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MICROBIAL PENETRATION THROUGH BEREA SANDSTONE AND THE EFFECT OF NITRATE ON BIOGENIC SULFIDE PRODUCTION: THEIR RELEVANCE TO MICROBIAL ENHANCED OIL RECOVERY

The University of Oklahoma PH.D. 1985

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GRADUATE COLLEGE

MICROBIAL PENETRATION THROUGH BEREA SANDSTONE AND THE EFFECT OF NITRATE ON BIOGENIC SULFIDE PRODUCTION: THEIR RELEVANCE TO MICROBIAL ENHANCED OIL RECOVERY

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

GARY EDWARD JENNEMAN

Norman, Oklahoma

1985

MICROBIAL PENETRATION THROUGH BEREA SANDSTONE AND THE EFFECT OF NITRATE ON BIOGENIC SULFIDE PRODUCTION: THEIR RELEVANCE TO MICROBIAL ENHANCED OIL RECOVERY

A DISSERTATION

APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

By

DISSERTATION COMMITTEE

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DEDICATION

This dissertation is dedicated to my wife, Betty, whom has supported my every effort to complete this manuscript and degree. Both her inspiration and unselfish sacrifice of time has made this academic venture possible.

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MICROBIAL PENETRATION THROUGH BEREA SANDSTONE AND THE EFFECT OF NITRATE ON BIOGENIC SULFIDE PRODUCTION: THEIR RELEVANCE TO MICROBIAL ENHANCED OIL RECOVERY

CHAPTER I

INTRODUCTION

As of December, 1978 it was estimated that the discovered, original oil in place for the United States totaled 453 billion barrels (3). Of these barrels, only 118 billion had been produced by this time. By 1984 it was estimated that 136.9 billion barrels of oil had been produced (4), or roughly 30% of the discovered original oil in place. Current estimates predict that about one-third of the discovered oil is recoverable through primary (natural gas drive) and secondary (water-flooding) recovery techniques (3). In January 1984 it was estimated that proven reserves (recoverable oil) for the United States totaled about 27.3 billion barrels of oil (4). In the world there are an estimated 669.3 billion barrels of oil in proved reserves, but since 1980 the rate of production has declined and the rate of new discoveries has been declining sharply since 1970 (4). The United States alone consumes roughly 15 million barrels of oil a day of which about 25% is imported from other countries (4). If our present daily consumption rate is continued it would take approximately five years (1989) before present U. S. reserves are

depleted. Therefore, it is of paramount importance to exploit our total petroleum resources through enhanced oil recovery (EOR) technology in order to maintain our present reserves.

It should be no surprise that interest in enhanced oil recovery has increased in the past ten years. Of course, political and economic issues also play a vital role in research directive but they will not be discussed in this text because of their unpredictable and sometimes unscientific nature. It has been predicted that EOR could account for a final recovery of about 45% of the light oil in place (3), which would make available to the U.S. roughly an additional 100 billion barrels of oil. This is a fairly sizable amount considering our present proven reserves are only about one-fourth of this amount.

EOR entails some recently developed technologies, as well as, ones that have been in development since the 1950's. These technologies include polymer and surfactant flooding, steam and in-situ combustion, caustic flooding, CO_2 flooding, etc. (9). All these methods are designed to recover residual oil trapped in the reservoir after water-flooding (if applicable) has taken place. They are for the most part uneconomical (i.e. without tax incentive) at crude oil prices below about $30.00 per barrel (2). Furthermore, the increase in cost of this technology often parallels that of the price of oil. Therefore, it is of interest to search for EOR methods that are not necessarily linked to increases in the price of a barrel of oil. For this reason, as well as others, MEOR (microbial enhanced oil recovery) or MORE (microbial oil recovery enhancement), often used by industrial concerns for obvious reasons, has recently garnered the attention of the U. S. Department of Energy.

The history of MEOR is long in years but extremely short with respect to the amount of published research available. Beckman (1), in 1926, reported the

potential use of bacterial enzymes to recover oil based on his work involving the action of microorganisms on mineral oil. He also suggested using microorganisms to break oil-water emulsions caused by bitumens and coal. It was not until the 1940's that the American Petroleum Institute enlisted the help of C. E. ZoBell whom performed his pioneering work on the origin of petroleum, and subsequently in 1946 was issued a patent involving the use of a hydrocarbon-utilizing, sulfate-reducing bacterium that was claimed to cause chemical or physical changes in the reservoir useful to recover oil (10). These chemical and physical changes were thought to be a result of the bacterial transformation of the hydrocarbon itself or some change in the surface properties of the rock as a result of bacterial metabolites. ZoBell's creative ideas inspired subsequent investigators in the 1950's and 1960's such as Updegraff and Wren (8), Hitzman (5), as well as Jones (6) to propose further methods of using microorganisms to recover oil.

However, a real urgency to develop this technology has only emerged recently (i.e. since 1979) as a result of oil embargos, rising cost of crude oil and other reasons previously mentioned. The U. S. Department of Energy, through the Bartlesville Energy Technology Center and under the direction of E. C. Donaldson, began a program designed to understand the underlying mechanisms by which microorganisms could be utilized to recover oil, as well as, to develop the technology and understanding necessary to apply the process to the field.

The mechanisms presently under investigation include selective plugging of high permeability rock strata to reduce reservoir heterogeneity, modification of light and heavy oils, via biosurfactants, to improve their mobility and recovery, and the in situ production of biogenic gas to reduce oil viscosity and provide a repressurization of the reservoir. It is also clear that success of these mechanisms depends on the availability in the reservoir of bacteria capable of

performing these tasks. Therefore, research efforts were also directed towards understanding how indigenous or injected microorganisms can penetrate rock. Chapters II-IX of the following dissertation focus on this latter aspect of MEOR research.

Chapters II-VI explain how rock permeability and length can affect the penetration of a bacterium in nutrient-saturated sandstone (i.e. Berea sandstone). These chapters also propose mechanisms by which bacteria (motile and non-motile) could penetrate rock and discusses how the results of this study support or refute proposed models for bacterial penetration.

Chapters VII-IX deal with rock sterilization and how treatment effects due to autoclaving and dry-heat sterilization have an impact on microbial penetration rates in Berea sandstone. Understanding the effects of these treatments leads to the development of ideas and theories as to which physical properties of the rock have an effect on microbial penetration.

As mentioned previously, the studies involving microbial penetration are performed in rock saturated with nutrient solutions. This is important because bacterial cells require nutrients to produce energy for useful work. This work may be referred to in physiological terms as motility and gas production, as well as biosurfactant and biopolymer production. These functions are all potentially important to mechanisms for oil release. In order to most efficiently supply this energy it is necessary to optimize the potential energy that is available to the organisms under the conditions to be employed (i.e. oil reservoir).

Since MEOR processes are to be employed in subterranean environments, it will be necessary to supply externally to these reservoirs the required nutrients for cellular energy production. These nutrients must contain all essential elements necessary for cell reproduction and metabolism. Especially important

will be the addition of elements that are limiting in the injected brines. Most notably will be the addition of carbon, nitrogen, and phosphorus. But also of importance will be the limitation of oxygen in the brine. Oxygen provides the best bio-oxidant available for the conversion of organic matter into chemical energy. In the absence of oxygen, nitrate provides the best oxidant for cellular energy yielding processes.

The addition of nitrate to nutrient supplements in MEOR processes can serve in the absence of oxygen as a means of coupling the oxidation of organic matter into useful chemical energy for the cell. This of course implies that the bacteria used, whether indigenous or injected, be able to use nitrate as a terminal electron acceptor in the absence of oxygen. Denitrifying bacteria are capable of performing this function at the expense of oxidizing both organic or inorganic substrates (7). These organisms are ubiquitous in nature being found in soil, sewage, marine sediments, groundwater, and reservoir brines. Under anoxic conditions they are in direct competition with anaerobes (strict or facultative) that utilize the same energy sources. Therefore, anaerobes such as sulfate-reducers, acetogens, and methanogens pose a problem as potential competitive partners for energy sources in an anaerobic habitat.

Chapters X-XIV deal with the effect of nitrate and sulfate addition to an anaerobic sample on the preferential reduction of either of these electron acceptors. Since many petroleum reservoirs contain sulfate-reducing bacteria it is important to know what effect the addition of exogenous nutrients can have on stimulating or suppressing sulfate reducing activity. This is important because the reduction of sulfate to sulfide is an undesirable end-product of any MEOR process due to the following properties of sulfide: corrosiveness, toxicity, and its propensity to precipitate heavy metal ions. Therefore, it is of interest to study

the fate of sulfate in the presence of nitrate and denitrifying bacteria which are capable of competing for the same energy sources as sulfate-reducers (i.e. acetate, hydrogen, and intermediates of glucose metabolism).

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CHAPTER II

ABSTRACT

Microbial Penetration Through Nutrient-saturated Berea Sandstone

Penetration times, and penetration rates, for a motile Bacillus strain growing in nutrient-saturated Berea sandstone cores were determined. The rate of penetration was essentially independent of permeability above 100 millidarcys (mD) and rapidly declined for permeabilities below 100 mD. It was found that these penetration rates could be grouped into two statistically distinct classes consisting of rates for permeabilities above 100 mD and those below 100 mD. Instantaneous rates of penetration were found to be zero-order with respect to core length for cores with permeabilities above 100 mD and first-order with respect to length for cores below 100 mD permeability. The maximum observed penetration rate was 0.47 $cm \cdot h^{-1}$ and the slowest was 0.06 $cm \cdot h^{-1}$; however, these rates may be underestimates of the true penetration rate since the observed rates included the time required for growth in the flask as well as the core. The relationship of penetration time to the length of the core squared suggested that cells penetrated high permeability cores as a band and low permeability cores in a diffuse fashion. The motile Enterobacter aerogenes penetrated Berea sandstone

cores 3-8 times faster than the non-motile Klebsiella pneumoniae strain when cores of comparable length and permeability were used. A penetration mechanism based entirely on motility predicted penetration times that were in agreement with the observed penetration times for motile strains. The fact that non-motile strains penetrate suggested that filamentous and/or unrestricted growth may also be important.

CHAPTER III

INTRODUCTION

In recent years, increased attention has focused on the microbiology of subterranean aquifers and petroleum reservoirs. This interest has been generated by a desire to understand how microorganisms can be used to recover petroleum from reservoirs and how they influence the fate of wastes accidentally or intentionally released into fresh-water or salt-water reservoirs (10,27). The use of microorganisms in the recovery of oil is the main concern of this paper. Microbially enhanced oil recovery (MEOR) has been the topic of several recent books and reviews (7,8,28). Microorganisms, either indigenous to the subsurface or injected from above ground, are fed a carbohydrate-based medium (e.g. cattle-feed molasses) with the intention of stimulating in situ microbial growth. The in situ growth of microorganisms can be used to produce cells and associated extracellular polymer for the improvement of reservoir sweep efficiency (18) or be used to form gases, solvents or biosurfactants to increase oil mobility in the reservoir (12,17).

MEOR has stimulated a desire to understand how injected nutrients or chemicals can be metabolized by microorganisms in porous media (i.e. rock). It is also of interest to know how water-soluble carbohydrate solutions will affect the spread of microbes throughout the porous medium and therefore govern the

availability of biological products potentially useful for MEOR. An understanding of the rates at which these processes occur is needed in order to evaluate the economic and practical feasibility of MEOR. Therefore, it is necessary to understand the kinetics and mechanisms of microbial processes in porous media.

Little information exists on the penetration of microbes under growth conditions through rock. Craw (6) studied the penetration of bacteria out of different filters (Berkefeld, Coulter, etc.) saturated with nutrients and tried to correlate penetration times with grain size of the filters. However most work has involved the introduction of dead bacteria or bacteria under non-growth conditions into porous media in order to study the kinetics of bacterial adsorption and filtration (13,19). Recent work concerned with plugging rates involving the injection of bacteria under growing conditions were performed in highly permeable rock models (15). However, much residual oil and disposed wastes reside in less permeable rock (e.g., consolidated sandstone). It was the purpose of this study to examine the kinetics of microbial growth as it relates to microbial penetration times through a consolidated Berea sandstone core. It is shown that relationships exist between penetration time, penetration rate, permeability, and core length. Furthermore, these relationships allow us to speculate as to the importance of growth, motility, and chemotaxis in bacterial penetration.

CHAPTER IV

MATERIALS AND METHODS

Bacterial Strains. Strain BCI-1NS was isolated from a Berea sandstone core. It is a gram-positive, spore-forming rod occurring both as single cells and in chains. Spores are located subterminally. Individual cells are approximately 4.0 um long and 0.8 µm wide and they are motile.

Strain 140 was isolated from a water sample obtained from the aquifer underlying the Norman Municipal landfill. Morphologically, its characteristics are virtually identical to those described for strain BCI-1NS.

Enterobacter aerogenes and Klebsiella pneumoniae strains used in this study were obtained from the University of Cklahoma culture collection.

Media and Growth Conditions. E. aerogenes and K. pneumoniae were each grown in Nutrient Broth (Difco) amended with 1.0% (w/v) NaCl and 0.1% (w/v) $NaNO_3$. Cultures were grown at 36°C under static conditions.

Strains BCI-INS and 140 were each grown in Medium E amended with 0.05% (w/v) Yeast Extract (Difco) and 0.1% (w/v) $NaNO_3$. Medium E contained (all w/v): 5.0% NaCl, 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 1.0% sucrose in 100 mM phosphate buffer (pH 7.0) supplemented with a 1.0% (v/v) trace metals solution (4). BCI-INS and 140 strains were grown at 50°C under static conditions and had growth rates of 0.94 and 1.14 h^{-1}, respectively.

Core Preparation. Cores were cut from 12x12x6 inch blocks of Berea sandstone obtained from Cleveland Quarries, Amherst, OH. Unless otherwise indicated, cores were cut into 1.9 cm diameter cylinders. Each core was steam cleaned for 2 weeks to remove humic substances and then dried in an oven for 24 hours at 125°C. The core was cooled in a dessicator and coated on the sides with Conap Easy-poxy (Fisher). The core was then cut to the desired length.

Growth Chambers. Cores used in growth-related experiments were initially autoclaved at 121°C for 30 min while submerged in a 5% (w/v) NaCl solution. It was found that this step was necessary to remove residual toxic compounds from the epoxy which inhibited the growth of bacteria used in this study. After cooling, the core was then placed in a permeability-measuring apparatus (Fig. 1) and the initial permeability (K_o) was determined using the same brine solution that had been previously filtered through a 0.2 μm Millipore filter to remove small particulate matter.

Each core was then flushed with at least 3 pore volumes (PV) of the respective nutrient medium while still attached to the permeability apparatus. For strains BCI-1NS and 140, Medium E with 0.05% (w/v) yeast extract and 0.1% (w/v) $NaNO_3$ was used. Nutrient Broth with 1% (w/v) NaCl and 0.1% (w/v) $NaNO_3$ was used for the enteric bacteria.

The core was then placed in a growth chamber similar to the one used by Chang and Yen (3) which consisted of two 250 ml Ehrlenmeyer flasks with a 1.9 X 2.5 cm glass nipple attached to the bottom of each flask (Fig. 2). The core was then fastened into place by means of hose clamps and autoclaved for an additional 20 min at 121°C. After cooling, trace metals and $MgSO_4$ were added aseptically to 100 ml of Medium E in flasks A and B. The growth chambers were then incubated overnight at the respective incubation temperature. Flask A was then

Figure 1. Permeability Apparatus.

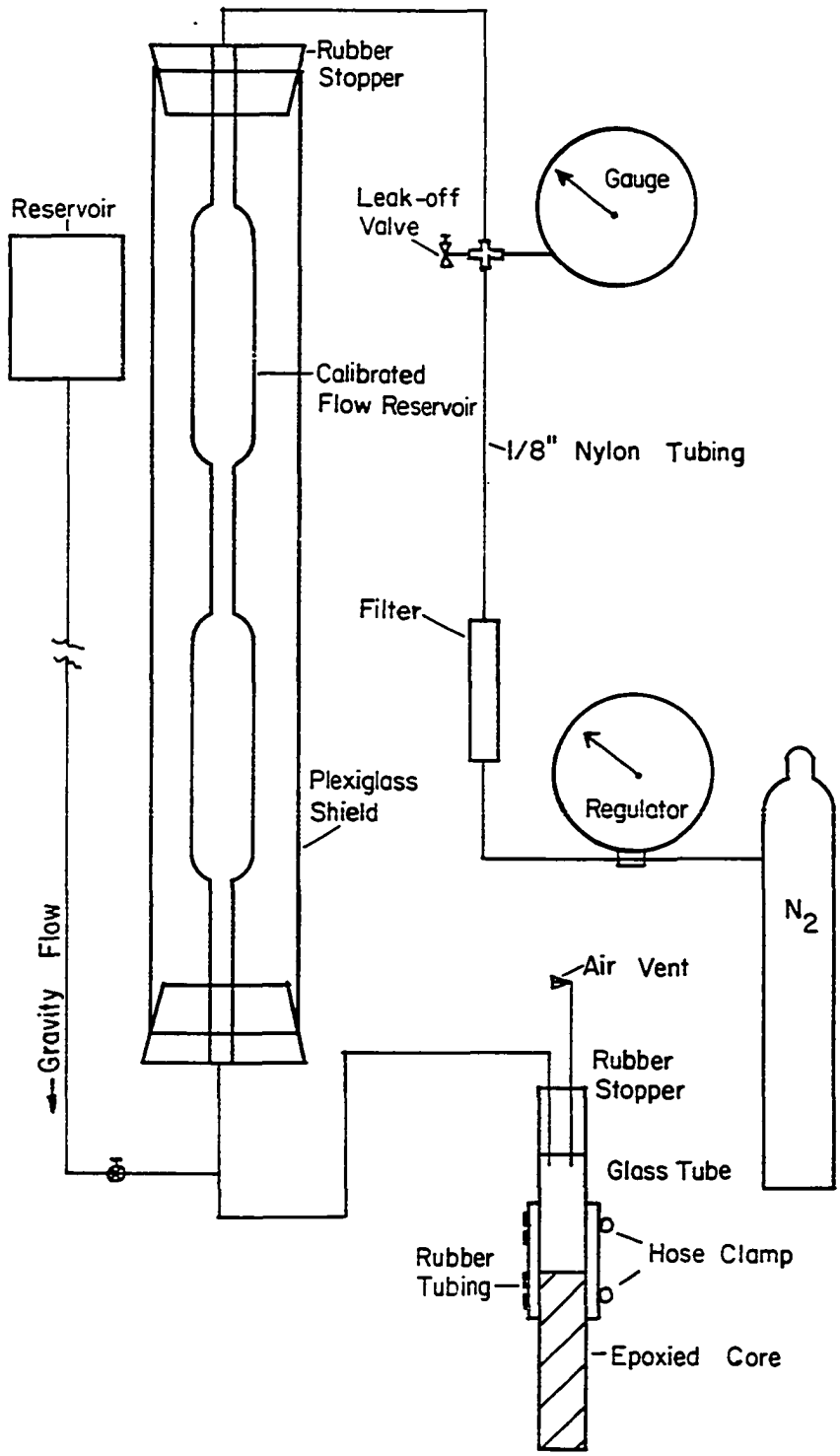
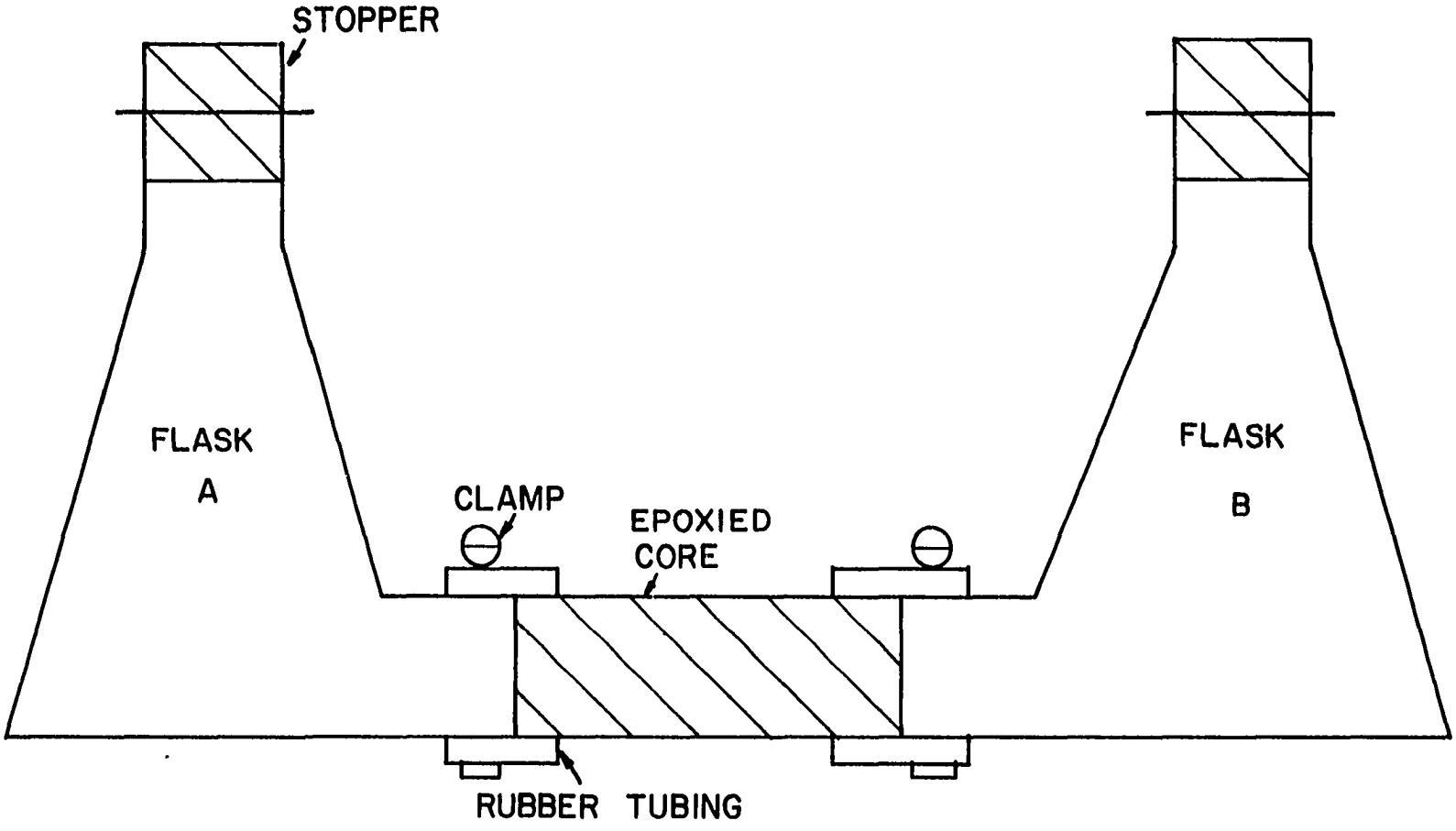

