mg/L, a carbon to nitrogen (C:N) ratio of 0.72 was present.

The time line of nitrate reduction in the sandstone-packed column is shown in Figure 4.1. Measurable nitrate in the effluent was seen as early as day 2 and then rapidly decreased to undetectable limits after day 22. The nitrate remained at zero concentration for approximately 115 days without any variations. After day 140, slight increases in nitrate concentration appeared, possibly caused by an unplanned shutdown due to in-line plugging. At day 200,

an additional carbon substrate, lactate, was added and a slight increase in nitrate concentration was observed. On day 240, the lactate concentration was increased to 700 mg/L and acetate was entirely omitted from the medium. Switching carbon substrates caused nitrate levels to sharply increase to approximately 200 mg/L before decreasing to zero concentration again for 20 days. The nitrate influent concentration experienced constant variations throughout the experiment, possibly due to change-out of medium, IC disturbances, medium evaporation during the autoclaving process, or plugging/channeling resulting in local consortium variations.

Nitrate Reduction at Sample Ports 1 and 2

Effluent samples were extracted from sample port 1 (5-cm from inlet) and sample port 2 (15-cm from inlet) for approximately 80 days to observe the nitrate and nitrite reduction gradients within the sandstone-packed column (Figure 4.2). Complete nitrate reduction in both sample ports 1 and 2 occurred after day 20 and continued to remain near zero concentration with minimal variation through day 80. The nitrate concentration at sample port 1 was slightly higher. The nitrate influent concentration in Figure 4.2 is the same as the influent concentration in Figure 4.1. On day 18 the nitrate concentration excursion found within the sandstone-packed column at sample port 1 and sample port 2 is also displayed in similar effluent patterns to Figure 4.1.

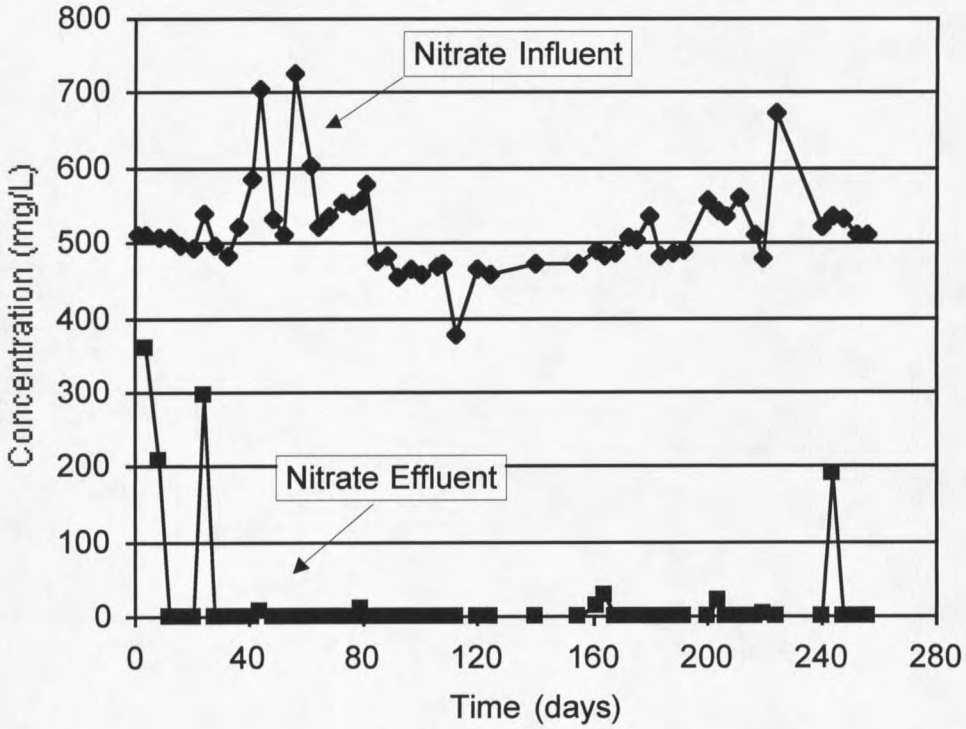

Figure 4.1 Timeline comparison between the nitrate influent concentration of approximately 500 mg/L and nitrate effluent levels (distance=30 cm) from the sandstone-packed column. Sample readings are displayed only for every fourth day.

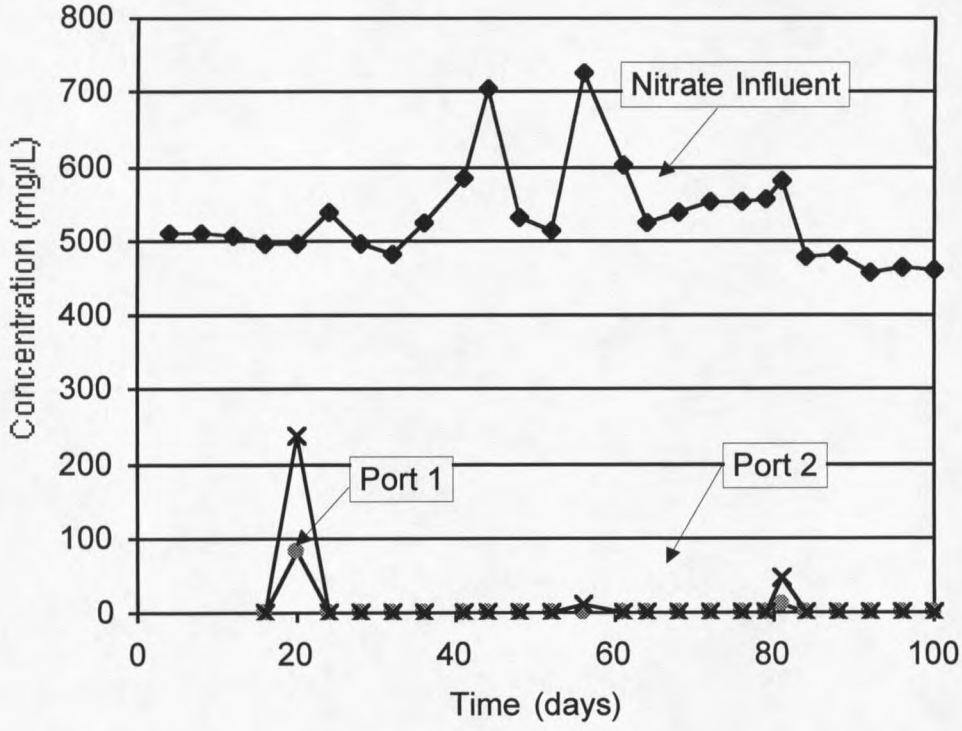

Figure 4.2 Comparison between the nitrate influent concentration (approximately 500 mg/L) and nitrate concentrations found within the column at sample port 1 (5-cm from inlet) and sample port 2 (15-cm from inlet) of the sandstone-packed column. Sample readings are displayed for every fourth day.

Nitrite Reduction

Figure 4.3 shows the timeline for nitrite accumulation in the column; nitrite accumulation appeared to vary over the course of the experiment. On day 18 the nitrite concentration increased to approximately 100 mg/L simultaneous to the increase in nitrate effluent concentration of 300 mg/L observed in the timeline of Figure 4.1. There appears to be no correlation between the nitrate influent and the nitrite effluent.

After the addition of 400 mg/L of lactate at day 170 the nitrite concentration oscillated sharply increasing and decreasing for several weeks. After the omission of acetate from the influent medium on day 240, nitrite sharply increased and remained at that concentration for at least 10 days before showing a decline towards zero concentration. Once zero concentration was reached for several days, the sandstone-packed column was shut down and dismantled.

Nitrate and Nitrite Reduction Patterns

The sandstone-packed column did appear to reach "steady-state," which is defined as the stabilization of nitrate and nitrite effluents near zero concentration. By day 20, the nitrite concentration decreased below 75 mg/L by the first sampling port (5-cm from inlet). A zero concentration was finally reached and continued to remain at this level for several weeks. Prior to day 80, another fluctuation appeared, increasing the nitrite concentration to 100 mg/L (Figure 4.4).

The nitrate levels reached zero concentration in both sample ports 1 and 2 after day 20 and remained at that level for the remainder of the 100-day sample port extraction experiment.

Organic Acid Utilization

Acetate was the primary substrate used for nitrate reduction in the sandstone-packed column. Consumption of acetate was detected in the effluent after day 2. By day 22 acetate was rapidly depleted reaching a concentration of zero (See Figure 4.5). The acetate effluent remained near zero concentration for an additional 80 days eventually reaching a high fluctuation of 75 mg/L. The acetate effluent concentration began to erratically increase throughout the remainder of the experiment. Increases in acetate effluent did seem to correlate with nitrite effluent concentrations.

A second organic acid, lactate, was added at a concentration of 400 mg/L on day 170. The lactate appeared to be consumed at a faster rate than acetate. Both organic acid concentrations did not reach steady state consumption. Acetate was eventually removed from the medium by day 240 (See Figure 4.5).

However, the acetate concentration increased when lactate was added to the medium indicating incomplete oxidation. Variations were seen with the acetate substrate and may have been due to temporary interruptions in the nitrate reducing activity (change-out of medium and removal of biomass plugging on the inlet side of the column). Competition among microbial

species for available electron acceptors and redox conditions is another important factor that may influence organic contaminant transformation.

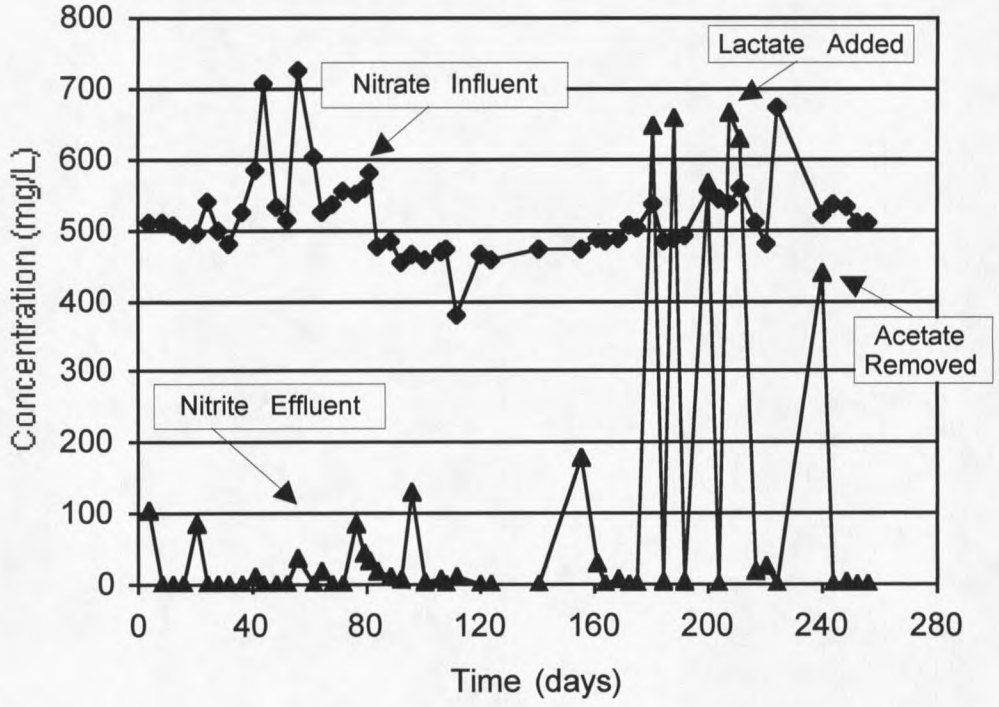

Figure 4.3 Overall comparison between the nitrate influent at approximately 500 mg/L and the nitrite accumulation found in the effluent (distance=30-cm) of the sandstone-packed column. Sample readings are displayed for every fourth day.

