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AEROBIC DENITRIFICATION AS AN INNOVATIVE METHOD FOR IN SITU BIOLOGICAL REMEDIATION OF CONTAMINATED SUBSURFACE SITES

L.N. BRITTON

TEXAS RESEARCH INSTITUTE, INCORPORATED
9063 BEE CAVES ROAD
AUSTIN TX 78733

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The objective of this project was to conduct laboratory testing of an aerobic denitrification concept for the enhancement of *in situ* bioremediation of subsurface sites. Microbial enrichments using phenol, dodecane, toluene, naphthalene, and methylene chloride demonstrated that many soil organisms classically associated with degradation of these organics are also capable of respiring with nitrate. Chemostat experiments with an isolate from the enrichments were performed to follow phenol degradation at varying oxygen concentrations. Nitrate respiration and aerobic phenol degradation could occur simultaneously at oxygen concentrations as high as 10 percent in the sparging gas. Thus the concept of aerobic denitrification was proven. An enhancement of biodegradation of phenol occurred when the isolate was provided nitrate, in lieu of oxygen, as a final electron acceptor for respiration. This enhancement was attributed to the decreased oxygen demand for phenol oxidation.

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19. Tentative evidence using a mixed culture demonstrated that nitrate could increase mineralization of certain organics in limiting oxygen.

The study provided proof of concept that the addition of nitrate could enhance *in situ* biodegradation of organic contaminants in subsurface sites by decreasing the oxygen demand.

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EXECUTIVE SUMMARY

The objective of this project was to provide laboratory testing of an aerobic denitrification concept for the enhancement of *in situ* bioremediation of contaminated subsurface sites. The rationale behind this approach is that oxygen, which is essential for rapid biodegradation of typical organic contaminants such as hydrocarbons, is limiting in the subsurface. The addition of nitrate under aerobic conditions would provide an alternate final electron acceptor for microbial respiration (the major consumer of oxygen) and thereby decrease the oxygen requirement for catabolism of the organics. The end result would be enhancement of biodegradation by increasing the rate and/or extent of oxidation of the organic contaminants by soil microorganisms.

The project was conducted to answer three questions. The first question was does the addition of nitrate enrich for microorganisms that are qualitatively different than those associated with aerobic catabolism of selected organics? Enrichments on phenol, dodecane, toluene, naphthalene, and methylene chloride were performed with and without added potassium nitrate and under anaerobic, 0.5 percent and 21 percent oxygen. There were no qualitative differences in the population with respect to degradation of the selected organics, and it was concluded that many soil microorganisms classically associated with degradation of the organics are also capable of respiring with nitrate (i.e., they are nitrate reducers).

The second question was does aerobic denitrification (dissimilative reduction of nitrate in the presence of oxygen) occur at levels of oxygen adequate for aerobic catabolism of organics? An isolate, designated T-33, isolated from enrichment experiments was used in chemostat studies to follow phenol degradation at varying oxygen concentrations. The concept of aerobic

denitrification was demonstrated. That is, T-33 suspended cells could aerobically degrade phenol while simultaneously carrying out nitrate respiration, a process that classically is considered to be anaerobic. A window of aerobic denitrification was established for this culture in which both nitrate respiration and phenol degradation could occur. The window extended from near zero percent to approximately ten percent oxygen in the sparging gas.

The third question was does the addition of nitrate to oxygen-limited environments enhance biodegradation of organic contaminants? Studies with T-33 demonstrated that nitrate enhanced the biodegradation of phenol at limiting oxygen concentrations. This enhancement of degradation was the result of nitrate acting as final electron acceptor for respiration and thus, for a given amount of phenol oxidized, less oxygen was required. Tentative evidence using a mixed culture demonstrated that nitrate could increase mineralization of certain organics in limiting oxygen.

The project provided proof of concept that the addition of nitrate could enhance *in situ* biodegradation of organic contaminants in subsurface soils by decreasing the oxygen demand. A number of advantages are expected from this approach including:

- reliance on indigenous microbial populations;
- expected good results with biofilms, the presumed subsurface conditon;
- more rapid mineralization of organics and secondary metabolites;
- decrease in the biochemical oxygen demand and the requirement for intense oxygenation in order to enhance bioremediation.

PREFACE

This report was prepared by Texas Research Institute, Austin, Texas, under Contract Number FO 8635-86-C-0158 for the Air Force Engineering and Services Center, Engineering and Services Laboratory, (AFESC/RDVW), Tyndall Air Force Base, Florida 32403-6001.

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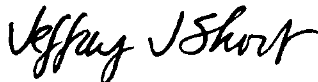

JEFFREY J. SHORT, GS-12
Project Officer

THOMAS J. WALKER, Lt Col, USAF, BSC
Chief, Environics Division

F. THOMAS LUBOZYNSKI, Maj, USAF, BSC
Chief, Environmental Engineering Branch

JAMES R. VAN ORMAN
Deputy Director of Engineering and Services Laboratory

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SECTION I

INTRODUCTION

A. OBJECTIVE

The objective of this project was to provide preliminary laboratory testing of an innovative site-remediation technology for use at Air Force facilities. Specifically, the concept of "aerobic denitrification" was examined as a method for enhancing the biodegradation of organic contaminates found in subsurface sites. Oxygen is often the limiting factor for biodegradation in subsurface environments, and therefore, enhancement of biodegradation requires either that the oxygen concentration be increased or that the demand for oxygen be lowered. Aerobic denitrification refers to the use of nitrate as a final electron acceptor in respiration in spite of the presence of oxygen. In practical terms, nitrate could be added to contaminated underground sites and theoretically could decrease the total biochemical oxygen demand by providing an alternative electron acceptor (i.e., nitrate) for respiration, one of the two main processes that typically consume oxygen. This Phase I project was a proof-of-concept approach to determine whether aerobic denitrification could improve the current technology.

B. BACKGROUND

The contamination of soil and groundwater with hazardous substances has created unusual problems for containment and remediation. An attractive

treatment technology is *in situ* remediation, whereby the indigenous microbial population is stimulated to promote degradation of organic contaminants. Table 1 lists examples of subsurface remediations and specifies whether active or passive approaches were used. The passive approach means doing nothing and letting nature takes its course. The active approach includes the stimulation of microbes *in situ*, the addition of special microorganisms, and/or the removal of contaminated groundwater followed by treatment above ground.

With *in situ* remediation, oxygen is often needed for rapid biodegradation of organics, especially hydrocarbons. Because of the sparing solubility of oxygen in water and because large amounts of oxygen are being consumed at contaminated subsurface regions, oxygen is a limiting factor in subsurface environments. Raymond (References 3-6) reported that oxygen was the limiting factor for *in situ* gasoline bioremediation although the injected nutrient solution was vigorously aerated by sparging. The American Petroleum Institute funded research to resolve this problem during *in situ* biological cleanup. Britton (Reference 30) examined the use of hydrogen peroxide as a source of oxygen. The catalytic decomposition of hydrogen peroxide by bacterial catalases releases molecular oxygen, making oxygen available for respiration and substrate oxidation. An advantage of using hydrogen peroxide, versus adding oxygen by sparging, is that hydrogen peroxide is freely soluble in water, therefore making it easier to achieve higher concentrations of oxygen at the target site. Hydrogen peroxide was found to be stabilized by phosphates and was shown to enhance gasoline biodegradation when employed in tolerable concentrations (<2000 ppm). The use of hydrogen peroxide for *in situ* biorestoration has since become popular, and specific examples of its application are given in Table 1.

TABLE 1. CASE HISTORIES OF RENOVATION OF SUBSURFACE SITES BY BIOLOGICAL PROCESSES

	INCIDENT	CLEANUP PROCEDURES/COMMENTS	REFERENCES
1.	Gasoline leakage in urban California area.	Passive. 250-fold increase in gasoline degraders.	1
2.	Gasoline pipeline rupture near Barrow, Alaska.	Passive. Enrichment of hydro-carbon - utilizing bacteria observed in contaminated lake sediments.	2
3.	Gasoline pipeline rupture in Ambler, Pennsylvania.	Active. Enhancement of gasoline degraders by addition of nitrogen, phosphorus and oxygen.	3, 4, 5
4.	Gasoline spill from storage tank in Millville, N.J.	Active. Enhancement of gasoline degraders by addition of nitrogen, phosphorus and oxygen.	6
5.	Gasoline leak from storage tank in La Grange, Oregon.	Active. Enhancement of gasoline degraders by addition of mineral growth medium and oxygen.	7
6.	Subsurface spill of a mixture of methylene chloride, acetone, n-butyl alcohol and dimethyl aniline at the Biocraft Laboratories in Waldwick, N.J.	Active. Aboveground biotreatment of groundwater followed by injection of aerated nutrients into underground contaminated area.	8
7.	Rhode Island Superfund site contaminated with phenol and substituted phenols including chlorinated phenols and nitrophenols.	Active. Biostimulation with plowing and nutrient additions followed by cosubstrate addition.	9
8.	Hydrocarbons in groundwater from a petroleum products spill at a rail yard in Karlsruhe, West Germany.	Active. Withdrawal of groundwater, ozonation, and reinjection. Increased oxygen levels resulted in increased bacterial counts and decreased DOC concentrations.	10
9.	Contamination of soil and groundwater from an acrylonitrile spill in Indiana.	Active. Aboveground treatment of groundwater with commercial source of bacteria (Polybac Corp.).	11
10.	Groundwater contamination from a spill of phenol and chlor-inated derivatives in central Michigan.	Active. Injection of mutant bacteria into soil and runoff containment pond.	12

TABLE 1. CASE HISTORIES OF RENOVATION OF SUBSURFACE SITES BY BIOLOGICAL PROCESSES (CONTINUED)

	INCIDENT	CLEANUP PROCEDURES/COMMENTS	REFERENCES
11.	Contamination of soil and shallow groundwater from a spill of various organics including ethylene glycol and propyl acetate.	Active. After physical methods reduced the contaminant levels, biostimulation was conducted by injecting special bacteria, nutrients, and air.	13
12.	Groundwater contamination with dichlorobenzene, methylene chloride, and trichloroethane.	Active. After an air-stripping primary treatment, commercial hydrocarbon - degrading bacteria and nutrients were injected into the contaminated underground area.	14
13.	Contamination of shallow aquifer from a mixed organic spill.	Active. Polishing step consisting of injection of specific hydrocarbon degrades, nutrients and oxygen.	14
14.	Contamination of groundwater with volatile organics, phenols, aromatics and other compounds at the Ott/Story site in Muskegon, Michigan.	Active. Aboveground treatment of groundwater using a commercial microbial culture.	15
15.	Contamination of soil and groundwater from gasoline.	Active. Air sparging to remove volatiles and oxygenate; nutrients added to reinjected water.	16, 17
16.	Contamination of soil and groundwater from gasoline.	Active. Stimulation with nutrients and hydrogen peroxide.	18
17.	Contamination of soil and groundwater from gasoline.	Active. Several sites renovated using combinations of nutrients and dissolved oxygen additions via air stripping or hydrogen peroxide.	19, 20
18.	Contamination of soil and groundwater from gasoline.	Active. Oxygen supplied to the unsaturated zone by soil venting.	21
19.	Contamination from waste solvents and alkanes from a leaking tank.	Active. Stimulation with nutrients plus hydrogen peroxide.	19, 22 23, 24
20.	Contamination of groundwater with methylene chloride.	Active. Withdrawal and treatment above ground with air stripping followed by treatment in an activated sludge bioreactor and and subsequent reinjection.	25, 26

TABLE 1. CASE HISTORIES OF RENOVATION OF SUBSURFACE SITES BY BIOLOGICAL PROCESSES (CONCLUDED)

	INCIDENT	CLEANUP PROCEDURES/COMMENTS	REFERENCES
21.	Contamination of groundwater with ethylene glycol.	Active. Treatment above ground with degraders and subsequent reinjection.	26, 27
22.	Soil and groundwater contamination with isopropanol, acetone, and tetrahydrofuran.	Active. Treatment above ground with acclimated culture followed by nutrient addition and reinjection.	28
23.	Two separate incidences of subsurface contamination, one with an aliphatic plasticizer and the other with chloroform.	Active. Treatment in an above ground activated sludge bioreactor followed by reinjection of water amended with bacteria and nutrients.	29

Other methods for providing oxygen in the subsurface have been tried. Sparging of injection solutions, ozonation, and soil venting have been mentioned in case histories cited in Table I. A microdispersion of air, known as colloidal gas aphrons, has been shown to provide effective removal of hexadecane in laboratory biodegradation experiments and has potential for *in situ* applications (Reference 31). These 25-50 micron-sized gas bubbles within a surfactant encapsulant would be pumped through the pore spaces of sand and ultimately to the contaminated region.

Both real and potential problems of all of these oxygenation techniques point to the need to minimize the microbial utilization of oxygen without sacrificing rates or extent of biodegradation of organics. Decreasing the biochemical oxygen demand for degradation of subsurface organic contaminants can be used to decrease the intensity, costs and problems of subsurface oxygenation. This project was a feasibility effort to do exactly this - that is, to increase the availability of molecular oxygen for substrate oxidation reactions by establishing denitrifying conditions.

A number of benefits could be gained by using this approach. One is to decrease or possibly eliminate the added oxygen requirement for *in situ* bioremediation of sites containing a variety of organic contaminants. A second benefit is the possibility of enhanced biodegradation of organic mixtures in comparison to an intensely aerobic approach, since some organics may be more readily degraded under anaerobic conditions. Such is the case with halogenated organics. Although there are a number of papers reporting the dehalogenation of halogenated organics under aerobic conditions (References 32-43), anaerobic biotransformation by methanogenic bacterial cultures appears to be the most significant dehalogenation process in nature (Refer-

ences 32, 44-50). Intense aeration or peroxidation could decrease (but probably not eliminate) the contributions by this anaerobic group of microbes. This creates a problem because oxygen is needed for transformation of those organics that are metabolized obligatorily by an aerobic route. Oxygen may inhibit the strictly anaerobic catabolism, and more important, the lack of oxygen (or other suitable electron acceptors for respiration) can retard complete mineralization of organics. One resolution to this predicament may be found by examining an aerobic denitrification approach which uses nitrate in respiration, in an environment where oxygen is also present in limiting quantites.

This approach can be understood if one considers the fate of oxygen during oxidation of organics by aerobic microbial cultures. Typically, these cultures require molecular oxygen for two processes. Molecular oxygen is a substrate for oxygenases that act upon aromatic compounds as well as many other organics (e.g., aliphatics, heterocyclics). Molecular oxygen is also required as final electron acceptor in aerobic respiration. The establishment of denitrifying conditions by substituting nitrate for oxygen as the final electron acceptor may "free" the limited amounts of oxygen for use by oxygenases rather than being consumed by the cytochrome oxidases of the respiratory chain. The end result would be the oxidation of organic pollutants without the requirement for intense oxygenation of underground sites.

Biological denitrification refers to the dissimilatory reduction of nitrate and/or nitrite by microbes to the gaseous oxides nitric oxide (NO) and nitrous oxide (N_2O) which may further be reduced to dinitrogen (N_2). Many reviews on the subject (see References 51-54) stress the overall importance of denitrification in the nitrogen cycle and specifically the role of this

process in agriculture and waste treatment. Denitrification is an important concept in the ecology of soil and aquatic systems, and nitrate-reducing bacteria are ubiquitous in nature (References 55-57), occurring in great diversity in soil and water. Of the 20 major divisions of procaryotes described in the eighth edition of Bergey's Manual of Determinative Bacteriology (Reference 58), including the cyanobacteria, 16 contain one or more genera of nitrate reducers. Table 2 lists the genera of bacteria containing strains reported to reduce nitrate dissimilatively. The nitrate-reducing bacteria listed in Table 3 were compiled from Bergey's Manual and only include those nitrate reducers found in soil and water. The list is far from complete. Many denitrifying species, such as those from the genera *Moraxella* and *Achromobacter*, are commonly found in soil and water, but are not described or are listed in Bergey's Manual as "*species insertae sedis*".