Rubber Stopper
Gauge
Reservoir
Leak-off Valve
Calibrated Flow Reservoir
1/8" Nylon Tubing
Filter
Plexiglass Shield
Regulator
N_2
Gravity Flow
Air Vent
Rubber Stopper
Glass Tube
Hose Clamp
Rubber Tubing
Epoxied Core

Figure 2. Growth Chamber. Modified from Jang et al. (16).


STOPPER
FLASK
A
CLAMP
EPOXIED
CORE
FLASK
B
RUBBER TUBING

inoculated with 1.0% (v/v) of the appropriate strain grown in the same medium. The growth chambers were incubated at 50°C for BCI-1NS and 140 and at 36°C for the enteric bacteria. Penetration or breakthrough time (t_p) is the time (h) elapsed from the time flask A was inoculated until faint visible growth appears in flask B. The penetration rate is the length of the core divided by the penetration time ($cm \cdot h^{-1}$) and thus is representative of average rates and not instantaneous rates. Visible growth refers to the point at which faint turbidity appeared in flask B. The cells in flask B were periodically checked by plating on Medium E with yeast extract and nitrate, to insure that these cells were of same type as those inoculated into flask A. No growth was observed in uninoculated growth chambers.

Permeability and Porosity Measurements. Permeability, which is a measure of the fluid conductivity of a porous material, was calculated using Darcy's Law:

$$K = \frac{q\mu}{A(\Delta P/L)}$$

where "q" is the volumetric flow rate ($cm^3 \cdot sec^{-1}$), "μ" is the dynamic viscosity of the fluid in centipoise (cp), "A" is the cross-sectional area of the core in cm^2, "L" is the length of the core in cm, and "ΔP" is the differential pressure across the core in atm. The permeability (K) is given in units of Darcies.

The permeability apparatus (Fig. 1) maintains a constant pressure differential across the core. Thus, by measuring flow rate the permeability of the core can be calculated. Each core was injected under pressure with a 5% (w/v) NaCl brine solution (pre-filtered through a 0.22 μm Millipore filter) and the time for a fixed volume of brine to exit the core was measured. The viscosity of the 5% (w/v) NaCl solution was determined to be 0.91 cp at 38°C. The K_o was

determined after the permeability of the core had stabilized (i.e. permeability values of three successive readings within 5% of each other). At times, it was found that flow rate would continuously decrease with increasing volume of brine flushed through the core. When this occurred, the core was backflushed with several PV of brine. After the backflush, the core was flushed in the original direction of fluid flow and the permeability was calculated using data obtained with the first or second PV of fluid flushed through the core. In most cases, it was found that permeability increased after backflushing, which indicated that the reductions in permeability were probably due to fines migration (11,20). The Reynolds number for all cores used in these experiments was below 10^{-2}, therefore, permeability measurements were in the region of viscous flow and Darcy's Law is valid (5). Initial permeability values also indicated that the epoxy coating provided a good seal between outside wall of core and epoxy, since permeabilities measured were in close approximation to permeability quoted by Cleveland Quarries. This is very important since it is desirable to not have bacteria growing between rock and epoxy coating.

Porosity was measured by first saturating the core overnight with 5% (w/v) NaCl solution under a vacuum. The core was weighed (bulk volume) and then dried in a microwave oven (Amana, 700 watts) for 12 minutes at full power and allowed to cool in a desiccator. The core was then weighed again to determine the dry weight. The weight of the brine-saturated core minus the weight of the dry core gave the weight of a pore volume of fluid. The porosity (ϕ) was calculated as:

$$\frac{W_{PV}/\rho_B}{\text{Bulk volume}} = \phi \qquad (2)$$

where W_{PV} is the weight of a PV of fluid, ρ_B is the density of the 5% NaCl solution (1.02 g/ml) and the bulk volume can be calculated from core dimensions

(length x cross-sectional area). The PV was calculated using equation 3:

$$W_{PV}/\rho_B = PV \qquad (3)$$

Regression Analysis. A multiple regression model was developed to establish whether the penetration rates obtained from cores with permeabilities above 100 md (n=24) were significantly different from those rates obtained in cores with permeabilities below 100 md (n=14).

$$y = B_o + B^I x \qquad (4)$$

$$y = B_o + B^{II} x \qquad (5)$$

where "y" is the penetration rate ($cm \cdot h^{-1}$), "x" is the independent variable, length (cm), B_o is the y-intercept, and B^I and B^{II} are the regression coefficients for rates in cores with permeabilities above 100 mD and below 100 mD, respectively. The y-intercepts were assumed to be equal. The actual values obtained for the y-intercept in each case were very close. The hypothesis to be tested was whether the slopes in equation 4 and equation 5 are equal (H_o: $B^I = B^{II}$). The hypothesis was tested by combining equations 4 and 5 and establishing a "dummy variable" to explain the difference in the slopes ($B^{II} - B^I$). If this difference was significantly different from B^{II} or B^I then the null hypothesis was rejected and B^I and B^{II} were assumed to be from different populations. The significance test was performed using a two-tailed t-test. The regression routine used also provided information to determine if B_o, B^I and B^{II} were significantly different from zero. Also, an overall fit of the data to the dependent variable rate, regressed against both length and permeability class was given by an F-test. The R^2 value represented the amount of variance that the overall model explained in terms of the two independent variables. The total analysis was performed using the REG routine of the Statistical Analysis Systems program (23).

CHAPTER V

RESULTS

Effect of length and permeability on penetration. The effect of the rock permeability on rate of microbial penetration for strain BCI-1NS is shown in Figure 3. Rates were determined for the same bacterium using Berea sandstone rock of varying lengths. The lengths of cores sampled were evenly distributed within the range of permeabilities tested. The results showed that the rate of penetration was fastest and relatively independent of permeability above 100-200 mD and decreased rapidly for cores with permeabilities below this value. The maximum penetration rate measured was 0.47 $cm \cdot h^{-1}$ in an 11.4 cm long core with a K_o of 182 mD, while the slowest rate measured was 0.06 $cm \cdot h^{-1}$ in an 11.3 cm long core with a K_o of 70.7 mD. The lowest permeability examined in this study was approximately 55.0 mD and the highest was 520 mD. At least two other cores of less than 100 mD permeabilities with lengths of 11.4 cm were tried, but nc growth was observed in flask B for these cores within a two week period.

Penetration rates were divided into two classes based on the permeability of the cores. One class contained cores with permeabilities greater than 100 mD and another with permeabilities below 100 mD. When these two classes of penetration rates were plotted as a function of the core length two linear relationships were found (Figure 4). An overall fit of the entire data set to

Figure 3. Effect of permeability on rate of bacterial penetration by strain BCI-1NS. Rates were calculated by dividing penetration times in Figures 5A and 5B by the length of rock used.

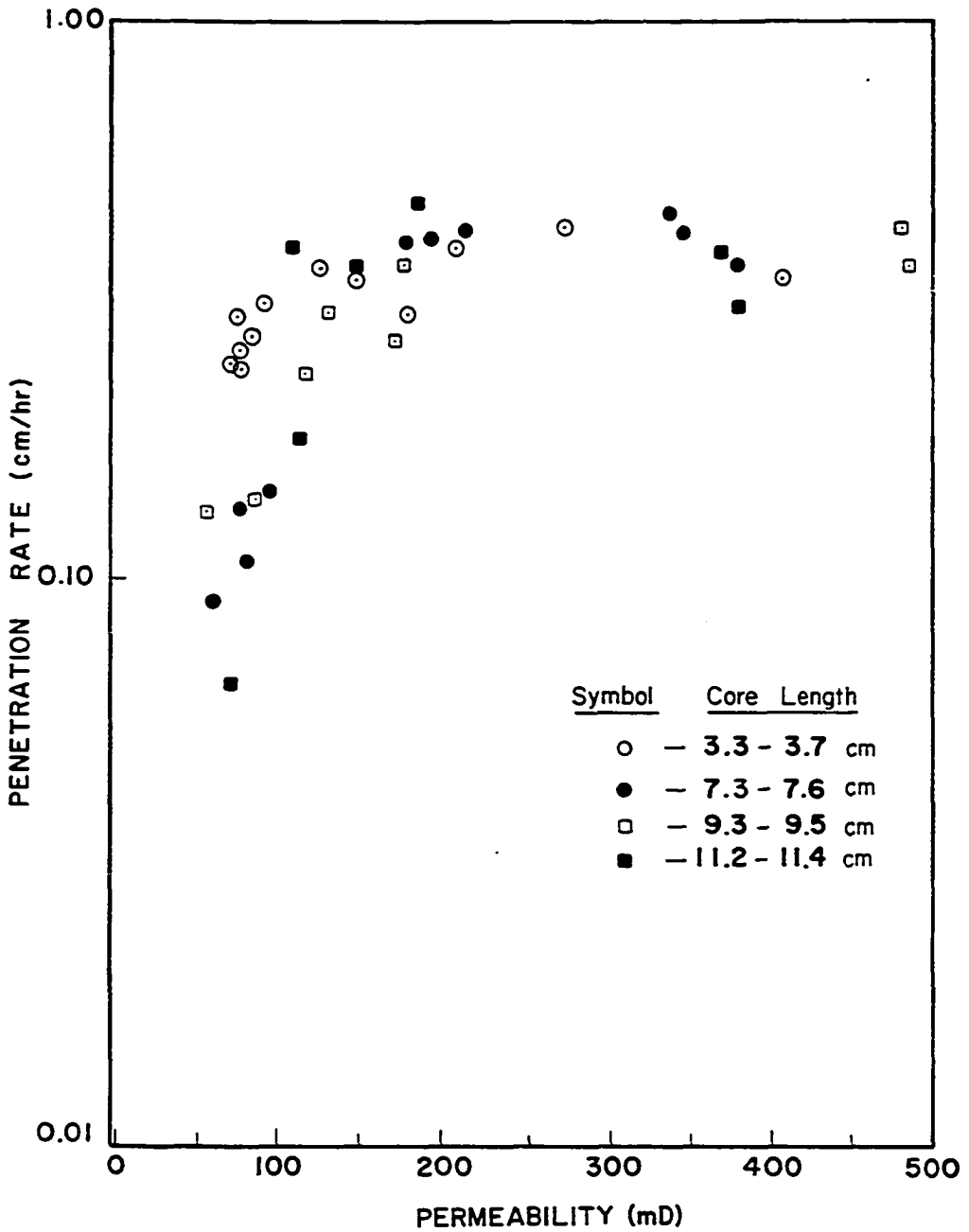
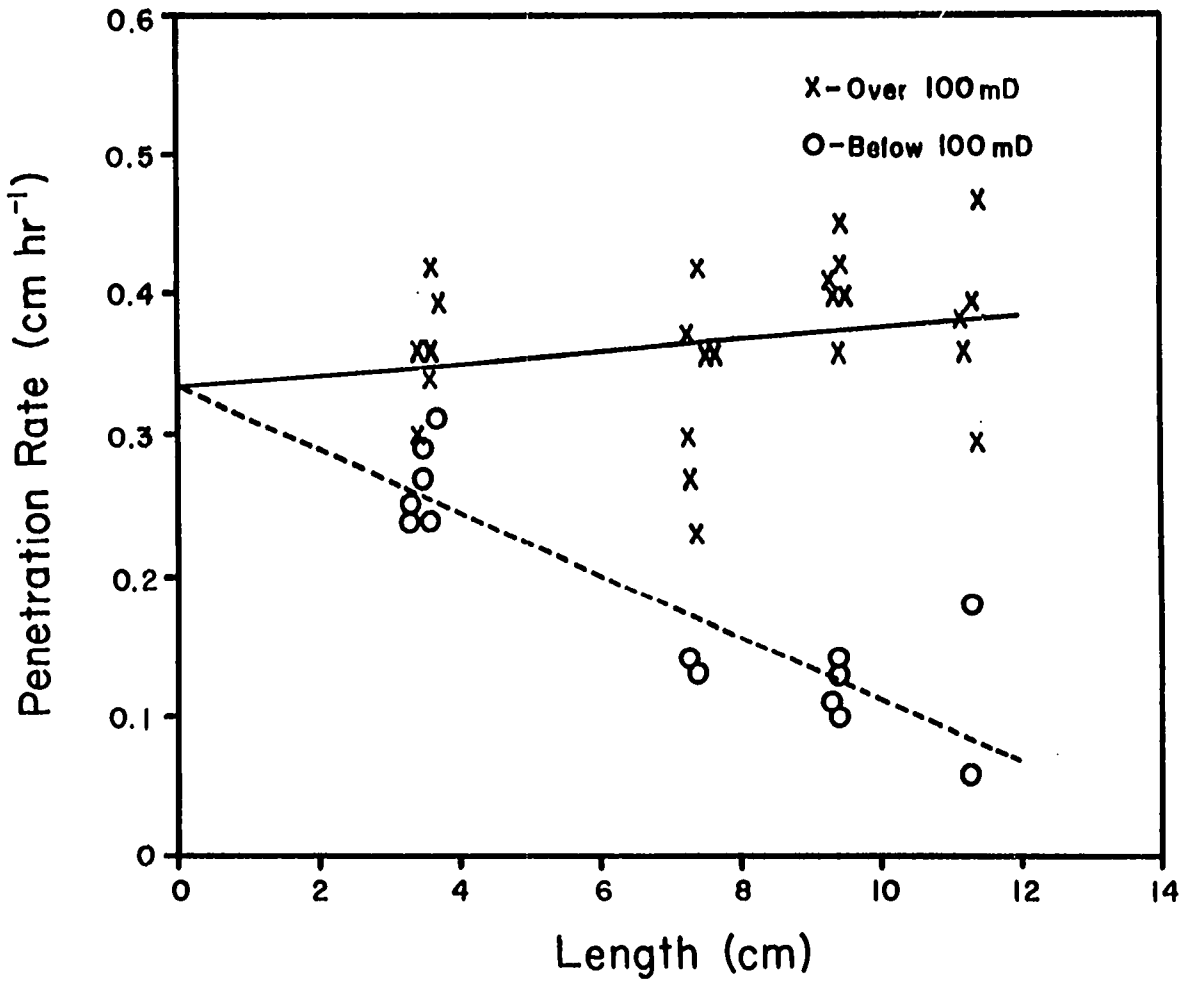

1.00
0.10
0.01
PENETRATION RATE (cm/hr)
0
100
200
300
400
500
PERMEABILITY (mD)
Symbol Core Length
○ — 3.3 - 3.7 cm
● — 7.3 - 7.6 cm
□ — 9.3 - 9.5 cm
■ — 11.2 - 11.4 cm

Figure 4. Effect of core length on rates of bacterial penetration represented in Figure 3 for bacterial strain BCI-1NS growing in Medium E amended with 0.05% (w/v) yeast extract and 0.1% (w/v) sodium nitrate. The solid line represents a least-squares, best fit line for cores above 100 mD, (X), and the dashed line a best fit line for cores below 100 mD, (O). These points were replotted from Figure 3.


X-Over 100 mD
O-Below 100 mD
Penetration Rate (cm hr^{-1})
Length (cm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14

the multiple regression model described in Materials and Methods resulted in a highly significant F-value of .0001 (Table 1). This indicates that the model accounts very well for the behavior of the dependent variable. Furthermore, an R^2 value of 0.8032 for the overall model was obtained. Thus, approximately 80% of the observed variance in the penetration rates can be explained by the independent variables of core length and permeability class. The remainder of the variance may be explained as experimental error or some other independent variable of the core that was not measured. More importantly, the model indicates that the difference between the slopes (B^{II}-B^{I}) is significantly different from zero which implies that the penetration rates can be grouped into two distinct classes based on permeabilities above and below 100 mD. In the model chosen, the y-intercepts for both permeability classes were assumed to be equal since at a length equal to zero the penetration rate should be independent of the core used when the same bacterium and same nutritional conditions were used in all experiments.

If penetration time is plotted as a function of core length for cores with permeabilities above 100 mD, a linear relationship is obtained as shown in Figure 5A. The slope of the line is equal to 2.5 $cm \cdot h^{-1}$ and the y-intercept is equal to 2.0 h. Therefore, an equation that describes the penetration of BCI-INS through Berea sandstone above 100 mD is:

$$t_p = 2.5L + 2.0 \qquad (6)$$

where t_p is the penetration time (h) and L is the length of rock (cm). The average rate of penetration of BCI-INS calcuated from the reciprocal of the slope of equation 6 was 0.4 $cm \cdot h^{-1}$.