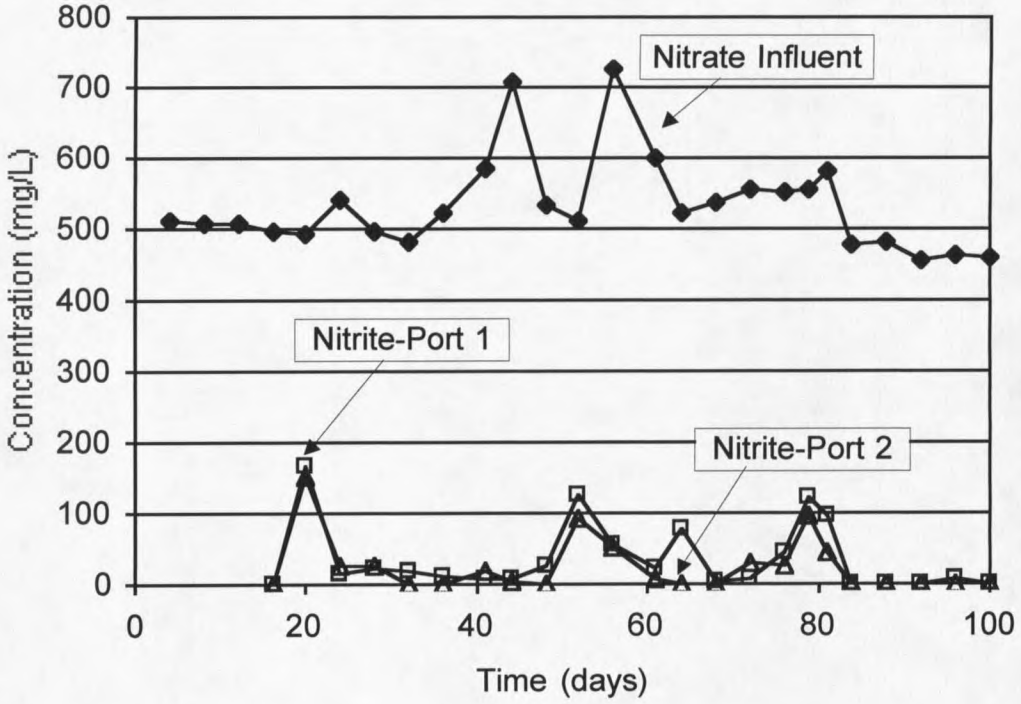

Figure 4.4 Comparison between the nitrate influent concentration (approximately 500 mg/L) and nitrite accumulation found within the column at sample port 1 (5-cm from inlet) and at sample port 2 (15-cm from inlet) of the sandstone-packed column. Sample readings are displayed for every fourth day.

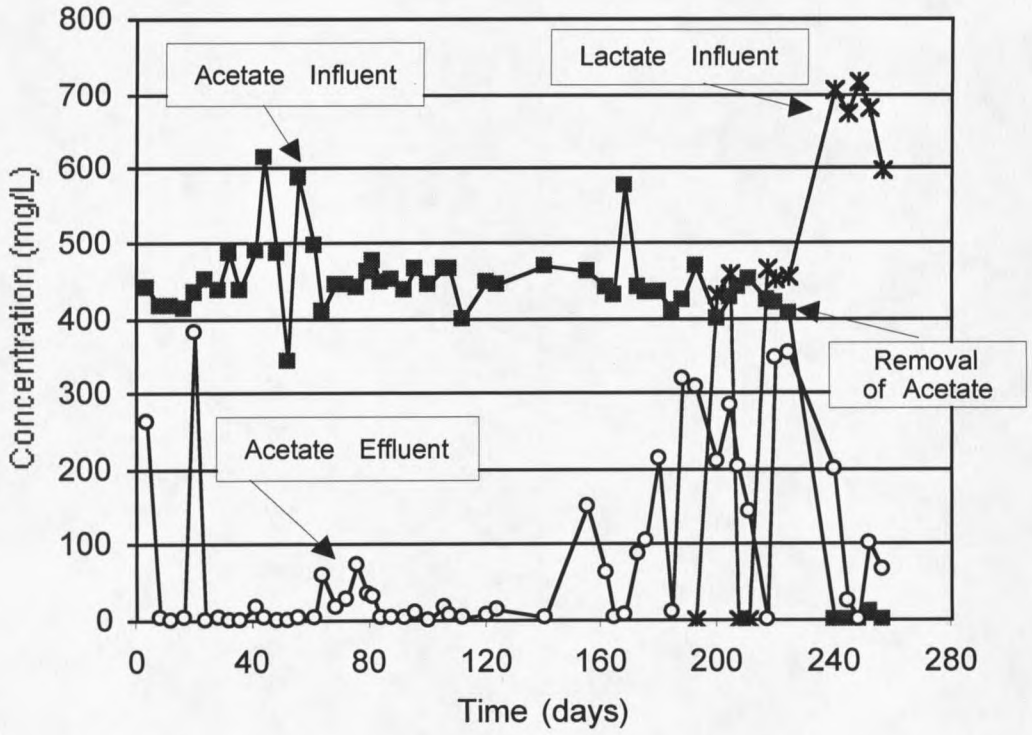

Figure 4.5 Overall comparison between the acetate influent concentration of approximately 400 mg/L and acetate levels in the effluent (distance=30-cm). Sample readings are displayed for every fourth day.

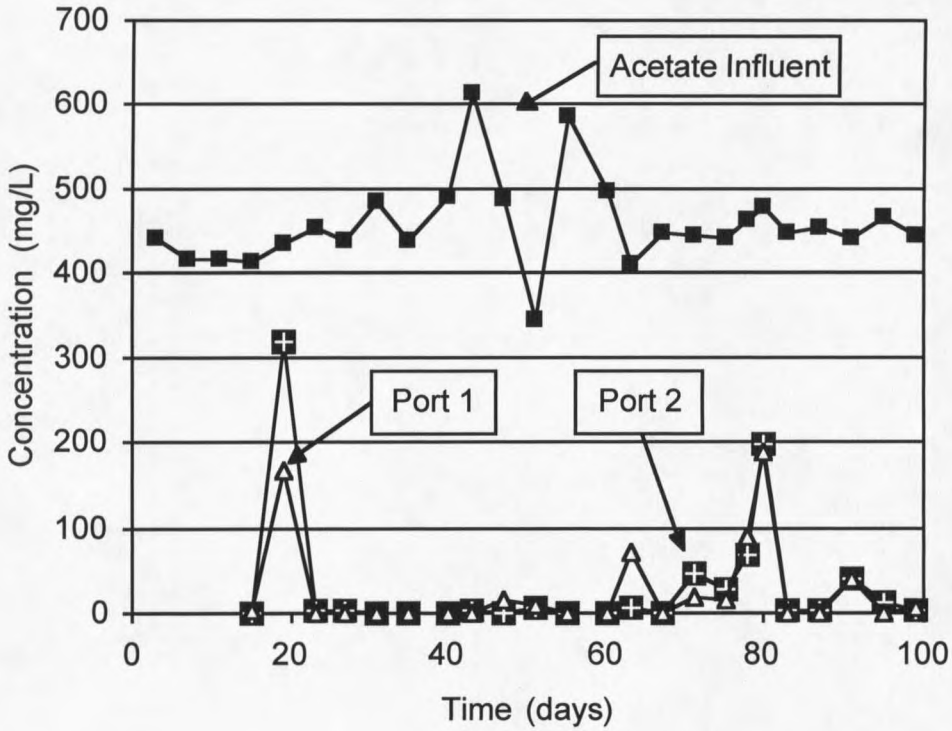

Figure 4.6 Comparison between the acetate influent (approximately 500 mg/L) and acetate levels found within the column at sample port 1 (5-cm from inlet) and at sample port 2 (15-cm from inlet) in the sandstone-packed column. Sample readings are displayed for every fourth day.

Biomass

Total cell counts were performed on the effluent sample throughout the course of the experiment. Cells were present in the effluent of the column as early as day 5 after influent flow resumed (See Figure 4.7). Total cell counts were also performed on samples obtained from sample ports 1 and 2 (Figure 4.8). An increase in cells/mL were found in the aqueous phase withdrawn from these sampling ports within the column than in the effluent (30-cm). This may possibly be due to some biofilm detachment when extracting samples within the column.

Most-Probable-Number

A common method used for enumeration of denitrifiers is the most-probable-number (MPN) procedure. At the end of a 4-week incubation period, the last three logarithmic tubes near the dilution to extinction were read and removed for analysis. Turbidity was observed as a sign of growth as well as an indicator of possible denitrifiers existing. Other indicators are the presence of nitrite, which is indicative of nitrate-respiring bacteria, while no nitrate or nitrite is presumptive evidence of denitrifiers. In addition, when the formation of gas is noted with subsequent disappearance of nitrate, the presumption of denitrification is greatly strengthened. The MPNs resulted in dilution tubes that showed turbidity with no detection of nitrate and nitrite. The tubes that were negative for both nitrate and nitrite were then sampled for nitrous oxide

production. Gas chromatography analysis resulted in no detection of nitrous oxide, supporting the possibility that nitrate reducers are present.

Estimation of Column Biomass

The total biomass, which includes the active biomass within the sandstone-packed column, was also measured. The sandstone-packed column was dismantled at the end of the experiment. The biomass was measured by sectioning the sandstone-packed core into 6-cm lengths, dried at 100°C, and then ashed at 400°C. The difference in weight between what was originally sectioned and what was ashed determines the total biomass value. Attached biomass (mg biomass per g sand) was found to be fairly consistent throughout the column (Figure 4.9). The total biomass equaled approximately 10.88 g (See Appendix 5) of cells attached to the surface area available in the sandstone-packed column.