The nitrate-reducing bacteria take on added significance when one realizes that these genera are frequently studied oxidizers of organic compounds including the hazardous organics at Air Force sites. It is not a coincidence that these denitrifying bacteria are involved in the degradation/transformation of organics in soil and water. In environments with a surplus of organic substrates, oxygen is in short supply, as in most contaminated underground environments and in lakes undergoing eutrophication. Even though there may be an excess of nonfermentable organics, their decomposition by microbial processes will decline when oxygen is exhausted and there are no other sources of final electron acceptors. If nitrate is available in sufficient concentrations, denitrification can be a vehicle for soil and water microorganisms to continue oxidizing organic substances as oxygen becomes depleted. Nitrate salts are freely soluble (unlike oxygen) and are probably

TABLE 2. GENERA OF BACTERIA CONTAINING STRAINS REPORTED TO REDUCE NITRATE DISSIMILATIVELY

Actinobacillus
Actinomyces
Aeromonas
Agrobacterium
Alcaligenes
Arachnia
Arthrobacter
Bacillus
Bacteroides
Bacterionema
Beneckea
Bordetella
Branhamella
Brucella
Campylobacter
Cellulomonas
Chromobacterium
Citrobacter
Clostridium
Corynebacterium
Cytophaga
Dactylsporangium
Enterobacter
Erwinia
Escherichia
Eubacterium
Flavobacterium
Fusobacterium
Gemella (NO_2, not NO_3)
Geodermatophilus
Haemophilus
Halobacterium
Halocuccus
Hyphomicrobium
Hyphomonas
Klebsiella
Lactobacillus
Leptothrix
Listeria
Lucibacterium
Microbispora
Micrococcus
Micromonspora
Moraxella
Mycobacterium
Neisseria
Nocardia
Paracoccus
Pasteurella
Peptococcus
Photobacterium
Planobispora
Planomonspora
Plesiomonas
Propionibacterium
Proteus
Pseudomonas
Rhizobium
Rothia
Salmonella
Selenomonas
Serratia
Shigella
Simonsiella
Spirillum
Sporosarcina
Staphylococcus
Streptomyces
Streptosporangium
Thiobacillus
Thiomicrospira
Veillonella
Vibrio

From: Reference 57

TABLE 3. NITRATE-REDUCING BACTERIA FROM SOIL AND WATER

Pseudomonas aeruginosa
Pseudomonas stutzeri
Pseudomonas mendocina
Pseudomonas fluorescens
Pseudomonas chloraphis
Pseudomonas aureofaciens
Pseudomonas pseudomallei
Pseudomonas mallei
Pseudomonas caryophylli
Pseudomonas solanacearum

Halobacterium marismortui

Alcaligenes faecalis
Alcaligenes eutrophus

Chromobacterium violaceum

Flavobacterium capsulatum
Flavobacterium lutescens
Flavobacterium rigense

Paracoccus denitrificans
Paracoccus halodenitrificans

Acinetobacter calcoaceticus

Arthrobacter globiformis
Arthrobacter simplex
Arthrobacter tumescens
Arthrobacter citreus
Arthrobacter terregens
Arthrobacter flavescens
Arthrobacter duodecadis

Cellulomonas flavigena

Mycobacterium phlei
Mycobacterium smegmatis
Mycobacterium flavescens
Mycobacterium fortuitum
Mycobacterium peregrinum

Bacillus subtilis
Bacillus licheniformis
Bacillus cereus
Bacillus thuringiensis
Bacillus megaterium
Bacillus stearothermophilus
Bacillus coagulans
Bacillus firmus
Bacillus laterosporus
Bacillus brevis

Nocardia farcinica
Nocardia brasiliensis
Nocardia asteriodes
Nocardia formicae
Nocardia polychromogenes
Nocardia paraffinae
Nocardia rhodnii
Nocardia vaccinii
Nocardia minima

Nocardia cellulans
Nocardia rubropertincta
Nocardia corollina
Nocardia salmonicolor
Nocardia opaca

Nocardia atlantica

Streptomyces (all species)

Micrococcus roseus
Micrococcus varians

From: Bergey's Manual, Eighth Edition (Reference 58)

more accessible to underground environments than oxygen because diffusion of oxygen through soil strata is retarded.

The term "aerobic denitrification" would appear to be contradictory considering that denitrification is generally accepted as a process requiring anaerobic conditions. However, this generalization is simplistic and belies the complexity of the genetics and biochemistry of denitrification. Furthermore, "anaerobic" is a subjective term that does not necessarily mean the total absence of oxygen. There are notable exceptions to the dogma that denitrification is a strictly anaerobic process (References 53, 54).

It is intuitive that the processes of aerobic degradation of organics and denitrification can occur simultaneously, and the literature supports this contention. Sachs and Barker (Reference 59) in 1949 observed that *Pseudomonas denitrificans* reduced nitrate at oxygen tensions as high as 5 percent. Similar observations with *P. fluorescens* (Reference 60), *P. aeruginosa* (References 61, 62), *Haemophilus parainfluenza* (Reference 63), *Bacillus stearothermophilus* (Reference 64), and *Flavobacterium* sp (Reference 65) appear to prove that denitrification in many organisms does not require strict anaerobiosis.

Although the synthesis of nitrate reductase has been reported to be repressed by oxygen, aeration by shaking does not necessarily lead to repression of nitrate reductase, especially if the potential rate of oxygen consumption is greater than the rate in which oxygen is supplied (Reference 54). As Collins (Reference 62) explained, the high oxygen demand by actively metabolizing bacteria creates anoxic environments. Thus, anything that affects aeration, such as flask size and shape, would affect denitrification rates. It is expected that there is a critical level of oxygen tension (directly

related to oxygen demand of the culture) below which denitrification can occur. This was seen in steady-state growth of *Paracoccus denitrificans* (Reference 66).

In complex environmental systems, like trickling filters and soil, denitrification in the presence of oxygen also occurs. This has been observed in sewage treatment systems and in soil as exemplified by simultaneous nitrification (an oxygen requiring process) and denitrification (Reference 67). In laboratory-scale solid columns Misra *et al*. (Reference 68) found that the rate constant for nitrate reduction was independent of oxygen concentration.

Many factors influence the oxygen concentration in soil, including: (1) the oxygen consumption rate by the microbes; (2) the oxygen diffusion rate; (3) the geometry of the diffusion path, and probably many other factors (Reference 54). Anaerobic zones develop even though the bulk phase is aerobic. The growth of microorganisms as a film attached to soil components could account for the majority of the denitrification activity that occurs in aerobic environments. Through laboratory simulations and mathematical models, Strand *et al*. (References 69, 70) showed that dissolved oxygen in the bulk fluid had little effect on the denitrification rate in microbial films if the film was sufficiently thick for an anoxic layer to develop. The oxygen concentration in the biofilms is greatest at the interface with the aerobic fluid, which is where aerobically metabolizing cells are found. Aerobic respiratory activity results in oxygen depletion in the deeper recesses of the biofilms with subsequent denitrification. In the intermediate transition area of biofilms both aerobic metabolism and denitrification are possible. Therefore, aerobic denitrification in biofilms can be easily envisioned.

C. SCOPE AND TECHNICAL TASKS

The establishment of aerobic denitrification as an innovative method for *in situ* biological remediation of subsurface sites requires that certain questions be answered. These are listed below:

1. Does the addition of nitrate to oxygen-limited subsurface environents enrich for a qualitatively different group of microorganisms, in comparison to the usual aerobic enrichment? Stated in another way, what is the relationship of nitrate reducing and denitrifying soil microorganisms and their response to organic contaminants and varying oxygen concentrations?
2. Does denitrification occur at levels of oxygen that are adequate for aerobic catabolism of organics?
3. Does the addition of nitrate to oxygen-limited environments (i.e., aerobic denitrification) enhance biodegradation of organic contaminants? The term enhance can mean increases in rates and/or extent of degradation.

This Phase I study attempts to answer these basic research questions. The experimental work to answer these questions was grouped into five technical tasks:

1. Obtaining bacterial cultures.
2. Characterizing mixed cultures isolated by enrichment on organics in high oxygen and oxygen-limited plus nitrate environments.
3. Determining the effects of varying oxygen levels on denitrifying activity of pure and mixed cultures.

4. Determining the effects of oxygen on the induction of oxygenases.

5. Determining the effectiveness of aerobic denitrification in static cultures.

These technical tasks were originally proposed for the project and identified in the Laboratory Plan. It is difficult to present the results of these tasks without losing the overall goal of the project, namely to answer the three questions above. Also, some of the tasks overlapped or were combined in the experiments. Therefore, this report is organized in sections that address each question rather than each individual task.

Although this project examined the use of nitrate to enhance *in situ* bioreclamation, it did not look at the benefits of nitrate addition in the total absence of oxygen. There are some positive findings on the anaerobic catabolism of organic contaminants with nitrate present. The biochemical mechanisms are yet to be determined for some organics, and it is uncertain in some cases if oxygen was totally absent. On the other hand, the aerobic denitrification approach demonstrated in this project is easily explained with our current understanding of microbial biochemistry.

SECTION II

RELATIONSHIP OF NITRATE REDUCING AND DENITRIFYING SOIL MICROORGANISMS AND THEIR RESPONSE TO ORGANIC CONTAMINANTS

A. RATIONALE

An untested presumption of the aerobic denitrification concept is that the aerobic soil bacteria credited with biodegradation of the organics are also nitrate reducers. It is expected that some (but certainly not all) of the degraders in a population that arise in response to enrichment culturing with an organic compound will be nitrate reducers. However, an answer was needed to the question of whether or not the addition of nitrate to oxygen-limited environments enriches for a qualitatively different group of microorganisms in comparison to the usual aerobic enrichment. Therefore, experiments were designed to characterize mixed cultures from enrichments on organics in anaerobic, low, and high oxygen environments plus or minus the addition of nitrate.

B. MATERIALS AND METHODS

1. Selection of Organic Substrates

Toluene, n-dodecane, naphthalene, phenol, and methylene chloride were used individually as carbon and energy sources for enrichment of degraders. Because methylene chloride alone would not support growth, it was tested as a

cosubstrate. Acetate (0.1 percent) was added to vessels containing methylene chloride. The decision to select these compounds was based on: (1) their presence at contaminated Air Force sites; and (2) their representation of chemical classes (e.g., dodecane represents aliphatic contaminants like jet fuel; toluene and phenol represent aromatics; naphthalene represents poly nuclear aromatics; methylene chloride represents chlorinated aliphatics).

2. Enrichment Vessels and Their Operation.

Enrichment culturing was performed with each of the five substrates with 0 percent, 0.5 percent, and 21 percent molecular oxygen. Vessels for these experiments are depicted in Figure 1 and are referred to as continuous cycling, closed-loop biofilm columns. The bottom portion is a 4-ounce jar equipped with a perfluoroethylene-lined screw cap. A column is attached through the cap to the jar using polypropylene tube fittings and is connected via a greaseless ground-joint fitting to a filling tube and an exit tube for gases. Ninety milliliters (mL) of sterile mineral salts medium, designated MSB (Reference 71), with or without 0.1 percent (w/v) potassium nitrate were added to the reservoir of each sterile enrichment vessel. The column portion of each vessel contained 35 grams of sterile, acid-washed, 0.5 millimeter diameter, glass beads and was plugged with glass wool to keep the glass beads in place.

All vessels were inoculated identically into the glass bead sections with 0.5 grams composite soil from a petroleum sludge landfill, and 1 mL each of activated sludge from a petrochemical treatment facility and from a petroleum refinery, and groundwater from a site contaminated with petroleum

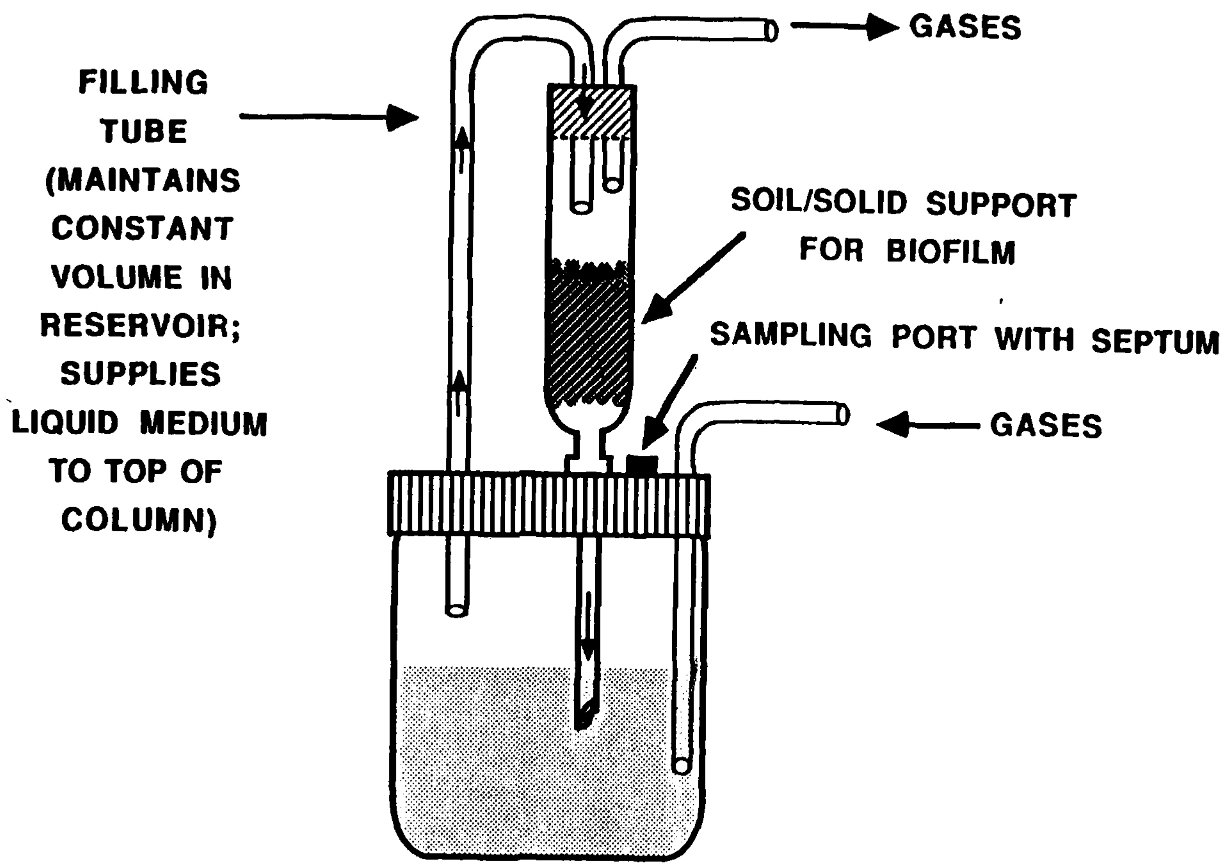


Figure 1. CONTINUOUS CYCLING, CLOSED LOOP BIOFILM COLUMN
(NOT TO SCALE)

refinery wastes. All samples were likely sources of microbial degraders of the substrates being examined.

After inoculation the vessels were supplied with the respective gases (100 percent nitrogen, 0.5 percent oxygen in nitrogen, or 21 percent oxygen in nitrogen) at a rate of approximately 250 mL/min. The buildup of pressure in the reservoir from the incoming gases would force liquid medium up the filling tube and into the column section, resulting in a continuous perfusion of the column with MSB medium. This perfusion would cycle in a closed-loop fashion, with the volume of liquid proportioned between the reservoir and column being dependent on the positioning of the filler tube in the reservoir. The columns were allowed to cycle for 18 hours before the carbon sources were added as vapors.

Upstream from the entrance of gases into the vessels, the liquid or solid forms of the five, individual organic substrates were placed in the gas streams. For dodecane, which has a comparatively low volatility, the gases were bubbled through an impinger containing the liquid dodecane. For the other substrates, gases were metered over, rather than through, the compounds. The concentrations of the organics in the gas streams were set at approximately 200 ppm for phenol, naphthalene, and dodecane and 50 ppm for methylene chloride and toluene. These concentrations were confirmed by gas chromatography. Since the water solubilities of these compounds differ, the concentrations in the media were quantified using a Tracor/Microtek (registered trademark) gas chromatograph equipped with a 25-meter BP-10 capillary column and flame ionization detection and operated isothermally at 70°C, 100°C , or 150°C depending on the substrate. The concentrations of the

organics in the MSB medium were: methylene chloride, 30 ppm; toluene, 15 ppm; phenol, 30 ppm; naphthalene, 10 ppm; and dodecane, 5 ppm.

To make the experiments manageable, 10 vessels were set up at a time for each of the oxygen concentrations (0, 0.5, or 21 percent oxygen). After the initial 18-hour equilibration time, the microbial population was examined in the medium. Then each of the substrates/carbon sources was supplied to two vessels, one with and one without 0.1 percent potassium nitrate. The microbial population was reexamined at 5, 11, and 15 days after substrate exposure. In addition, the columns were dismantled after 15 days, and the microbial population in the glass beads (i.e., biofilm) was examined.

3. Microbiological Methods

At each time point the following data were obtained:

- Nitrate concentrations by ion chromatography (Dionex 2000i);
- Total aerobic heterotrophs from plate counts on half-strength trypticase soy agar (TSA) (Difco Laboratories);
- Total anaerobic heterotrophs from plate counts on half-strength TSA and on nutrient agar (Difco) incubated anaerobically;
- Total nitrate reducers from plate counts on anaerobic nitrate agar (Difco);
- Total denitrifiers by most probable number (MPN) tube dilutions in nitrate broth tubes equipped with inverted Durham tubes to trap and score for nitrous oxide and molecular nitrogen;
- Total degraders of each of the five substrates from plate counts on MSB agar plates exposed to vapors of the appropriate carbon source.

All analyses and counts were run in duplicate, and all incubations were at room temperature (22 ± 2°C). A Coy Anaerobic Chamber was used to incubate plate media and broth tubes anaerobically.

Distinguishable colony types on TSA were cloned and characterized based on cell morphology, Gram staining, oxidase and catalase tests and biochemical properties established in API-NFT Rapid Test Strips.

C. RESULTS AND DISCUSSION

1. Distinction of Nitrate Reduction and Denitrification

Before the results of the enrichment experiments are presented, it is appropriate to distinguish the terms nitrate reduction and denitrification. The dissimilative reduction of nitrate proceeds by the following pathway:

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

Denitrification refers to the reduction of nitrate to the gaseous products nitrous oxide (N_2O) and/or molecular nitrogen (N_2). Nitrate reduction, on the other hand, specifies only that nitrate is reduced, leaving open the possibility that nitrite (NO_2^-) is the final product. All denitrifiers are nitrate reducers, but not all nitrate reducers are denitrifiers. This subtle difference takes on great significance in the soil since it is probable that a microbial population much larger than just the denitrifiers can utilize nitrate in respiration. To illustrate this point, 20 pure cultures of *Pseudomonas* species were tested for nitrate reduction (the ability to grow

anaerobically on nitrate agar) and denitrification (the production of nitrous oxide and/or nitrogen gas in nitrate broth). These results are presented in Table 4. Seven of the cultures reduced nitrate and grew anaerobically, yet they did not reduce nitrate to nitrous oxide or nitrogen. Although not indicated in the table, anaerobic plates without nitrate were included to demonstrate that nitrate was essential for anaerobic growth on nutrient agar. This simple experiment reinforces the assertion that nitrate respiration is more widespread in soil isolates than just among those classically defined as denitrifiers.

2. Enrichment Experiments

The enrichment vessels were designed and constructed to simulate conditions expected in the soil. The carbon source was supplied in the continuous gas stream and was unlimited, although it was supplied at a reduced concentration. Growth of microbes occurred in the bulk liquid phase as well as a biofilm attached to the glass beads in the column. As liquid medium percolated through the beads and came in contact with the biofilm, a gradient of carbon source and oxygen could form just as in the soil. In the absence of microbial oxygen consumption, the theoretical dissolved oxygen concentration would be 240 μM oxygen (7.7 ppm) in the 21 percent oxygen experiments and 3 μM oxygen (0.1 ppm) in the 0.5 percent oxygen experiments.