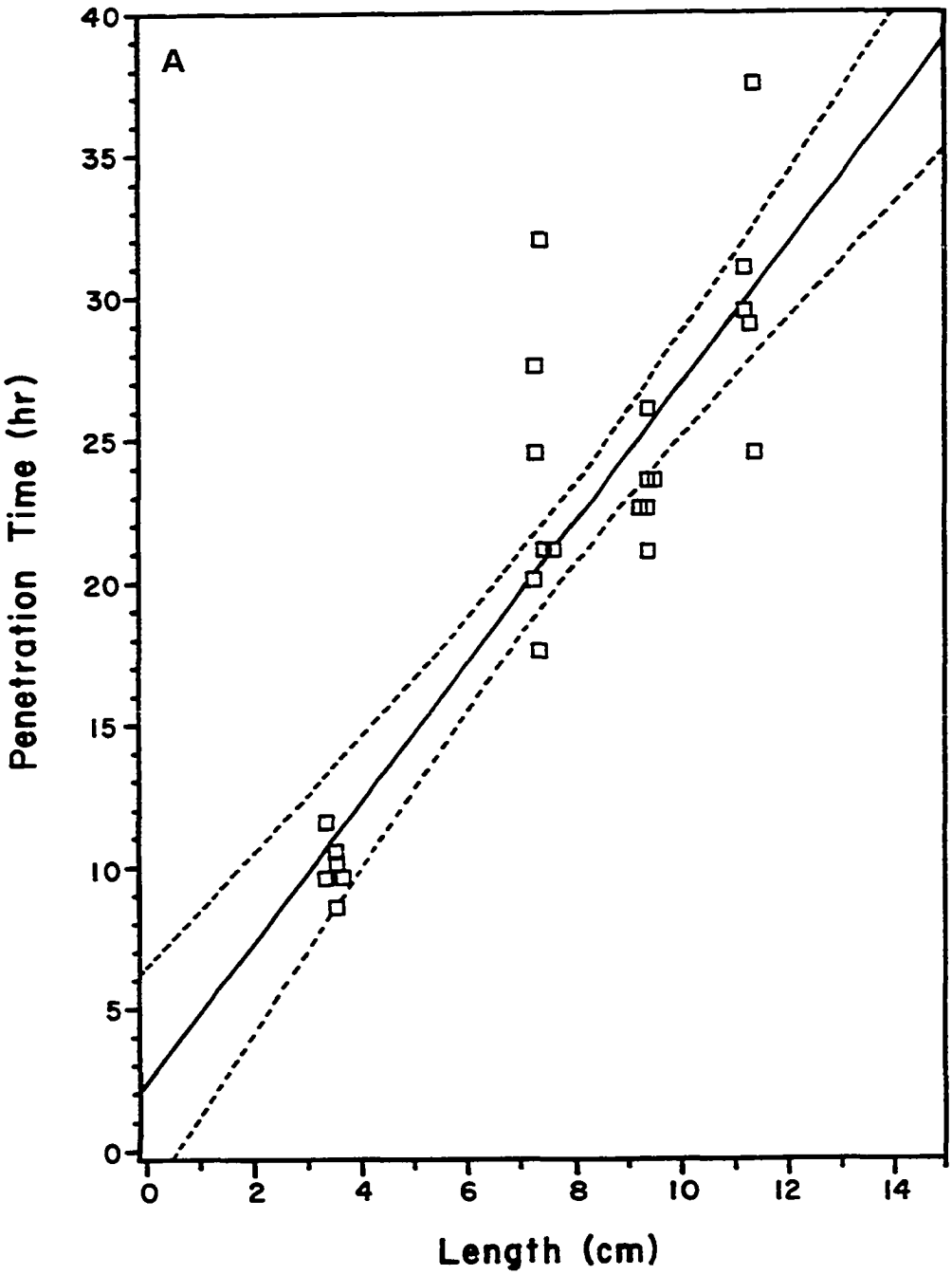
Table 1. Statistical parameters for multiple regression analysis of rate vs. length data[a]

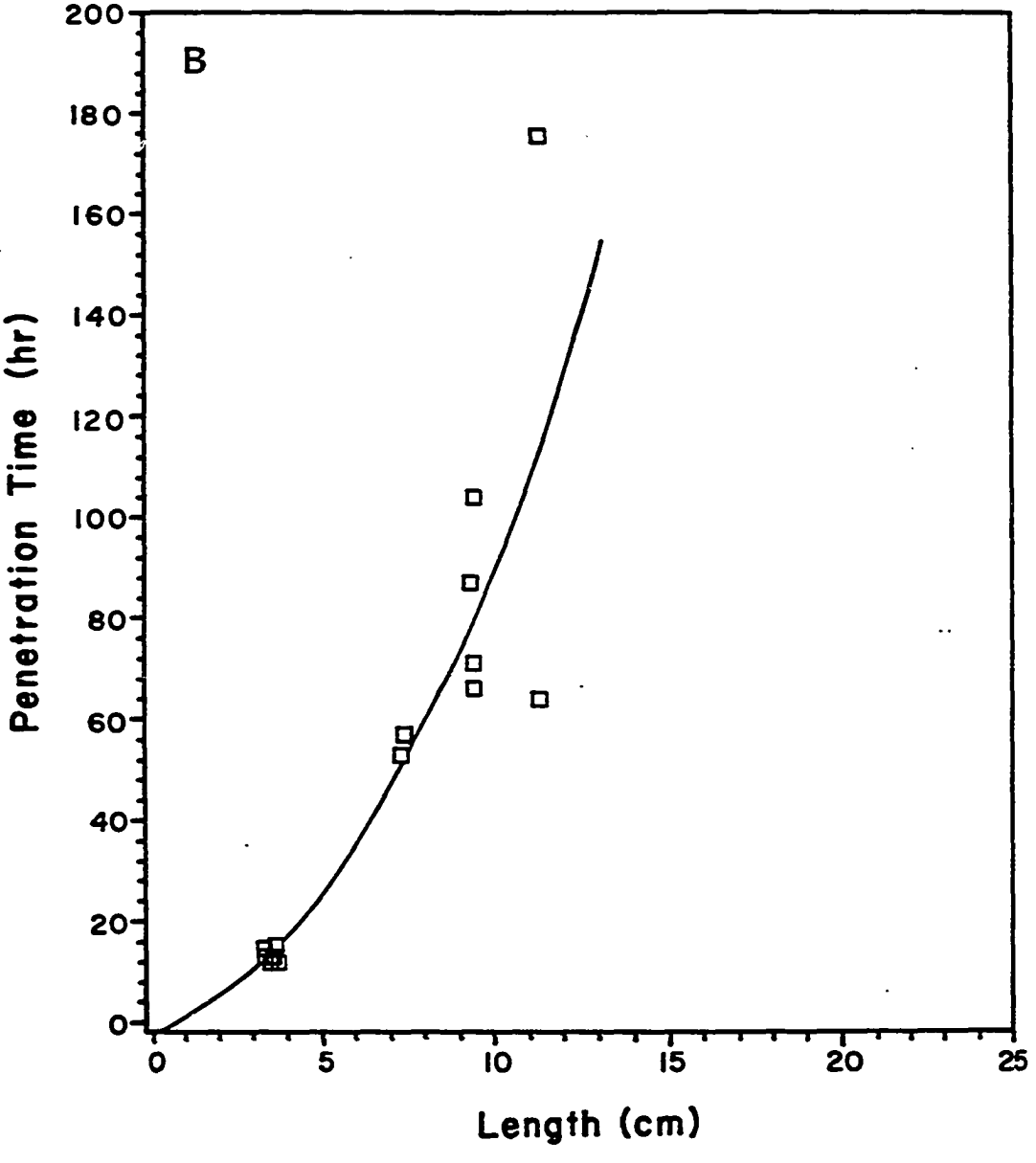
Parameter	Overall model	Bo	B^I	B^{II}	$B^{II}-B^I$
Degrees of Freedom	37	1	1	1	1
Probability > F	.0001	-[b]	-	-	-
Probability > /T/	-	.0001	0.1149	.0001	.0001

[a]The overall model tested is a multiple regression model with the dependent variable rate regressed against two independent variables representing permeability above 100 mD and permeability below 100 mD. B^I and B^{II} are the regression coefficients representing these independent variables while Bo is the y-intercept and was assumed to be the same for both permeability classes. The prob >F for the overall model tests how well this model accounts for the dependent variables behavior (penetration rate) after adjusting for mean. The prob >/T/ represents the significance of Bo, B^I, and B^{II} from zero as well as the significance of whether B^I is equal to $B^{II}(B^{II}-B^I)$, using a two-tailed t-test. See Figure 6 for a plot of data and materials and methods for additional explanation.

[b]not applicable.

Figure 5. Effect of core length on penetration time in cores above 100 mD for strain BCI-INS grown in Medium E amended with 0.05% (w/v) yeast extract and 0.1% (w/v) sodium nitrate. A. A least squares linear regression routine was used to plot the line and its 95% confidence intervals are represented by the dashed lines. A log-anova (Scheffe'-Box test) test for homogeneity of variances was run on the data and found not to be significant at P 0.05 and therefore the variances were considered homogeneous. B. Effect of core length on penetration time in cores below 100 mD for strain BCI-INS grown in Medium E amended with 0.05% (w/v) yeast extract and 0.1% (w/v) sodium nitrate. The curve was fit to the data using a curve fitting routine designed for an HP-41C (24). The curve is of the general form $y = ax^b$ with a coefficient of determination (R^2) of .938, where a = 1.395 and b = 1.808. A log-anova test of the log transformed y-variable indicated that the transformed variances were not heterogeneous at a significance level of P 0.05. Also a linear least squares fit of the transformed data yield a R^2 value of 0.92 indicative that a curvilinear relationship for t_p vs L is valid.


A
Penetration Time (hr)
Length (cm)
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12
14


B
Penetration Time (hr)
Length (cm)
0
20
40
60
80
100
120
140
160
180
200
0
5
10
15
20
25

On the other hand, a plot of penetration time vs core length for sandstone below 100 mD yields a curvilinear relationship (Fig. 5B). A fit of the data to a power curve of the general form $y = ax^b$ yields an R^2-value of 0.938. An equation for the curve in Figure 5B is:

$$L = .8318\ (t_p)^{.5531} \qquad (7)$$

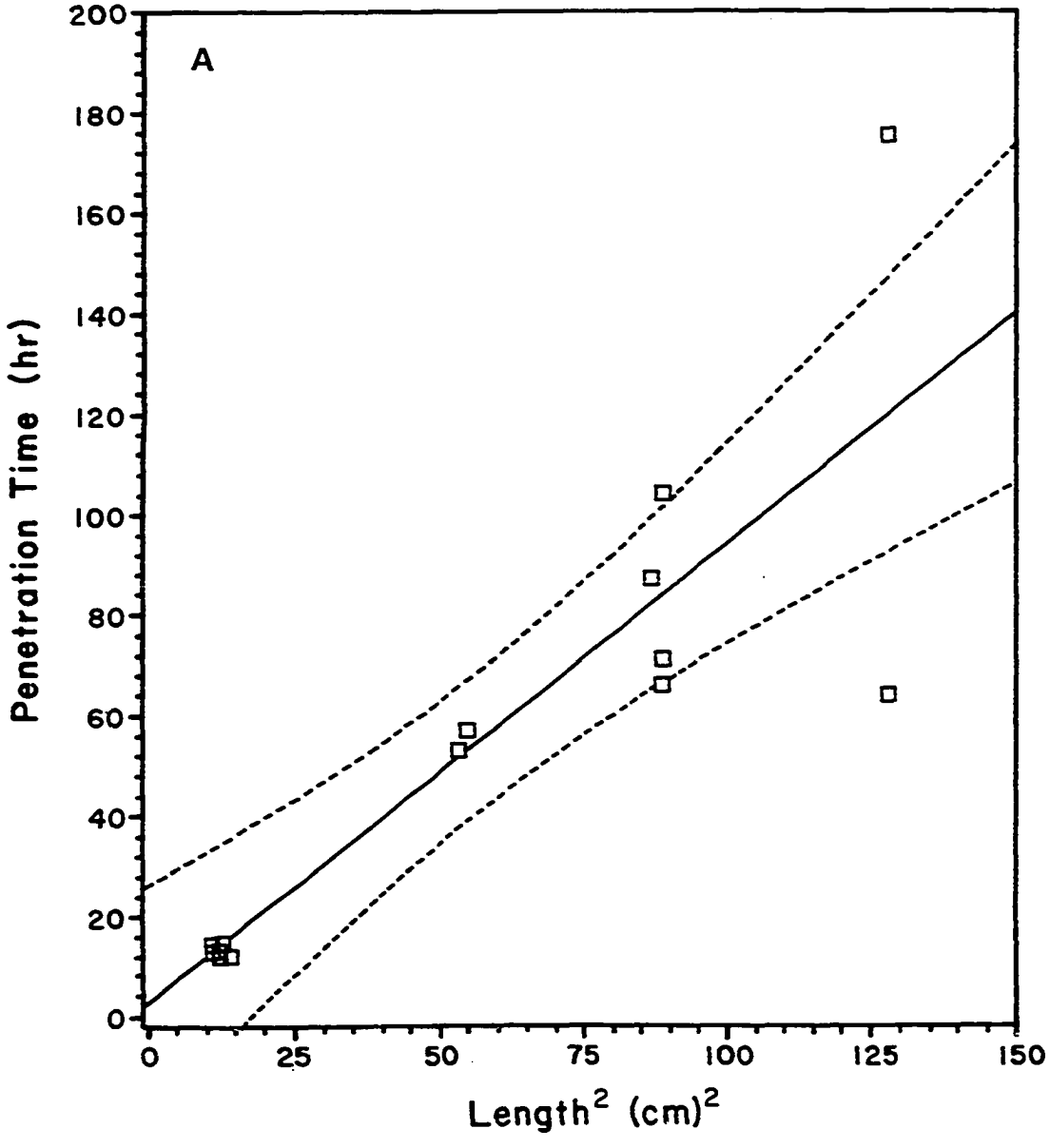
where L is core length (cm) and t_p is penetration time. If equation 7 is solved as a function of L, the following equation is obtained:

$$t_p = 1.395L^{1.808} \qquad (8)$$

which implies that t_p increases in a nearly linear fashion if plotted as a function of L^2 (Figure 6A). The possible significance of this is explained later.

Importance of cell motility, diffusion, and growth. The effect of cell motility on the penetration time was investigated using both a motile and a non-motile enteric bacterium (Table 2). Two taxonomically similar organisms were selected for this experiment, one of which is a motile bacterium (E. aerogenes) and the other a non-motile bacterium (K. pneumoniae). The growth rate of these two organisms under nitrate-respiring conditions (O_2-limited) was 1.38 h^{-1} and 1.11 h^{-1} for E. aerogenes and K. pneumoniae, respectively. The motile strain penetrated the rock at a rate 3-8 times faster than the non-motile strain (Table 2). Permeability was not a factor since penetration time for the non-motile strain in the highest permeability core was still approximately 4 times longer than the slowest penetration time for the motile strain.

Figure 6. The relationship between the penetration time and the square of the core length. A. For cores with permeabilities below 100 mD. Solid line is a least-squares fit of the data with the dashed lines representing the 95% confidence intervals. Data were replotted from Figure 5B. Although the data display severe heteroscedasticity of variance a log transformation of the y-variable will eliminate this heterogeniety to a non-significant level ($P < 0.05$). The rationale for selecting a linear least-squares fit for this data is derived from the fact that equation 8 implies that the relationship of t_p vs L2 should be fairly linear and should pass through the origin. B. For cores with permeabilities above 100 mD. The data were fit to a power curve of the general form $y = ax^b$ using a curve fitting routine (24). This equation had the best fit ($R^2 = 0.86$ when $a = 3.152$ and $b = 0.466$) out of the four equations tested, linear ($R^2 = .71$), logarithmic ($R^2 = .78$), exponential ($R^2 = .71$) and power. Data were replotted from Figure 5A.


A
Penetration Time (hr)
Length^2 (cm)^2
0
20
40
60
80
100
120
140
160
180
200
0
25
50
75
100
125
150

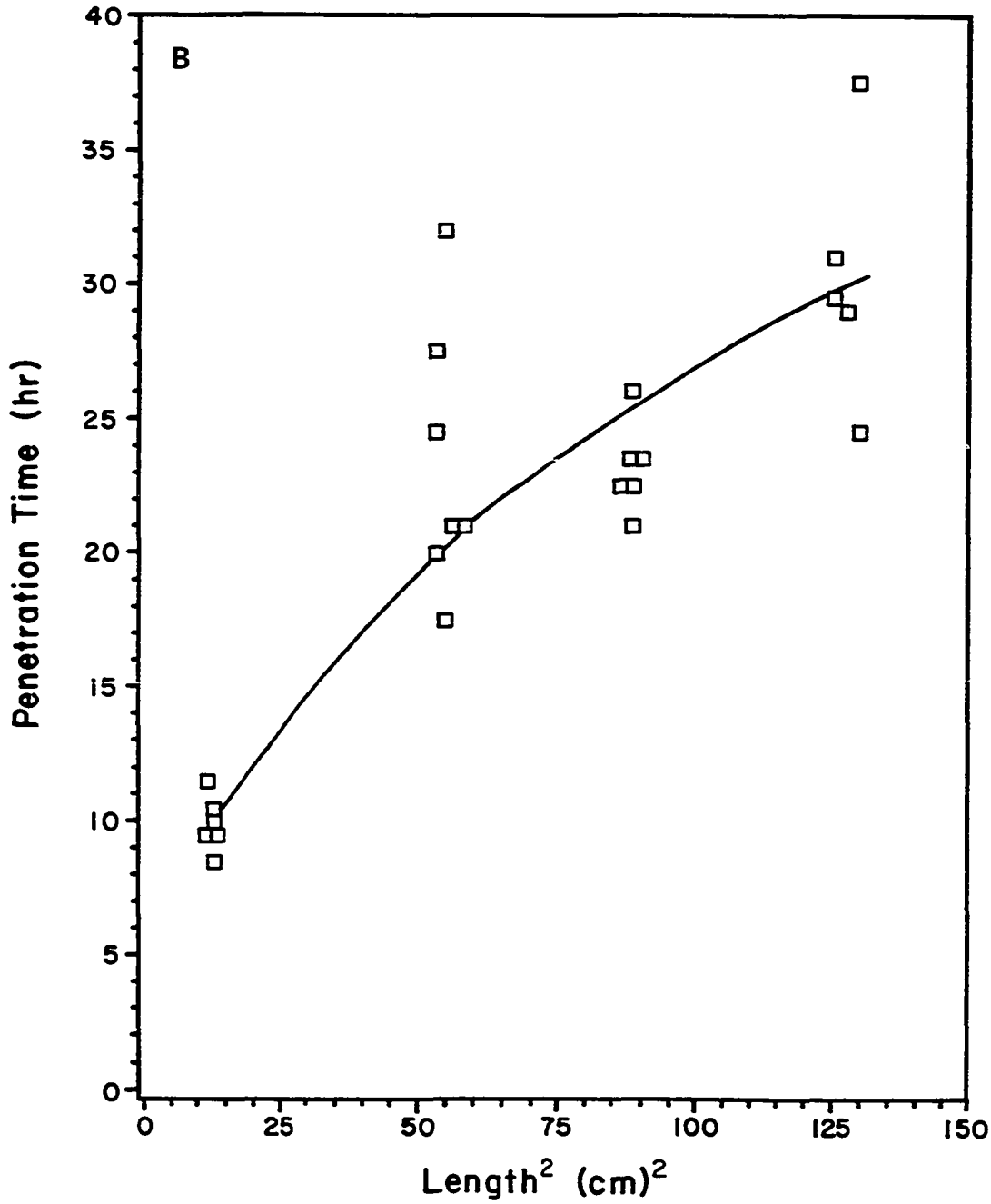

B
Penetration Time (hr)
Length^2 (cm)^2
0
5
10
15
20
25
30
35
40
0
25
50
75
100
125
150

Table 2. Penetration times of E. aerogenes (motile) and K. pneumoniae (non-motile) through Berea sandstone cores

Organism	Core No.	Growth Rate hr^{-1}	K_o md	Length cm	Penetration Time[a] hrs
E. aerogenes[b]	150		518	4.8	9.5
	151	1.38	611	4.8	9.5
	152		628	4.9	13.5
K. pneumoniae[b]	153		565	4.8	40
	154	1.11	611	4.8	48-55
	155		466	5.0	83

[a]Penetration time was the time from inoculation in flask A until visible growth occurs in flask B.