Yields

Yields were calculated using two different methods:

1) Effluent Cell Yield: Assuming that the cell counts were at steady state when data was taken means that cell detachment (which was measured as cells in suspension) equals cell growth. Active biomass quantities can be calculated using the cell counts and an average cell size of 0.30 μm^3 (Mueller, 1994). The product of cell count, cell size, wet cell specific gravity of 1.09×10^{-12} g wet cell/μm^3 (Doetsch et al., 1973) and the ratio of 0.22 g dry cell/g wet cell

(Luria, 1960) gives the concentration of dry cell mass in suspension (Reinsel, 1995). Cell yields for nitrate reducing bacteria were lower for this consortium (Table 4.1(A)) as compared to the results of Reinsel (1995) and Mueller (1994). All these yields are relatively low as compared to literature values for most anaerobic bacteria.

2) Adsorbed Cell Yield: A second method calculating the concentration of dry cell mass accumulation on the sandstone is determined from the product of the column volume, sand density of 1.53 g/cm^3 and the ratio 2 mg dry biomass/g sand. Next, the overall nitrate and carbon source consumption was determined to obtain the denominator for the yield ratio of dry biomass to nitrate and dry biomass to acetate. Cell yields for nitrate reducing bacteria were higher for this particular consortium when compared to the results obtained from method 1) above. The results in part 2) show cell yields, which do not depend on steady state but assume that flow effluent is negligible. The results from the cell yields obtained in part 1) indicates that steady state had not been reached, i.e., account for the lower cell yields obtained.

Maximum Growth Rate Comparisons

A maximum growth rate value, μ_{max}, was calculated to determine if the cell concentrations and growth seen in the effluent was similar to literature values. A "triple-Monod" kinetics expression was utilized to calculate μ_{max} at the various sandstone-packed column lengths of 6-cm, 18-cm, and 30-cm using the corresponding dry cell mass concentrations found at those lengths.

Using a model equation, the concentrations of cells, electron acceptor (nitrate), and growth substrates (acetate, lactate), were analyzed by two methods. The first method, assuming steady state, used the cell concentrations exiting in the liquid phase (Equation i); the second method used the adsorbed cells and ignored the cells in the liquid phase as negligible (Equation ii). The concentrations of cells inside the biofilm are expressed in terms of g dry cell mass. The balance on the cells in the liquid phase is

$$\frac{dX_e}{dt} = \mu X_e - bX_e \qquad \text{(i)}$$

where the left term represents steady state effluent of suspended cells in the sandstone-packed reactor and equals the effluent rate. The first right term is growth and the last term is decay. In the following calculation, the decay term in the column is assumed to be zero. In Equation (ii), at the end of the experiment, the accumulation rate, dX_a/dt, is assumed to be equal to the biomass on the surface divided by the time (days) to accumulate the biomass.

$$\frac{dX_a}{dt} = \mu X_a \qquad \text{(ii)}$$

Microbial growth is dependent upon the substrate concentration. The "triple-Monod" kinetics equation is dependent on the concentration of both the substrate (C_s) and the electron acceptor (C_e). Non-competitive inhibition kinetics is also included in the model equation. The inhibition term is multiplied

by the standard double-Monod kinetic expression, so the kinetic expression for the growth rate μ is of the following form

$$\mu = \left(\frac{\mu_{max} C_s}{K_s + C_s}\right)\left(\frac{C_e}{K_e + C_e}\right)\left(\frac{K_i}{I + K_i}\right)$$

Inhibition effects have been seen for NO_2^- acting against the nitrate-reducing bacteria. Therefore, to determine the μ_{max} coefficient, substitution of the appropriate terms into the previous Equation (i) gives the following

$$QX_e = \mu_{max}\left[\left(\frac{C_s}{K_s + C_s}\right)\left(\frac{C_e}{K_e + C_e}\right)\left(\frac{K_i}{I + K_i}\right)\right]X_e \qquad \text{(i-a)}$$

Using the literature values for K_s, K_e, K_i (Mueller, 1994; Reinsel, 1995) and the outlet measured values for C_s, C_e, C_i, QX_e exiting, and X_e from the effluent total cell counts, a μ_{max} can be calculated from Equation (i-a). The μ_{max} coefficient from Equation (ii) depends on the surface microbial concentrations. In Equation (ii-a), $\langle \overline{Xa} \rangle$ is an order-of-magnitude estimate of the cell biomass adsorbed on the surface averaged over time (see Equation (ii-b).

$$\frac{X_a}{\Delta t} = \mu \langle \overline{X_a} \rangle \qquad \text{(ii-a)}$$

$$\langle \overline{X_a} \rangle = \frac{X_a}{2} \qquad \text{(ii-b)}$$

The μ_{max} calculations determined in both cases are theoretically lower bound values. The actual value would be higher and may serve to obtain a maximum rate if all the cells were reacting. It is probable that most of the

reactivity occurs in the first 6-cm entrance of the column and so $\langle \overline{X_a} \rangle$ which reacts should be smaller. Also C_s, and C_e, should be point values or average reactor values, and not effluent values.

Table 4.2 shows the tabulated μ_{max} comparisons between the cited literature values and the herein calculated values of μ_{max} for cases (i) and (ii) at different column lengths. The μ_{max} coefficients calculated for the full column length in case (i) 0.07-0.66 hr^{-1}, and in case (ii) 0.01-0.02 hr^{-1} appeared to fall within range when compared to the literature values obtained by Mueller, (1994) and Reinsel, (1995). For the first 6-cm, the μ_{max} coefficients of range 0.31-8.32 hr^{-1} calculated for case (i) was higher in comparison to the results obtained by Mueller, (1994) and Reinsel, (1995). However, for case (ii), a range of 0.01-0.02 hr^{-1} fell within the μ_{max} values obtained by Mueller, (1994) and Reinsel, (1995). Case (i), as shown by errors in yield-value calculations, is probably not a theoretically sound estimate of μ_{max}.

Table 4.1(A) Nitrate reducing bacteria yields based on the difference between influent and effluent concentrations of cell counts, nitrate and organic carbons.

	Y_{xs}-acetate (g dry cell/g total substrate)	Y_{xs}-lactate (g dry cell/g total substrate)	Y_{xe} (g dry biomass/g nitrate)
Effluent	0.0002	0.0002	0.0001
Sample Port 1	0.0003	Not determined	0.0003
Sample Port 2	0.0003	Not determined	0.0002

Table 4.1(B) Nitrate reducing bacteria based on cell mass accumulation on the sandstone and on the difference between influent and effluent concentrations of nitrate and organic carbons.

	Y_{xs}-acetate (g dry cell/g total substrate)	Y_{xs}-lactate (g dry cell/g total substrate)	Y_{xe} (g dry biomass/g nitrate)
Effluent	0.11	0.42	0.08
Sample Port 1	0.02	Not determined	0.02
Sample Port 2	0.05	Not determined	0.04

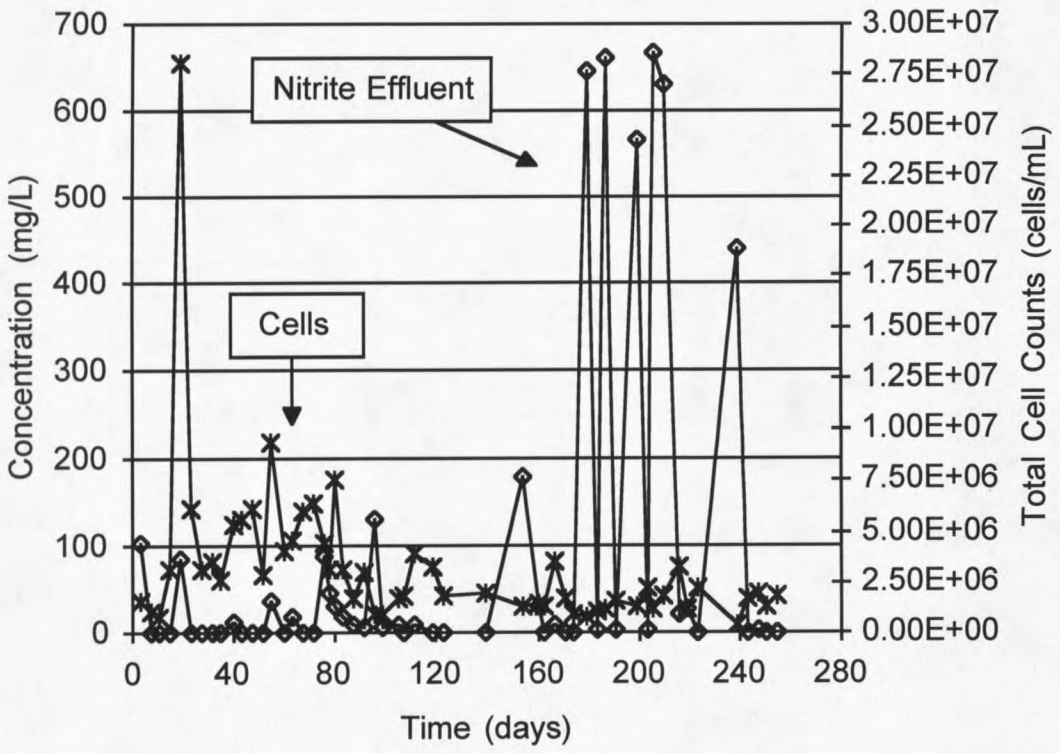

Figure 4.7 Presentation of the production of nitrite and total cell counts over the lifetime of the sandstone-packed column to examine time correspondence of behavior. Timelines display sample readings for every fourth day of cells and nitrite accumulation found in the effluent (distance=30-cm).