After the vessels were inoculated, they were cycled without carbon source for 18 hours before the Day 0 counts were determined. This permitted

TABLE 4. DISTINCTION OF NITRATE REDUCTION AND DENITRIFICATION AMONG PURE CULTURES OF PSEUDOMONAS[1]

Organism	Denitrifier	Nitrate Reducer
1. *Pseudomonas aeruginosa* (PAO-1, Holloway)	+	+
2. *P. aeruginosa* (pocon)	+	-[2]
3. *P. aeruginosa* (103)	+	+
4. *P. aeruginosa* (10145)	+	+
5. *P. aeruginosa* (HT 15540)	-	+
6. *P. aeruginosa* (16938)	-	+
7. *P. aeruginosa* (28576)	-	+
8. *P. aeruginosa* (30395)	-	+
9. *P. aeruginosa* (HT 32486)	+	+
10. *P. aeruginosa* (35455)	-	+
11. *P. aeruginosa* (HT 36495)	-	+
12. *P. aeruginosa* (36739)	+	+
13. *P. aeruginosa* (HT 37523)	-	+
14. *P. aeruginosa* (40866)	+	+
15. *P. fluorescens* (6130)	-	-
16. *P. fluorescens* (6380)	-	-
17. *P. pseudomallei* (like)	-	-
18. *P. pseudomallei* (papin)	-	-
19. *P. putrifaciens* (protease VII)	-	-
20. *P. stutzeri* (IM 604)	+	+

[1]Pure cultures were tested for denitrification and for the ability to grow anaerobically with nitrate as final electron acceptor (i.e., nitrate reducer).

[2]This species could not grow anaerobically; however, it was obvious that nitrate could be reduced.

the metabolism and growth from endogenous reserves and substrates added with the inoculum.

The data for these experiments are presented in Appendix A. Although the enrichments were examined for 15 days, the microbial populations stabilized after 5-9 days. Therefore, data from time points 0 and 5 or 9 days were most useful for comparisons of populations and the effects of nitrate and oxygen levels. The succeeding sections summarize the results for each of the three oxygen levels, and a final comparison is presented. However, before these results are given, the data from the methylene chloride enrichments must be qualified.

Since methylene chloride is normally metabolized as a cosubstrate, the enumeration of degraders is difficult. The plate medium for enumerating "degraders" from methylene chloride enrichment vessels was MSB plus 0.1 percent acetate plus methylene chloride vapors. Growth on the medium did not necessarily indicate the presence of methylene chloride degraders, but rather was a measure of acetate utilization in the presence of, and tolerance to, moderate levels of methylene chloride. To determine if methylene chloride utilization was occurring in the enrichment vessels, liquid samples from the 21 percent oxygen enrichments ($\pm$ nitrate) were mixed and used as an inoculum for an experiment to measure the utilization of methylene chloride during growth with acetate present. Triplicate culture tubes for each of four incubation conditions and two time points (0 and 6 days) were prepared with 20 ppm methylene chloride. The tubes were filled so that there was no headspace and the only oxygen available was dissolved in the medium. At 0 and 6 days the tubes were extracted with octane, and the methylene chloride quantified by GLC. The results are presented in Table 5. Loss of methylene chloride from

TABLE 5. FATE OF METHYLENE CHLORIDE IN A MIXED CULTURE OBTAINED FROM METHYLENE CHLORIDE ENRICHMENTS UNDER 21 PERCENT OXYGEN[1]

Sample/Condition	Methylene Chloride (ppm) T-0 Days	Methylene Chloride (ppm) T-6 Days
Control (Mineral Medium Only)	20	19.8
Mineral Medium + Inoculum	20	18.7
Mineral Medium + 0.1 Percent Acetate + Inoculum	20	13.0
Mineral Medium + Acetate + Nitrate + Inoculum	20	5.3

[1]Results are averages of triplicate samples for each condition.

the control tubes (MSB, MSB + inoculum) was minimal, while loss of methylene chloride from the tubes containing acetate and acetate plus nitrate was significant. The presence of nitrate resulted in an increased disappearance of methylene chloride (13 ppm vs. 5.3 ppm at 6 days). This difference was not due to differences in total cell mass and partitioning of methylene chloride in cell membranes, since the amount of growth in the two series was similar (data not shown). The beneficial effect of nitrate can be explained by permitting increased metabolic activities, at the expense of acetate, in an otherwise oxygen depleted condition and, thereby, providing for an increased "opportunity" to cometabolize the methylene chloride. The lack of oxygen should not hinder methylene chloride degradation since a likely mechanism that does not require molecular oxygen has been demonstrated in eucaryotic systems (Reference 72). This proposed pathway is shown in Figure 2. Although the enzyme mechanism(s) for methylene chloride degradation in bacteria is unclear, commonality may exist in procaryotic and eucaryotic systems for methylene chloride degradation.

The results from this methylene chloride degradation experiment indicate that methylene chloride degraders can arise in the enrichment vessels. However, the quantification of the numbers of degraders is difficult, and the reader should be aware that the numbers in Appendix A actually reflect acetate utilizers tolerant to methylene chloride and that some portion of this population can catabolize methylene chloride.

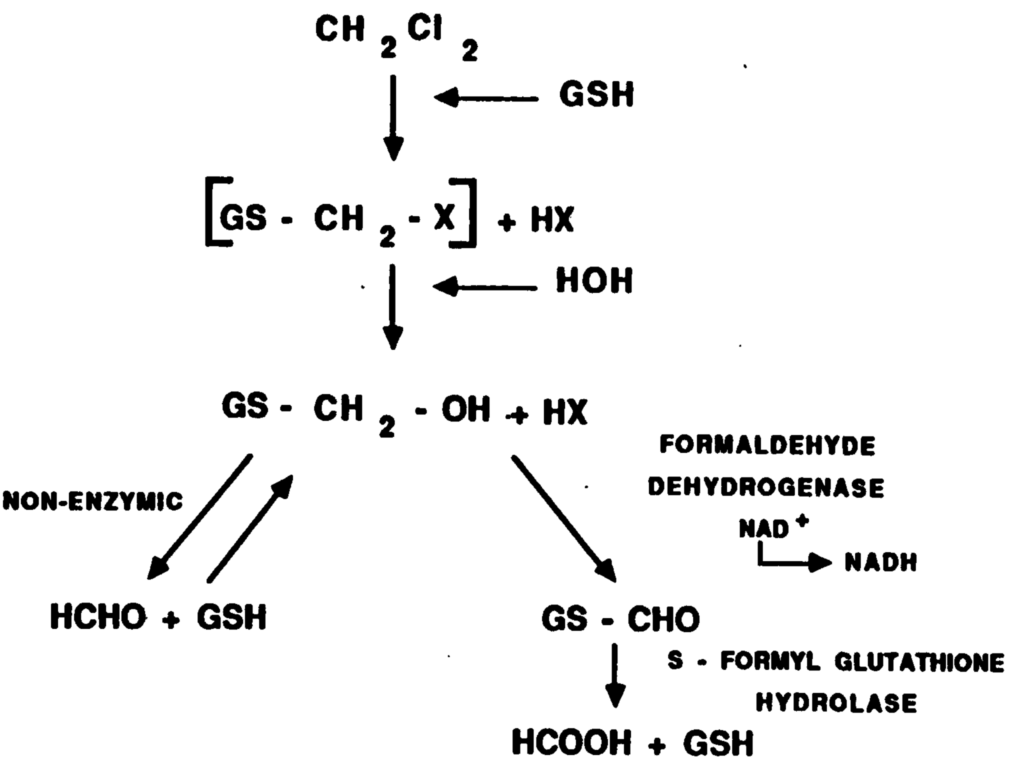


Figure 2. PROPOSED MECHANISM FOR METABOLISM OF METHYLENE CHLORIDE

a. 21 Percent Oxygen Enrichments

This set of enrichments provided a baseline for compaiisons with anaerobic and low oxygen conditions ± nitrate. The comparison of data for 21 percent oxygen apparatuses cycled with MSB versus those cycled with MSB + nitrate showed that enrichment of total degraders (excluding methylene chloride counts) was similar in most cases, and slightly greater for the substrates dodecane and toluene. Increases in the total numbers of denitrifiers or nitrate reducers were minimal in all experiments except for phenol and naphthalene. Of course, enrichment for nitrate-reducing bacteria was not expected at high oxygenation, and the results from the naphthalene enrichment experiment may be because the majority of naphthalene degraders were nitrate reducers as well.

b. Anaerobic Experiments

In comparison to aerobic enrichments, the anaerobic experiments did not demonstrate a dramatic increase in degraders. This was expected since the utilization of these as sole carbon sources is favored under aerobic conditions. In all of the vessels containing nitrate, growth was more pronounced than in the counterparts without nitrate. As a consequence, the number of degraders was considerably higher. The columns of vessels containing nitrate developed gas-containing voids, presumably due to voluminous production of nitrous oxide and/or nitrogen from denitrification. The increases in total heterotrophs and degraders under denitrifying conditions were more pronounced than expected since the growth had to be at the expense

of the organics introduced in the gas streams and the metabolites produced from their degradation. These sole carbon sources generally require aerobic conditions for degradation. Phenol and acetate/methylene chloride are exceptions. This does not imply that anaerobic degradation of these substrates cannot occur - indeed some reports have demonstrated this. However, rapid degradation is associated with aerobic conditions.

The possible introduction of oxygen into the liquid-tight vessels was examined, and it was found that the integrity of the vessels was tight and that only negligible amounts of oxygen would have diffused into the vessels. However, the instrument grade nitrogen that was constantly flushing through the vessels at approximately 250 mL/min contained measurable concentrations of oxygen (< 5 ppm gaseous oxygen). It is suggested that this extremely low concentration of oxygen could promote the aerobic catabolism of these organics, particularly if nitrate was in excess of that needed for respiration by aerobes. In essence, this was a crude demonstration of the concept that nitrate can promote metabolic activities in limiting oxygen and lead to utilization of organic contaminants, even those that are aerobically catabolized.

c. 0.5 Percent Oxygen Enrichments

As in the anaerobic experiments, the columns from vessels with nitrate developed extensive gas bubbles (evidence of denitrification). Also, the numbers of degraders were high, but in comparison with the complementary vessels without nitrate, the differences were not readily apparent. To put these results and those from the 21 percent oxygen and anaerobic experiments

into perspective, all of the data were analyzed on the basis of ratios of populations. This is explained in the following section.

d. Ratios of Populations

All of the vessels started with the same inoculum and continuous supply of carbon and energy source in the form of the selected organics. The differences in oxygen supply, or lack thereof, plus the presence and absence of nitrate resulted in the development of populations and subpopulations. The comparisons of just the concentrations of these microbial members among the differing enrichment conditions can be misleading *if the objective is to simply evaluate the relationship of nitrate reducing and denitrifying soil microorganisms and their response to organic contaminants*. Figure 3 helps in explaining the hierarchical arrangement of heterotrophic organisms responding to an organic carbon and energy source. In a heterotroph population there will be degraders and non-degraders of any particular organic carbon and energy source. The introduction of a carbon source results in the increase of degraders since they can utilize this carbon source. The number of non-degraders can be expected to increase because of the availability of other metabolites, and the numbers can be less or greater than the degraders. The degraders and nondegraders can be further classified by their ability to reduce nitrate in a dissimilatory manner, and the nitrate reducers can likewise be categorized as denitrifiers and nondenitrifiers.

The question that was posed at the beginning of this project was "does the addition of nitrate to an oxygen-limited environment enrich for a qualitatively different group of microorganisms in comparison to the usual

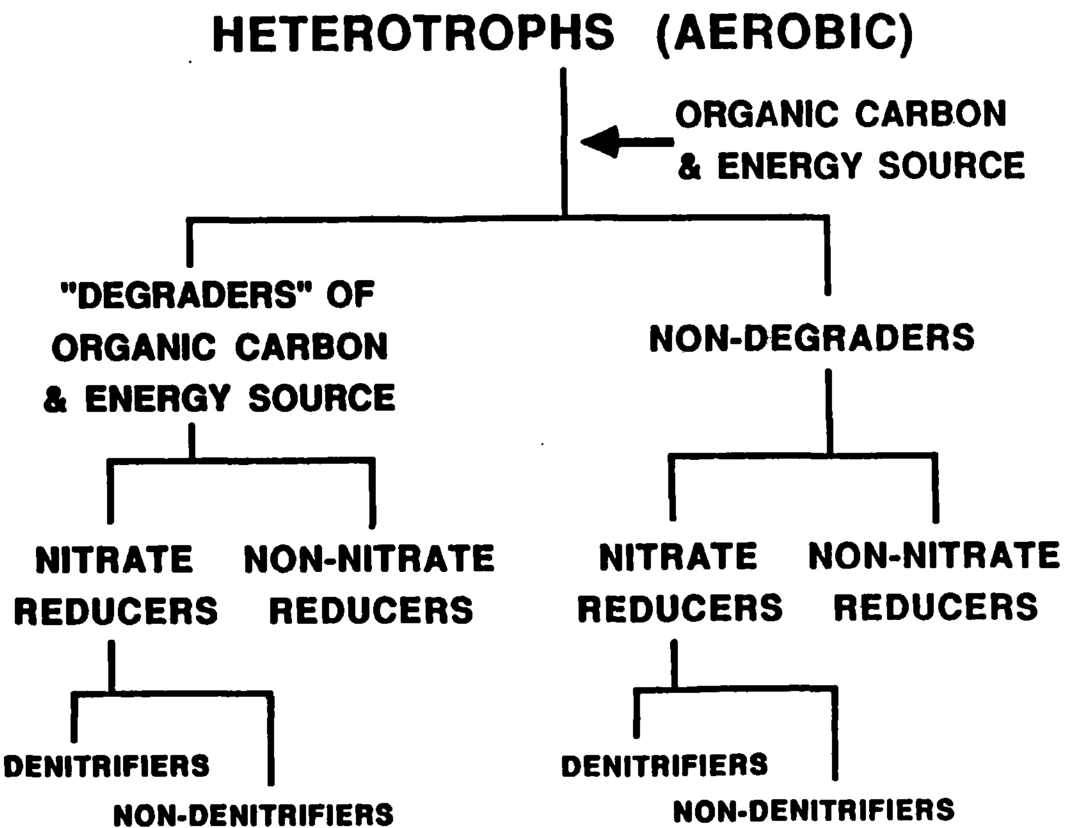


Figure 3. HIERARCHICAL ARRANGEMENT OF HETEROTROPHS

aerobic enrichments"? To answer this, the data from the enrichment experiments at Day 5 were plotted in the following three ratios:

- Ratio of degraders to heterotrophs;
- Ratio of nitrate reducers to degraders;
- Ratio of denitrifiers to degraders.

These ratios with dodecane, phenol, toluene, and naphthalene as carbon source and at 0, 0.5, and 21 percent oxygen are presented in Figures 4-7. A general conclusion can be drawn from these results. The presence of nitrate in oxygen-limited conditions generally results in a higher ratio of degraders to heterotrophs that can be quantified on the chosen media. There is no evidence to suggest that the addition of nitrate results in a qualitatively different population. In fact, a large portion of degraders are nitrate reducers. Also, as expected, there is a larger population of organisms that can respire on nitrate (i.e., nitrate reducers that probably accumulate nitrite) than those classically identified as denitrifiers.