[b]The motility of each strain was checked by inoculating MIO Broth (Difco).

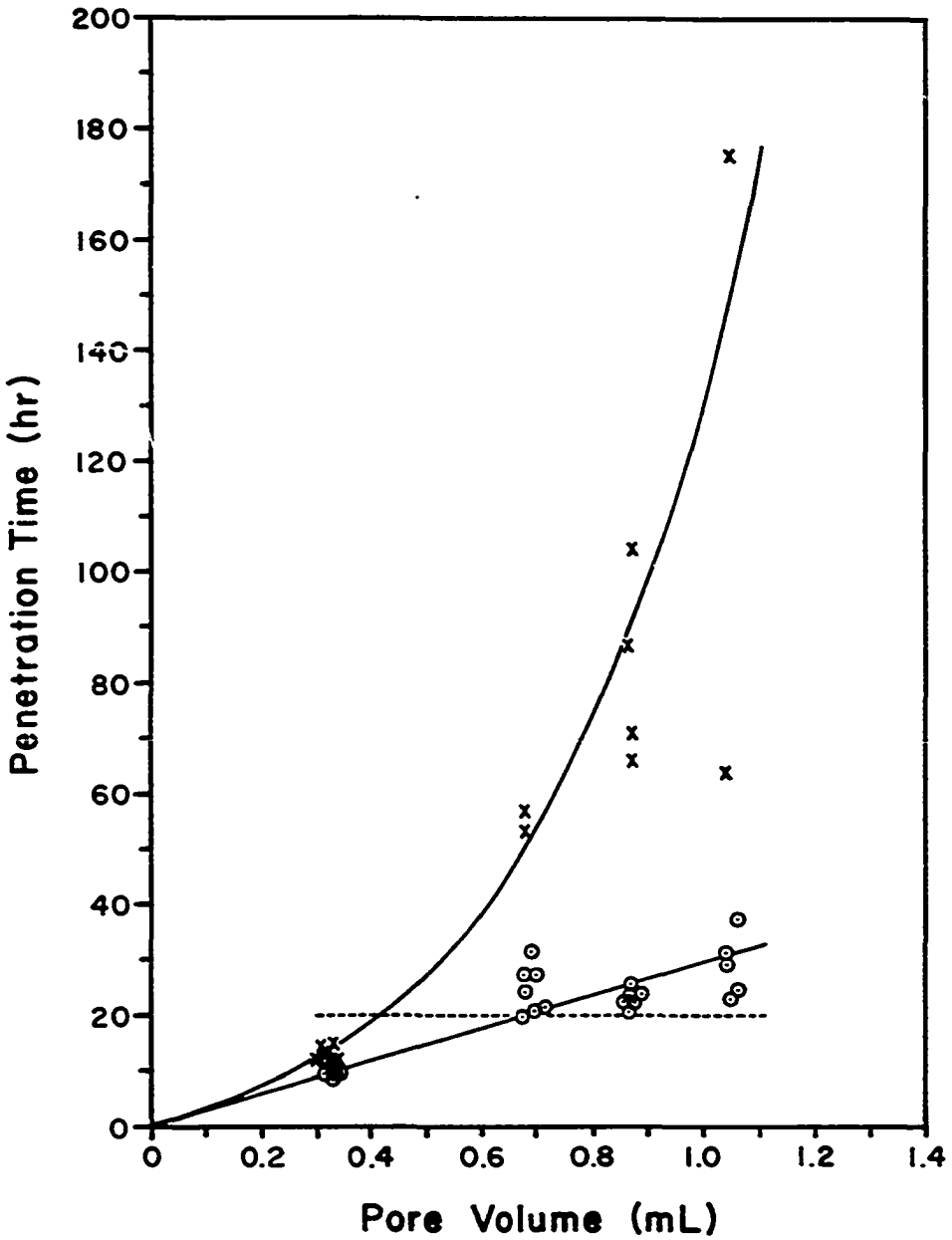
To examine the role of diffusion or random movement, penetration times were plotted vs length. If a core is assumed to be composed of a bundle of capillaries then the movement of cells through a pore should approximate the behavior of cells through a single capillary. Adler and Dahl (1) demonstrated that cell behavior could be described by an equation for diffusion of a thin-layer in a column of liquid, where a plot of time vs the square of the distance that the fastest cells have moved from the origin (L^2) gives a straight line with a y-intercept of zero. Our assay is similar to the "frontier assay" used by Adler and Dahl (1) in which the penetration time is a function of the time for the fastest cells to penetrate some known distance of rock. In Figure 6B for cores with permeabilities above 100 mD, the relationship between t_p and L^2 is curvilinear and therefore does not resemble a diffusion process. However, for cores with permeabilities below 100 mD (Figure 6A) the relationship is linear with a y-intercept near zero and therefore, penetration of cells, at least qualitatively, resembles diffusion.

The relationship between penetration time and pore volume of the core for both permeability classes is shown in Figure 7. In both cases, the penetration time is dependent on the pore volume. For cores with permeabilities greater than 100 mD, the penetration time increased linearly with pore volume while penetration time increased exponentially with pore volume for cores with permeablities below 100 mD. These data exclude the possibility that growth occurs inside the core as it does outside the core, i.e., that the time required to observe visible growth is dependent only on the liquid volume. If growth is not restricted by the presence of the core and penetration time is a function only of total liquid volume, one would predict that the observed penetration times would be independent of the pore volume of the cores (usually less than 1 ml) since this

Figure 7. The relationship between pore volume of the core and penetration time for cores with permeabilities below 100 mD (x) and above 100 mD (0). The dashed line represents the theoretical relationship when penetration through the core is assumed to occur as unrestricted growth. The theoretical relationship was calculated using the following equation:

$$t_p = \frac{\ln X_t - \ln X_o}{\mu}$$

where X_t is the number of cells needed to give faint turbidity in flask B (estimated at 10^8 cells per 100 ml), plus the number of cells contained in a pore volume of the core (estimated to be insignificant compared to the number of cells in flask B), X_o is the number of cells entering the core from flask A (assumed to be one) and u is the specific growth rate of BCI-INS (0.94 h^{-1}) experimentally determined in a flask containing Medium E with $NaNO_3$ and yeast extract.


Penetration Time (hr)
200
180
160
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Pore Volume (mL)

observed penetration time for a core of this length was approximately 12 h (at least 2.5 times faster).

If motility is assumed to be the mechanism for microbial penetration then the observed results should be predicted by the following equation:

$$t_p = l_x/v_m + (\ln X_t - \ln X_0)/\mu \qquad (11)$$

where v_m is the actual velocity of the motile cell ($cm \cdot hr^{-1}$) and l_x is the length (cm) of the path from flask A to flask B (i.e. core length), and again the second term explains the time for a single cell in flask B to reach faint visible turbidity. If an actual velocity of 10 $cm \cdot h^{-1}$ (actual velocity of E. coli as measured by Adler and Dahl (1)) is assumed for BCI-INS then it would be predicted that a single cell would take 0.35 h + 19.6 h or 19.96 h to reach faint visible turbidity in flask B, however, the observed time is almost twice as fast.

In another experiment, a washed cell preparation of strain BCI-1NS was suspended in a 5% NaCl and 100 mM phosphate buffer contained in flask A. The cell suspension in flask A had a final concentration of 10^7 $cells \cdot ml^{-1}$ and penetration of these cells through a 3.5 cm x 0.75 cm, 200 mD, cylindrical, Berea core into flask B was monitored for a 24 h period by plating 0.1 ml of B on Medium E containing yeast extract and nitrate. No cells were detected in core B within the period tested. This was twice the time required for penetration to occur if nutrients were present, however, it is possible that less than 10 $cells \cdot ml^{-}$ penetrated the core within the time period checked since this would be below the detection limits of the plating method used.

CHAPTER VI

DISCUSSION

Myers and Samiroden (21), while studying the penetration of ^{32}P-labeled S. marsecens in oil-containing sandstone and limestone cores of differing permeability and porosity, found no relationship between rate of penetration and either permeability or porosity of the rock. However, our results indicate that in nutrient-containing, brine-saturated Berea sandstone the rate of bacterial penetration in cores above approximately 100 mD is essentially independent of permeability, whereas below 100 mD the rate decreases rapidly with decreasing permeability. Conditions favoring inhibition of cells and brine were used by Myers and Samiroden (21) as a means of cell penetration while our results were achieved under static conditions favoring growth, chemotaxis, and motility. Therefore, it would appear that if bacterial cells are allowed to penetrate rock by their own locomotion that their rate of penetration can be predicted based on the rock permeability. Craw (6), while investigating the penetration of bacteria out of nutrient-saturated Doulton, Pasteur, Berkefeld, and Slack and Brownlow filters, concluded that the grain size of the filter mass was the most important factor governing the growth of bacteria through the filters. For a relatively homogeneous rock like Berea sandstone, it is plausible to assume that with decreasing permeability or grain size of rock that the median pore throat size of the rock

decreases with decreasing permeability, however in many instances rock is highly stratified with respect to permeability and this assumption would not necessarily be the case. Therefore, it is probable that one of the major factors controlling rate of penetration is pore throat size. Updegraff (25), as well as Updegraff and Wren (26), found that pore throat size of rock should be at least twice the cell size to allow passage of cocci or bacilli through rock when cells suspended in brine were pumped into the rock. Kalish, et al. (19) using Berea sandstone of different permeability ranges (278-400 mD, high; 130-162 mD, medium; and 17.7-48.3 mD, low) found that the median pore size distribution of the cores was 3.5-4.0 μm for the low permeability range, 4.5-5.0 μm for the medium permeability range and 5.5-6.0 μm for the high permeability cores. Nazarenko et al. (22), as well as Zvyagintsev and Pitryuk (29), found that while studying the growth of bacteria and yeast in small capillaries of rectangular cross-section, growth rates and cell size decreased and lag times increased with decreasing rectangular cross-section. In fact, in rectangular cross-sections with dimensions of 8 x 4 μm, no increase in cell number was observed for Bacillus subtilis when growing under nutrient-rich conditions. Therefore, it seems that pore throat size and permeability should influence bacterial penetration in sandstone.

Mechanisms for penetration of motile cells could include: growth, motility, chemotaxis, or a combination of these modes of locomotion. Our results suggest that the mode of penetration must be some function of the core's length. Therefore an unrestricted growth model as described in Figure 7 cannot entirely explain our results since penetration time for this model is essentially independent of length and pore volume for the length of rock examined. The penetration times for the filamentous growth model are a function of the length of the core; however, it is conceptually difficult to visualize a chain of cells elongating at an

exponential rate along a tortuous path. Furthermore this model predicts that penetration times should be 2.5 times longer than those we observed. Therefore, the theoretical model which best explains the observed penetration times is one based on motility. As mentioned in the Results, equation 11 predicts that it takes approximately 20 h for a cell to travel 3.5 cm if we assume equation 11 is valid. This value is still almost two times longer than the observed penetration time of 9-12 h. However, shorter penetration times are predicted by equation 11 if more than a single cell simultaneously enters flask B or at least enters within the time required for one doubling of the cell population. Jang et al. (16) recorded a penetration time of 24 h for a B. subtilis isolate to penetrate 3.81 cm of Berea rock with a permeability of approximately 400 mD, using time to reach visible turbidity in the outlet flask as part of the total penetration time. Chang and Yen (3) found that only 45 minutes was required in a similar growth chamber for about 10^2 cells$\cdot$ml^{-1} of E. coli B to be present in flask B after penetrating a 2.5 cm length of Berea sandstone (400 mD permeability). Our results of t_p vs length^2 for high permeability cores (Fig. 6) is consistent with the kind of movement that Adler and Dahl described as "band-like" which means the cells move as a front such that more than one cell could reach flask B simultaneously. However, Adler and Dahl (1) were working with auxotrophic mutants capable of chemotaxis and motility but not capable of growth in the test medium. If we were to assume that 10^1-10^2 cells$\cdot$ml^{-1} entered flask B within the time for a single doubling to occur, as was found by Chang and Yen (3), then equation 11 would predict penetration times of 10-12.5 h, which is well within our observed times. However, at longer core lengths, equation 11 would predict that shorter penetration times would occur than those observed in our results, but possibly these longer penetration times result from fewer and fewer cells entering flask B simultaneously due to an

increasing number of pore throat constrictions that restrict cell passage at longer core lengths.

In low permeability cores, penetration times increased exponentially with length, whereas in high permeability cores a linear relationship with length was found. This does not preclude the possibility that at shorter lengths of rock, cells in both high and low permeability cores could not have penetrated the entire length in similar time periods. This would be possible if it is assumed that fewer cells reach flask B simultaneously in low permeability cores which would increase the time required to reach faint visible turbidity. This possibility is further substantiated by the fact that penetration of cells through these low permeability cores, at least qualitatively, resembles diffusion. Adler and Dahl (1) demonstrated that cells that exhibited a diffusion-like penetration in glass capillaries were non-chemotatic cells that moved randomly and not as a uniform band of cells. Since in our experiments no attempt was made to eliminate chemotaxis, the diffusion-like movement in low permeability cores is most likely due to some property of the rock. If it is assumed that rock is made up of a network of capillaries and that in general as permeability decreases the pore diameter of these capillaries decreases then it is evident that as permeability decreases and length increases the probability becomes greater that a larger number of these capillaries will reach some constriction inaccessible to bacterial penetration. Therefore, the fact that penetration time in low permeability cores is directly proportional to the $(\text{length})^2$ could imply that the actual path taken by the bacterium is longer than the sample path. This phenomenon is often described in petrophysical terms as tortuosity.

If motility is indeed the major mode of penetration for cells through rock the problem of how non-motile cells penetrate rock is a puzzling one. Our results

(Table 2) indicated that non-motile cells do penetrate Berea sandstone although at a slower rate than motile cells. This slower rate could be explained by the filamentous growth model. However as previously mentioned, it is conceptually difficult to envision a filament of cells expanding exponentially along a tortuous path. It is more likely that a mode of penetration consisting of both filamentous and non-filamentous (i.e. unrestricted growth) growth would occur. It is not likely that diffusion, convection, and Brownian movement play a significant role in movement because of the large size of bacterial cells. Hirsch and Christensen (14) found that filamentous growth, such as occurs with actinomycetes, enables these organisms to penetrate 0.22 μm membrane filters (Millipore) while non-filamentous bacteria could not penetrate the same filters. Also, it is possible that the cells generate enough gas inside these rock pores that localized pressure increases may act to push these non-motile cells through larger pores. Experiments using non-motile cells penetrating varying lengths of rock could help to further elucidate the mechanism.

Information regarding the instantaneous rate of bacterial penetration would also be very helpful in increasing our understanding of bacterial penetration of porous rock. We have already mentioned penetration rate and its relationship to permeability, however, these rates are representative of average rates and not instanteous rates. In order to measure instantaneous rates the experiments measuring penetration time vs length would have to be measured on the same rock sample. Such measurements would be very difficult if not impossible to perform on intact pieces of consolidated rock. If we can assume that the cells will behave similarly in a whole piece of rock as they would if the whole was divided into separate lengths then we could derive an apparent rate equation from figures (5A) and (5B) for cores above and below 100 mD. This assumption may not be

unreasonable since tests were performed on a relatively homogenous rock such as Berea sandstone. Therefore, a rate equation for penetration in cores with permeabilities above 100 mD is the derivative of equation 6 with respect to t_p:

$$d\,t_p = 2.5\,dL \qquad (12)$$

$$dL/dt_p = 0.4 \qquad (13)$$

where t_p is penetration time and L is core length. Equation 13 represents a zero order rate expression and thus penetration rate would be independent of length at permeabilities greater than 100 mD. This implies that neither pore size nor tortuosity is restrictive to bacterial penetration in high permeability cores. For low permeability cores the apparent rate equation is the derivative of equation 8 with respect to t_p:

$$d\,t_p = 2.522\,L^{0.808}dL \qquad (14)$$

$$dL/dt_p = 0.396L^{-0.808} \qquad (15)$$

This rate expression is very close to a first-order rate equation and further indicates that penetration rate decreases with increasing core length. This supports our conclusion that in low permeability cores decreasing pore throat sizes result in the presence of a greater number of constrictions that are restrictive to bacterial penetration. The number of these constrictions will increase with increasing length of rock, meaning that the cell will have to travel a path which could be much longer than the sample path. Also, fewer and fewer

cells will be found per area of rock as length increases since many cells will be retained in constrictions at the proximinal end of the core with respect to inoculation. There is also an increasing probability with decreasing pore size that many cells will not even enter the core due to fewer accessible pores. Therefore, the apparent decrease in penetration rate with length may not actually reflect that cells are slowing down as they penetrate longer lengths of rock but may simply indicate that fewer and fewer cells are found per unit area as cells penetrate further into the rock. Thus, only a few cells, if any, will reach flask B at any given moment which results in an increased time until faint visible growth occurs in flask B than if the cells were arriving as a front or band.

As far as MEOR is concerned, these results have interesting implications for processes which rely on the ability of microorganisms to preferentially grow and plug high permeability regions of a reservoir (i.e. selective plugging) while hopefully not plugging the low permeability zones which have higher oil saturations. According to our results, penetration should occur to the greatest extent in these higher permeability regions and cause the greatest permeability reduction in these areas, while plugging in the low permeability zones (higher oil saturation) will be more superficial and occur more slowly. It has often been stated (9,25,26) that around 100 mD is the lowest permeability at which effective cell penetration can occur and our results further substantiate this claim. However, it should be remembered that permeability is not always a good indicator of pore throat size and thus 100 mD may not be limiting for all types of rocks or reservoirs. Instead it would probably be more correct to correlate bacterial penetration with pore throat size and tortuosity, which is a measure of the interconnectedness of flow paths along the length of a rock. Other types of sandstone along with limestone need to be tested in order to determine if similar

relationships of penetration with permeability and length exist. We can conclude from our results that cells that are best suited for MEOR processes should be motile, however, it is hoped that future work with chemotatic and motility mutants (2) can further elucidate the mechanism of bacterial penetration for both motile and non-motile cells.

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CHAPTER VII

ABSTRACT

The Effect of Sterilization by Dry-Heat or Autoclaving on Bacterial Penetration Through Berea Sandstone

Bacteria were found to penetrate lengths of consolidated sandstone (Berea) faster when the sandstone was sterilized by autoclaving versus dry-heat (150°C, 3h). Changes in permeability, porosity, and pore throat size of the rock as a result of autoclaving were not sufficient to explain the difference in penetration times observed, but energy dispersion spectroscopy and scanning electron microscopy of the rock revealed changes in mineral content and clay morphology. The change in mineral content of the autoclaved cores revealed a higher chloride content than that of dry-heated cores and the clays of autoclaved cores were fused and irregularly shaped. Therefore, it is suspected that differences in bacterial penetration rates due to dry-heat versus autoclave sterilization are a result of a change in surface charge of the pores of the rock as well as a reduction in surface area of clays available for adhesion. The results imply that dry-heat sterilization may be preferred to autoclaving when examining the effect of biotic and abiotic interactions in a "native-state" rock model.