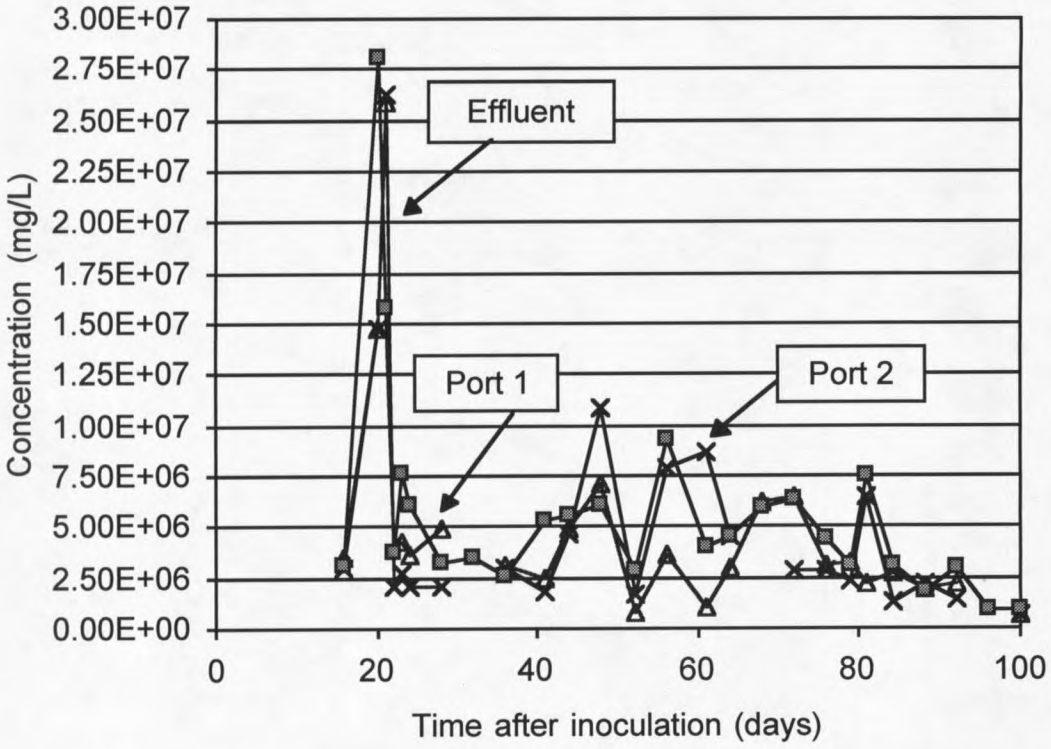

Figure 4.8 **Presentation of total cell counts found at sample port 1 (distance=5-cm), sample port 2 (distance=15-cm), and in the effluent (distance=30-cm) of the sandstone-packed column. Sample readings are displayed for every fourth day.**

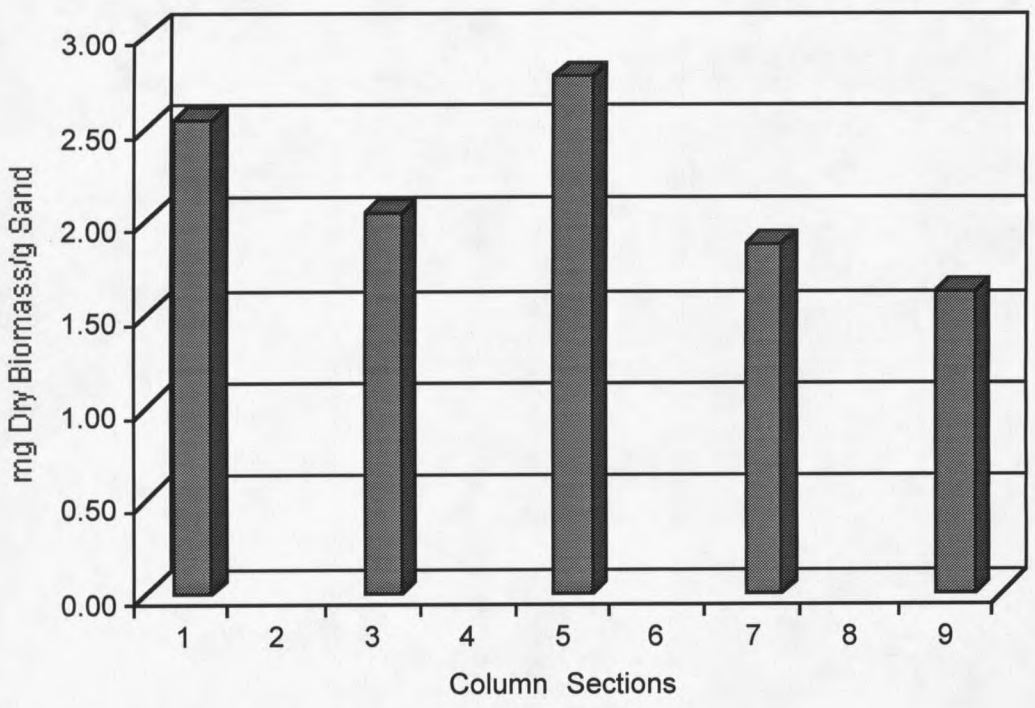

Figure 4.9 Measurement of cumulative biomass by section of the sandstone-packed column at the conclusion of the continuous experiment.

Table 4.2 Comparison between the cited literature information for μ_{max} and the calculation of μ_{max}. The coefficient μ_{max} was obtained by examination of effluent cell concentration and dry biomass measurement using the triple Monod kinetics and the half-saturation constants obtained from literature.

Literature	dX_e/dt	dX_a/dt	K_s (mg/L)	K_e (mg/L)	K_I (mg/L)	Xe (cells/mL)	Xa (g biomass)	μ_{max}-lit (1/h)	μ_{max} (i) calc (1/h)	μ_{max} (ii) (calc (1/h)
Case 1: L=30 cm:										
Mueller (1994)	9.86E+04	0.04	5.5	9	2	3.16E+06	5.44	**0.04**	**0.08**	**0.02**
Mueller (1994)	9.86E+04	0.04	5	5	50	3.16E+06	5.44	**0.06**	**0.10**	**0.01**
Reinsel (1995)	9.86E+04	0.04	5	5	133	3.16E+06	5.44	**1**	**0.07**	**0.01**
Reinsel (1995)	9.86E+04	0.04	2	5	4.4	3.16E+06	5.44	**0.04**	**0.66**	**0.01**
Case 2: L=18 cm:										
Mueller (1994)	2.24E+05	0.03	5.5	9	2	4.03E+06	3.69	**0.04**	**3.14**	**0.02**
Mueller (1994)	2.24E+05	0.03	5	5	50	4.03E+06	3.69	**0.06**	**0.18**	**0.01**
Reinsel (1995)	2.24E+05	0.03	5	5	133	4.03E+06	3.69	**1**	**0.12**	**0.01**
Reinsel (1995)	2.24E+05	0.03	2	5	4.4	4.03E+06	3.69	**0.04**	**1.17**	**0.01**
Case 3: L=6 cm:										
Mueller (1994)	5.96E+05	0.01	5.5	9	2	4.05E+06	1.27	**0.04**	**8.32**	**0.02**
Mueller (1994)	5.96E+05	0.01	5	5	50	4.05E+06	1.27	**0.06**	**0.48**	**0.01**
Reinsel (1995)	5.96E+05	0.01	5	5	133	4.05E+06	1.27	**0.04**	**0.31**	**0.01**
Reinsel (1995)	5.96E+05	0.01	2	5	4.4	4.05E+06	1.27	**0.04**	**3.10**	**0.01**

CHAPTER 5

DISCUSSION

In the batch enrichment cultures prepared to selectively enhance the nitrate reducers, it was observed that nitrate and nitrite were both reduced to zero concentrations. The anaerobic enrichment cultures also generated small amounts of nitrous oxide within the headspace of the 1-L vessel. Limited amounts of data were collected, mainly as a check to see if the enrichment cultures had actually been stimulated.

The sampled well water consortia contained a broad variety of microorganisms. It has been found that denitrifiers were the most abundant species found in the well water. Per 100 mL bacteria, 4600 turned out to be nitrate reducers (CeRAM, 1996).

Due to the objectives of the experimental program at that time, it was imperative to have a denitrification sandstone-packed column set-up and operating. This study primarily investigated the microbial reduction of nitrate in a porous sandstone-packed column under simulated in situ bioremediation conditions with a native consortium. The results from Figures 4.2 and 4.4 indicate the majority of the reduction occurred in the entrance of the column once "steady state" was reached.

Nitrate reducing bacteria became stimulated once nitrate and other nutrients were added. Acetate was the prime carbon source utilized as the

electron donor. A second carbon source, lactate, was later used as well. The concentration of acetate began at 400 mg/L and was eventually increased to 600 mg/L. Growth of nitrate reducing bacteria was limited by an influent nitrate concentration of 500 mg/L, which remained at this level throughout the experiment.

Nitrate and nitrite were reduced, but it is unclear whether nitrous oxide was produced/reduced, and whether nitrogen was produced within the column. Present analytical procedures were not sensitive at these levels. The MPN incubation time prior to analysis may not have been long enough to generate N_2O. The MPNs resulted in no detection of nitrate and nitrite. Gas chromatography analysis of nitrous oxide resulted in negative tubes as well, supporting the possibility that nitrate reducers are present. However, if nitrous oxide is not observed, the culture may be considered to be a dissimilatory ammonia producer. Ammonia was not analyzed.

The cellular growth yields calculated by Method 1 for nitrate reducing bacteria were about an order-of-magnitude lower than what was obtained by Mueller, (1994). For method 2, the cell yields were an order-of-magnitude higher than those obtained by Mueller, (1994). Yields of 0.0055 g dry cell mass/g substrate utilized and 0.1089 g dry cell mass/g NO_3^--N reduced were reported by Mueller, (1994). When compared with data reported by Rusten, (1982), Mueller, (1994) also found his calculated yields to be uncharacteristically low.

From the data observed it was found that nitrate reducers utilized acetate well and eventually reached steady-state consumption for a 3-month period of the experiment. When a secondary substrate, lactate, was included in addition to acetate, utilization of lactate was gradual, but finally became preferentially utilized leaving amounts of acetate unreacted (Figure 4.5). Lactate may not have completely oxidized and may have contributed to the high acetate concentrations as observed in the IC analysis. Nitrate concentration was still reduced to zero levels (Figure 4.1). Nitrite accumulation was much greater with lactate as the carbon source. The effluent cell concentration was observed to have slightly decreased as well.

Why did nitrite effluent concentrations change? Some of the microorganisms induced on acetate may have converted to lactate as the carbon source. Other microorganisms may have become active with lactate, but were not yet reducing nitrite. This would cause nitrite to accumulate in higher concentrations initially. Some microorganisms continued to utilize the acetate and were still able to reduce the nitrate to nitrite, but at a much slower rate. Also, the organisms with nitrite reductase may have become less active, causing an accumulation of nitrite in the effluent, which may have acted as an inhibitor to nitrate reduction. It is possible the growth rate of the nitrite reducing bacteria is slower within the column. Other parameters which affected nitrite growth may have been temperature, pH, and possibly the absence of this particular reductase. The other explanation is that biomass accumulation

became high enough that some channeling occurred as porosity decreased and the pressure drop forced new channels. When a new channel was opened, organisms in that channel may not have been active. The lag time to fully activate the colony allowed for nitrite accumulation until full activity was developed. Thus oscillations would be observed whenever a new channel was forged due to biomass plugging of an old channel. Another mechanism to explain some of the acetate and lactate behavior may have been misinterpretations of the ion chromatography analysis. With both acetate and lactate within the same sample, a resolution problem occurred. These carbon substrates have close retention times, therefore confusing the conductivity detector and possibly giving false effluent concentration levels. These speculations should be further examined.

Acetate was eventually removed from the influent medium leaving lactate as the sole substrate. Increased fluctuations then occurred and remained throughout the course of the experiment. This fits with increased biomass accumulation. Nitrate was observed to decrease at this point, but reduction did resume and concentrations of nitrate gradually reached zero concentration towards the end of the experiment. Nitrite levels had slightly decreased along with cell counts, but these also rebounded eventually.

Medium contamination due to in-line plugging occurred several times throughout the course of the experiment. A sample of the influent was analyzed for substrate concentration and found it had decreased from its set level. New

medium was prepared and placed into the system whereupon nitrate reduction resumed. Plugging of the column inlet had also occurred, causing the influent lines and the flow break to become back-filled. The column was quickly dismantled and a thin layer of intact cell mass was physically removed. The influent medium continued to flow freely without further obstruction.