3. Characterization of Isolates from Enrichment Experiments

Isolates were selected from the plating media for further characterization. These are shown in Tables 6-8. Degraders of the respective organics used in the selection process were easily isolated except for methylene chloride degraders. Methylene chloride degraders could probably have been isolated (based upon the information previously presented in Table 5); however, this would have required more extensive methods. It should be noted that the inoculum used (sludge landfarming soil, sludges from petrochemical and refinery treatments, and groundwater contaminated with refinery

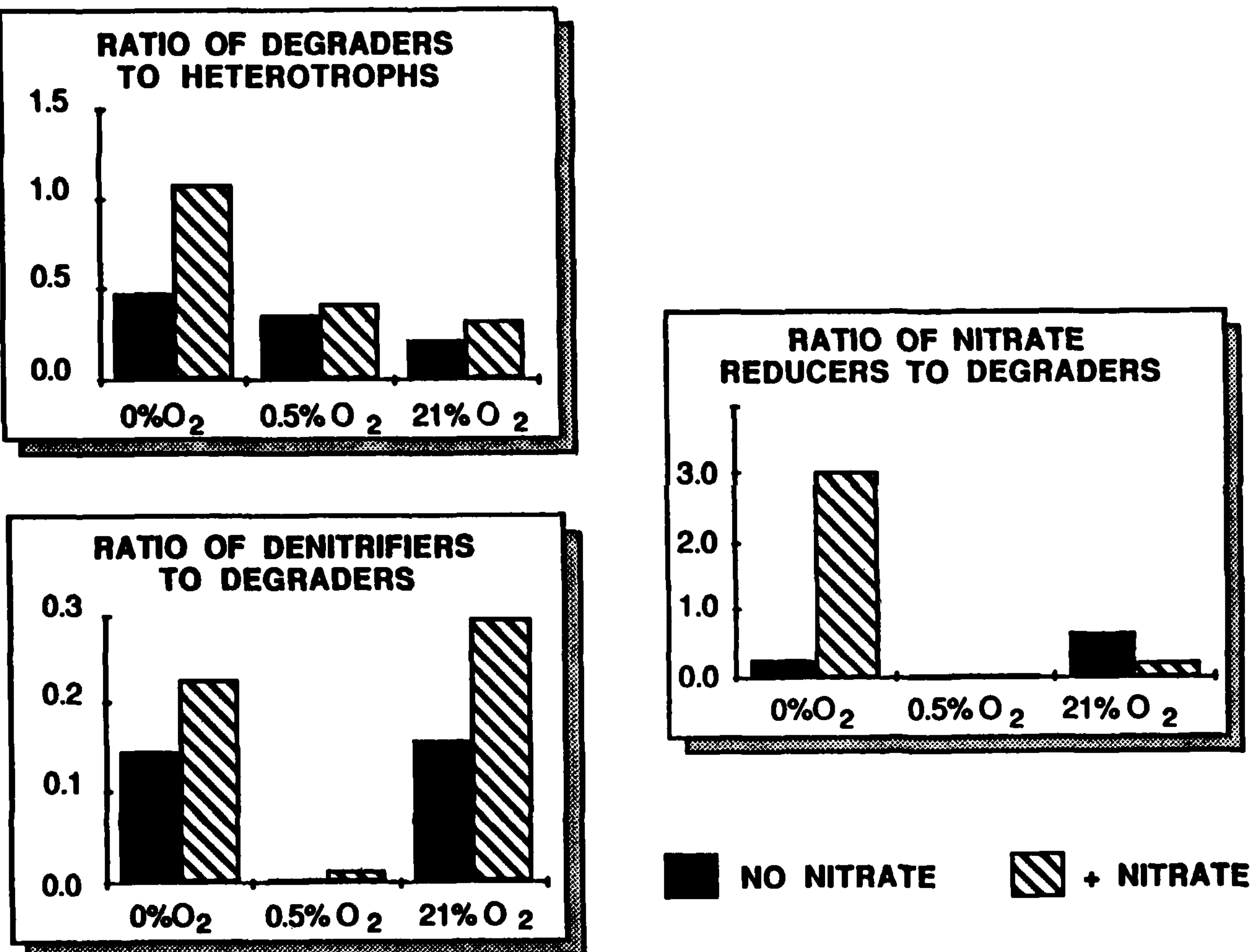


Figure 4. RATIOS OF POPULATIONS. PHENOL

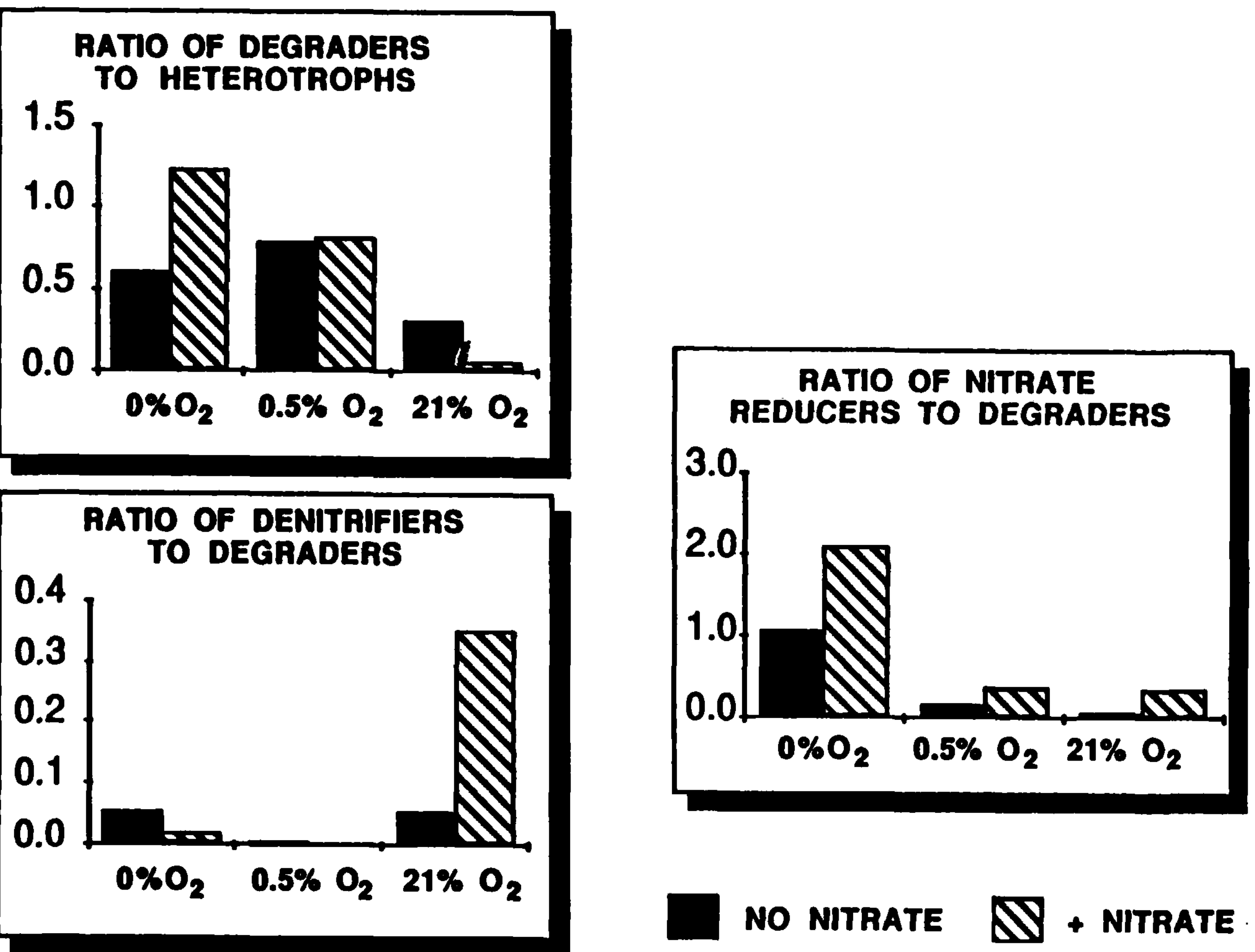


Figure 5. RATIOS OF POPULATIONS. DODECANE

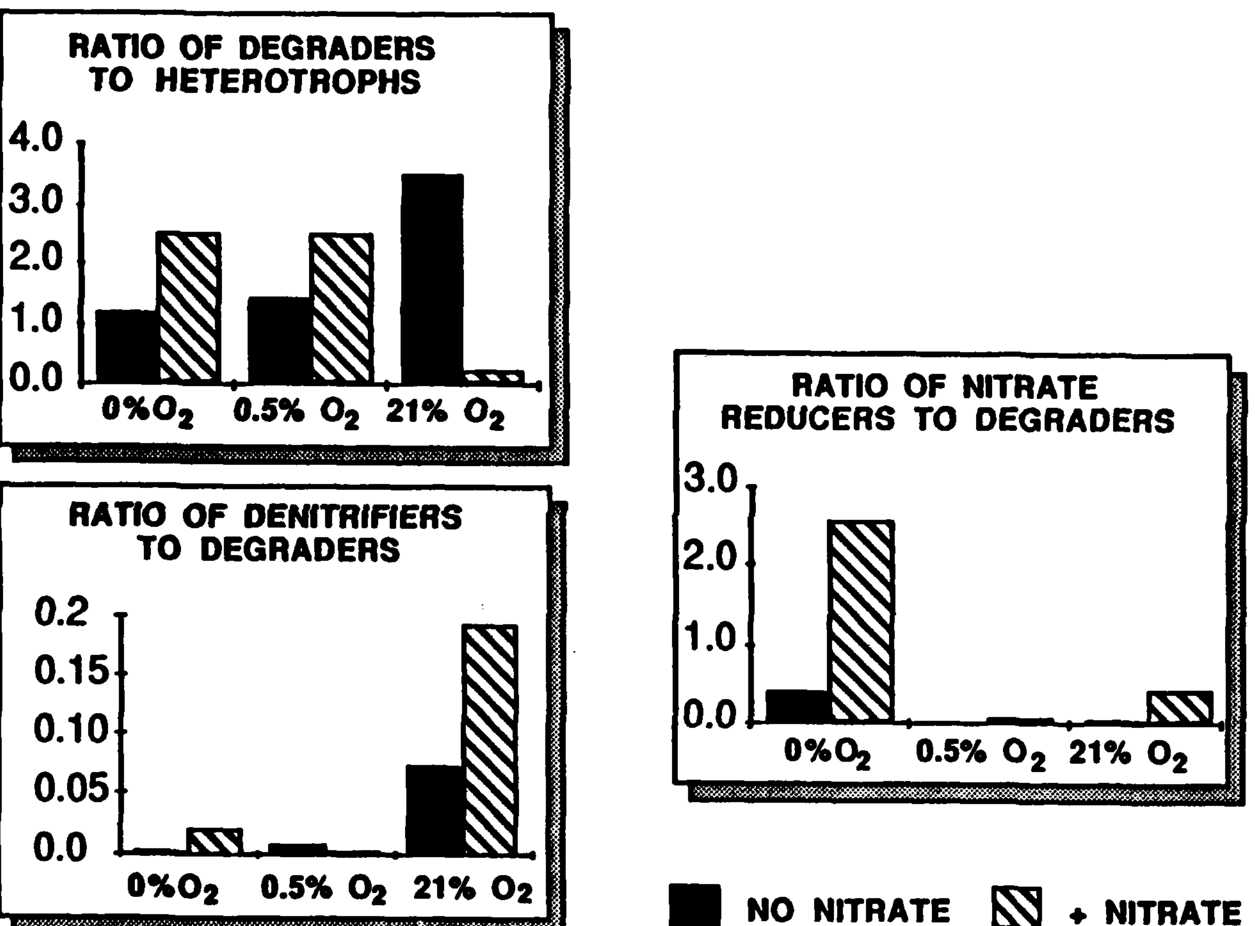


Figure 6. RATIOS OF POPULATIONS. TOLUENE

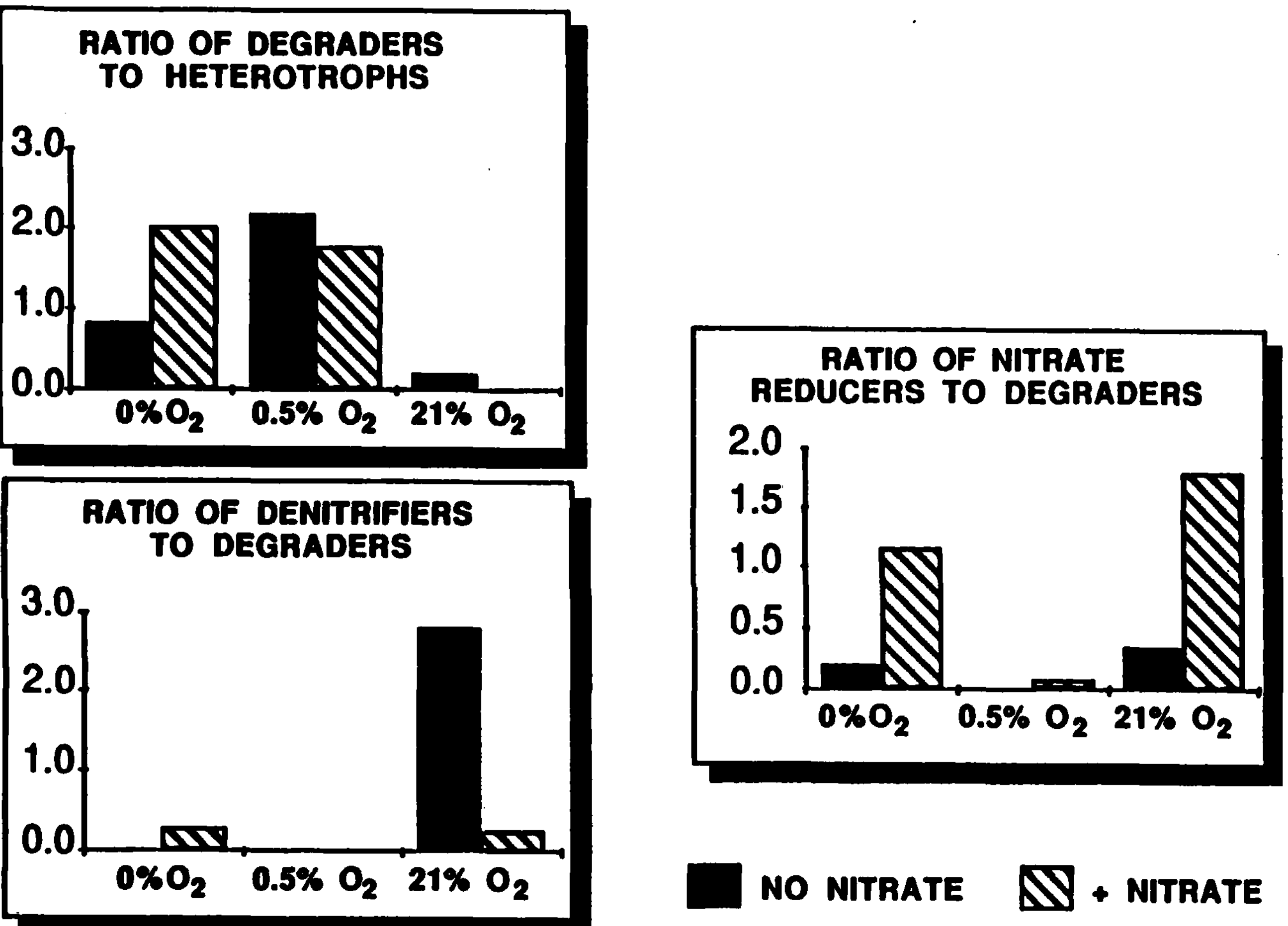


Figure 7. RATIOS OF POPULATIONS. NAPHTHALENE

TABLE 6. CHARACTERIZATION OF ISOLATES FROM ENRICHMENT EXPERIMENTS. ENRICHMENT UNDER 21 PERCENT OXYGEN CONDITIONS ± KNO_3

Isolate Designation	Source	Cellular Morphorlogy	TEST[1] Gram Rxn.	Cat.	Ox.	Degrader D	P	T	N	NRed.	DeN.	ID[2]
3b	D	rod	-	+	-	+	-	-	-	+	-	1
5	D	rod	-	+	-	+	-	-	-	-	-	
6	D	rod	-	+	-	+	-	-	-	-	-	
7	D	rod	-	+	-	+	-	-	-	-	-	
9	D	rod	-	+	+	+	-	-	-	-	-	
12	D+nit	coccoid	-	+	-	+	-	-	-	-	-	
17	P	coccoid	-	+	-	-	+	-	-	-	-	
18	P	rod	-	+	+	-	+	+	-	+	-	2
19	P	rod	-	+	+	-	+	+	-	+	-	
20	P	rod	+	+	-	-	+	-	-	-	-	1
21	P	rod	-	+	+	-	+	+	-	+	-	2
22	P+nit	rod	-	+	+	-	+	-	-	+	-	2
23	P+nit	rod	-	+	+	-	+		-	-	-	
24	P+nit	rod	-	+	+	-	+	-	-	-	-	3
27a	P+nit	rod	-	+	-	-	+	-	-	+	+	
31	M	coccoid	+	+	-	+	+	-	-	-	-	
34	M+nit	rod	-	+	+	-	-	-	+	+	+	
37b	M+nit	rod	-	+	+	-	+	-	-	+	+	4
40	T	rod	+	+	-	-	+	-	-	-	-	
41	T+nit	rod	-	+	+	-	-	-	-	+	+	
42a	T+nit	rod	-	+	+	-	-	-	-	+	+	5
42b	T+nit	rod	-	+	+	+	-	-	-	+	+	
43	T+nit	rod	-	+	+	-	+	-	-	-	-	
47	N	rod	-	+	+	-	-	-	+	+	+	2
49	N	rod	-	+	+	-	-	-	+	+	+	
50	N+nit	rod	-	+	+	-	-	-	+	+	+	2
52	N+nit	rod	-	+	+	-	-	-	+	-	-	
53	N+nit	rod	-	+	+	-	-	-	+	+	+	2

[1]Cat. = Catalase
Ox. = Oxidase
NRed. = Nitrate Reducers
DeN. = Denitrifiers
D = n-Dodecane
P = Phenol
M = Methylene Chloride
T = Toluene
N = Naphthalene
nit = Nitrate

[2]1 = *Acinetobacter calcoacetitus* var. *lwoffi*
2 = *Pseudomonas stutzeri*
3 = *P. paucimobilis*
4 = *Aeromonas hydrophilia*
5 = *P. picketti*

TABLE 7. CHARACTERIZATION OF ISOLATES FROM ENRICHMENT EXPERIMENT. ENRICHMENT UNDER ANAEROBIC CONDITIONS ± KNO_3

Isolate Designation	Source	Cellular Morphorlogy	TEST[1] Gram Rxn.	Cat.	Ox.	Degrader D P T N	NRed.	DeN.	ID[2]
A-16	P	rod	-	+	+	- - - +	-	+	1
A-18	P+nit	rod	-	+	+	- - - +	+	+	1
A-19a	P+nit	rod	-	+	+	- - + -	+	+	1
A-20	P+nit	rod	-	+	+	- - + -	+	+	1
A-26	T	rod	-	+	+	- - + -	+	+	1
M-10	P	rod	-	+	+	- + - -	+	+	2
M-13	N	rod	-	+	+	- - - +	+	-	1
M-14	N	rod	-	+	+	- + - -	+	-	1
M-15	N	rod	-	+	+	- - - +	+	-	3
M-20	N+nit	rod	-	+	+	- + - -	+	-	3
M-27	T+nit	rod	-	+	+	- - + -	+	-	1
M-31	T+nit	rod	-	+	+	- - - +	+	-	1
M-33	M	rod	-	+	+	- - - +	+	-	1
M-36	M	rod	-	+	+	- - - +	+	-	1
M-39	M+nit	rod	-	+	+	- - - +	+	-	1

[1]Cat. = Catalase
Ox. = Oxidase
NRed. = Nitrate Reducers
DeN. = Denitrifiers
D = n-Dodecane
P = Phenol
M = Methylene Chloride
T = Toluene
N = Naphthalene
nit = Nitrate