CHAPTER VIII

INTRODUCTION

Recent interest in subsurface environments with regard to metabolism of toxic wastes (10, 12), to biofilm formation (8), and to microbial enhanced oil recovery processes (MEOR, 3, 5) has created a need to develop experimental models to study the factors influencing the rate of microbial penetration through porous media. Some investigators have studied these factors in synthetic soil or rock systems by packing glass columns with sand, fused glass beads, iron filings, or even agar (11). These systems are useful and advantageous because of the ease of preparation, consistency between columns, and the limited number of abiotic variables. However, these experimental systems often do not adequately emulate the physical environment of deep subsurface environments. It is for this reason that much of the current research in MEOR has been performed using Berea sandstone. This rock is a consolidated sandstone outcropping which is quarried near Cleveland, Ohio. It is widely used in the petroleum industry as a model rock system for studying various oil recovery techniques because of its ready availability and its close resemblance in many respects to the physical properties of actual oil-bearing sandstones.

In order to study penetration of selected bacterial species in nutrient-saturated Berea sandstone it was necessary to sterilize the cores before

inoculating them with the bacterium under investigation. Autoclaving has most often been used in past studies to sterilize these sandstone cores (1,6,7). However, dry heat (i.e. 150°C for 3 h) is also an effective means of sterilization. It was the purpose of this study to examine the effects of dry-heat sterilization and autoclaving on bacterial penetration times and determine what physical changes through Berea sandstone in the rock occurred as a result of these treatments. It is hoped that by understanding these changes insight may be gained as to ways to increase penetration rates as well as improve sterilization methods for consolidated rock. The understanding of the interaction between biotic and abiotic factors governing bacterial penetration through porous media is paramount to the development of MEOR as well as other environmental bioengineering processes.

CHAPTER IX

MATERIALS AND METHODS

Organisms and Cultivation Medium. Strain 140 was isolated from a subterranean sample underlying the Norman Municipal Landfill. BCI-1NS was isolated from a Berea sandstone core (Cleveland Quarries, Amherst, Ohio). Both isolates are morphologically similar to cells of the genus Bacillus.

The growth medium (Medium E with 0.1% (w/v) $NaNO_3$ and 0.05% (w/v) yeast extract) as well as cultivation conditions for both strains have been previously described (Chapter IV).

Core Preparation. For experiments involving cell penetration, cores were epoxied as described in Chapter IV. Berea sandstone obtained from Cleveland Quarries was used in all experiments. The permeability of 3/4 inch diameter cores was determined using a permeameter and Darcy's Law as previously described (Chapter IV) and the results are given in millidarcies (.001 Darcy).

Sterilization of Cores (i) Autoclave. Expoxied cores used in penetration studies were presaturated under vacuum with 5% (w/v) NaCl solution. The saturated cores were submersed in the brine and autoclaved at 121°C and 15 psig

for 30 min. The initial permeability (K_i) was determined using 5% NaCl brine previously filtered through a 0.22 μm Millipore filter. The cores were then placed in growth chambers containing nutrients as previously described (Chapter IV) and autoclaved an additional 20 min.

(ii) Dry-Heat. Dry heat sterilization of epoxied cores was performed in a forced-draft oven (Fisher Scientific) set at 150°C. The epoxied core was presaturated in the 5% NaCl solution, its K_i determined with this solution, and then the core was placed in a glass petri dish and heated. The dry-heat sterilized core was aseptically removed from the petri dish and vacuum saturated with sterile Medium E containing 0.1% (w/v) $NaNO_3$ and 0.05% yeast extract and then aseptically placed in growth chambers.

Penetration Time. Penetration time was taken as the time elapsed from inoculation of one flask until faint visible turbidity occurred in the adjacent flask, as described previously (Chapter IV).

Pore Throat Size Distribution. The centrifugation method of Slobod et al. (8) as modified by Torbati et al. (9) was used to determine the capillary pressure profiles of the sandstone cores at increasing centrifugal forces. Centrifugation steps were performed using a Beckman Model L5-50B Uitracentrifuge. The pore throat size distribution was generated from the capillary pressure curve and physical data on the core's length, diameter, dry weight and wet weight using a computer program (E. C. Donaldson, personal communication).

Engery Dispersion Spectroscopy (EDS) and Scanning Electron Microscopy (SEM). Both SEM and EDS analysis of sandstone were performed as previously described by Crocker et al. (4). Three Berea cores (2 x 6 inch cylinders) were cut from a 12 x 12 x 6 inch block of sandstone and steam-cleaned for 2 weeks to remove humic substances. Each core was saturated under vacuum with 5% (w/v) NaCl solution. One core was sterilized by autoclaving at 121°C and 15 psig for 1 hour while submerged in brine, while another core was sterilized by heating in a forced-draft oven at 150°C for 3 hours. The third core was steam cleaned but not brine saturated or sterilized. Samples for analysis were then taken from both ends of each core as well as from a point midway between the two ends.

CHAPTER X

RESULTS AND DISCUSSION

It was found that the penetration time for strains BCI-1NS and 140 was dependent on the method of core sterilization used (i.e. dry-heated or autoclaved). The same three cores (132, 133, 134) were used for all penetration experiments shown in Table 3 to eliminate variations between different cores. Cores that were sterilized by dry-heat had longer penetration times compared to autoclaved cores of the same length. Both sterilization methods increased the permeability of inoculated and uninoculated cores indicating that changes in permeability cannot explain the difference in penetration times observed with the two sterilization methods. The times for the penetration of 7.4 cm of dry-heat sterilized rock were 103 h for both cores inoculated with strain 140, whereas, strain BCI-1NS took from 60-75 or 96 h to penetrate the same cores after additional dry-heat sterilization. When these same cores were then autoclaved, the time for penetration by BCI-1NS was 4-6 times faster. These penetration times for autoclaved cores are well within times previously reported for strain BCI-1NS under similar conditions (Chapter V). The data drawn in Table 4 show that successive autoclaving decreased the penetration times of BCI-1NS through Berea cores of 7.4-7.6 or 11.2-11.5 cm in length. The penetration times reported in Table 4 of 7.4-7.6 cm long cores are simlar to the penetration times reported in

Table 3. Effect of autoclaving or dry heat sterilization of sandstone cores on the rate of microbial penetration through Berea sandstone cores[a]

Strain[b]	Core No.	Dry Heat[c]			Autoclaved[c]		
		Penetration Time (hr)	K_i (mD)	K_f (mD)	Penetration Time (hr)	K_i (mD)	K_f (mD)
140[d]	132	103	213.8	-[e]	-	-	-
	133	103	314.5	-	-	-	-
	134	-	245.9	323.0	-	-	-
BCI-INS	132	96	365.6	-	16	399.2	-
	133	60-75	445.3	-	18	460.0	-
	134	-	429.3	409.3	-	421.6	510.2

[a]Each core was 7.4 cm long and 1.9 cm in diameter. The same cores were used throughout the experiment with core #134 serving as an uninoculated control. The sequence in which the treatments were applied was as follows: dry heat sterilization inoculated with strain 140; dry heat sterilization inoculated with BCI-INS; and autoclaved inoculated with BCI-INS.

[b]Both of these strains are motile and were grown at 50°C in medium E plus 0.1% $NaNO_3$ and 0.05% Yeast Extract.

[c]Dry heat sterilization was at 150°C for 3.0 hrs. Autoclaving was for 20 minutes at 121°C. K_i and K_f refer to core permeabilities measured before inoculation and after incubation, respectively.

[d]It was later found that strain 140 was capable of penetrating a 6.1 cm long Berea core (K_i, 202.0 md) in 27.5 hrs that had been sterilized by autoclaving.

[e]-, not determined.

Table 4. Effect of successive autoclave treatments on penetration time of BCI-INS through Berea sandstone cores

Core	Length cm	K_i[a] md	Penetration time after successive autoclaving (h)[b] 1	2	3
B60	3.6	269	8.5	9.0	10.0
B61	3.7	205	9.5	11.0	11.0
B62	3.6	405	10.5	11.0	11.0
B57	7.5	521	21.0	16.0	10.5
B58	7.4	478	17.5	16.0	--
B59	7.6	484	21.0	13.5	11.5
B63	11.4	379	37.5	23.0	18.0
B64	11.5	423	--	24.0	18.5
B65	11.2	367	29.5	27.0	18.5

[a]Permeabilities were measured after the initial 30 min autoclaving period (see Materials and Methods).

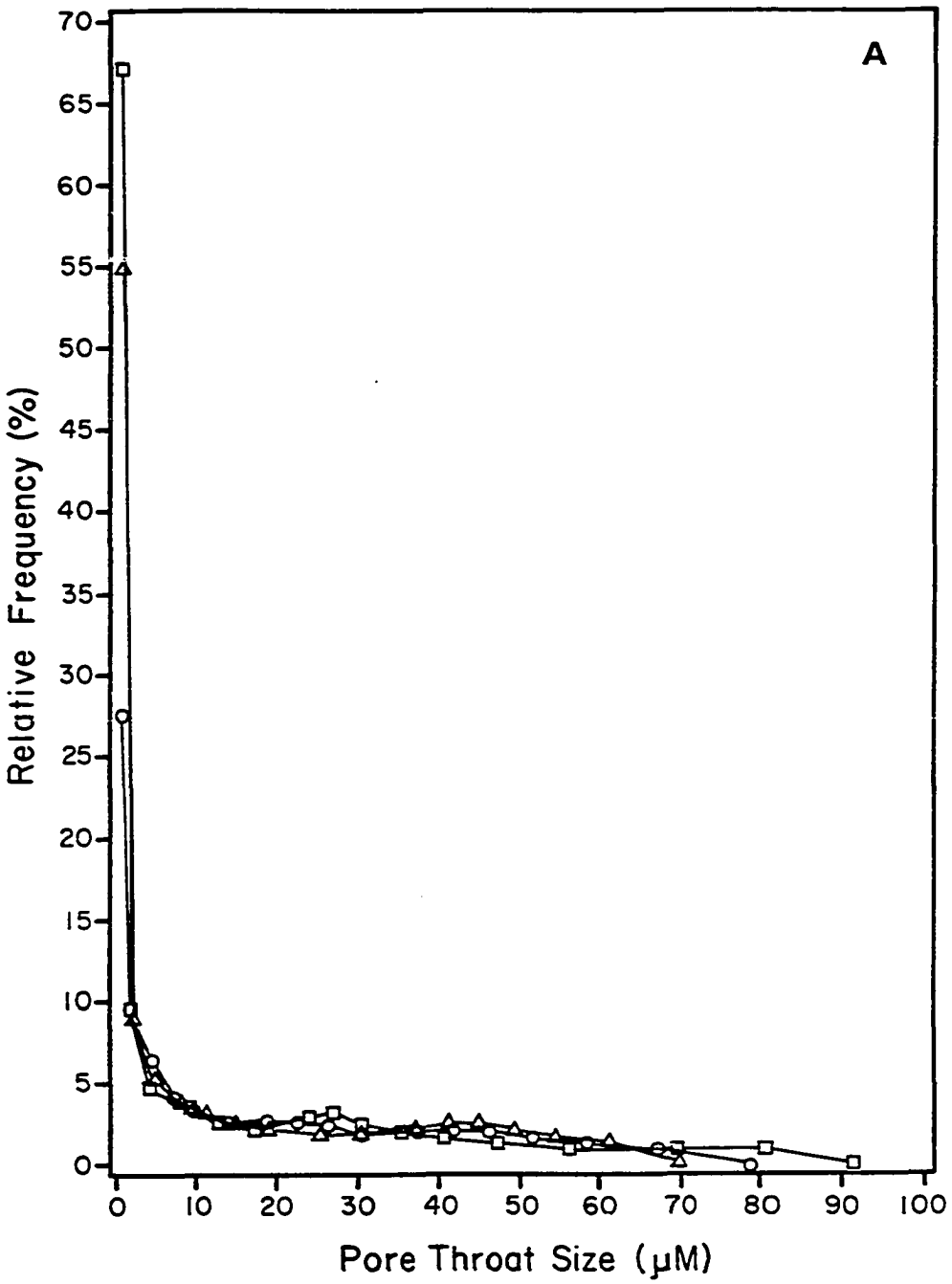
[b]Each core was first autoclaved for 30 min., flushed with Medium E with 0.05% yeast extract and 0.1% $NaNO_3$, mounted in the growth chamber and inoculated with BCI-INS. After growth was observed in flask B, the treatment was repeated except the core was autoclaved for 20 min, before mounting and inoculating.

Table 3 after autoclaving. The 3.6-3.7 cm long cores had faster penetration times that were unaffected by successve autoclaving.

Strain 140 penetrated a 6.1 cm long autoclaved Berea core (K_i of 202.0 mD) in 27.5 h or approximately 3.5 times faster than it took to penetrate a 7.4 cm long core that was dry-heat sterilized (Table 3). It was previously demonstrated in Chapter V that penetration rate was independent of length for cores with permeabilities above 200 mD using autoclaved Berea cores and with strain BCI-1NS. So the small difference in length (6.1 cm vs 7.4 cm) can not account for the large increase in penetration time observed when strain 140 was grown in an autoclaved core. The presence of either a dry-heat sterilized core or an autoclaved core did not inhibit growth of BCI-1NS in liquid culture. Therefore, it was apparent that some change occurred in the sandstone by autoclaving. The modest increases in permeability observed after autoclaving previously dry-heat sterilized cores (Table 3) did not seem sufficient to account for the large decreases observed in penetration times, since the penetration rate of BCI-1NS in Berea cores with permeabilities above 200 mD is not affected by increased permeability (Chapter V). Therefore, it was not necessary to examine what other physical changes in the rock occurred as a result of autoclaving and dry-heat sterilization.

It was thought that the autoclaving process might have increased the sizes of pore throats in the rock thereby allowing the cells easier access through the core. However, the results from pore throat size determinations of four different cores indicated that neither autoclaving nor dry-heat sterilization altered the distribution of pore throat sizes in the sandstone (Fig. 8). Furthermore, the order of treatment (dry-heat then autoclave (Fig. 8 a, b), or autoclave then dry-heat (Fig. 8 c, d) had little affect on the pore size distribution.

Figure 8. Effect of autoclaving and dry heat on pore throat size distribution of Berea sandstone. Pore size distribution determined on four different cores cut from a block of 400 mD Berea sandstone. For all four cores the pore throat size distribution of the untreated cores (□) were determined first and then the cores were either (a, b) autoclaved at 121°C and 15 psig for 30 min and then dry-heated at 150°C for 3 h (0), or (c, d) dry-heated and then autoclaved (Δ). Pore size distribution was determined subsequent to each heating step.


A
Relative Frequency (%)
70
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35
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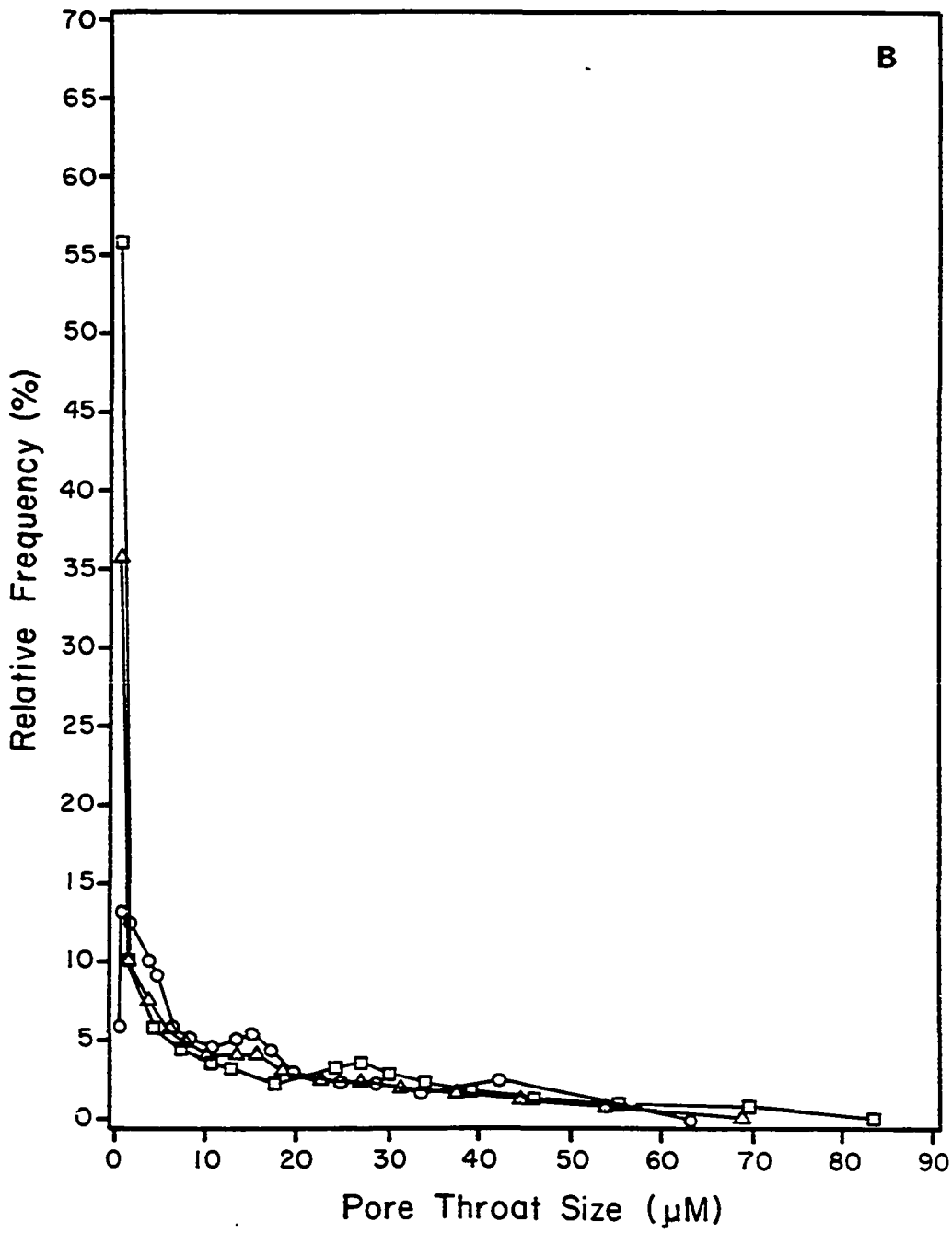
80
90
100
Pore Throat Size (μM)