(Roberts et al., 1991) and (Shouche et al., 1993) hypothesized that electron donor and acceptor pulsing can effectively distribute biomass away from the injection zone. Implications of this result for in situ bioremediation are that pulsing can provide a greater biologically active radius of influence for a given nutrient-injection well. With pulsing, less biomass accumulates near the injection point, therefore, biomass near the entrance will metabolize less of the injected substrate mass. This permits un-utilized substrate to travel further into the aquifer, thereby increasing the radius of influence and creating a larger volume of bioactive aquifer. This can increase the total amount of active biomass in the aquifer and may result in higher contaminant removal rates.

A second electron acceptor, sulfate, was added to induce the sulfate-reducing population. A similar concentration, 500 mg/L, used for nitrate was also used for the sulfate addition. The reduction of nitrate to any one of the intermediate products has a much more favorable Gibbs free energy than does sulfate reduction. When both denitrifying bacteria and sulfate-reducing bacteria are present within the same consortium, the denitrifying bacteria should dominate. If the two types of bacteria utilize a common substrate such as

acetate, the denitrifying bacteria should inhibit the sulfate-reducing bacteria if both nitrate and sulfate are present in excess, which could limit or eliminate souring activity (Hitzman et al., 1994; Reinsel, 1995). No sulfate reductions were observed in any of the samples obtained, therefore, no hydrogen sulfide was detected. The sulfate in the influent medium continued to remain near the set concentration level.

The nitrite concentrations appeared low enough to not inhibit SRB activity. Nitrite levels were observed to decrease to zero concentration with minor variability for a period longer than 30 days (Figure 4.3). Reinsel, (1995) found that 30 mg/L NO_2^- completely inhibits active souring and that 16 mg/L NO_2^- will maintain the inhibition.

CHAPTER 6

GENERAL CONCLUSIONS

An experimental program was performed to study the microbial degradation of nitrate in a porous sandstone-packed column. The following major conclusions were obtained:

Column Experiments

1. At room temperature, nitrate was reduced and nitrite produced with the consumption of volatile fatty acids in enrichment cultures inoculated with the produced water from the Tuba City site.
2. MPN bacterial enumerations in the highest dilutions showed turbidity but no nitrous oxide production, indicating that certain steps in the dissimilatory pathway may not be operative.
3. Nitrate reducing bacteria were present in the produced water from the Tuba City UMTRA site as evident from the prepared enrichment cultures and the reduction of nitrate in the column effluent.
4. Sulfate-reducing bacteria were present in small amounts in the produced water from the UMTRA site. Anaerobic enrichment cultures prepared for SRBs formed a black precipitate, which is an indicator of SRB content. The distinct H_2S odor present in extracted liquid samples also indicated SRB activity.

5. Nitrite levels were variable within the continuous flow sandstone-packed column.

6. High concentrations of nitrite were observed in the column effluent towards the end of the experiment. We believe that these high levels of nitrite could inhibit sulfate reduction to some degree.

7. Acetate and lactate were both utilized by the microbial consortium within the column, with the lactate being consumed preferentially.

8. Plugging of the influent lines occurred at various times during the operation. A "biofilm" had formed on the radial surface of the sandstone core on the inlet side of the column. In addition, formation of gas bubbles was observed to be present on this side of column entrance.

9. For Method 2 (Adsorbed Cells), cell yield values for nitrate reducing bacteria resulted in an order-of-magnitude higher than those obtained by Mueller, (1994) and Reinsel, (1995). Yields of 0.11 g dry cell mass/g substrate utilized and 0.08 g dry biomass/g NO_3^- reduced were obtained.

10. Maximum growth rate values, μ_{max}, for the adsorbed cells, case (ii), were determined to be in the same range as those found by Mueller, (1994) and Reinsel, (1995). For the full column and the first 6-cm, μ_{max} was found to be 0.01-0.02 hr^{-1}.

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APPENDICES

APPENDIX 1

BASAL MEDIUM COMPOSITION AND PREPARATION

Denitrifying Bacteria (DNB) Medium with Acetate and Lactate (Balch et al., 1979)

1. To 2 L of deionized water, add the following:

Components	g / 2 L
Sodium bicarbonate	5.0 g
Ammonium chloride	3.0 g
Potassium dihydrogen phosphate	1.2 g
Potassium chloride	0.2 g
Potassium nitrate (500 mg/L)	1.64 g
Sodium acetate (400 mg/L)	1.12 g
60% Sodium lactate syrup (400mg/L)	1.68 g
Buffer Components (20 mM):	
Potassium phosphate	4.64 g
Potassium dihydrogen phosphate	1.82 g
Trace vitamin solution	20 mLs
Trace Mineral solution	20 mLs

1. Autoclave for 40 minutes and allow to cool to room temperature.

2. Sparge with nitrogen for at least 20 minutes depending upon the volume and record pH. Medium is ready for use.

Denitrifying Bacteria (DNB) Medium with Lactate and Sulfate (Balch et al., 1979)

1. To 2 L of nanopure water, add the following:

Components	g / 2 L
Sodium bicarbonate	5.0 g
Ammonium chloride	3.0 g
Potassium dihydrogen phosphate	1.2 g
Potassium chloride	0.2 g
Potassium nitrate (500 mg/L)	1.64 g
Sodium sulfate (500 mg/L)	1.48 g
60% Sodium Lactate syrup (650 mg/L)	2.74 g
Buffer Components (20 mM):	
Potassium phosphate	4.64 g
Potassium dihydrogen phosphate	1.82 g
Trace vitamin solution	20 mLs
Trace mineral solution	20 mLs

2. Autoclave for 40 minutes and allow to cool to room temperature.

3. Sparge with nitrogen for at least 20 minutes prior to use.

4. Obtain subsample and label as feed.

Sulfate-reducing Bacteria (SRB) Medium for *Desulfovibrio* Species (Lovely, 1992)

1. To basic medium per 930 mL of nanopure water, add the following:

Component	Amount
Nanopure water	930 mLs
Potassium Chloride (KCl)	0.1 g
Ammonium Chloride	1.5 g
Sodium Phosphate (monobasic, anhydrous)	0.6 g
Sodium bicarbonate ($NaHCO_3$)	2.5 g
Magnesium Sulfate ($MgSO_4 \cdot 7H_20$)	1.5 g
Sodium Sulfate	1.5 g
60% Lactate Syrup	5 mL
Trace mineral solution (per 930 mL)	10 mL
Trace vitamin solution (per 930 mL)	10 mL

2. After autoclaving the above basal nutrient solution (950 mLs), add the designated mL of each of the following sterile, anaerobic stock solutions:

Components	Final Conc. (g/L)	Stock Solution (g/mL)	Vol. (mL)
Yeast extract	2	2/10	10
Proteose peptone	5	5/20	20
L-cysteine-HCl	0.25	2.5/10	1
Ferrous ammonium sulfate	0.1	1/10 (acidify with H_2SO_4, 1-2 drops)	1

3. Add 20 mL of inoculum (sample or transfer culture).

Composition of Standard Medium (Balch et al., 1979)

A. Trace Minerals

Contains, in grams per liter of distilled water (pH to 7.0 with KOH):

Constituent	g/L
nitrilotriacetic acid	1.5
$MgSO_4*7H_2O$	3.0
$MnSO_4*2H_2O$	0.5
NaCl	1.0
$FeSO_4*7H_2O$	0.1
$CoSO_4$ or $CoCl_2$	0.1
$CaCl_2*2H_2O$	0.1
$ZnSO_4$	0.1
$CuSO_4*5H_2O$	0.01
$AlK(SO_4)_2$	0.01
H_3BO_3	0.01
$Na_2MoO_4*2H_2O$	0.01

Dissolve nitrilotriacetic acid with KOH to pH 6.5, then proceed to add minerals.

B. Trace Vitamins

Contains, in mg/L of distilled water:

Constituent	mg/L
Biotin	2
folic acid	2
pyridoxine hydrochloride	10
thiamine hydrochloride	5
Riboflavin	5
nicotinic acid	5
DL-calcium pantothenate	5
vitamin B_{12}	0.1
p-aminobenzoic acid	5
lipoic acid	5

APPENDIX 2

DATA TABLES FOR FIGURES

Sandstone-Packed Column Data

Table A2.1 Influent Column Data					
Date	Sample Name	Nitrate Conc (mg/L)	Acetate Conc (mg/L)	Lactate Conc (mg/L)	Direct Count (# / ml)
1/24/97	Inoculum				2.39E+06
1/27/97	Feed	510.88	441.07		
1/31/97	NFeed	508.16	414.39		
2/4/97	Feed	506.94	416.38		
2/8/97	Feed	495.26	411.69		
2/12/97	Feed	494.39	434.06		
2/16/97	Feed	539.24	452.07		
2/20/97	Feed	497.18	437.17		
2/24/97	Feed	481.57	484.01		
2/28/97	Feed	523.15	435.93		
3/5/97	Feed	584.33	489.76		
3/8/97	Feed	705.86	614.07		
3/12/97	Feed	531.71	486.48		
3/16/97	Feed	512.73	343.35		
3/25/97	Feed	601.38	495.22		
3/28/97	Feed	523.13	407.79		
4/1/97	Feed	536.76	444.87		
4/5/97	Feed	553.77	443.32		
4/9/97	Feed	551.51	439.71		
4/12/97	Feed	557.23	460.39		
4/14/97	Feed	579.67	475.54		
4/17/97	Feed	476.71	445.66		
4/21/97	Feed	483.04	451.56		
4/25/97	Feed	455.77	438.35		
5/3/97	Feed	458.93	443.12		

Table A2.2 Influent Column Data (cont.)					
Date	Sample Name	Nitrate Conc	Acetate Conc	Lactate Conc	Direct Count
		(mg/L)	(mg/L)	(mg/L)	(# / ml)
5/9/97	Feed	467.07	462.90		
5/11/97	Feed	471.36	464.08		
5/23/97	Feed	466.87	445.83		
5/27/97	Feed	459.02	443.52		
6/12/97	Feed	473.18	468.28		
6/27/97	Feed	471.52	461.27		
7/3/97	Feed	488.58	438.55		
7/6/97	Feed	482.01	428.40		
7/10/97	Feed	487.05	576.56		
7/14/97	Feed	506.4	439.49		
7/17/97	Feed	502.58	432.30		
7/21/97	Feed	511.76	431.65		
7/25/97	Feed	496.46	424.58		
7/29/97	Feed	487.22	435.69		
8/3/97	Feed	489.84	469.81	0.00	
8/11/97	Feed	556.09	396.64	429.57	
8/15/97	Feed	544.23	425.44	456.28	
8/18/97	Feed	536.58	440.62	0.00	
8/22/97	Feed	559.94	450.93	0.00	
8/28/97	Feed	510.18	424.14	465.42	
8/31/97	Feed	478.25	417.69	450.88	
9/4/97	Feed	671.86	406.49	454.02	
9/20/97	Feed	521.8	0.00	705.46	
9/24/97	Feed	535.18	0.00	673.67	
9/28/97	Feed	533.23	0.66	716.98	
10/2/97	Feed	509.45	8.84	679.69	
	AVERAGE	**514.49**	**415.5**	**449**	