[2] 1 = *Pseudomonas stutzeri*
2 = *P. putida*
3 = *P. picketti*

TABLE 8. CHARACTERIZATION OF ISOLATES FROM ENRICHMENT EXPERIMENT. ENRICHMENT UNDER 0.5 PERCENT OXYGEN CONDITIONS ± KNO_3

				TEST[1]								
Isolate Designation	Source	Cellular Morphorlogy	Gram Rxn.	Cat.	Ox.	Degrader D	P	T	N	NRed.	DeN.	ID[2]
T-1	N+nit	rod	-	+	+	-	-	-	+	-	-	4
T-25	N	rod	-	-	+	-	+	-	-	-	-	1
T-26	N	rod	-	+	+	-	-	-	+	+	-	
T-33	T+nit	rod	-	+	+	-	+	-	+	+	+	5
T-34	T+nit	rod	+	+	-	-	+	+	-	-	-	2
T-37	T+nit	rod	-	+	+	-	+	-	+	+	+	
T-45	T	rod	-	+	+	-	-	-	+	-	-	4
T-46	T	rod	-	+	+	-	-	-	+	+	-	
T-49	M+nit	rod	+	+	-	-	+	-	+	+	-	
T-52	M	rod	-	+	+	-	+	-	-	-	-	
T-53	P+nit	rod	-	+	+	-	+	-	-	-	-	
T-54	P+nit	rod	+	+	-	-	+	-	-			
T-56	P+nit	rod	-	+	+	-	+	-	-	-	-	
T-58	P+nit	rod	+	+	-	-	+	-	-	-	-	
T-60	P+nit	rod	-	+	+	-	+	-	-	-	-	
T-65	P+nit	rod	-	+	+	-	-	-	+	-	-	
T-69	P	rod	-	+	+	-	-	-	+	-	-	4
T-87	D+nit	rod	-	+	+	-	-	-	+	+	+	
T-92	P	rod	-	+	+	-	-	-	+	+	-	
M-1	P	rod	-	+	-	-	+	-	-	-	-	1
M-2	P	rod	-	-	+	-	+	-	-	-	-	1
M-3	P	rod	-	+	+	-	+	-	-	+	-	
M-4	P	rod	-	+	+	-	+	-	-	+	+	2
M-5	P	rod	-	+	+	-	-	-	-	+	+	
M-6	P	rod	-	+	+	-	+	-	-	+	+	5
M-7	P	rod	-	+	+	-	+	-	-	+	+	4
M-8	P	rod	-	+	-	-	+	-	-	-	-	
M-9	N+nit	rod	-	+	+	-	-	-	+	+	+	5
M-10	N+nit	rod	-	+	+	-	+	-	-	-	-	4
M-12	N+nit	rod	-	+	+	-	-	-	+	+	-	4
M-18	P	rod	-	+	-	-	+	-	-	-	-	
M-21	P+nit	rod	-	+	+	-	-	-	-	-	-	
M-22	P+nit	rod	-	+	+	-	+	-	-	-	-	6
M-23	P+nit	rod	-	+	+	-	+	-	-	-	-	1
M-24	P+nit	rod	-	+	+	-	+	+	+	+	-	4
M-25	P	rod	-	+	+	-	+	-	-	-	-	4

TABLE 8. CHARACTERIZATION OF ISOLATES FROM ENRICHMENT EXPERIMENT. ENRICHMENT UNDER 0.5 PERCENT OXYGEN CONDITIONS ± KNO_3 (CONCLUDED)

[1]Cat. - Catalase
Ox. - Oxidase
NRed.- Nitrate Reducers
DeN. - Denitrifiers
D - n-Dodecane
P - Phenol
M - Methylene Chloride
T - Toluene
N - Naphthalene
nit - Nitrate

[2] 1 - *Aeromonas calcaligenes* var. *lwoffi*
2 - *Pseudomonas picketti*
3 - *P. mendocina*
4 - *P. stutzeri*
5 - *P. testosteroni*
6 - *Bordetella bronchiseptica*

wastes) was probably already enriched to some degree for degraders of the organics used in these experiments.

From a subjective viewpoint, the presence of nitrate did not influence the types of organisms present in enrichment vessels for each organic. The same organisms, including nitrate reducers, could be isolated equally well for a given substrate regardless of the presence of nitrate.

The "take home lesson" for this exercise is that the abilities to degrade selected organics and to respire with nitrate are not mutually exclusive. Soil populations are abundant in degraders that also can reduce nitrate, and their presence is not influenced by the addition of nitrate except to provide these degraders with a greater opportunity to proliferate.

D. CONCLUSIONS

The enrichment experiments provided information regarding the relationship of nitrate respiring organisms and their expected response to organic contaminants. Specific conclusions are:

1. The presence of nitrate in oxygen-limiting conditions generally resulted in a higher ratio of degraders to heterotrophs. From the standpoint of enhancing the more rapid *in situ* degradation of organic contaminants, this is a desirable feature.
2. The addition of nitrate did not appear to result in a qualitatively different population (with respect to degradation of selected organics) than enrichments in the absence of nitrate.
3. Many degraders of phenol, dodecane, toluene, and naphthalene isolated from the enrichments could also respire anaerobically with nitrate

(i.e., nitrate reducers). It is probably safe to assume that nitrate reducers are equally well represented among degraders of other organics, including those at contaminated Air Force sites.

4. Of the degraders that were isolated and examinined in more detail, there were more nitrate reducers than denitrifiers. This implies that the ability to respire with nitrate is not an exclusive property of denitrifiers, but may be more widespread in soil organisms.

SECTION III

ESTABLISHING THAT AEROBIC DENITRIFICATION OCCURS AT OXYGEN LEVELS ADEQUATE FOR AEROBIC CATABOLISM OF SELECTED ORGANICS

A. RATIONALE

This section addresses the question of whether or not denitrification occurs at oxygen concentrations that are adequate for aerobic catabolism of organic compounds. Figure 8 presents this concept in graphic form. This figure is a hypothetical plot of oxygen consumption from aerobic metabolism and nitrate reduction by suspended cells and biofilms, versus the oxygen absorption rate, a measure of aeration. Since the presence of oxygen has been documented to cause a decrease in nitrate reduction, it was desirable to determine the conditions that maximize the aerobic catabolism of an organic contaminant while still maintaining sufficient nitrate reduction for respiratatory purposes. The shaded area represents the "window" of aeration whereby nitrate reduction by suspended cells can occur concurrently with aerobic oxidation of a selected organic. This window would appear to be much larger with biofilms. Although the oxygen absorption rate (OAR) in the liquid phase may be high, the available oxygen decreases with increasing depth of the biofilm, thus allowing nitrate reduction to occur at a higher oxygen concentration in the bulk phase than is seen with suspended cells. The answer to the question of whether or not aerobic denitrification occurs was experimentally translated into efforts to establish the boundaries of this window.

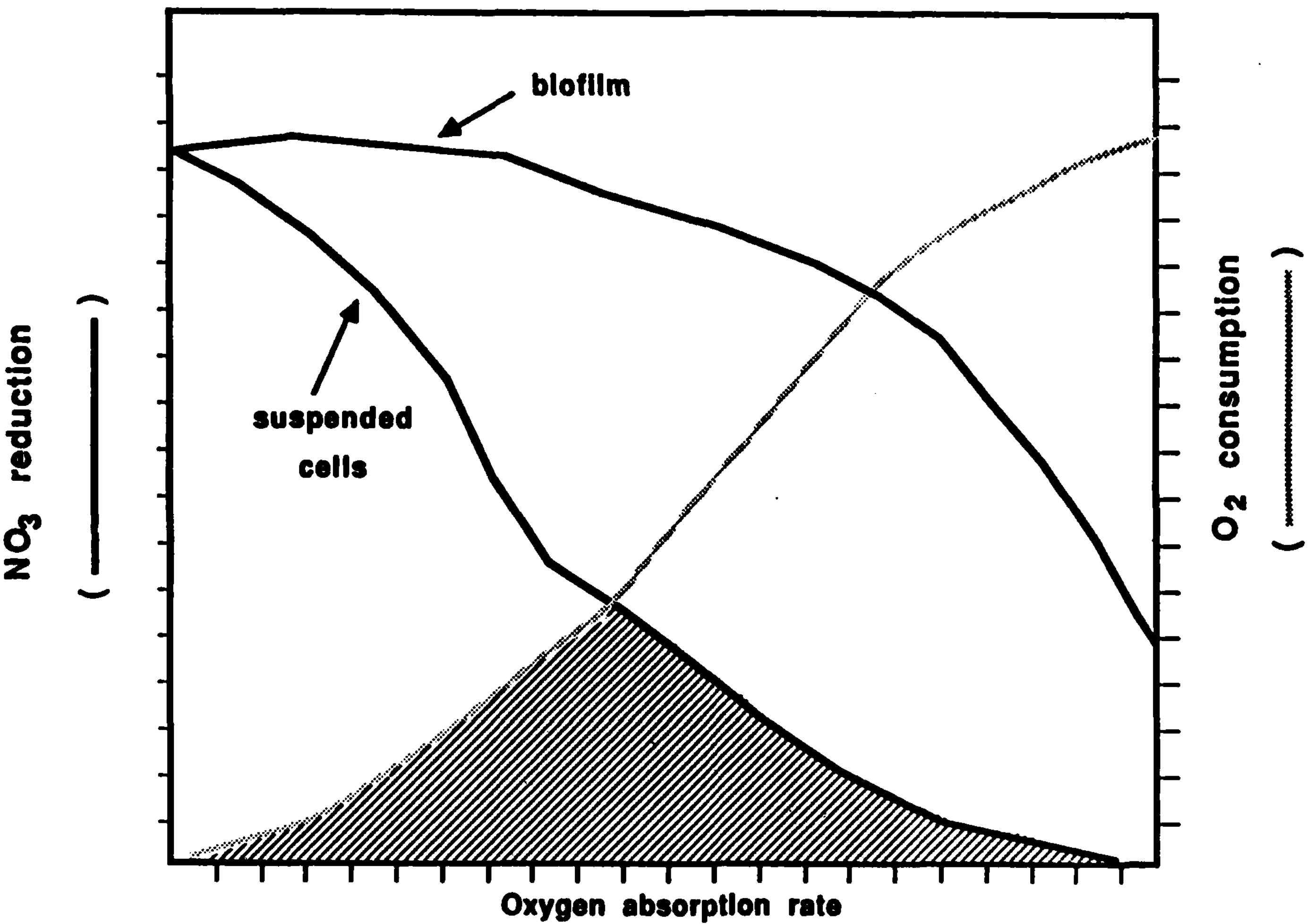


Figure 8. THEORETICAL RELATIONSHIP OF DISSIMILATORY NITRATE REDUCTION AND RESPIRATORY METABOLISM

B. MATERIALS AND METHODS

The determination of the window of aerobic denitrification was accomplished with the aid of a chemostat constructed from a 6-inch diameter by 6-inch-tall section of Corning glass process pipe equipped with a top and bottom of 3/8-inch thick Type 316 stainless steel. Nitrogen gas with varying concentrations of oxygen was sparged into the chemostat with a fritted glass sparger, and the medium was constantly stirred with a magnetic stir bar suspended in a Teflon (registered DuPont trademark) cage. The medium was MSB containing 0.2 percent succinate, 500 ppm phenol, and 0.1 percent potassium nitrate. The medium was pumped into the chemostat at a constant dilution rate of 0.072 per hour, and the temperature was closely maintained at 34°C. The chemostat was equipped with dissolved oxygen and oxidation/reduction potential probes; however, the dissolved oxygen levels were essentially nil during the experiments, and the redox measurements were a more reliable measurement of aeration.

C. RESULTS AND DISCUSSION

Experiments were originally designed to study both pure and mixed cultures, with the mixed culture grown and maintained as a biofilm in the chemostat. However, adequate methods for chemostat-type growth of biofilms could not be accomplished. Therefore, a single pure culture was chosen for these experiments.

The culture chosen was designated T-33 and was originally isolated from 0.5 percent oxygen enrichments with toluene plus nitrate. T-33 is a non-

fermentative, Gram-negative denitrifier that keys out by API RAPID NFT tests to be *Pseudomonas testosteroni* or *Pseudomonas alcaligenes*. It could utilize naphthalene, toluene, acetate, succinate, and phenol as sole carbon and energy sources.

T-33 could grow anaerobically on succinate or acetate as sole carbon sources when nitrate was present. However, T-33 could not grow under rigorously anaerobic conditions with phenol as the sole carbon source, even if nitrate was supplied as an alternative electron acceptor; growth was seen only when succinate was supplied as a second carbon source, and even then, phenol was not cometabolized anaerobically. The significance of these results is that phenol degradation by T-33 requires oxygen. Therefore, in subsequent experiments with the chemostat, when both nitrate and phenol are utilized, the concept of concurrent denitrification and aerobic metabolism of an organic substrate will have been demonstrated.

Table 9 presents respirometry data examining T-33 cells grown under rigorously anaerobic conditions, sometimes referred to as grown under Hungate procedures. Phenol-dependent oxygen uptake is an indirect measure of phenol hydroxylase, the first enzyme in the oxidation of phenol by an aerobic pathway, and catechol-dependent oxygen uptake is an indirect measure of the second enzyme, catechol 2,3-dioxygenase. (The distinction between the ortho and meta pathways for phenol catabolism was made based upon spectrophotometric assays of cell-free extracts which revealed that only the catechol 2,3 dioxygenase activity was present.) Under anaerobic conditions, phenol hydroxylase was not induced, even when phenol was present. Therefore, T-33 requires oxygen not only as a substrate in the pathway responsible for phenol oxidation, but also to induce the phenol hydroxylase directly or to permit the

TABLE 9. SUBSTRATE-DEPENDENT RESPIRATORY ACTIVITIES OF T-33 CELLS GROWN UNDER RIGOROUSLY ANAEROBIC CONDITIONS

	Oxygen Uptake (μmoles/hr/mg cells)	
	MSB Medium + Nitrate + Succinate	MSB Medium + Nitrate + Succinate + Phenol
Phenol-dependent	0 (none detected)	0
Catechol-dependent	2.89	1.30
Succinate-dependent	7.12	6.62

formation of an oxidation intermediate, such as catechol, that acts as an inducer of this enzyme. On the other hand, catechol 2,3-dioxygenase is apparently constitutive in T-33 since activity was detected in anaerobically and aerobically grown cells even in the absence of phenol.

Phenol was chosen as the substrate for the chemostat studies because its oxidation by T-33 is mediated by oxygenases as discussed above and because of its greater solubility which allowed it to be added directly in the medium rather than in the gas lines. This latter convenience made it easier to control the concentration of phenol supplied to the culture.

Succinate was added as a co-substrate (with phenol) in the chemostat medium because under anaerobic and limiting oxygen conditions, succinate was the major utilizable substrate. Without succinate, the minimal growth due to phenol would have been washed out at the set dilution rate. Preliminary experiments had demonstrated that the addition of succinate did not lead to catabolite repression of phenol oxidation. On the other hand, acetate did cause catabolite repression.

Table 10 summarizes the chemostat experiments. The chemostat contained 500 mL of medium, and nitrogen containing varying concentrations of oxygen from 0 to 21 percent was sparged through the stirred medium at 250 mL/min. The chemostat was operated for up to 2 weeks at each of 8 oxygen concentrations (0, 1.3, 4.5, 6.3, 7.7, 9.1, 10.4, and 20.8 percent). The cultures were allowed to reach steady-state growth at each of these oxygen levels and at the constant dilution rate 0.072/hr. The enzyme activities in Table 10 are from cells harvested from each of the experimental runs.

As expected, nitrate reductase activity was high under anaerobic conditions (0 percent oxygen). The percent removal of phenol was 12.7 percent of

TABLE 10. RESULTS OF CHEMOSTAT STUDIES

Parameter	Percent Oxygen Concentration 0	1.3	4.5	6.3	7.7	9.1	10.4	20.8
Colony-Forming Units (CFU) ($x10^9$)	0.14	1.3	2.5	1.8	1.7	2.1	2.1	3.4
Oxidation-Reduction Potential (mV)	-92	-100	-151	-160	-150	+22	+15	+239
Oxygen Uptake (umoles/hr/mg)								
Phenol-dependent	0.6	7.6	24.7	ND[1]	ND	8.2	ND	8.3
Catechol-dependent	6.1	15.2	48.2	ND	ND	15.0	ND	24.1
Succinate-dependent	ND	5.2	7.6	ND	ND	3.6	ND	4.9
Nitrate Reductase (nmoles/min/mg)	437	309	96.5	ND	ND	109	ND	106
Nitrite Reductase (nmoles/min/mg)	10.4	12.0	6.6	ND	ND	0	ND	0
Percent Phenol Utilized[2]	12.7	30	99.4	100	100	100	100	99.7
Percent Nitrate Reduced[2]	99	100	100	100	99	90.1	13	6

[1]ND - Not Determined
[2]In comparison to concentrations in feed medium

the original 500 ppm even though phenol-dependent oxygen uptake from harvested cells was a barely perceptible 0.57 moles/hour/mg cells. This decrease in phenol under anaerobic conditions may have been partly attributable to air stripping and/or uptake without catabolism by cells growing at the expense of succinate. Another likely explanation is that trace levels of oxygen in the instrument-grade nitrogen permitted aerobic catabolism of phenol. Because phenol hydroxylase activity is normally absent in rigorously anaerobically grown T-33, it was concluded that the low level of this oxygenase in the chemostat-grown cells was due to trace oxygen (<5 ppm) in the nitrogen.

In determining the window where one observes both nitrate reduction (respiration) and aerobic oxidation of the selected organic "contaminant" (phenol), the aeration level that results in induction of hydroxylases and other oxygenases is very important. At least with T-33 and phenol as the substrate, the window starts at very low oxygen levels.

As oxygen was increased in the next experiments, oxygenase activities and phenol utilization increased. Nitrate and nitrite reductase activities decreased, but nitrate reduction did not cease even with sparging at 20.8 percent oxygen. At 20.8 percent oxygen the redox potential was high (+289 mV), yet nitrate reductase was 24 percent of the anaerobic levels. Nitrite reductase, on the other hand, was more severely diminished with increasing oxygenation of the chemostat.