B
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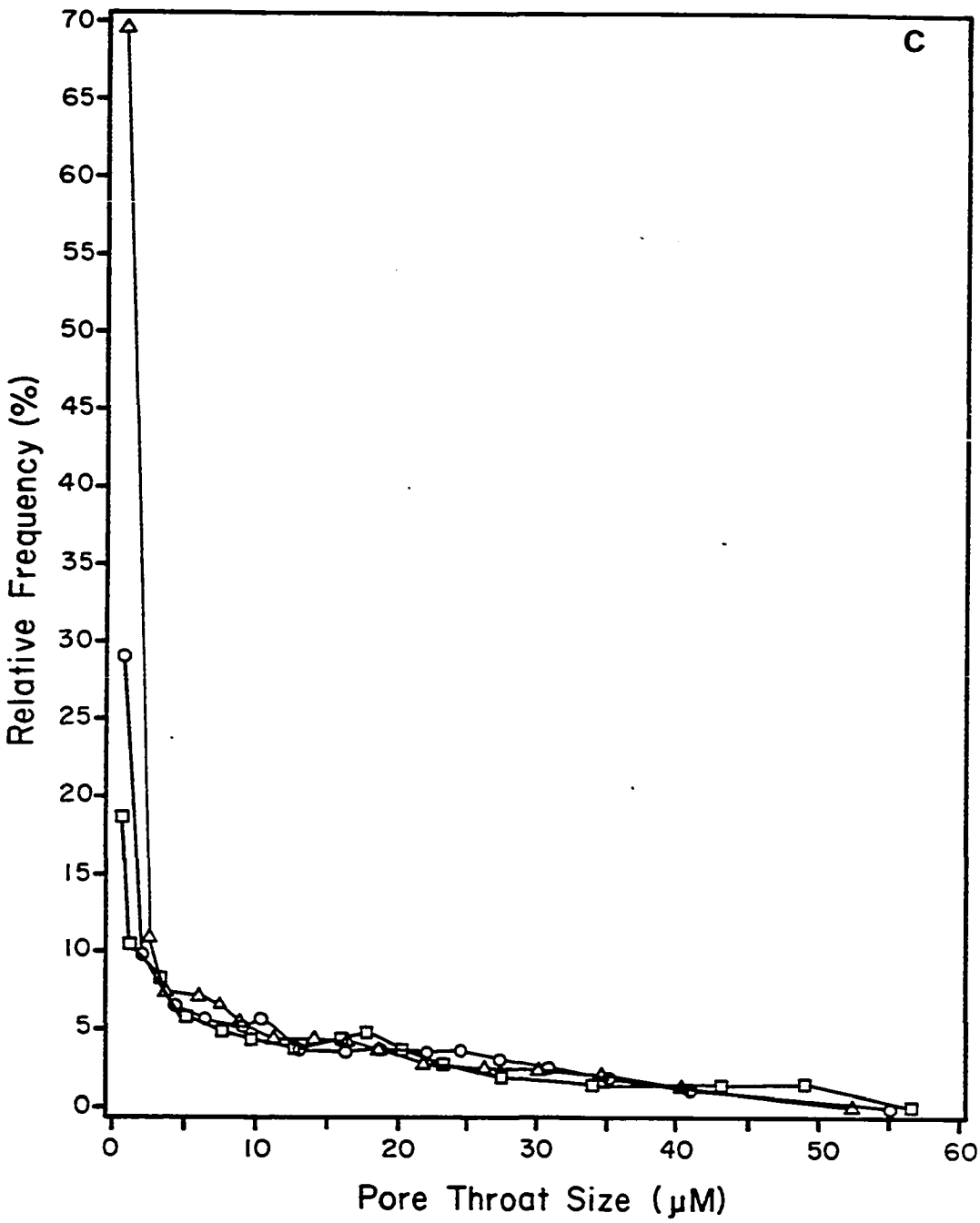
Pore Throat Size (μM)


C
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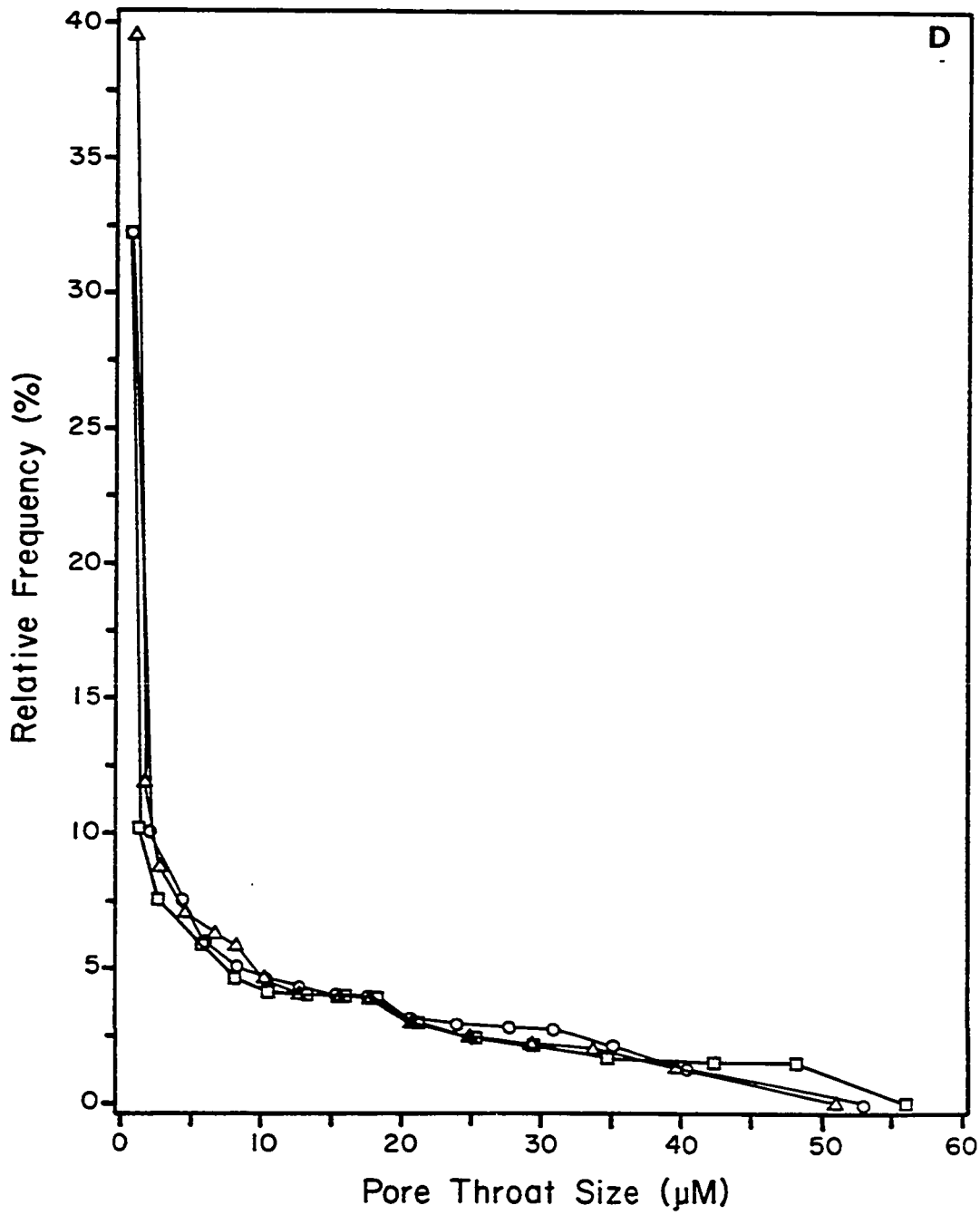
Pore Throat Size (μM)


D
Relative Frequency (%)
Pore Throat Size (μM)
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40
50
60

However, there were differences in distribution of pore throat sizes between the different cores indicative of the inherent heterogeneity of consolidated rock samples.

Another physical property of rock that might have been affected was porosity. Porosity is a measure of the fraction of void space to bulk volume in a porous medium. Table 5 shows the effect of autoclaving and dry-heat sterilization on the static and non-static porosity. Non-static porosity refers to the porosity determined after 5% NaCl solution was flushed through the core under a differential pressure of about 2.5 atmosphere, whereas static porosity was measured without fluid flow through the core. Dry-heat sterilization did not appear to significantly change the static or non-static porosity of the cores tested, nor did autoclaving have a significant affect on the static porosity of these same cores. However, the non-static porosity of autoclaved cores resulted in an average percentage increase in porosity of 7.3% above values for the dry-heat sterilized cores. This increase compared to the static porosity differences observed between dry-heated and autoclaved cores was not statistically significant at $p < 0.1$ when a two-tailed t-test for the difference between two means was used. Additional autoclaving had little or no affect on subsequent non-static porosity measurements as indicated in Table 5.

Crocker et al. (4) have shown that the clay minerals in Berea sandstone act to cement together the silica grains lining the pores as opposed to other sandstones in which the clays are dispersed freely within the pores. Therefore, it is possible that heating of these cores in the presence of a hydrating phase could cause dissolution of the clay and silica particles which would result in a change in the degree of cementation and hence porosity. This effect would be especially pronounced if cores were subjected to a subsequent brine flush as performed when

Table 5. Effect of dry heat and autoclaving on the static and non-static porosity of Berea sandstone cores.

Core Length (cm)	Static Porosity[a]			Core Length (cm)	Non-Static Porosity[b]			
	Untreated	Dry Heat[c]	Autoclaved[c]		Untreated	Dry Heat[c]	Autoclaved[c] 1	2
3.7	.214	.212	.212	7.2	.209	.201	.218	.217
3.6	.214	.205	.204	7.4	.204	.200	.211	.211
3.6	.214	.211	.208	7.3	.203	.200	.216	.214
2.8	.227	.220	.220	-	-	-	-	-

[a]Cores used for static porosity measurements were not epoxied and were 2.54 cm in diameter. All cores were cut from a 400 ± 50 mD block of Berea sandstone.

[b]Cores used for non-static porosity were epoxied on the sides and were 1.90 cm in diameter. Flow through cores was performed after each treatment at a differential pressure of 1.02 atm. Porosities were determined after flowing brine (5% NaCl) through core.

[c]Dry heat treatment was for 3 hours at 150°C and autoclaving was for 20 min at 121°C while the core was submerged in 5% NaCl brine.

measuring non-static porosity. Baking sandstone cores (usually at 600 ^{o}C) tends to stabilize clays and fines reducing their movement in the presence of a flowing fluid (4). This may explain why non-static porosity did not increase with dry-heat cores.

EDS analysis of the surface of the dry heat sterilized cores vs the autoclaved cores revealed some interesting differences (Table 6). The surfaces of the non-treated and dry-heated Berea sandstone cores had higher silica, aluminum, and potassium content than the autoclaved cores. The dry-heated core had a much lower chloride content than the autoclaved core. The other elements (Fe^{+3}, Ca^{+2}, and Ti^{+4}) made up only a small or negligible part of the surface elements. In all cases, the total amount of elemental oxides detected were near 100% of the total oxide measured in the sample so that if other elements were present, they would be present in very small quantities. These data are consistant with a change in porosity after autoclaving since a reduction in silica content indicate a loss in cementation between clays and the grain particles. Furthermore, decreases in amounts of aluminum and potassium on the pore surfaces of the autoclaved core are also consistent with a loss in clay content, since clays are composed of these elements. More importantly, this analysis indicates that a change in surface charge resulted from autoclaving. The large increase in chloride content and the reduction in the content of aluminum and potassium could shift the surface charge to a more negatively charged state. This could cause a greater electrostatic repulsion between bacterial cells which tend to have net negative charges at their surface when suspended in fluids near a pH of 7.0. Chang and Yen (2) found that the addition of the anion, pyrophosphate, to cell suspensions decreased the degree of cell retention inside of Berea sandstone. They attributed this to a higher repulsive energy induced at the surface of either

Table 6. Effect of dry heat and autoclaving on elemental composition of the pore surface of Berea sandstone as determined by energy dispersive spectroscopy.

Treatment[a]	Oxide Percent							
	SiO_2	Al_2O_3	Fe_2O_3[c]	CaO[c]	TiO_2[c]	K_2O	Na_2O	ClO_x
none	88.7 ± 2.1[b]	8.7 ± 1.4	0	1.0	0	2.3 ± 0.3	0	ND[d]
dry heat	84.9 ± 0.2	8.9 ± 0.4	0	0.3	0	2.2 ± 0.2	0	4.0 ± 0.4
autoclave	72.8 ± 10.9	3.6 ± 1.6	0	0	0	0.25 ± 0.4	0	23.5 ± 12.6

[a]Each core was prepared as described in the Materials and Methods section.

[b]Mean ± standard error of the values of the three subsamples taken along the length of the core.

[c]Values obtained from only one or two subsamples taken along the length of the core.

[d]ND, not determined.

the bacterial cell or the particle surface therby reducing the possibility of irreversible adhesion of cells.

Scanning electron micrographs of untreated, dry-heated, and autoclaved Berea cores also showed that autoclaving changed the nature of the clay particles inside the core. The untreated and dry heated cores contained clay particles that were flat and plate-like with irregularly shaped edges. The clay particles in the autoclaved core appeared to be fused and clumped with smooth and rounded edges.

The evidence for the treatment effect due to autoclaving appears at first to be dichotomous. Increases in the porosity of the autoclaved core along with decreases in the amount of silica, aluminum, and potassium which are elements often associated with clays, imply that autoclaving results in the removal of clay particles. However, no changes were observed in pore throat size distribution, which would argue that these losses in cementation are not significant with respect to pore geometry. On the other hand, EDS analysis of the surface of the autoclaved core shows a large increase in the chloride content compared to dry-heat sterilized and non-treated cores. This could result in an increased negative charge at the surface of the rock which would cause a greater electrostatic repulsion between the negatively charge surfaces of the bacterial cell and the rock. Scanning electron micrographs, although they do not indicate any quantitative change in clay content, do show that the clays have been structurally altered from their natural sharp-edged appearance to a more smooth edge and fused arrangement. These fused particles would have less surface area available for adhesion of cells than the flat, plate-like clay particles observed in the non-treated and dry-heated samples.

Therefore, it is likely that autoclaving results in an alteration of clays as well as the surfaces lining the pores. These alterations include an increase in the content of negatively charged ions at the surfaces as well as in a reduction in surface area available for cell adhesion. The evidence for removal of clays or grains which would result in a loss in cementation and hence an increase in porosity was observed but it is not certain whether these changes are significant, since no change in pore throat size distribution was observed and the increases in porosity were not statistically significant. However, it is possible that the autoclaving acted merely to clean out clays and fines deposited within the pores without significant alteration of the pores themselves.

The importance of understanding the effect of sterilization on the physical properties of the rock and other porous media is crucial in explaining the role of biotic and abiotic factors in cellular penetration or retention. This information should prove useful in future comparisons of penetration times and rates reported for bacteria in different porous media, as well as, suggest improved sterilization procedures. It appears that dry-heat sterilization may be a better method than autoclaving to determine the penetration rate of bacteria in "native-state" cores. However, experiments with autoclaved cores can lead to an understanding of how surface modification and other changes in physical properties of rock affect the penetration rates of bacteria. This information could be useful for processes involving the cleanup of toxic wastes polluting groundwater as well as MEOR, both of which may require transport of injected microorganisms through consolidated rock. Whether sterilization methods will affect the physical properties of consolidated rock other than Berea sandstone remains to be determined.

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CHAPTER XI

ABSTRACT

The effect of nitrate addition on biogenic sulfide production

The addition of 58.8 mM nitrate was found to inhibit biogenic sulfide production in sewage sludge (10% v/v) amended with 20 mM sulfate and either acetate, glucose, or hydrogen as energy sources. This inhibition was observed for at least six months and was accompanied by the oxidation of the redox indicator, resazurin, from its colorless reduced state to its pink oxidized state. Lesser amounts of nitrate (5.9 mM and 19.5 mM), as well as, increasing amounts of sewage sludge resulted in only transient inhibition of sulfide production. The addition of 156 mM sulfate and 58.8 mM nitrate to 10% sewage sludge or pond sediment slurries resulted in no sulfide inhibition.

Residual concentrations of nitrate and nitrite were present throughout incubation periods in those instances where sulfide production was inhibited, while nitrate and nitrite were below detectable levels at the time sulfide production commenced. The oxidation of resazurin was attributed to nitrous oxide concentrations which were approximately 1.0 mM after five months incubation. After prolonged incubation of oxidized bottles, the numbers of sulfate-reducing organisms decreased from 10^6 cfu·ml^{-1} sludge to undetectable levels (<10 cfu·ml^{-}

sludge), while nitrous-oxide producing bacteria remained relatively constant at about 100 cfu $\cdot ml^{-1}$.

Therefore, the prolonged inhibition of sulfide production was attributed to an increase in oxidation-reduction potential due to production of biogenic nitrous oxide. Futhermore, these oxidized conditions appeared to have a cytotoxic affect on sulfate-reducing populations. The onset of these inhibitory conditions can be explained in terms of the energetics and kinetics of sulfate and nitrate utilization under anaerobic conditions.

CHAPTER XII

INTRODUCTION

Since 1929 (12) the use of nitrate was recognized in the abatement of odors caused by sulfate-reducing bacteria in conjunction with waste water treatment. Since this time, sporadic reports of nitrate usage with respect to odor control in sewage (1, 5, 9, 17), cannery wastes (34), pulp mill waste (25), and other industrial wastes have been reported. Takai and Kamura (39) reported using nitrate to control sulfide production in a rice paddy. Most of these tests were accompanied by some success in controlling these sulfurous odors, however, follow-up information on these tests is not available because it was felt that nitrate control is transient and that effective control of sulfide would involve prolonged treatments with nitrate that would result in high chemical costs. This reasoning was based on the observation that under anaerobic conditions nitrate was reduced preferentially to sulfate and, once nitrate was metabolized, the remaining organic matter would be used to reduce sulfate to sulfide unless additional nitrate was added.

Although the transient inhibition of sulfide production by nitrate was observed in many instances, in two cases a more prolonged effect of nitrate addition with regard to the control of sulfurous odors has been reported. For example, Heukelekian (17) found that sulfide production did not occur in sewage

for 3 days after the addition of 120 ppm nitrate even though all the nitrate had disappeared within 24 h. Likewise, Allen (1) found that 1,000 ppm nitrate amended to sewage eliminated sulfide production for at least 29 days, which was the longest period tested. In both of these cases it was felt that the inhibition of sulfide production was probably caused by the ability of nitrate to poise the oxidation-reduction potential (ORP) at a level high enough such that subsequent sulfate reduction was delayed until the redox potential lowered after nitrate was depleted. However, nothing was mentioned as to how the redox potential could be lowered without sulfate reduction.

Recently, Poduska and Anderson (32) found that using nitrate to control sulfide production in a wastewater lagoon was feasible if enough nitrate was added initially such that the redox potential of the lagoon remained high even after nitrate concentration was decreased. Once enough nitrate was added to poise the ORP at +300 mV then a positive redox potential was easily maintained with little or no additional nitrate. The increase in ORP was attributed to a reduction in the amount of organic matter and the production of N_2 gas by denitrification which allowed a means for mixing in the lagoon sediment.

Work in this laboratory is also concerned with the control of sulfide production during the input of large amounts of organic matter to environmental habitats such as groundwater and petroleum reservoirs. Microbially enhanced oil recovery (MEOR) processes rely on the ablity of indigenous or injected bacteria to anaerobically metabolize added nutrients and produce useful by-products such as gas, biosurfactants, polymers, etc. that result in additional oil recovery (11, 13). Nitrate is often added to nutrient mixtures to be injected into petroleum reservoirs because it enhances the energy-yielding capacity of certain bacteria under O_2-limited conditions. Therefore, the effect of added nitrate and nutrients

on these environments, some of which are already high in sulfates, as well as, sulfate-reducing organisms, is of paramount concern because sulfide is toxic, causes corrosion, and non-selectively plugs petroleum reservoirs (33).

This study examines the effect of nitrate addition on sulfate reduction in anaerobic habitats. Anaerobic sewage sludge was used most often in these studies because of its availability and because it is known to be a good source of denitrifiers and sulfate reducers. The approach was to add energy sources to an anaerobic sample that could be used by both nitrate-reducing or sulfate-reducing populations and determine under what conditions of nitrate and sulfate amendment that sulfide production would occur. From these experiments, an alternative explanation is presented for the increase in redox potential and the prolonged inhibition of sulfide production associated with high nitrate additions.