Table A2.2 Effluent Column Data						
Date	Sample Name	Nitrate Conc (mg/L)	Nitrite Conc (mg/L)	Acetate Conc (mg/L)	Lactate Conc (mg/L)	Direct Count (# / ml)
1/27/97	Effluent	207.24	103.69	263.59		1.61E+06
1/31/97	Effluent	0.00	0.00	2.20		1.02E+06
2/4/97	Effluent	0.00	0.00	0.73		7.49E+05
2/8/97	Effluent	0.00	0.00	3.59		3.07E+06
2/12/97	Effluent	296.98	84.09	381.02		2.80E+07
2/16/97	Effluent	0.00	0.00	0.58		6.09E+06
2/20/97	Effluent	0.00	0.00	4.90		3.18E+06
2/24/97	Effluent	0.00	0.00	1.40		3.53E+06
2/28/97	Effluent	0.00	0.00	1.74		2.54E+06
3/5/97	Effluent	7.57	12.10	17.65		5.30E+06
3/8/97	Effluent	0.00	0.00	2.36		5.55E+06
3/12/97	Effluent	0.00	0.00	0.61		6.05E+06
3/16/97	Effluent	0.00	0.00	0.00		2.81E+06
3/25/97	Effluent	0.00	0.00	2.97		4.03E+06
3/28/97	Effluent	0.00	16.91	59.01		4.55E+06
4/1/97	Effluent	0.00	0.00	16.86		5.95E+06
4/5/97	Effluent	0.00	1.49	26.22		6.38E+06
4/9/97	Effluent	8.93	87.08	73.02		4.40E+06
4/12/97	Effluent	0.00	44.12	35.57		3.15E+06
4/14/97	Effluent	0.00	31.71	30.45		7.47E+06
4/17/97	Effluent	0.00	16.82	3.51		3.10E+06
4/21/97	Effluent	0.00	9.39	3.37		1.75E+06
4/25/97	Effluent	0.00	7.42	3.04		2.96E+06
5/3/97	Effluent	0.00	4.80	0.23		8.62E+05
5/9/97	Effluent	0.00	8.65	15.73		1.63E+06
5/11/97	Effluent	0.00	0.00	6.68		1.88E+06
5/23/97	Effluent	0.00	0.00	8.49		3.26E+06
5/27/97	Effluent	0.00	0.00	15.45		1.83E+06
6/12/97	Effluent	0.00	0.00	3.99		1.96E+06
6/27/97	Effluent	12.54	180.11	149.54		

Table A2.2 Effluent Column Data (cont.)						
Date	Sample Name	Nitrate Conc	Nitrite Conc	Acetate Conc	Lactate Conc	Direct Count
		(mg/L)	(mg/L)	(mg/L)	(mg/L)	(# / ml)
7/3/97	Effluent	29.94	29.74	62.62		1.45E+06
7/6/97	Effluent	0.00	0.00	5.01		1.32E+06
7/10/97	Effluent	0.00	9.17	7.31		
7/14/97	Effluent	0.00	0.00	86.42		1.74E+06
7/17/97	Effluent	0.00	0.00	106.31		8.74E+05
7/21/97	Effluent	0.00	0.00	105.84		1.39E+06
7/25/97	Effluent	0.00	1.18	3.86		2.11E+06
7/29/97	Effluent	0.00	0.94	0.51		
8/3/97	Effluent	0.00	2.16	306.02	0.00	1.60E+06
8/11/97	Effluent	22.06	566.83	209.43	0.00	1.25E+06
8/15/97	Effluent	0.00	1.67	281.89	365.44	2.16E+06
8/18/97	Effluent	0.00	667.25	203.32	0.00	1.20E+06
8/22/97	Effluent	0.00	629.87	143.71	0.00	1.84E+06
8/28/97	Effluent	4.44	19.82	0.00	4.88	3.29E+06
8/31/97	Effluent	0.00	25.83	346.94	42.62	1.11E+06
9/4/97	Effluent	0.00	0.00	354.27	57.56	2.25E+06
9/20/97	Effluent	191.74	438.83	199.92	277.21	3.90E+05
9/24/97	Effluent	0.00	0.00	23.25	133.85	1.66E+06
9/28/97	Effluent	0.00	3.18	0.00	27.67	1.92E+06
10/2/97	Effluent	0.00	0.00	102.74	111.51	1.28E+06
	AVERAGE	**13.88**	**62.20**	**72.92**	**88.22**	**3.31E+06**

Table A2.3 Sample Port 1 Effluent Column Data					
Date	Sample Name	Nitrate Conc (mg/L)	Nitrite Conc (mg/L)	Acetate Conc (mg/L)	Direct Count (# / ml)
2/8/97	Effluent	0.00	0.00	2.91	3.48E+06
2/12/97	Effluent	82.02	165.59	166.51	1.48E+07
2/16/97	Effluent	0.00	16.08	0.00	3.61E+06
2/20/97	Effluent	0.00	21.06	0.00	4.92E+06
2/24/97	Effluent	0.00	17.28	0.00	4.23E+06
2/28/97	Effluent	0.00	9.61	0.00	3.15E+06
3/5/97	Effluent	0.00	6.79	0.24	2.48E+06
3/8/97	Effluent	0.00	7.36	0.23	4.88E+06
3/12/97	Effluent	0.00	25.82	16.30	7.10E+06
3/16/97	Effluent	0.00	125.72	8.74	7.83E+05
3/25/97	Effluent	0.00	20.46	0.00	1.01E+06
3/28/97	Effluent	0.00	78.57	70.30	3.00E+06
4/1/97	Effluent	0.00	2.90	0.76	6.17E+06
4/5/97	Effluent	0.00	5.91	19.87	6.41E+06
4/9/97	Effluent	0.00	43.45	14.40	3.09E+06
4/12/97	Effluent	0.00	121.50	90.08	3.24E+06
4/14/97	Effluent	9.25	96.87	187.91	2.16E+06
4/17/97	Effluent	0.00	0.00	0.95	2.68E+06
4/21/97	Effluent	0.00	0.00	1.13	1.93E+06
4/25/97	Effluent	0.00	0.00	39.15	2.24E+06
5/3/97	Effluent	0.00	0.00	6.90	7.03E+05
	AVERAGE	**7.89**	**28.04**	**23.73**	**4.26E+06**

Table A2.4 Sample Port 2 Effluent Column Data					
Date	**Sample Name**	**Nitrate Conc (mg/L)**	**Nitrite Conc (mg/L)**	**Acetate Conc (mg/L)**	**Direct Count (# / ml)**
2/8/97	Effluent	0.00	0.00	1.40	2.97E+06
2/12/97	Effluent	234.00	151.42	318.88	1.47E+07
2/16/97	Effluent	0.00	26.00	3.62	2.07E+06
2/20/97	Effluent	0.00	26.66	3.89	2.08E+06
2/24/97	Effluent	0.00	0.00	0.00	2.64E+06
2/28/97	Effluent	0.00	0.00	0.86	2.99E+06
3/5/97	Effluent	0.00	17.65	1.05	1.86E+06
3/8/97	Effluent	0.00	4.08	2.50	4.64E+06
3/12/97	Effluent	0.00	0.00	0.00	1.09E+07
3/16/97	Effluent	0.00	92.24	7.64	1.65E+06
3/25/97	Effluent	0.00	6.03	0.00	8.66E+06
3/28/97	Effluent	0.00	0.00	6.61	4.56E+06
4/1/97	Effluent	0.00	0.00	0.84	3.84E+06
4/5/97	Effluent	0.00	29.13	45.38	2.79E+06
4/9/97	Effluent	0.00	27.34	29.03	2.81E+06
4/12/97	Effluent	0.00	94.52	68.00	2.38E+06
4/14/97	Effluent	47.08	44.77	198.75	6.45E+06
4/17/97	Effluent	0.00	0.00	3.65	1.35E+06
4/21/97	Effluent	0.00	0.00	2.26	2.20E+06
4/25/97	Effluent	0.00	0.00	39.70	1.47E+06
5/3/97	Effluent	0.00	0.00	3.04	6.50E+05
	AVERAGE	**5.44**	**21.76**	**25.37**	**4.05E+06**