The critical information in Table 10 is the data on phenol utilization and nitrate utilization. The term utilization is used to denote the decrease in concentration in the chemostat relative to what was in the medium being fed into the chemostat, the assumption being that phenol was oxidized and nitrate was reduced to nitrite and other products of denitrification. Figure 9 is the

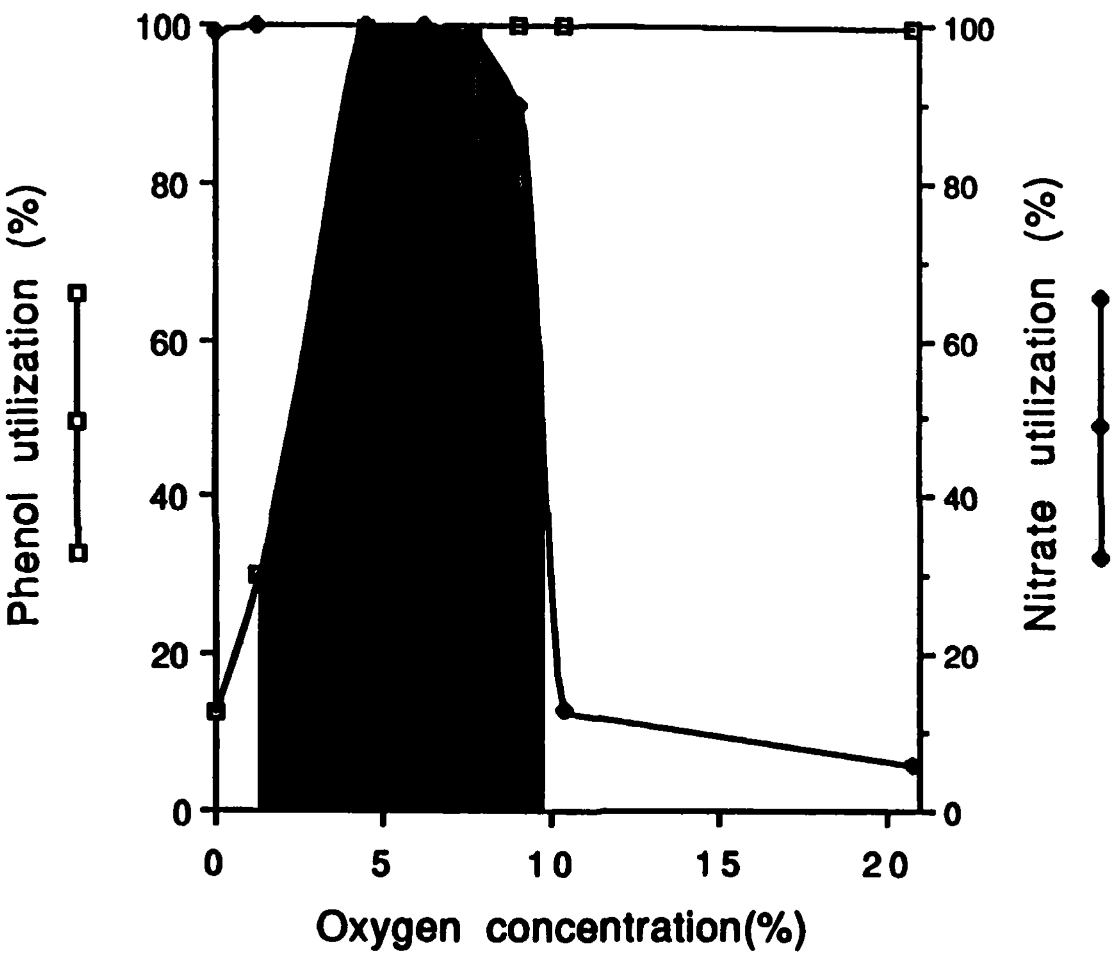


Figure 9. PHENOL AND NITRATE UTILIZATION VERSUS OXYGEN CONCENTRATION. WINDOW OF AEROBIC DENITRIFICATION

plot of phenol and nitrate utilization versus oxygen concentration in the sparging gases. The shaded area depicts the observed window of aerobic denitrification and extends from near zero to almost 10 percent oxygen. Phenol oxidation starts at extremely low oxygen levels, and nitrate reductase exhibits an abrupt decrease at approximately 10 percent oxygen even though complete cessation of nitrate utilization was not observed at 20.8 percent oxygen.

D. CONCLUSIONS

The chemostat studies with pure cultures of isolate T-33 demonstrated the following:

1. Suspended cells of this organism degraded phenol by a pathway with an obligate requirement for oxygen while simultaneously carrying out nitrate respiration, a process that is classically considered to require anaerobic conditions.
2. The window of aerobic denitrification extended from near zero percent oxygen in the sparging gas to approximately 10 percent. This window would expectedly be larger for biofilms, the condition that would occur in the subsurface. Since T-33 can also utilize toluene and naphthalene, it is expected that a similar window exits for these organics.
3. The results showed that the aerobic degradation of phenol by T-33 occurred even at very low oxygen levels. At least the enzymatic machinery for catabolism was in place. This observation may explain the published reports of reputedly "anaerobic" degradation under

denitrification conditions of organics that generally are believed to require molecular oxygen for catabolism.

SECTION IV

AEROBIC DENITRIFICATION AND THE ENHANCEMENT OF BIODEGRADATION

A. RATIONALE

The previous experiments established that denitrification and aerobic phenol catabolism can occur simultaneously in the soil isolate T-33. It is assumed that this capacity for aerobic denitrification is widespread in nature. At this point it is essential to determine if the aerobic denitrification concept can enhance biodegradation of organic contaminants when oxygen is limiting. Enhancement is defined as increases in *rate* or *extent* (or both) of degradation.

There are two mechanisms of aerobic denitrification that could explain enhancement of biodegradation. The first is the possibility that substitution of nitrate for oxygen could relieve the cell's respiratory requirement for oxygen, and the available oxygen would be funneled to the oxygenases rather than to cytochrome oxidases. If organic substrate oxidation were rate limited due to the lack of oxygen, this increase in available oxygen would result in a more rapid rate of substrate catabolism. If on the other hand the rate limiting step were independent of oxygen, then the addition of nitrate could still enhance the overall extent of biodegradation. By relieving the respiratory chain of its requirement for oxygen, more oxygenase reactions can be catalyzed per mole of oxygen avaiable. Therefore, there is an enhancement because the oxygen demand is decreased. In simple terms, it takes less oxygen to degrade a given amount of organic when nitrate is present. The experimen-

tal results presented below provide evidence that it is the latter type of enhancement that is gained by adding nitrate.

B. STUDIES WITH RESTING CELLS OF T-33

Previous experiments (data not shown) with mixed cultures growing on phenol ± nitrate failed to show any effect by nitrate at low oxygen, although it is now doubtful whether oxygen was limiting in these particular experiments. The inability of these static cultures to produce any encouraging data prompted us to redesign our approach to this problem. We elected to examine resting (nongrowing) cells under dynamic conditions whereby oxygen was maintained at limiting concentrations by continuously sparging with an oxygen/nitrogen mixture.

Resting cells of T-33, previously grown in the chemostat at 7.5 percent oxygen were placed in fresh MSB with 500 ppm phenol ± 0.1 percent potassium nitrate. Chloramphenicol was added to inhibit protein synthesis and growth. The suspensions were sparged continuously with 1 percent oxygen (dissolved oxygen = 10 μM). At designated time intervals, samples were removed and analyzed for remaining phenol and nitrate. Figure 10 presents the results.

There were no significant differences in the rates of phenol utilization in suspensions with or without nitrate. Since phenol was completely consumed in the presence or absence of nitrate, it was not possible to examine whether or not there was a difference in the extent of degradation. Yet nitrate was reduced at this low oxygen concentration, a result consistent with the chemostat experiments. If nitrate was reduced, less oxygen was consumed since biochemical oxidation/reduction reactions must balance.

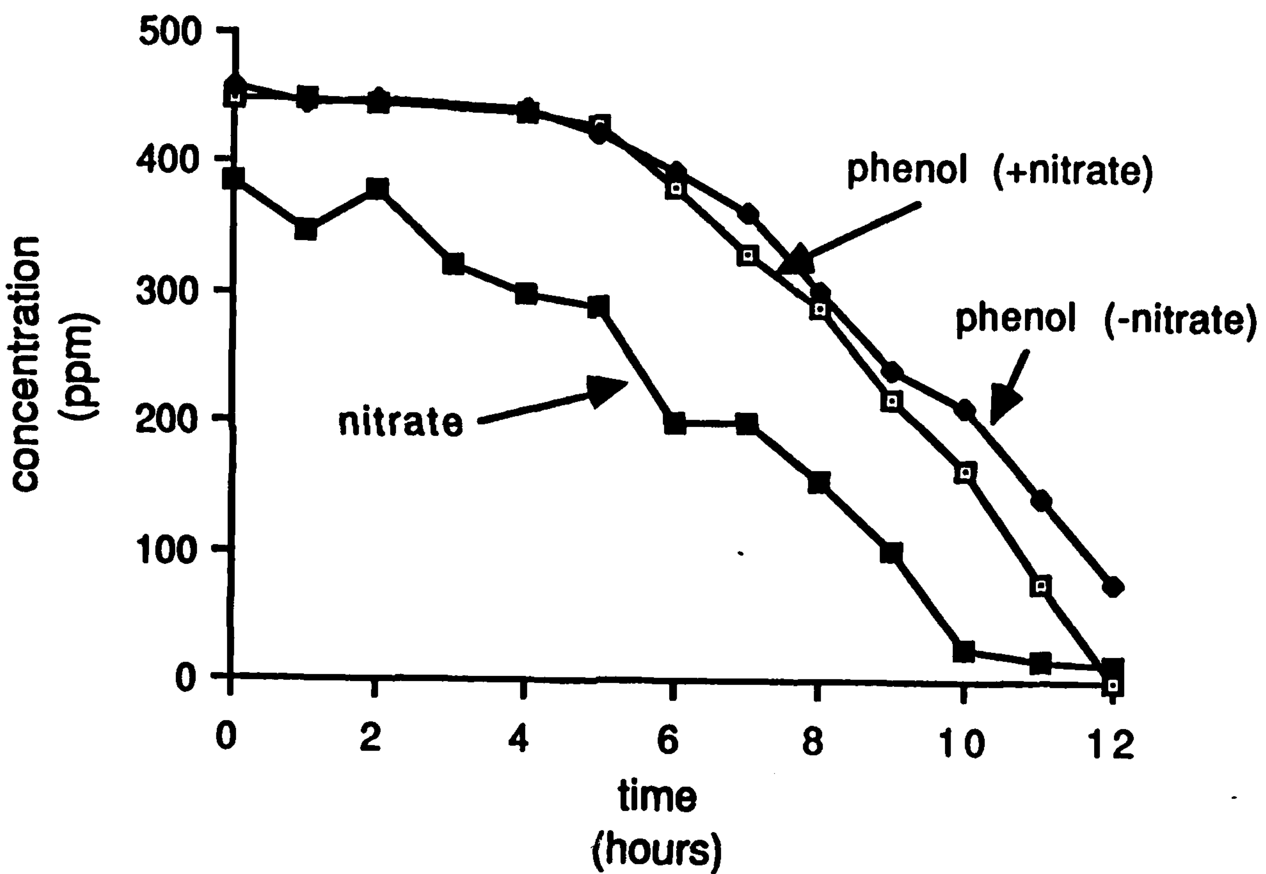


Figure 10. CONCOMITANT UTILIZATION OF PHENOL AND NITRATE REDUCTION BY T-33 UNDER LIMITING OXYGEN

Respirometry experiments were performed to prove that the addition of nitrate could result in a savings of oxygen. Figure 11 shows oxygen uptake tracings from T-33 cells and illustrates the results of these experiments. The lefthand panel, labeled "Phenol Oxidation", shows tracings in medium without (solid line) and with (dashed line) 0.1 percent potassium nitrate. If nitrate decreased the oxygen demand, it would be expected that for each increment of phenol added, less oxygen would be consumed. Small reproducible differences were observed, particularly at low (<24 μM) oxygen. The lack of dramatic differences between tracings with and without nitrate can be partially attributed (about half of the oxygen uptake) to phenol and catechol oxygenases which expectedly would not respond to nitrate. The righthand panel shows tracings using succinate as substrate. Oxygen uptake with succinate reflects the activity of the respiratory chain. As indicated within the dashed line, nitrate decreases the amount of oxygen consumed for the 50 nmole increments of succinate, particularly below 100 μM oxygen. These observations are consistent with the interpretation that nitrate is serving as an alternate electron acceptor, and therefore,for a given amount of succinate less oxygen is reduced (consumed).

Actually, the story is more complicated than this. Recent publications (References 73, 74) have elucidated another mechanism by which denitrification is affected by oxygen. Nitrate transport into the cell was shown to be inhibited by oxygen. Experiments with *Escherichia coli* and *Pseudomonas aeruginosa* have demonstrated that nitrate respiration is regulated at the transport level by trace levels of oxygen. T-33 apparently is not as exquisitely sensitive to oxygen, since chemostat studies showed that nitrate reduction occurs at moderate oxygenation. We do not believe that the selec-

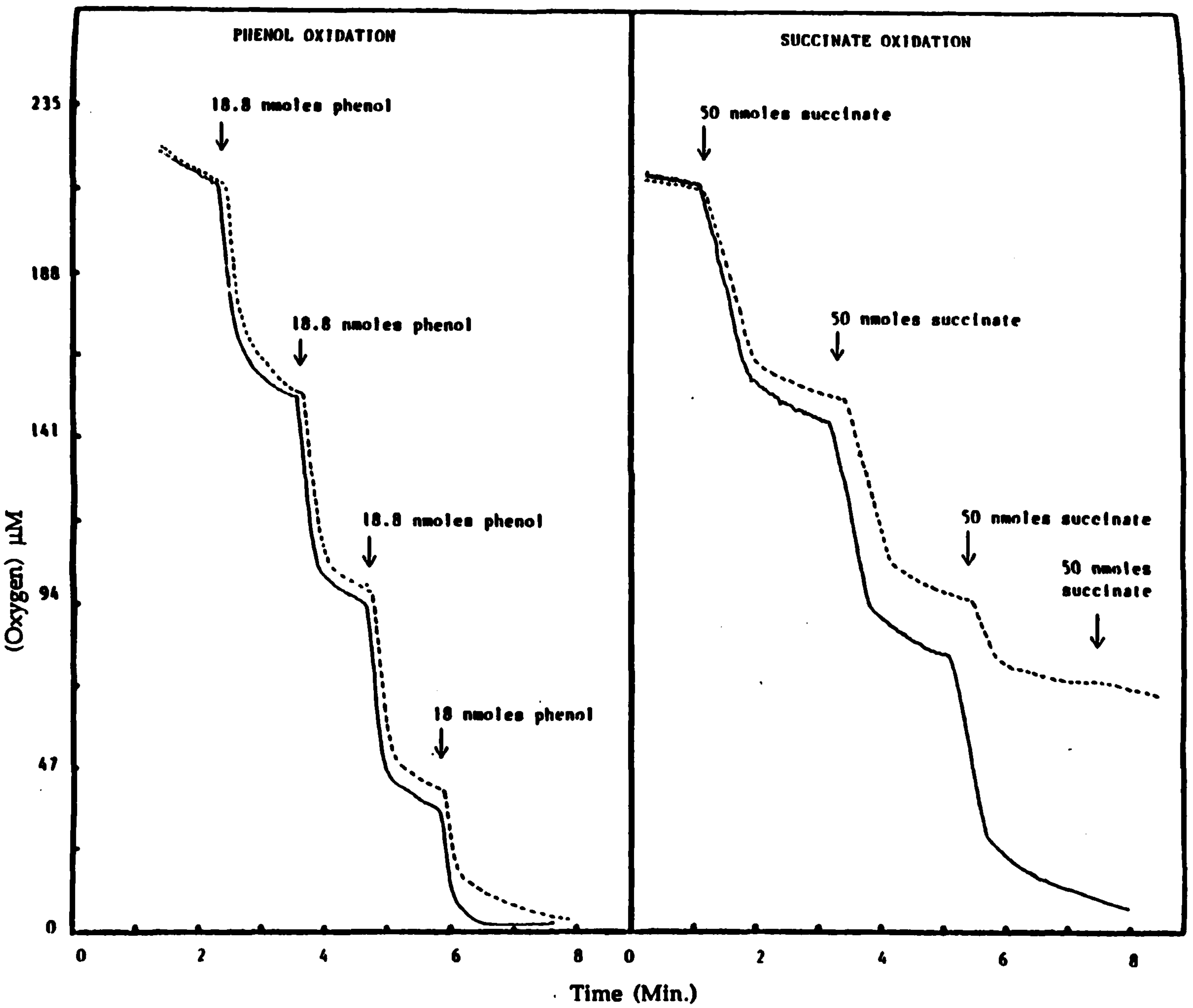
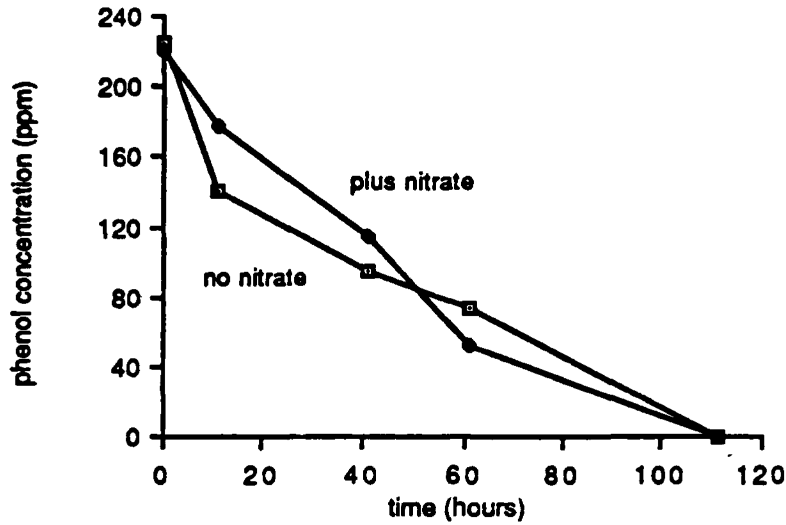


Figure 11: OXYGEN UPTAKE TRACINGS OF T-33 CELLS GROWN UNDER LIMITING OXYGEN

tion of T-33 is fortuitous, but rather think that this culture may be more typical of soil organisms than the laboratory strains used in the recently published studies.