CHAPTER XIII

MATERIALS AND METHODS

Sample Sites. Sewage samples were obtained from the secondary, anaerobic sewage slude digestor at the Norman, Oklahoma Municipal Sewage Treatment Facility. Sediment samples were collected at the University of Oklahoma Duck Pond and landfill samples from the aquifer underlying the Norman Municipal Landfill. Oil reservoir samples were co-produced brine and oil sampled at the well-head of producing wells.

Growth Media and Incubation Conditions. Samples were immediately brought to the lab and placed in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI; 90% N_2, 10% H_2) and pre-incubated overnight at 25°C. Samples were dispensed into sterile 160 ml serum bottles or Balch tubes containing the following (g/l): Na_2SO_4, 2.84; KH_2PO_4, 0.68; $MgCl_2$ $6H_2O$, 0.41; NH_4Cl, 0.32; $CaCl_2$ $2H_2O$, 0.09; Resazurin, 0.00001; and 10 mM MOPS (3- N-Morpholino propane sulfonic acid, final pH = 7.0). Trace metals and vitamins, as described by Balch, et al. (3), were added separately at 0.1% (v/v) and 0.5% (v/v), respectively. Sodium nitrate was added at a final concentration of 5.9, 19.6, or 58.8 mM. Sodium acetate, glucose, or H_2 were added at concentrations of 15 mM, 5 mM,

and 1.22 atm, respectively. A reducing agent consisting of 2.5% (w/v) cysteine HCl - 2.5% (w/v) $Na_2S \cdot 9H_2O$ was added at a final concentration of 0.05% (w/v) of each component. Serum bottles were then capped with black rubber stoppers and aluminum crimp-seals, and removed from the glove-box. The bottles were evacuated and then purged (1.22 atm) with oxygen-free nitrogen gas three consecutive times. Bottles or tubes containing hydrogen (1.22 atm) as electron donor were purged three times with H_2 instead of N_2.

Bottles or tubes were incubated in the dark at 25°C without agitation. Samples were withdrawn at 1 wk intervals for 4 wks using a 5 ml sterile glass syringe that was previously degassed with O_2-free nitrogen. Samples were to be assayed immediately for sulfide while a portion of the sample would be frozen until other analyses were performed.

Enumeration of Sulfate-reducers (SRB) and Nitrous oxide-Producing Bacteria (NOB). Sulfate reducers were counted by plate counting techniques using media amended with acetate or glucose. Media, as described above, were amended with 15 g/l Bacto-Agar (Difco) and 0.5 g/l $FeSO_4 \cdot 7H_2O$ and then boiled (approx. 10 min) while O_2-free nitrogen was bubbled through the medium. The reducing agent (1 mM-dithiothreitol) was added and the medium dispensed into test tubes previously degassed with O_2-free nitrogen. The tubes were stoppered with butyl rubber stoppers and autoclaved for 20 minutes. After cooling to 60°C the tubes were placed inside the glove-box and the contents poured into petri dishes containing 0.1 ml aliquots of 10-fold serial dilutions of the appropriate environmental sample. Diluent consisted of 9.0 ml of 10 mM MOPS (pH = 7.0) buffer preincubated overnight in the anaerobic chamber.

Enumeration of sulfate-reducers with hydrogen as the electron acceptor was determined by three-tube most probable number method (MPN). The medium, without agar or $FeSO_4 \cdot 7H_2O$ was dispensed anaerobically into Balch tubes containing a small amount of reduced iron powder (J. T. Baker). Each tube was inoculated with 1 ml of the appropriate dilution, then was stoppered, evacuated, and purged three consecutive times with H_2 (1.22 atm). Positive tubes were identified by a blackening of the medium.

SRB from oil reservoir brines were assayed according to API methods (2). Nitrous oxide-producing bacteria were enumerated by plate counting methods in the same medium without $FeSO_4 \cdot 7H_2O$ or dithiothreitol and with a 2.0% (v/v) cysteine-sulfide solution (described above) and either 58.8 mM, 19.6 mM, 5.9 mM, or no sodium nitrate. Positive identification of nitrous oxide-producing microorganisms was made by the presence of a pink, diffusable halo surrounding a colony due to localized oxidation of resazurin (unpublished results). For samples using hydrogen as electron donor MPN tubes were prepared as above for SRB detection except that nitrate was added at appropriate concentrations. Positive tubes were identified by the presence of a pink coloration of the medium which is indicative of gaseous nitrogen oxide formation. Most probable numbers were calculated using the computer program described by Hurley and Roscoe (20).

Analytical Techniques. (i) Sulfide. A modified colorimetric method of Truper and Schlegel (43) using 0.02% zinc acetate in 0.2% acetic acid was used to measure sulfide concentrations. A standard curve was prepared with $Na_2S \cdot 9H_2O$ crystals that were washed with distilled water and blotted dry before being weighed. A new standard curve was prepared whenever fresh reagents were made.

(ii) Sulfate: The turbidimetric procedure as described by Hach Co. (Ames, IA) was used to measure sulfate (15). Sulfate standards purchased from Hach Co. were used to prepare a calibration curve.

(iii) Nitrate and Nitrite. Nitrate was assayed by using a nitrate ion-specific electrode (Corning) and an expanded scale pH/ion meter to record millivolt potentials used to construct a calibration curve. Standard addition of NO_3^- standards (Hach Chemical Co., Ames, IA) indicated no interfering substances in the samples tested. Analysis for NO_2^- was performed according to the colorimetric method described by Hanson and Phillips (16).

(iv) Glucose. The determination of glucose was done according to the colorimetric glucose-oxidase method (Sigma Chemical Co., St. Louis, MO).

(v) Gases. Nitrous-oxide, oxygen, CO_2, CH_4, and H_2 were assayed using a Packard 427 gas chromatograph equipped with a thermal conductivity detector and a Porapack Q column. The oven temperature, detector temperature, and injector temperature were 70°C, 90°C, and 90°C, respectively. Peak identification was made by comparing peak retention times with external standards prepared from high purity gases (Supelco or other suppliers). For quantitation, calibration curves were prepared by adding high purity gases, via gas-tight syringe, to 160-ml serum bottles flushed with O_2-free nitrogen and sealed with a black rubber stopper. Peak areas were determined by either an HP 3390A integrator or by use of an Apple IIe computer equipped with an Apple Graphics Tablet and electronic planimeter.

(vi) Acetate. Samples were first centrifuged (12,100 x g, 10 min) to remove cells and debris and then frozen at -20°C in sealed tubes until analyzed. Samples were then acidified by adding 0.1 ml of 6N HCl to 0.9 ml of sample. A 1.0 µl sample was then injected in a Varian 3400 Gas Chromatograph equipped

with a flame ionization detector and a fused silica Ultrabore column (530 um diameter, 10 ft. long) packed with Carbowax 20 M (Hewlett Packard, Palo Alto, CA). The carrier gas was helium (11.5 $ml \cdot min^{-1}$) and the oven temperature was programmed as follows: 60°C for 3 min., 4.0°C min^{-1} increase until 100°C is reached, then 7.5°C min^{-1} increase until 200°C is reached, hold at 200°C for 5 min. Acetate was quantitated by comparing peak area to that of known standards.

(vii) Total Residue. Three milliliter samples were dried in aluminum weighing dishes to constant weight in an oven set at 50°C. Reported values were determined from three replicate samples.

CHAPTER XIV

RESULTS

The addition of 58.8 mM nitrate to anaerobic sewage slude samples in the presence of 20 mM sulfate resulted in the complete inhibition of biogenic sulfide production after a two week incubation period (Table 7). However, if additional sulfate was added at a final concentration of 156 mM then no inhibition was observed. A similar result was observed if eutrophic sediment material from the University of Oklahoma Duck Pond was used, except that in one case (replicate D) sulfide production was not inhibited even at the lower sulfate concentration. In fact, in most instances when sulfide was produced in the presence of added nitrate, the production of sulfide was enhanced. Sulfide production was also inhibited for up to 28 days in two petroleum reservoir brines amended with acetate, nitrate, and sulfate. However, similar amendments to aquifer and groundwater samples did not inhibit sulfide production. This suggests that the relative populations of SRB and NOB, as well as, concentrations of sulfate and nitrate are probably important in determining if sulfide inhibition will occur.

Since anaerobic sewage sludge provided an easily accessable source of anoxic material containing both sulfate-reducers and denitrifiers, the remainder of the study used sewage sludge as the inoculum source. If different

Table 7. Effect of nitrate and sulfate addition on biogenic production of sulfide in the presence of 15 mM acetate

Site[a]	Replicate	Sulfide ($mg \cdot l^{-1}$)[a] in samples amended with: Nitrate + Sulfate 58.8 mM / 20.0 mM	Sulfate 20.0 mM	Nitrate + Sulfate 58.8 mM / 156 mM	Sulfate 156 mM
Sewage Sludge	A	0[c]	45	92.3	66.4
	B	0	41	140.7	58.9
	C	0	27	58.9	45.7
	D	0	29	54.6	55.4
	E	0	64	-[b]	-[b]
Duck Pond Sediment	A	0	170	146.6	44.1
	B	0	205	180.4	30.9
	C	0	59	232.4	41.9
	D	150	89	364.4	178.6

[a] Final concentration of sulfide in tubes at the time of inoculation was approximately 2.0 $mg \cdot l^{-1}$ for sewage samples and less than 1.5 $mg \cdot l^{-1}$ for duck pond samples. The final concentration of sample was 10% (v/v). Sulfide was determined after 2 weeks of incubation. The growth medium described in Materials and Methods was used except that $NaHCO_3$ (0.35 g/100 ml) was added as a buffer with a gas phase containing 80% N_2 and 20% CO_2 (pH = 7.0).

[b] Not Determined.

[c] 0; Below 15 $mg \cdot l^{-1}$.

concentrations of sewage sludge were amended with 20 mM sulfate and 15.0 mM acetate, in the presence and absence of 58.8 mM nitrate, two different effects with regard to sulfide production were observed (Table 8). When 50% and 100% sewage sludge were used, the addition of 58.8 mM nitrate temporarily delayed sulfide production; however, when 10% sewage sludge was used, no detectable sulfide was observed for over 56 days as compared to the control without nitrate, which had detectable sulfide concentrations after 12 days.

In all cases where sulfide production was inhibited for long periods of time it was also observed that resazurin changed from its colorless reduced state to its pink oxidized state. It was at first thought that this was due to oxygen contamination. However, the progress of oxidation occurred from the bottom of the tube or bottle (i.e. at the liquid/sediment interface) and then spread throughout the entire sample, unlike oxygen contamination which would diffuse downward from the air/liquid interface. Therefore, it was assumed that some metabolic activity occurring at the sediment/liquid interface must be responsible for the oxidation. Since in bottles without nitrate this oxidation was never observed, it most likely was the result of biological metabolism of nitrate. In the absence of oxygen and in the presence of ammonium, the nitrate would most likely be used as an electron acceptor for respiration. Therefore, metabolic by-products of nitrate metabolism might include NO_2^-, NO, N_2O, and N_2. The NO_3^-/NO_2^- redox couple has an E_o of +433 mV while the NO/N_2O and N_2O/N_2 redox couples have E_o's of +1175 and +1355 mV, respectively (40). The addition of 0.2 mM N_2O and 0.5 nM NO oxidized resazurin, but addition of up to 72.5 mM NO_2^- and 58.8 mM NO_3^- to the medium did not oxidize resazurin (data not shown).

In another experiment, 10% (v/v) sludge was incubated in the presence of 0to 58.8 mM nitrate as well as 15.0 mM acetate and 20.0 mM sulfate (Figure 9).

Table 8. Effect of sewage sludge concentration on sulfide production in presence and absence of nitrate, 15 mM acetate, and 20 mM sulfate.

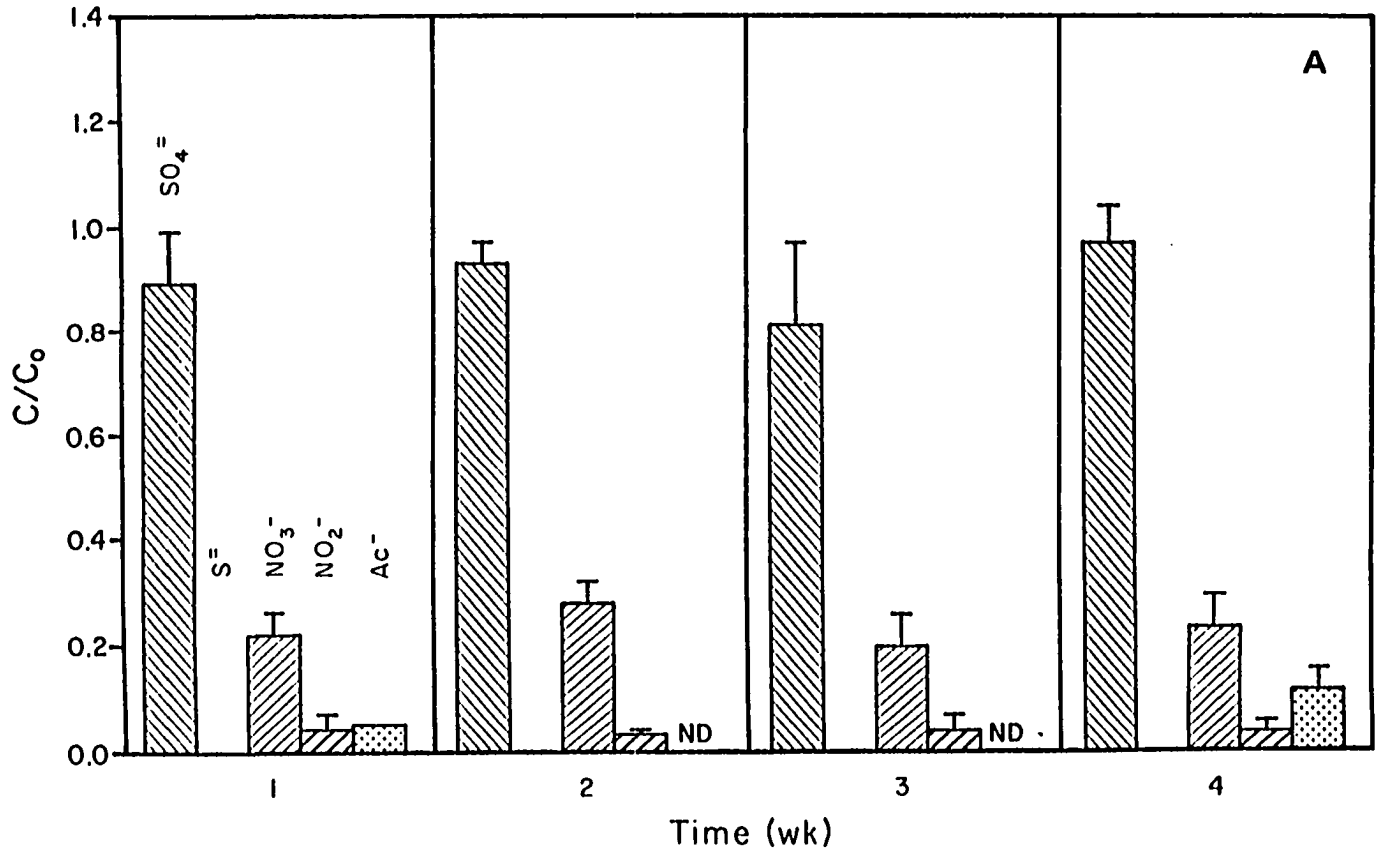
Time (days)	Sulfide ($mg \cdot l^{-1}$)					
	10% (v/v) Sewage Sludge[a]		50% (v/v) Sewage Sludge		100% (v/v) Sewage Sludge[b]	
	No Nitrate	58.8 mM Nitrate	No Nitrate	58.8 mM Nitrate	No Nitrate	58.8 mM Nitrate
3	-[c]	-	5.3	-	12.5	-
6	-	-	-	-	11.5	-
9	-	-	5.3	-	12.1	-
12	7.7	-	18.6	-	28.2	20.1
15	11.8	-	27.8	-	28.7	67.5
19	11.2	-	26.2	-	7.7	32.2
21	21.5	-	49.6	-	57.4	>75
28	>75	-	ND[c]	13.4	ND	ND
36	>75	-	ND	ND	ND	ND
49	ND	-	ND	50.3	ND	ND
56	ND	-	ND	>75	ND	ND

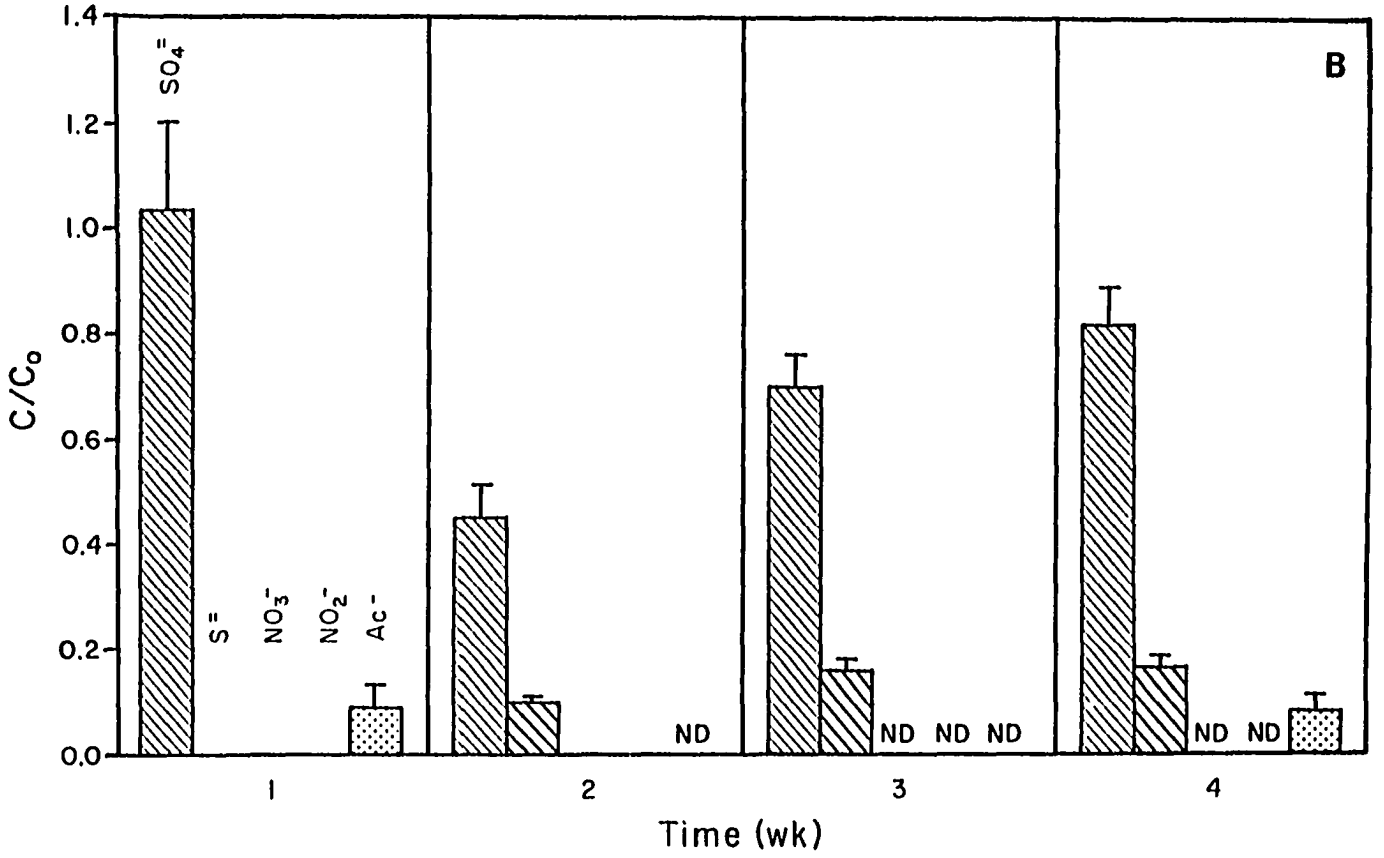
[a]Average total residue = 3.2% (w/v)

[b]Initial sulfide concentration of sewage sludge was 15.1 $mg \cdot l^{-1}$.