APPENDIX 3

STANDARD CURVES

Liquid Chromatography Analyses

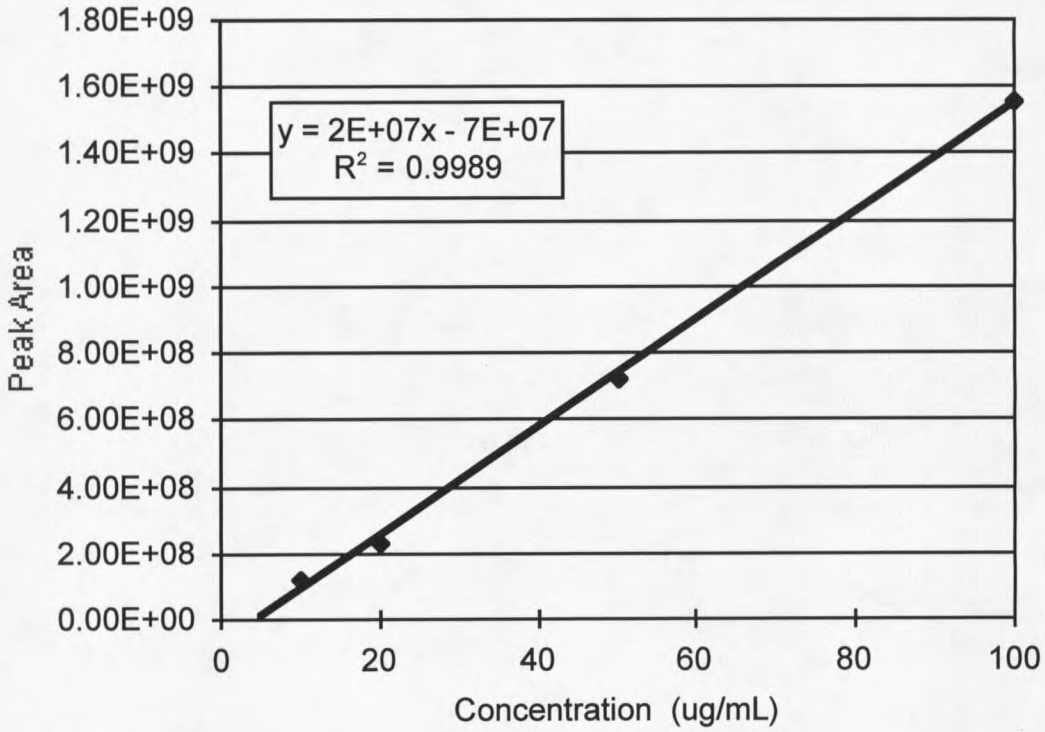

Figure A3.1 Nitrate IC Standard Curve

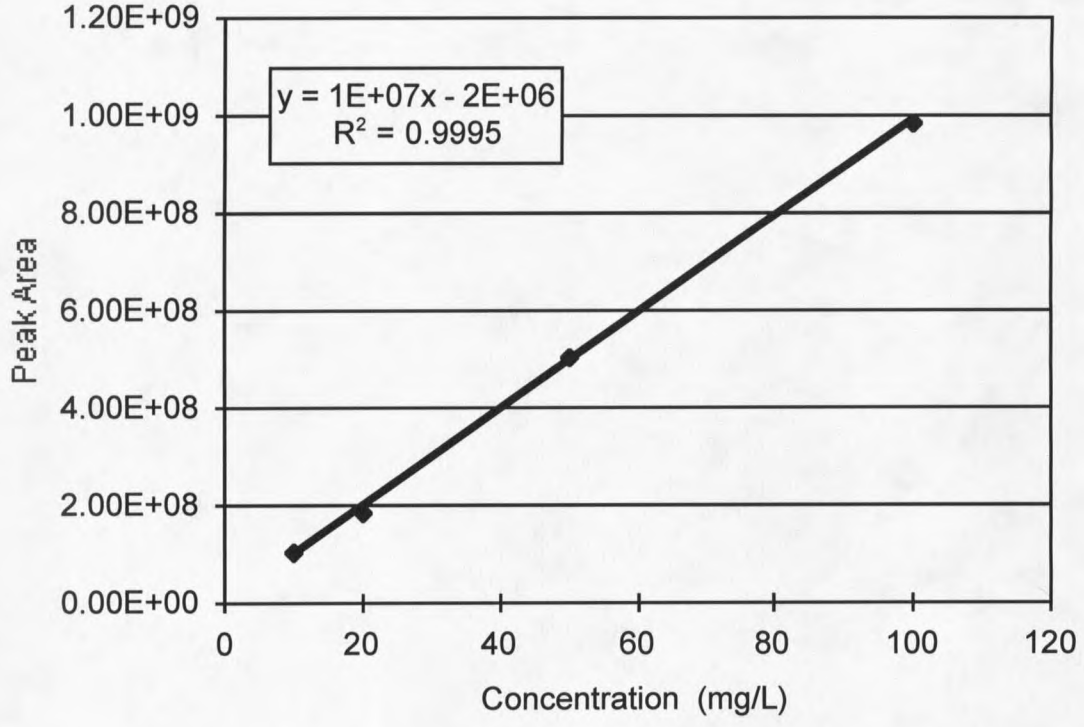

Figure A3.2 Nitrite Spectrophotometric Standard Curve

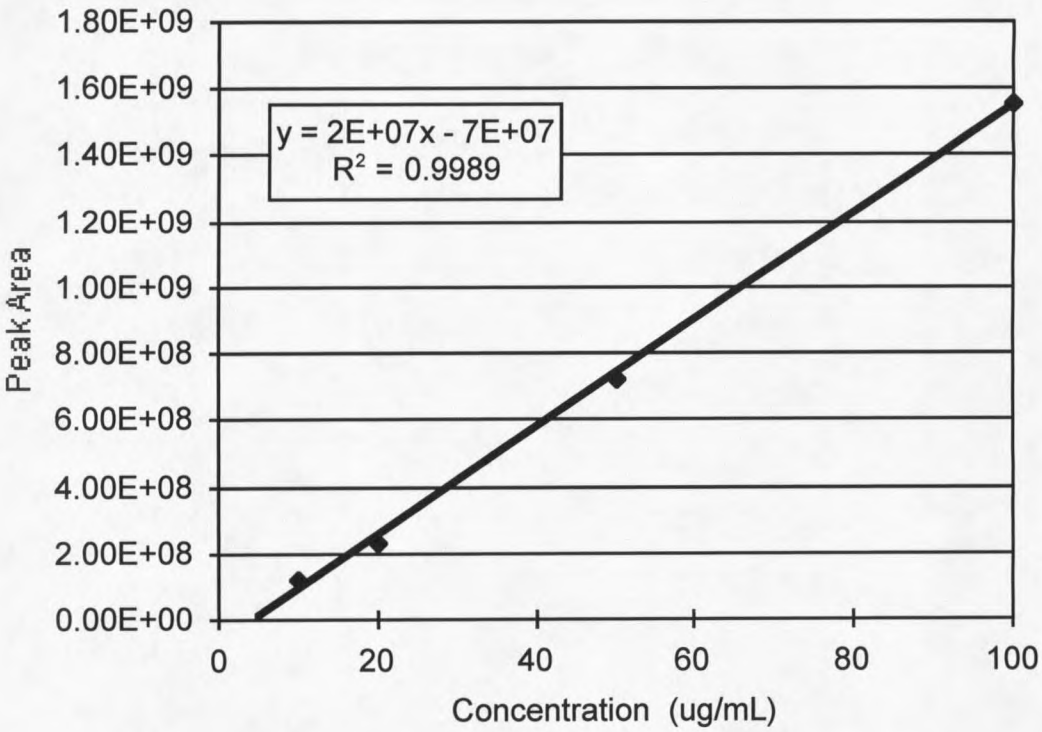

Figure A3.3 Sulfate IC Standard Curve

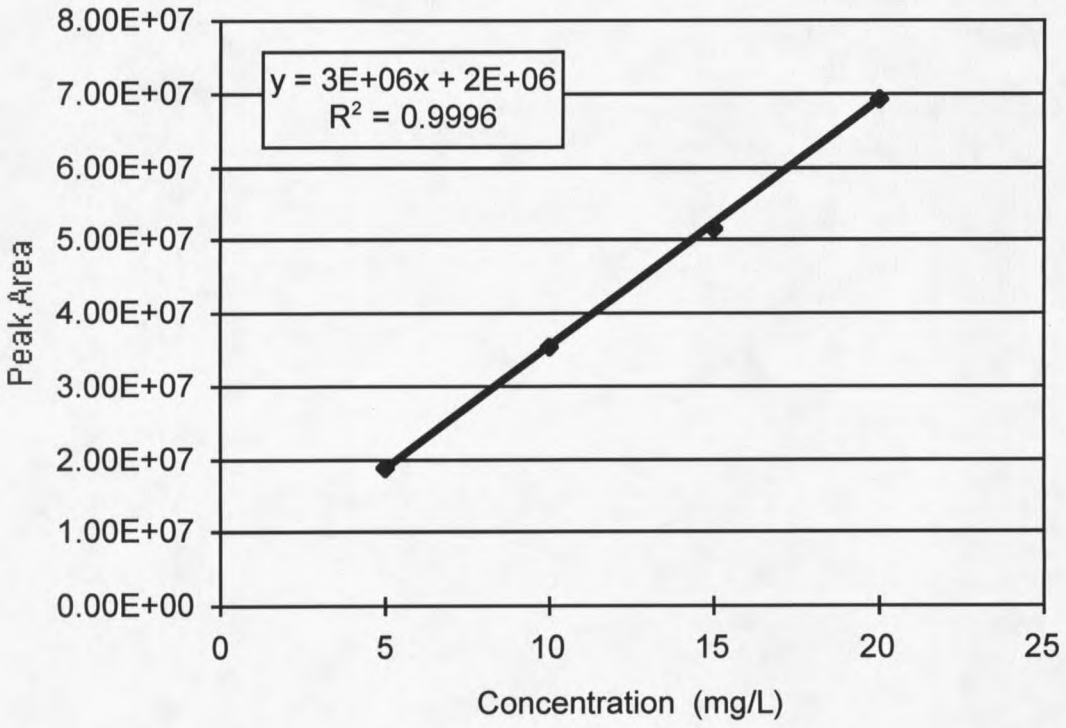

Figure A3.4 Acetate IC Standard Curve

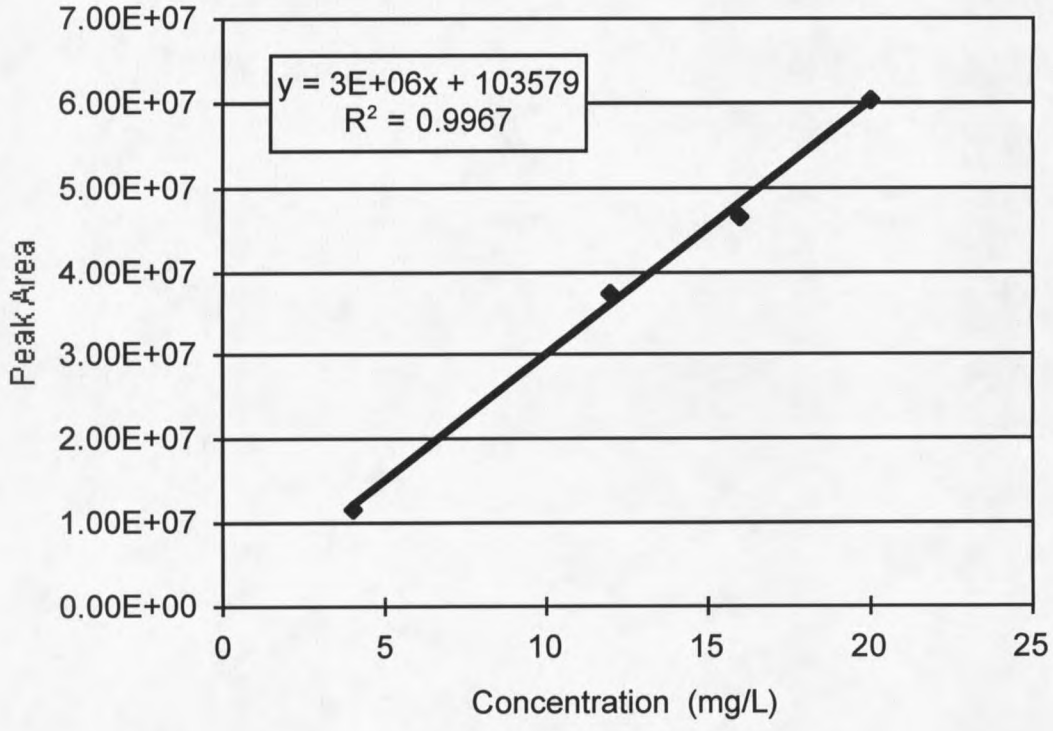

Figure A3.5 Lactate IC Standard Curve

APPENDIX 4

CALCULATIONS

Partitioning of Nitrous Oxide (N_2O) Between Aqueous and Vapor Phases

Variables:

K_H = Henry's Law Constant
C_{N2O} = measured N_2O vapor concentration (ppm)