Since the respirometry experiments only followed oxygen uptake by T-33 cells and not phenol utilization, experiments were performed to evaluate both phenol utilization and oxygen consumption. Resting cells of T-33 grown in the chemostat at 7.5 percent oxygen were placed in fresh MSB medium containing chloramphenicol. These suspensions, $\pm$ nitrate and with phenol or phenol plus succinate were placed in septa-sealed bottles with a headspace of air containing 4.4 percent oxygen. Samples were removed periodically and analyzed for the remaining phenol in the medium and gaseous oxygen in the headspace. The results are shown in Figure 12 for media containing phenol only and Figure 13 for media containing phenol and succinate.

The top panel in Figure 12 (labelled "Phenol Utilization") plots remaining phenol over time for duplicate runs. The rates of phenol utilization are similar and perhaps too close to conclude a significant enhancement by added nitrate. Since there was enough oxygen for T-33 to consume 100 percent of the phenol even in the absence of nitrate, an increase in the extent of phenol degradation would have been obscured. However, a big difference was observed in the amount of oxygen consumed as shown in the lower panel. When oxygen reached approximately 2.5 percent (~40 hr) in the headspace, the disparities of oxygen consumption in bottles with and without nitrate became prominent. It is assumed that nitrate transport and reduction rates became pronounced at this oxygen level. This is consistent with the respirometry studies. By 90 hours the savings in oxygen was almost 40 percent.



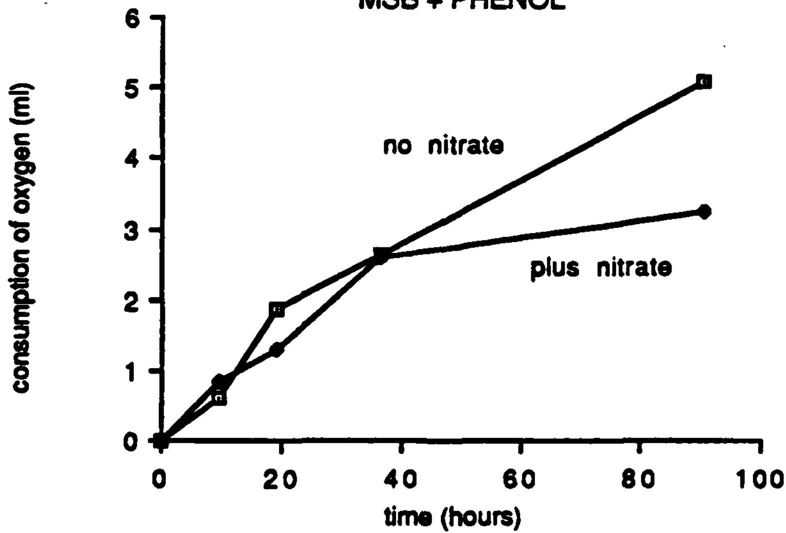
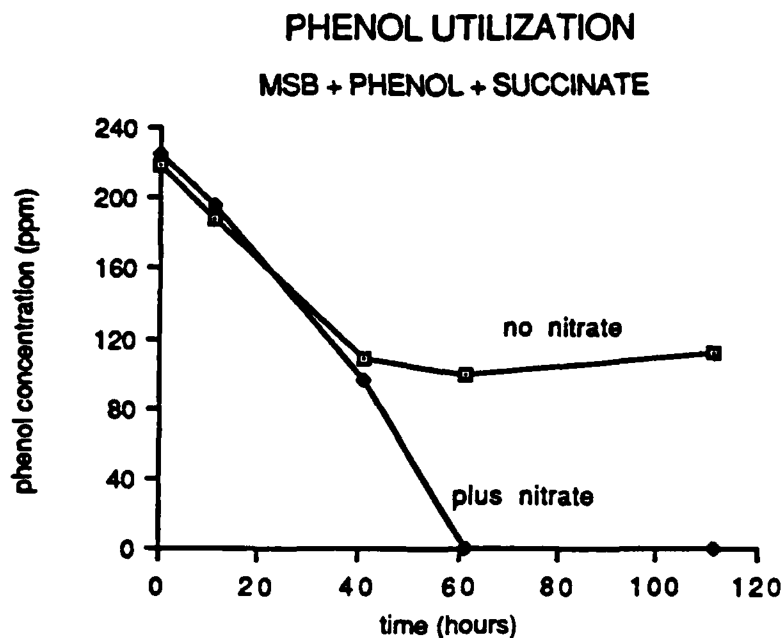


Figure 12. PHENOL UTILIZATION AND OXYGEN CONSUMPTION BY RESTING CELLS OF T-33. MINERAL MEDIUM PLUS PHENOL +/- NITRATE

Respirometry experiments had suggested that readily oxidizable substrates other than phenol (specifically succinate) would show even more impressive differences. This was tested in bottles containing both phenol and succinate. Reproducible enhancement of phenol degradation by nitrate was observed in the presence of succinate (Figure 13). Again, at approximately 2.5 percent oxygen in the headspace (~40 hr), phenol levels continued to decrease rapidly as nitrate became the final electron acceptor, in competition with oxygen. A 40 percent savings in oxygen resulted.

Why was there an enhancement of phenol utilization by nitrate when succinate was present, and how does this relate to *in situ* bioremediation? To answer the first part of this question, one should understand that the oxidation of phenol, as well as other substrates, is a carefully balanced series of oxidation/reduction reactions. The reducing equivalents generated are oxidized primarily via oxygenase- and oxidase-catalyzed reactions. The addition of a secondary oxidizable substrate, such as succinate, could increase the availability of reducing equivalents. This increase could result in nitrate-mediated enhancement of phenol oxidation by two possible ways. First, excess reducing equivalents could serve in the reactivation of oxygen-sensitive nitrate transport protein(s). Second, the excess reducing equivalents could shift electron transport in the respiratory chain so that nitrate reduction becomes the dominant pathway. The second part of the question, on the relevance to *in situ* bioremediation, can be answered by pointing out that all contaminated sites are expected to have secondary oxidizable substrates from multiple contaminants, from transformation of primary contaminants to intermediary metabolites, and from natural organic sources. Therefore, nitrate could provide an enhancement of organics at actual contaminated sites similar to the results with phenol plus succinate.



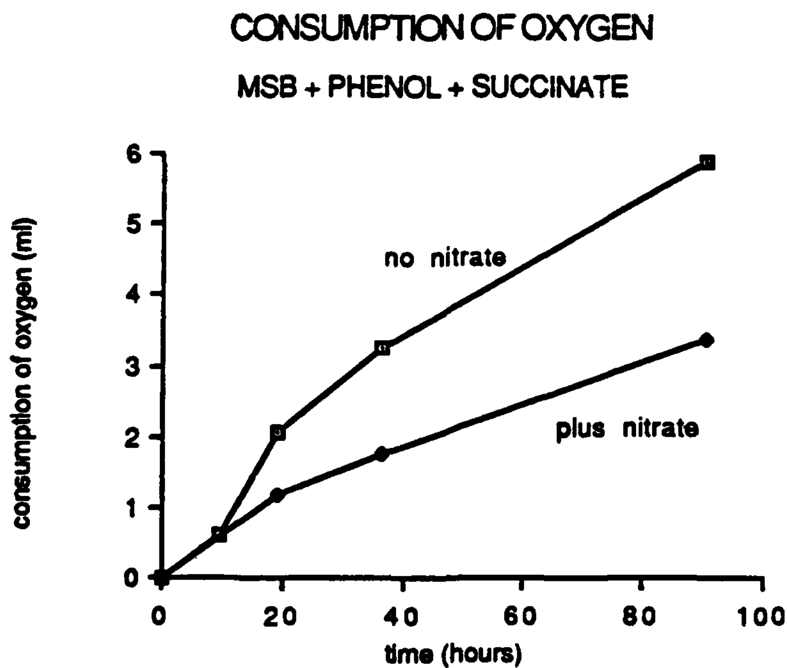


Figure 13. PHENOL UTILIZATION AND OXYGEN CONSUMPTION BY RESTING CELLS OF T-33. MINERAL MEDIUM PLUS PHENOL PLUS SUCCINATE +/- NITRATE

C. THE EFFECT OF NITRATE ON MINERALIZATION OF SELECTED ORGANICS BY MIXED CULTURES

The final experiments were performed similarly to the enrichment experiments in Section II, except that the inoculum was from 0.5 percent oxygen enrichments and that carbon dioxide (from mineralization of the individual organics phenol, naphthalene, and dodecane) was trapped from the gases exiting the enrichment vessels and quantified. The carbon dioxide was trapped as barium carbonate in solutions saturated with barium hydroxide. The barium carbonate precipitates were collected and quantified by titration with HCl. The results of these experiments are presented in Figures 14, 15, and 16.

The oxygen content of the continuous stream of nitrogen that was flowing through the vessels was 1 percent. The addition of nitrate made no difference with mineralization of dodecane, although growth rates on dodecane and the resulting carbon dioxide evolution were minimal. Nitrate improved slightly (20 percent) the mineralization of phenol, and increased it considerably (250 percent) with naphthalene. Additional experiments are needed to understand the different effects of nitrate on mineralization of different organics.

D. CONCLUSIONS

The following conclusions are made from the experiments in this section:

1. Based on studies with isolate T-33, nitrate enhances the biodegradation of phenol.
2. Nitrate enhances degradation of phenol by T-33 by providing a final electron acceptor for respiration other than oxygen and thereby

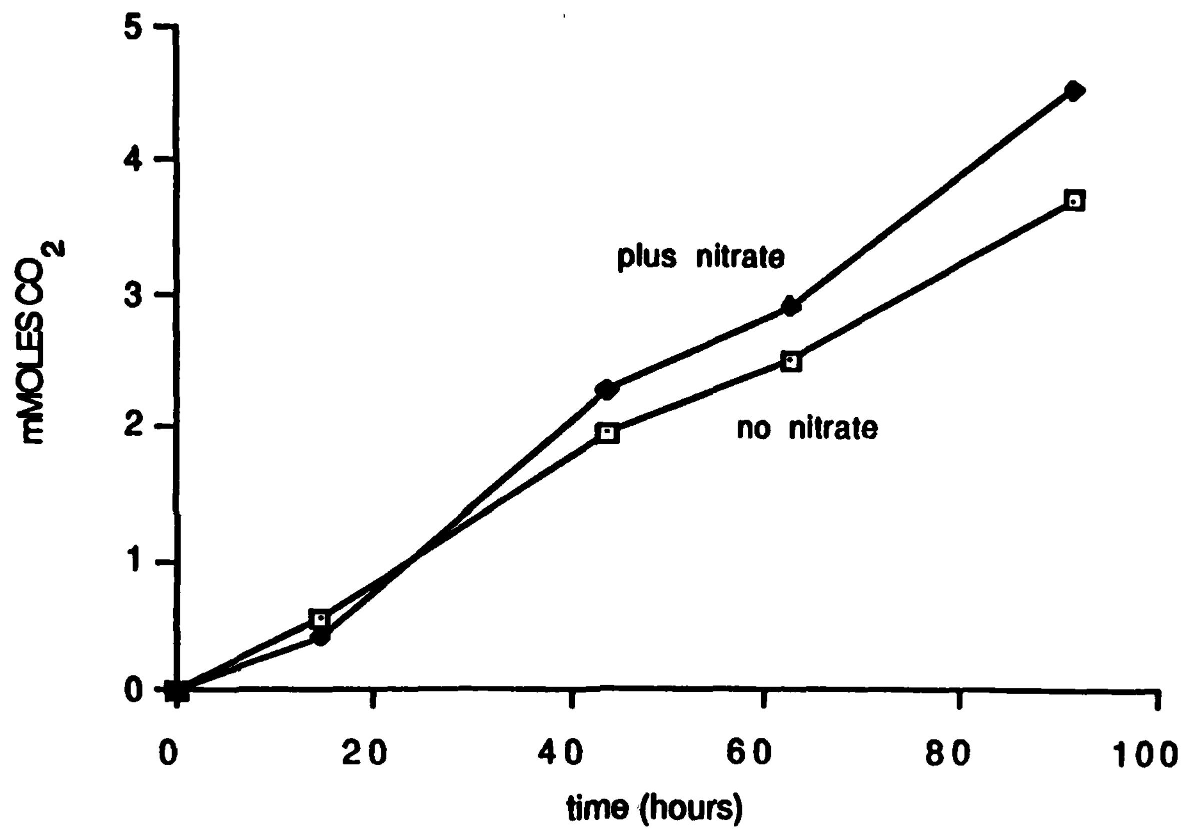


Figure 14. MINERALIZATION OF PHENOL UNDER LIMITING OXYGEN +/- NITRATE

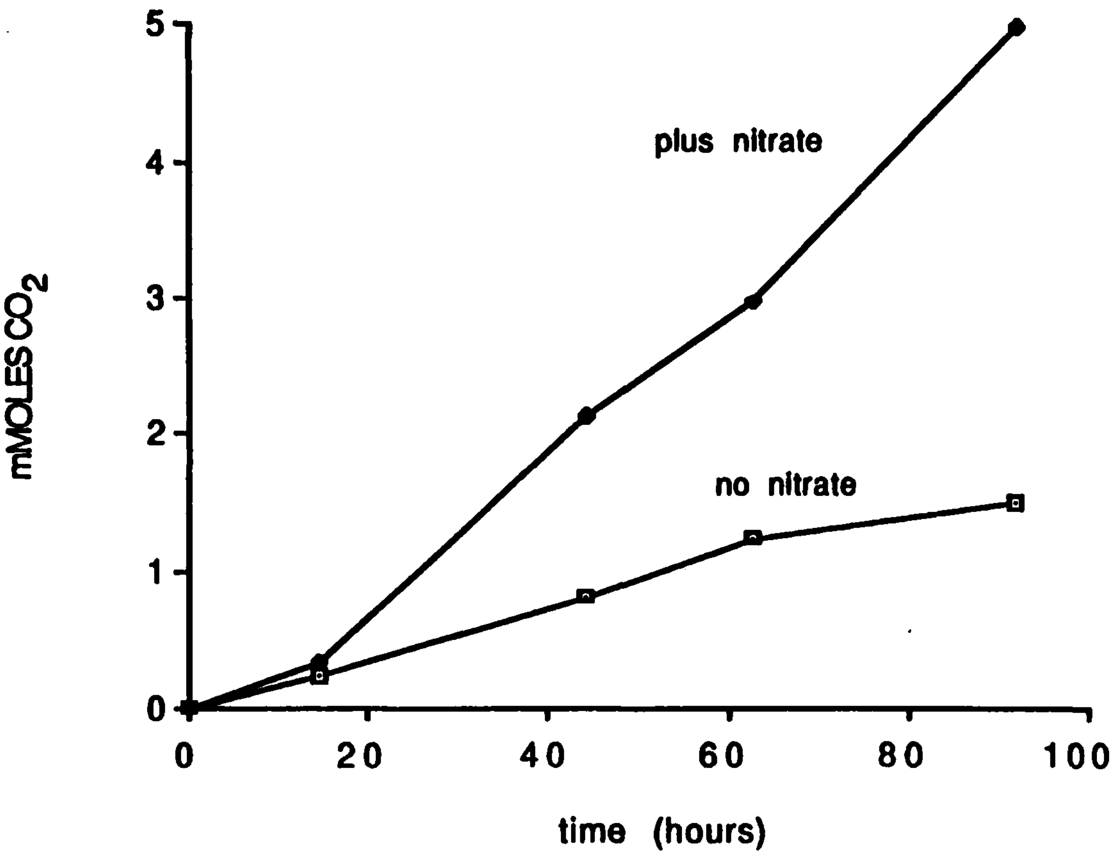


Figure 15. MINERALIZATION OF NAPHTHALENE UNDER LIMITING OXYGEN +/- NITRTATE

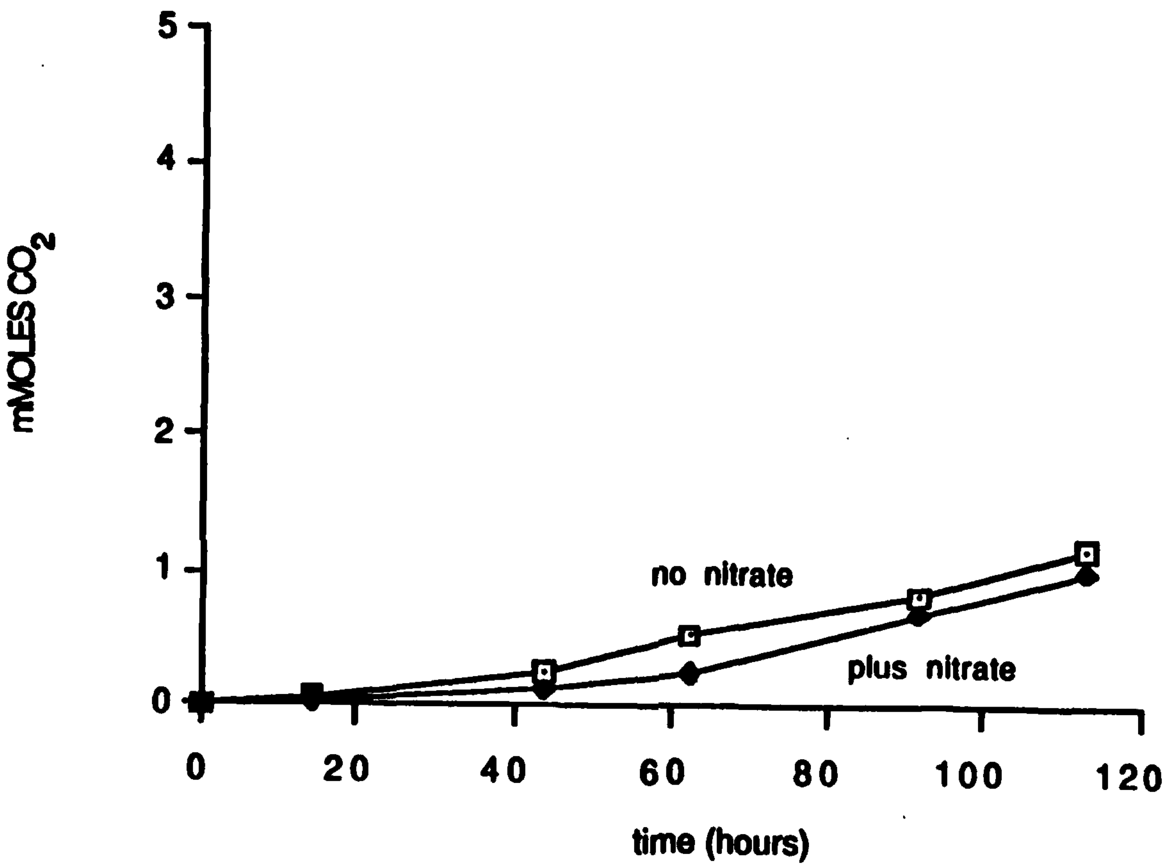


Figure 16. MINERALIZATION OF DODECANE UNDER LIMITING OXYGEN +/- NITRATE

decreasing the demand for oxygen for phenol oxidation. Thus, for a given amount of phenol, less oxygen is required.

3. Tentative evidence was presented that nitrate can increase mineralization of certain organics in limiting oxygen. However, additional studies are needed.

SECTION V

CONCLUSIONS AND RECOMMENDATIONS

In situ bioremediation is a promising technology for cleanup of subsurface sites contaminated with a variety of organic compounds. Aerobic oxidation of the majority of man-made organics, such as solvents and petroleum fuels, is readily accomplished by soil and water microorganisms. However, oxygen is usually a limiting factor in contaminated subsurface sites, and its addition via sparging of groundwater or injection of hydrogen peroxide is rarely adequate or without problems. A partial solution to this problem is to decrease the biological oxygen demand by providing nitrate in lieu of oxygen as the final electron acceptor for respiration.

This study provided preliminary laboratory evidence that nitrate could enhance biological remediation by decreasing the oxygen demand for oxidation of selected organics, typical of those at contaminated U.S. Air Force sites. This decrease in oxygen demand, in turn, resulted in a greater extent of organic removal. That is, more organic substrate was oxidized per unit of oxygen consumed when nitrate was present.

The application of this technique in the field has the following advantages:

1. It relies on the indigenous microbial population. This study demonstrated that the presence of nitrate did not enrich for a distinct population of microbes. The soil is replete with nitrate respirers that are capable of degrading organic contaminants.

2. Good results are expected with biofilms, the presumed subsurface condition. Suspended-cell experiments were conducted in this study that successfully demonstrated the aerobic denitrification concept. Denitrification does occur at oxygen levels sufficient for aerobic catabolism of organics by cells that are suspended in the bulk liquid phase. Since oxygen levels can range from the extremes of high to low in biofilms, the degradation of organics via aerobic denitrification may be greater than with suspended cells.
3. The addition of nitrate decreases the biochemical oxygen demand for oxidation of organics.
4. The additon of nitrate would result in the more rapid mineralization of organic carbon and secondary metabolites. This was illustrated in this study with naphthalene.
5. The low oxygen requirement with nitrate present can promote unique anaerobic metabolism of organic contaminants such as halogenated organics. In some cases anaerobic microbial transformations may be the only route for degradation of certain organics, such as halogenated compounds, in a complex mixture of contaminants. The aerobic denitrification concept is favorable for preserving the obligate anaerobes in the microbial consortium.
6. The end products of nitrate reduction (N_2O and N_2) are innocuous.

It is recommended that further studies be conducted to evaluate the efficacy of nitrate addition for oxidation of organic contaminants. This aerobic denitrification concept needs further proof-of-concept studies in scaled up laboratory experiments. Specifically, the use of biofilms in soil

matrices should be examined at varying oxygen levels with selected organic compounds plus and minus nitrate. These types of studies would be a prelude to *controlled* studies in the field.

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APPENDIX A

ENUMERATION OF MICROBIAL POPULATIONS FROM ENRICHMENTS (CFU/ML OR CFU/G)

MICROBIAL POPULATIONS IN ENRICHMENTS WITH PHENOL

Anaerobic Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$8.9x10^4$	$1.9x10^5$	$9.2x10^5$	$4.5x10^5$	$7.2x10^5$	$7.8x10^7$
Nitrate Reducers	$4.7x10^4$	$4.2x10^4$	0.00	$4.8x10^4$	$2.2x10^6$	$1.2x10^7$
Denitrifiers	$2.4x10^3$	$2.8x10^4$	$2.3x10^2$	$2.4x10^3$	$1.6x10^5$	$2.8x10^6$
Aerobic Heterotrophs	$1.1x10^5$	$4.0x10^5$	$2.3x10^6$	$1.3x10^6$	$6.6x10^5$	$7.6x10^6$
Anaerobic Heterotrophs	$3.0x10^3$	$1.1x10^5$	$4.5x10^5$	$4.0x10^3$	$1.6x10^5$	$5.6x10^5$

0.5 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=9 Days	Column	T=0 Days	T=9 Days	Column
Degraders	$6.4x10^3$	$1.6x10^8$	$1.9x10^8$	$7.7x10^3$	$2.2x10^8$	$1.6x10^8$
Nitrate Reducers	$3.9x10^3$	$1.1x10^7$	$8.2x10^6$	$1.2x10^4$	$2.4x10^7$	$4.2x10^7$
Denitrifiers	$6.0x10^1$	$1.7x10^5$	$4.0x10^5$	$3.5x10^3$	$1.6x10^5$	$9.4x10^6$
Aerobic Heterotrophs	$9.7x10^3$	$1.5x10^8$	$2.7x10^8$	$1.9x10^4$	$1.2x10^8$	$2.7x10^8$
Anaerobic Heterotrophs	$9.8x10^2$	$1.1x10^7$	$2.4x10^7$	$3.6x10^3$	$5.2x10^7$	$5.9x10^7$

21 percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$1.3x10^5$	$3.4x10^5$	$6.6x10^7$	$1.0x10^5$	$8.8x10^5$	$9.4x10^6$
Nitrate Reducers	$1.1x10^5$	$2.2x10^5$	$3.5x10^6$	$2.4x10^5$	$1.2x10^5$	$1.2x10^7$
Denitrifiers	$1.6x10^5$	$5.4x10^4$	$1.6x10^3$	$1.6x10^5$	$1.6x10^5$	$1.6x10^7$
Aerobic Heterotrophs	$1.2x10^7$	$1.6x10^6$	$3.5x10^7$	$7.0x10^6$	$1.7x10^6$	$2.8x10^7$
Anaerobic Heterotrophs	$4.3x10^5$	$1.5x10^6$	$4.5x10^5$	$3.6x10^5$	$2.0x10^5$	$3.6x10^6$

MICROBIAL POPULATIONS IN ENRICHMENTS WITH DODECANE

Anaerobic Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$7.1x10^4$	$5.4x10^5$	$9.0x10^5$	$6.1x10^4$	$8.7x10^6$	$1.9x10^7$
Nitrate Reducers	$4.7x10^4$	$5.7x10^5$	$6.1x10^5$	$4.8x10^4$	$1.8x10^7$	$3.8x10^7$
Denitrifiers	$2.4x10^3$	$2.8x10^4$	$6.0x10^3$	$2.4x10^3$	$1.6x10^5$	$2.8x10^6$
Aerobic Heterotrophs	$1.1x10^5$	$8.8x10^5$	$9.5x10^5$	$1.3x10^6$	$7.0x10^6$	$3.8x10^7$
Anaerobic Heterotrophs	$3.0x10^3$	$2.4x10^4$	$1.2x10^5$	$4.0x10^3$	$1.4x10^6$	$5.9x10^5$

0.5 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=9 Days	Column	T=0 Days	T=9 Days	Column
Degraders	$3.2x10^4$	$1.7x10^8$	$9.3x10^8$	$2.4x10^4$	$6.6x10^8$	$5.1x10^8$
Nitrate Reducers	$3.9x10^3$	$1.6x10^7$	$1.6x10^7$	$1.2x10^4$	$2.3x10^7$	$3.9x10^7$
Denitrifiers	$6.0x10^1$	$1.6x10^5$	$1.1x10^6$	$3.5x10^3$	$1.6x10^5$	$1.6x10^8$
Aerobic Heterotrophs	$9.7x10^3$	$6.7x10^7$	$1.1x10^9$	$1.9x10^4$	$1.3x10^8$	$5.5x10^8$
Anaerobic Heterotrophs	$9.8x10^2$	$4.6x10^7$	$1.1x10^9$	$3.6x10^3$	$3.4x10^7$	$6.9x10^6$

21 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$2.2x10^3$	$3.0x10^6$	$6.8x10^6$	$2.3x10^3$	$5.4x10^5$	$4.4x10^6$
Nitrate Reducers	$1.1x10^5$	$2.0x10^5$	$2.3x10^7$	$2.4x10^5$	$1.8x10^5$	$1.0x10^7$
Denitrifiers	$1.6x10^5$	$1.6x10^5$	$9.1x10^5$	$1.6x10^5$	$1.6x10^5$	$7.9x10^4$
Aerobic Heterotrophs	$1.2x10^7$	$9.4x10^6$	$1.7x10^8$	$7.0x10^6$	$8.0x10^6$	$1.6x10^8$
Anaerobic Heterotrophs	$4.3x10^5$	$1.0x10^6$	$9.5x10^6$	$3.6x10^5$	$2.1x10^5$	$6.4x10^6$

MICROBIAL POPULATIONS IN ENRICHMENTS WITH TOLUENE

Anaerobic Conditions.

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	7.5×10^{4}	5.0×10^{5}	4.7×10^{5}	6.8×10^{4}	6.5×10^{6}	1.6×10^{7}
Nitrate Reducers	4.7×10^{4}	2.1×10^{5}	0.00	4.8×10^{4}	1.7×10^{7}	1.3×10^{7}
Denitrifiers	2.4×10^{3}	1.7×10^{3}	0.00	2.4×10^{3}	1.6×10^{7}	1.4×10^{7}
Aerobic Heterotrophs	1.1×10^{5}	4.2×10^{5}	9.4×10^{5}	1.3×10^{6}	2.6×10^{6}	2.3×10^{7}
Anaerobic Heterotrophs	3.0×10^{3}	5.2×10^{4}	4.5×10^{5}	4.0×10^{3}	3.4×10^{5}	5.5×10^{5}

0.5 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=9 Days	Column	T=0 Days	T=9 Days	Column
Degraders	3.3×10^{4}	4.3×10^{8}	7.5×10^{8}	3.3×10^{4}	8.6×10^{7}	6.3×10^{8}
Nitrate Reducers	3.9×10^{3}	3.0×10^{6}	3.3×10^{7}	1.2×10^{4}	5.3×10^{6}	3.6×10^{7}
Denitrifiers	6.0×10^{1}	3.5×10^{4}	2.0×10^{5}	3.5×10^{3}	1.6×10^{5}	1.4×10^{7}
Aerobic Heterotrophs	9.7×10^{3}	7.3×10^{7}	4.2×10^{8}	1.9×10^{4}	1.3×10^{7}	5.2×10^{8}
Anaerobic Heterotrophs	9.8×10^{2}	8.0×10^{6}	5.7×10^{7}	3.6×10^{3}	7.8×10^{6}	9.6×10^{6}

21 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	1.8×10^{4}	2.2x107	8.8×10^{7}	7.6×10^{4}	1.4×10^{6}	5.3×10^{6}
Nitrate Reducers	1.1×10^{5}	9.1×10^{5}	1.1×10^{7}	2.4×10^{5}	6.4×10^{5}	1.4×10^{7}
Denitrifiers	1.6×10^{5}	1.7×10^{6}	6.0×10^{5}	1.6×10^{5}	2.8×10^{5}	1.6×10^{6}
Aerobic Heterotrophs	1.2×10^{7}	6.5×10^{6}	3.3×10^{9}	7.0×10^{6}	7.0×10^{6}	4.4×10^{9}
Anaerobic Heterotrophs	4.3×10^{5}	1.7×10^{8}	1.2×10^{7}	3.6×10^{5}	5.0×10^{5}	5.0×10^{7}

MICROBIAL POPULATIONS IN ENRICHMENTS WITH NAPHTHALENE

Anaerobic Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$6.5x10^4$	$9.9x10^4$	$1.1x10^6$	$6.6x10^4$	$5.8x10^5$	$4.3x10^6$
Nitrate Reducers	$4.7x10^4$	$1.9x10^4$	$3.7x10^5$	$4.8x10^4$	$6.8x10^5$	$1.8x10^6$
Denitrifiers	$2.4x10^3$	0.00	0.00	$2.4x10^3$	$1.6x10^5$	$6.0x10^1$
Aerobic Heterotrophs	$1.1x10^5$	$1.8x10^5$	$1.2x10^6$	$1.3x10^6$	$2.9x10^5$	$7.4x10^6$
Anaerobic Heterotrophs	$3.0x10^3$	$2.7x10^4$	$8.1x10^5$	$4.0x10^3$	$1.1x10^4$	$9.9x10^4$

0.5 Percent Oxygen Conditions

	MSB			MSB + NITRATE		
	T=0 Days	T=9 Days	Column	T=0 Days	T=9 Days	Column
Degraders	$1.8x10^4$	$2.5x10^7$	$2.1x10^8$	$2.7x10^4$	$8.7x10^7$	$2.8x10^8$
Nitrate Reducers	$3.9x10^3$	$1.5x10^5$	$4.8x10^6$	$1.2x10^4$	$1.4x10^7$	$2.5x10^7$
Denitrifiers	$6.0x10^1$	$2.2x10^5$	$2.0x10^3$	$3.5x10^3$	$1.6x10^6$	$7.9x10^5$
Aerobic Heterotrophs	$9.7x10^3$	$1.2x10^7$	$7.6x10^7$	$1.9x10^4$	$3.5x10^7$	$2.1x10^8$
Anaerobic Heterotrophs	$9.8x10^2$	$8.1x10^5$	$6.2x10^6$	$3.6x10^3$	$1.1x10^7$	$1.3x10^7$

21 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$6.9x10^4$	$3.2x10^7$	$3.2x10^8$	$2.1x10^4$	$1.3x10^7$	$3.5x10^7$
Nitrate Reducers	$1.1x10^5$	$1.1x10^7$	$3.8x10^6$	$2.4x10^5$	$2.3x10^7$	$3.3x10^7$
Denitrifiers	$1.6x10^5$	$9.1x10^7$	$1.7x10^6$	$1.6x10^4$	$3.4x10^6$	$2.3x10^7$
Aerobic Heterotrophs	$1.2x10^7$	$1.4x10^8$	$1.0x10^9$	$7.0x10^6$	$7.6x10^8$	$1.0x10^9$
Anaerobic Heterotrophs	$4.3x10^5$	$1.0x10^7$	$5.1x10^8$	$3.6x10^5$	$1.5x10^7$	$3.3x10^7$

MICROBIAL POPULATIONS IN ENRICHMENTS WITH METHYLENE CHLORIDE

Anaerobic Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	$3.8x10^4$	$5.9x10^5$	$1.1x10^6$	$3.8x10^4$	$5.7x10^6$	$7.5x10^7$
Nitrate Reducers	$4.7x10^4$	$6.3x10^5$	$1.8x10^6$	$4.8x10^4$	$2.5x10^7$	$1.4x10^7$
Denitrifiers	$2.4x10^3$	$1.6x10^5$	$1.4x10^3$	$2.4x10^3$	$1.6x10^5$	$3.4x10^3$
Aerobic Heterotrophs	$1.2x10^5$	$4.1x10^5$	$1.4x10^6$	$1.3x10^6$	$9.1x10^6$	$2.9x10^8$
Anaerobic Heterotrophs	$3.0x10^3$	$1.3x10^4$	$1.4x10^6$	$4.0x10^3$	$6.5x10^5$	$2.7x10^5$

0.5 Percent Oxygen conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=9 Days	Column	T=0 Days	T=9 Days	Column
Degraders	$4.3x10^3$	$3.5x10^7$	$6.4x10^7$	$1.0x10^2$	$3.4x10^7$	$5.5x10^6$
Nitrate Reducers	$3.9x10^3$	$1.0x10^7$	$4.8x10^7$	$1.2x10^4$	$6.5x10^6$	$6.1x10^6$
Denitrifiers	$6.0x10^1$	$1.6x10^5$	0.00	$3.4x10^3$	$1.6x10^5$	$1.6x10^8$
Aerobic Heterotrophs	$9.7x10^3$	$1.3x10^8$	$5.5x10^8$	$1.9x10^4$	$2.2x10^8$	$1.9x10^8$
Anaerobic Heterotrophs	$9.8x10^2$	$8.1x10^6$	$5.7x10^7$	$3.6x10^3$	$2.2x10^8$	$1.6x10^6$

21 Percent Oxygen Conditions

	MSB			MSB + Nitrate		
	T=0 Days	T=5 Days	Column	T=0 Days	T=5 Days	Column
Degraders	0.0	0.0	$8.5x10^6$	0.0	0.0	$5.8x10^6$
Nitrate Reducers	$1.1x10^5$	$1.6x10^4$	$9.5x10^6$	$2.4x10^5$	$7.1x10^5$	$1.1x10^7$
Denitrifiers	$1.6x10^5$	$5.4x10^4$	$2.4x10^6$	$1.6x10^5$	$9.1x10^4$	$3.3x10^5$
Aerobic Heterotrophs	$1.2x10^7$	$8.3x10^5$	$1.6x10^8$	$7.0x10^6$	$9.8x10^5$	$1.3x10^8$
Anaerobic Heterotrophs	$4.3x10^5$	$3.5x10^4$	$3.5x10^5$	$3.0x10^5$	$1.9x10^6$	$1.1x10^5$