Equations:

$N_2O\ \{g\} = N_2O\ \{aq\}$ (Stumm et al., 1996)
$H = K_H RT$
$y_{N2O}{}^*P = \chi_{N2O}{}^*H_{N2O}$ (Henry's Law)

Constants:

@ 25 C: $K_H = 2.57\times10^{-2}$ M atm^{-1}
R = 0.082057 L atm K^{-1} mol^{-1}
T = 293.15 K
MW_{N2O} = 44
P_T = 1 atm (assumption)

Solve for H:

H = 0.6182 (N_2O dissolution in water)

Solve for y_{N2O} from C_{N2O} = 0.34 ppm = 0.34 μg/mL—

(0.34 μg/mL) (1200 mL) = 408 μg N_2O in headspace volume
(4.08×10^5 mg N_2O) (1 mol/44 mg N_2O) = 9.27×10^3 mol N_2O

Solve for N_2O from Henry's Law above—

x_{N2O} = [(9.27×10^3 mol N_2O) (1 atm)]/(0.6182)
= 1.5×10^4 mol N_2O in aqueous phase

Determine ppm concentration in aqueous phase--

(1.5×10^4 mol N_2O) (44 mg N_2O/1 mol) = 6.6×10^5 mg N_2O = 6.6×10^8 μg N_2O
(6.6×10^8 μg N_2O) (1/1000 mL) = 6.6×10^5 ppm N_2O (aqueous phase at 20°C)

Determination of Biomass Volume

Sandstone-Porous Column ρ_{sand} =1.53 V_{sand}= 589 cm^3

			Before 500°C		After 500°C	
Tare #	Mass (g)	Section	Tare & Sand (g)	Wet Sand (g)	Tare & Sand (g)	Dry Sand (g)
1	67.07	1	321.38	254.31	268.5	201.43
M2	67.56	2	272.75	205.19	234.35	166.79
3	48.69	3	326.44	277.75	276.47	227.78
4	47.26	4	235.88	188.62	201.58	154.32
5	43.15	5	205.62	162.47	176.62	133.47
				1088.34		883.79

Biofilm vs. Suspended Cells

Section	Sample volume (ml - ρ_{sand})	mg volatile solids/L	mg fixed solids/L	Biofilm attached cells (g)
1	166.22	318.14	1211.86	2.54
2	134.11	286.33	1243.67	2.05
3	181.54	275.26	1254.74	2.78
4	123.28	278.23	1251.77	1.89
5	106.19	273.10	1256.90	1.62
				10.88

Biofilm versus Suspended Cells

Sect	Sample volume (ml - (sand)	mg volatile solids/L	mg fixed solids/L	Biofilm attached cells (g)	Sample ports	Avg. cells/mL	Biomass suspended cells (g)
1	131.65	401.66	1530.00	2.01	Port 1	4.05E+06	3.84E-05
1-2	240.67	159.56	693.03	3.68		ND	ND
1-3	389.54	128.28	584.74	5.96	Port 2	4.03E+06	1.13E-04
1-4	490.41	69.94	314.68	7.50		ND	ND
1-5	577.64	50.20	231.06	8.84	Effl	3.16E+06	1.31E-04

Sect	sample volume (ml - (calc)	mg volatile solids/L	mg fixed solids/L	Biofilm attached cells (g)	Sample ports	Avg. cells/mL	Biomass suspended cells (g)
1	117.75	449.09	1710.66	1.80	Port 1	4.05E+06	3.43E-05
1-2	235.50	163.06	708.24	3.60		ND	ND
1-3	353.25	141.46	644.81	5.40	Port 2	4.03E+06	1.02E-04
1-4	471.00	72.82	327.64	7.21		ND	ND
1-5	588.75	49.26	226.70	9.01	Effl	3.16E+06	1.34E-04

ND=No Data

Biofilm versus Suspended Cells

Calculation of biofilm cells (all 5 sections of column):

$\rho_{sand} = 1.53 g/cm^3$

$V_{sand} = \frac{5}{5}(30cm)(\pi)(2.5cm)^2 = 589cm^3$

$(1.53\frac{\text{g sand}}{\text{cm}^3\text{ sand}})(589cm^3)(2.18\frac{\text{mg biomass}}{\text{g sand}})(\frac{g}{1000mg}) = 1.96$ g dry biomass

Calculation of suspended cells:

$$(3.16\times10^6\frac{\text{cells}}{\text{ml}})(0.30\frac{\mu m^3}{\text{cell}})(\frac{1\text{ml}}{1\text{cm}^3})(589cm^3)(1.09\times10^{-12}\frac{\text{g wet cell}}{\mu m^3})$$
$$(0.22\frac{\text{g dry cell}}{\text{g wet cell}}) = 1.34\times10^{-4}\text{g dry cell}$$

Therefore, the biomass of attached cells is about four orders-of-magnitude greater than the biomass of the suspended cells.

Calculation of biofilm thickness:

$$\rho_{biomass} = (1.07\times10^{6}\frac{\text{g wet cell}}{\text{m}^3})(0.22\frac{\text{g dry cell}}{\text{g wet cell}}) = 2.35\times10^{5}\frac{\text{g dry cell}}{\text{m}^3}$$

$$\rho_{sand} = (1.53\frac{\text{g}}{\text{cm}^3})(100\frac{\text{cm}}{\text{m}})^3 = 1.53\times10^{6}\frac{\text{g sand}}{\text{m}^3}$$

$$\rho_{sandparticle} = \left(1.53\times10^{6}\frac{\text{g sand}}{\text{m}^3}\right)\left(\frac{1}{1-0.22}\right) = 1.96\times10^{6}\frac{\text{g sand}}{\text{cm}^3}$$

$$\frac{\left(\frac{1.96\text{g dry biomass (cells)}}{2.35\times10^{5}\text{ dry cell/m}^3}\right)}{\left(\frac{(1.53\text{g sand/cm}^3\text{ sand})(589\text{cm}^3)}{1.96\times10^{6}\text{ g sand/m}^3}\right)} = 0.018\frac{\text{m}^3\text{ dry cell}}{\text{m}^3\text{ sand}}$$

$$0.018\ \frac{\text{m}^3\text{dry cell}}{\text{m}^3\text{ sand}} = \left(\frac{V_2}{V_1}\right)\left(\frac{1}{1-0.22}\right)$$

$$= \frac{4\pi(110\times10^{-6}\text{m})^2 L_f}{4/3\pi(110\times10^{-6}\text{m})^3}\left(\frac{1}{1-0.22}\right)$$

$$0.018\ \frac{\text{m}^3\text{dry cell}}{\text{m}^3\text{ sand}} = \frac{3L_f}{110\times10^{-6}}(1.28)$$

Solving for L_f :

$$L_f = 0.52\times10^{-6}\text{m} = \underline{\underline{0.52\ \mu\text{m}}}$$

Solving for the biofilm thickness results in L_f = 0.52 μm for a measured porosity of 0.22 and a measured biomass of 2.18 mg biomass/g sand.

Determination of yields for entire column length (Method 2):

Calculate formation of biomass within column:

$$\pi(2.5\ \text{cm}^2)^2(30\ \text{cm})(1.53\frac{\text{g}}{\text{cm}^3})(2\frac{\text{mg dry biomass}}{\text{g sand}})(\frac{\text{g}}{1000\ \text{mg}}) = 1.8\ \text{g biomass}$$

Calculate overall nitrate consumed:

$$(0.13\frac{\text{ml}}{\text{min}})(60\frac{\text{min}}{\text{hr}})(24\frac{\text{hr}}{\text{day}})(256\ \text{days})(500\frac{\text{mg}}{\text{L}})(\frac{\text{L}}{10^3\text{mL}})(\frac{\text{g}}{10^3\text{mg}}) = 23.9\ \text{g}\ NO_3^-$$

Calculate overall acetate consumed:

$$(0.13\frac{\text{mL}}{\text{min}})(60\frac{\text{min}}{\text{hr}})(24\frac{\text{hr}}{\text{day}})(225\ \text{days})(400\frac{\text{mg}}{\text{L}})(\frac{\text{L}}{10^3\ \text{mL}})(\frac{\text{g}}{10^3\ \text{mg}}) = 16.9\ \text{g}\ CH_3COO^-$$

Calculate overall lactate consumed:

$$(0.13\frac{\text{mL}}{\text{min}})(60\frac{\text{min}}{\text{hr}})(24\frac{\text{hr}}{\text{day}})(57\ \text{days})(400\frac{\text{mg}}{\text{L}})(\frac{\text{L}}{10^3\ \text{mL}})(\frac{\text{g}}{10^3\ \text{mg}}) = 4.27\ \text{g}\ CH_3CH_2COO^-$$

Calculate yield for dry biomass to nitrate:

$$Y_{xe} = \frac{1.8\ \text{g dry biomass}}{23.9\ \text{g}\ NO_3^-} = 0.08\ \frac{\text{g}}{\text{g}}$$

Calculate yield for dry biomass to acetate:

$$Y_{xs} = \frac{1.8\ \text{g dry biomass}}{16.9\ \text{g}\ CH_3COO^-} = 0.11\ \frac{\text{g}}{\text{g}}$$

Determination of yields up to Sample Port 1:

Calculate yield for dry biomass to lactate:

$$Y_{xs} = \frac{1.8 \text{ g dry biomass}}{4.27 \text{ g } CH_3CH_2COO^-} = 0.42 \frac{g}{g}$$

Calculate formation of biomass within column:

$$\pi(2.5 \text{ cm}^2)^2(6 \text{ cm})(1.53\frac{g}{cm^3})(2\frac{\text{mg dry biomass}}{\text{g sand}})(\frac{g}{1000 \text{ mg}}) = 0.36 \text{ g biomass}$$

Calculate yield for dry biomass to nitrate:

$$Y_{xe} = \frac{0.36 \text{ dry biomass}}{23.9 \text{ g } NO_3^-} = 0.02 \frac{g}{g}$$

Calculate yield for dry biomass to acetate:

$$Y_{xs} = \frac{0.36 \text{ g dry biomass}}{16.9 \text{ g } CH_3COO^-} = 0.02 \frac{g}{g}$$

Determination of yields up to Sample Port 2:

Calculate formation of biomass:

$$\pi(2.5 \text{ cm}^2)^2(15 \text{ cm})(1.53\frac{g}{cm^3})(2\frac{\text{mg dry biomass}}{\text{g sand}})(\frac{g}{1000 \text{ mg}}) = 0.90 \text{ g biomass}$$

Calculate yield for dry biomass to nitrate:

$$Y_{xe} = \frac{0.9 \text{ dry biomass}}{23.9 \text{ g } NO_3^-} = 0.04\frac{g}{g}$$

Calculate yield for dry biomass to acetate:

$$Y_{xs} = \frac{0.9 \text{ g dry biomass}}{16.9 \text{ g } CH_3COO^-} = 0.05\frac{g}{g}$$

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