[c]-, Sulfide concentration below 4.4 $mg \cdot l^{-1}$; ND, Not Determined.

Figure 9. Effect of acetate and nitrate concentration on sulfide production in 10% sewage sludge. The concentrations of sulfate, sulfide, nitrate, nitrite and acetate were monitored over a four week period. Sulfate and sulfide, as well as nitrate and nitrite, are represented as a fraction of the initial added concentration (Co) of sulfate-S (20 mM) and nitrate-N, respectively. Acetate is represented as a fraction of acetate added initially (i.e. 15 mM). Standard error bars of the mean of three replicates are given for each component measured. Jagged horizontal lines indicate that concentrations were actually in excess of this amount, whle a blank space indicates the component was below limits of detection and ND; not determined. In all cases at least 90% of the added component was detectable. (9A) 58.8 mM NO_3^-; (9B) 19.6 mM NO_3^-; (9C) 5.88 mM NO_3^-; (9D) no nitrate.


A
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C/Co
SO4=
S=
NO3-
NO2-
Ac-
ND
ND
1
2
3
4
Time (wk)


B
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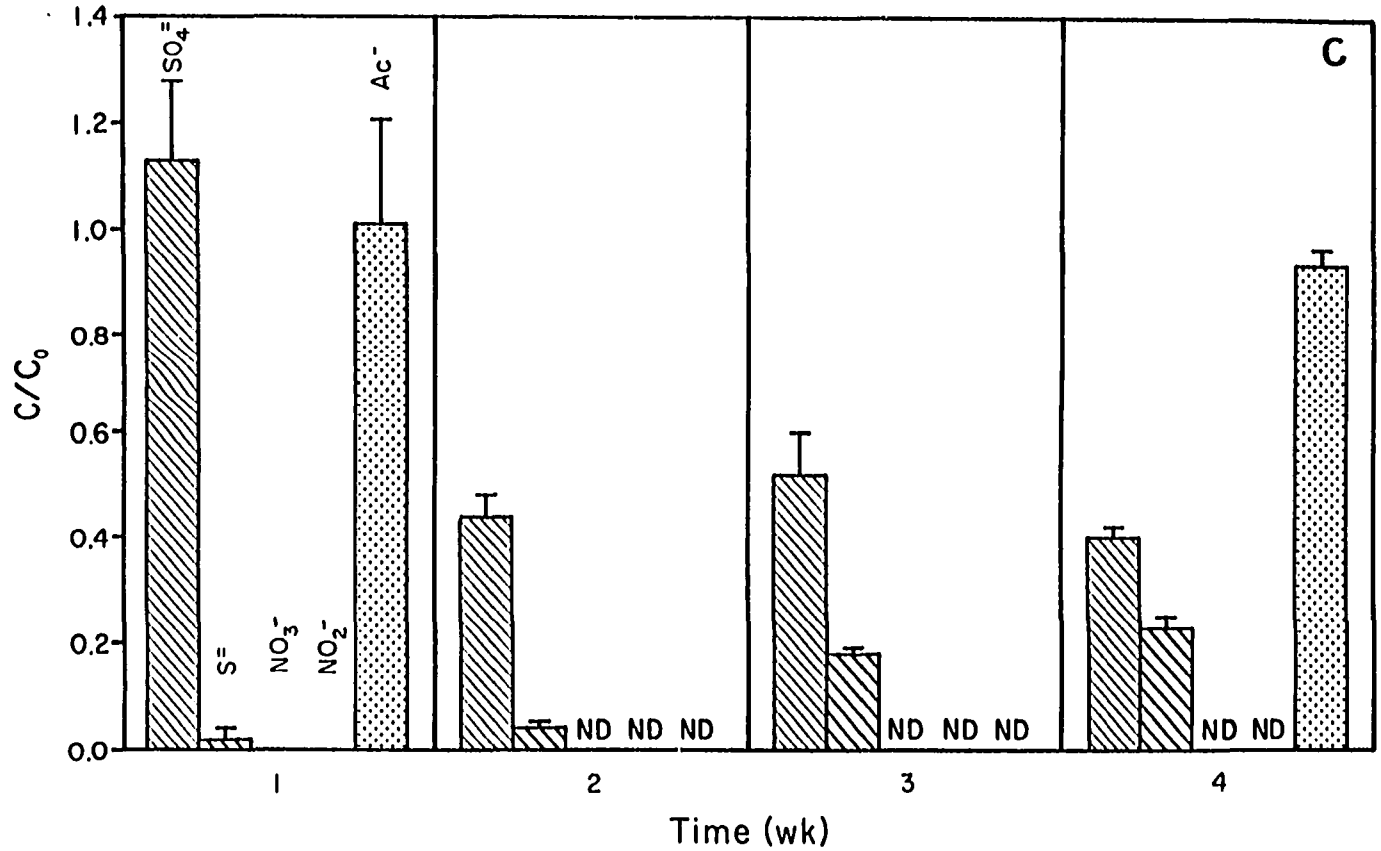
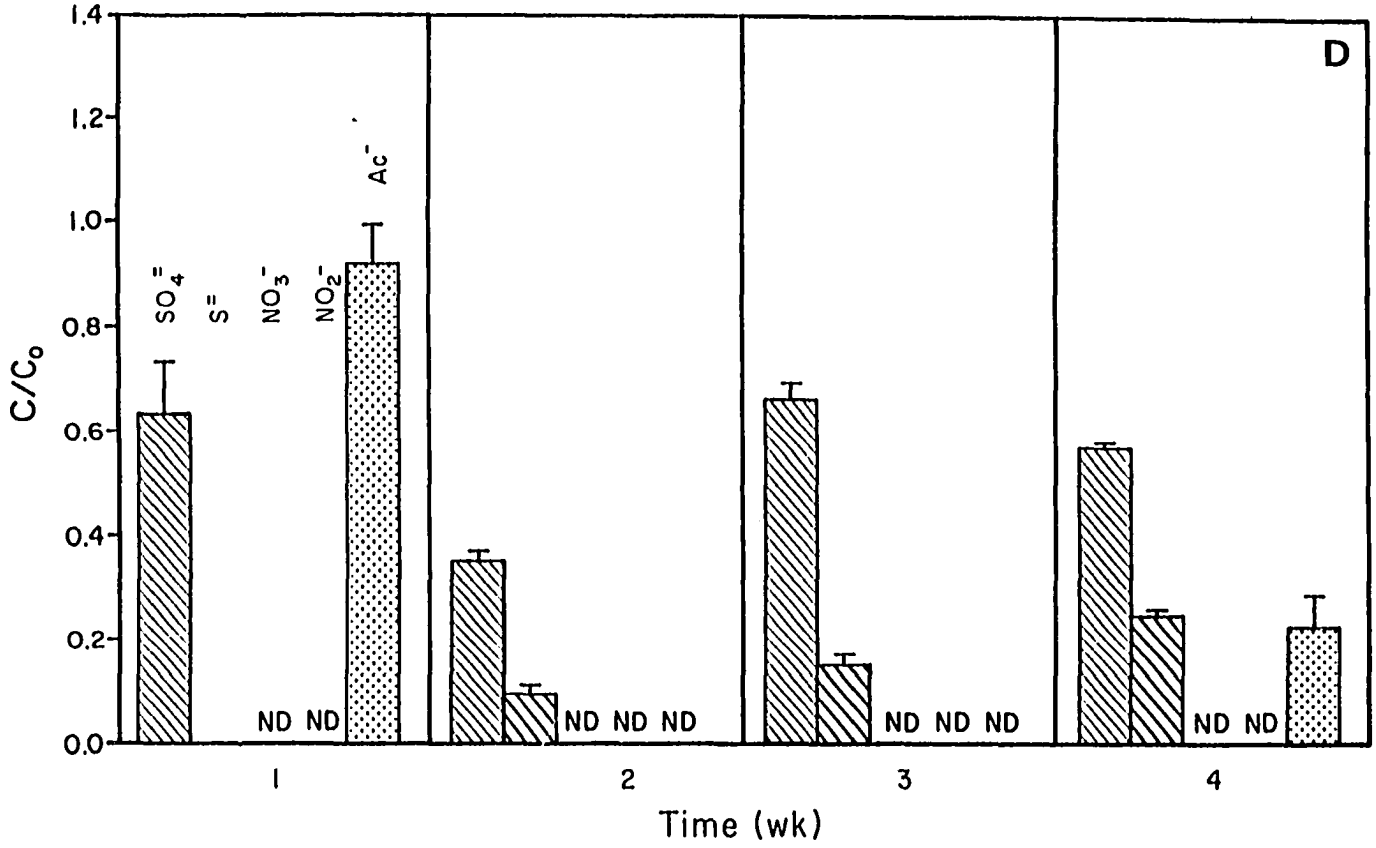
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C/Co
SO4=
S=
NO3-
NO2-
Ac-
ND
ND ND ND
ND ND
1
2
3
4
Time (wk)

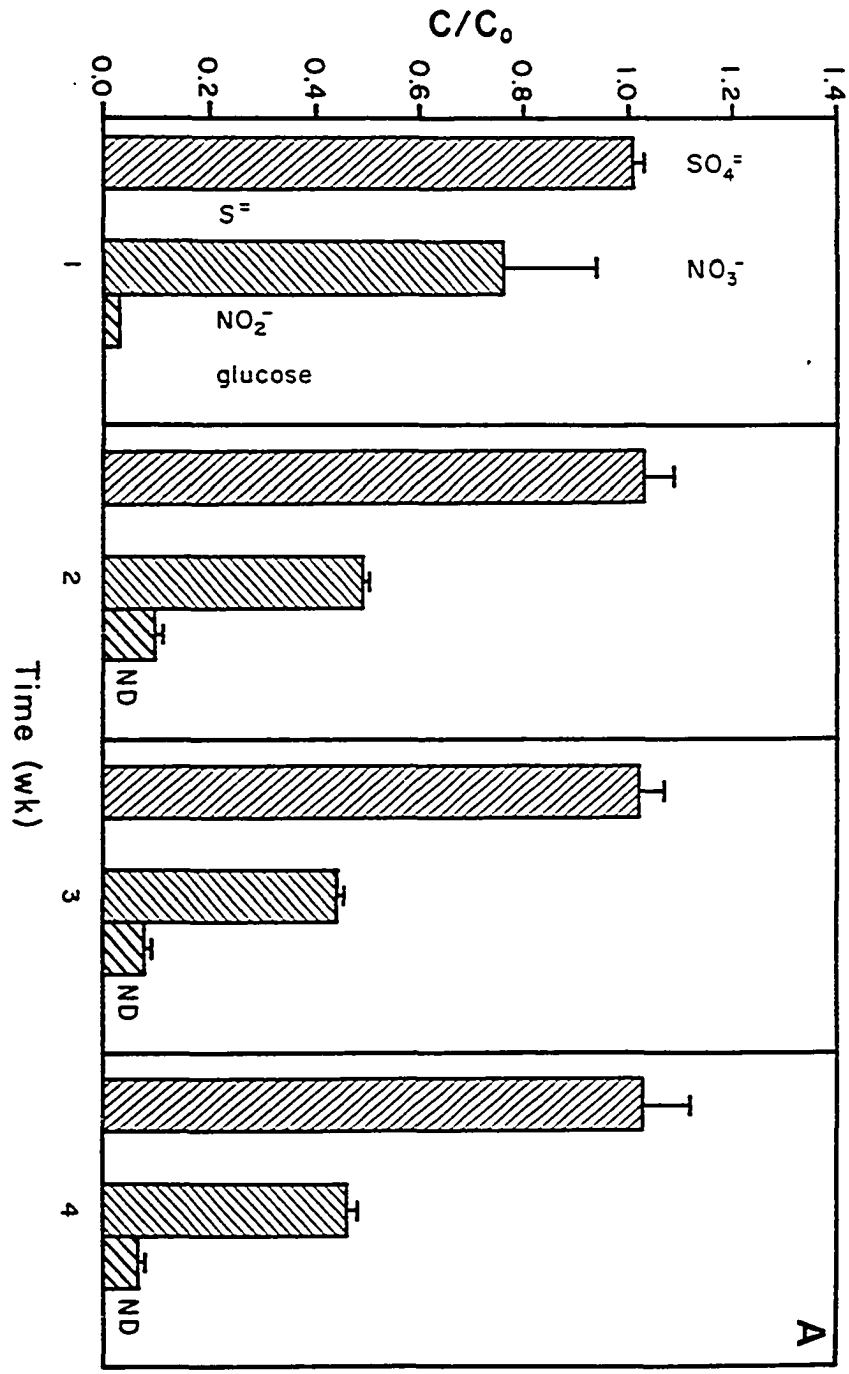

C
C/C_0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
$SO_4^=$
$S^=$
NO_3^-
NO_2^-
Ac^-
ND ND ND
ND ND ND
ND ND
1
2
3
4
Time (wk)


D
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C/Co
SO4=
S=
NO3-
NO2-
Ac-
ND ND
ND ND ND
ND ND ND
ND ND
1
2
3
4
Time (wk)

In bottles containing 19.6 mM NO_3^- or less (Figure 9; b, c and d) the onset of sulfide production began by the first or second week; however, for the bottles containing 58.8 mM NO_3^- (Figure 9, a) no sulfide was detected during the four-week testing period. In those cases where sulfide was produced in the presence of added nitrate, no NO_3^- or NO_2^- was detectable at the time of sulfide production; however, residual levels of NO_3^- and NO_2^- remained throughout the sampling period for bottles containing 58.8 mM NO_3^-. A similar trend was observed if either glucose (Figure 10) or hydrogen (Figure 11) instead of acetate was added as the energy source, except that NO_2^- was not detected in bottles amended with H_2. The added energy source was used after 1 week and the level of sulfate remained high until sulfide production commenced.

In all bottles amended with nitrate, regardless of the energy source, a sequence coloration of the medium was also observed. Initially the medium turned a green or yellowish-green color which was followed by a pink color, then a white cloudy appearance. The medium would clear and then turn gray. In bottles with acetate added, a yellowish-green color often followed the pink color and the pink and white cloudy appearance would occur simultaneously. The white cloudy stage was not observed when 1 mM dithiothreitol instead of cysteine sulfide was used as the reductant. The onset and duration of each color stage depended on the sample, energy source, and amount of nitrate added. Usually by the end of the second week of incubation the bottles containing 19.6 mM NO_3^- or less completed the sequence of colorations and were turning gray indicative of the onset of sulfide production. However, all bottles containng 58.8 mM NO_3^- remained pink throughout the four week period. Bottles containing sewage that had been autoclaved for 30 min (121°C and 15 psig) prior to addition of electron donors and acceptors never displayed any color change throughout the four week

Figure 10. Effect of glucose and different nitrate concentrations on sulfide production in 10% sewage sludge (10A) 58.8 mM NO_3^-; (10B) 19.6 mM NO_3^-; (10C) 5.88 mM NO_3^-; (10D) no nitrate; (see Figure 9 for additional comments).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0.2
0.4
0.6
0.8
1.0
1.2
1.4
SO₄⁼
S⁼
NO₃⁻
NO₂⁻
glucose
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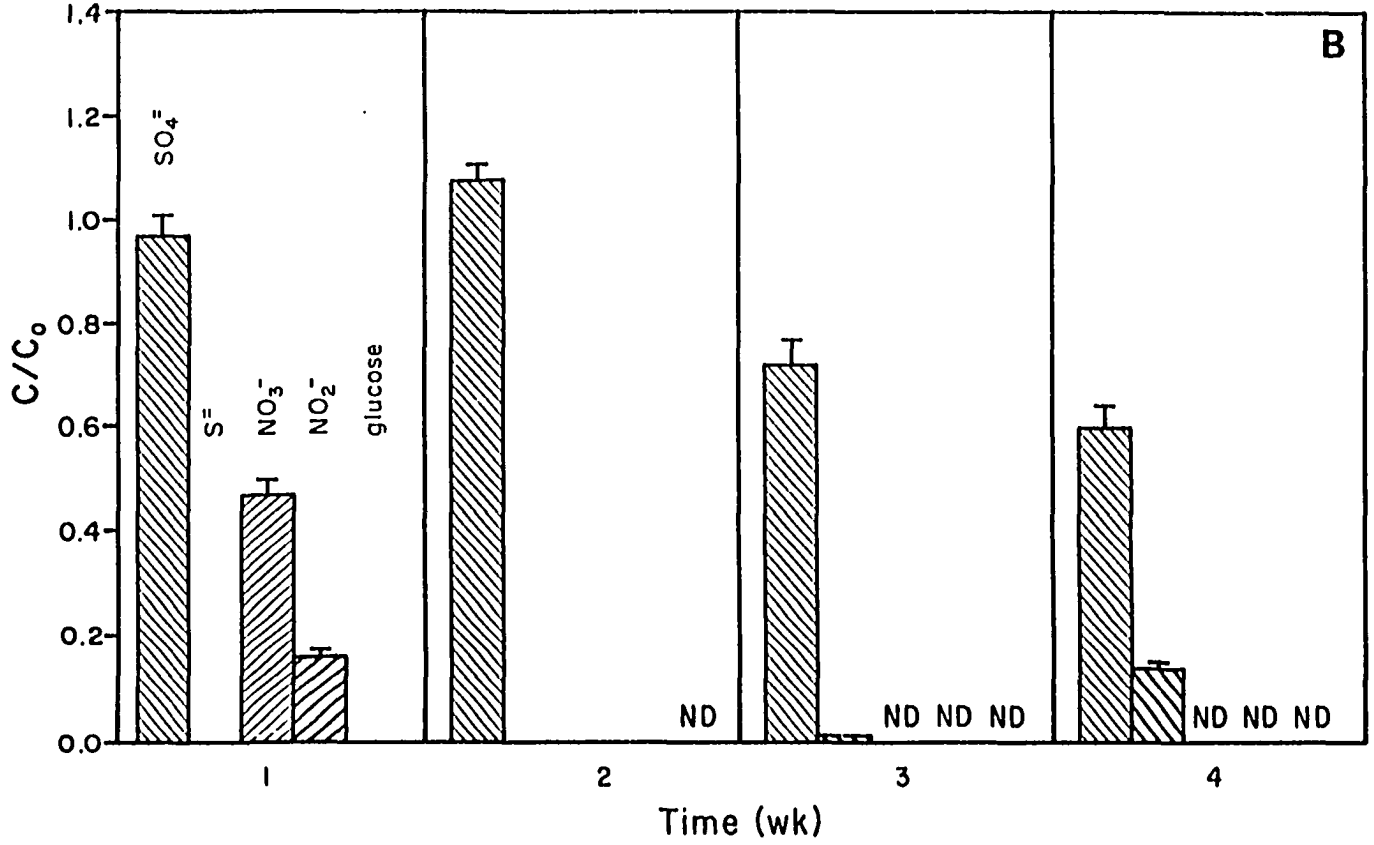
2
3
4
ND
ND
ND
Time (wk)
A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1.2
1.0
0.8
0.6
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0.2
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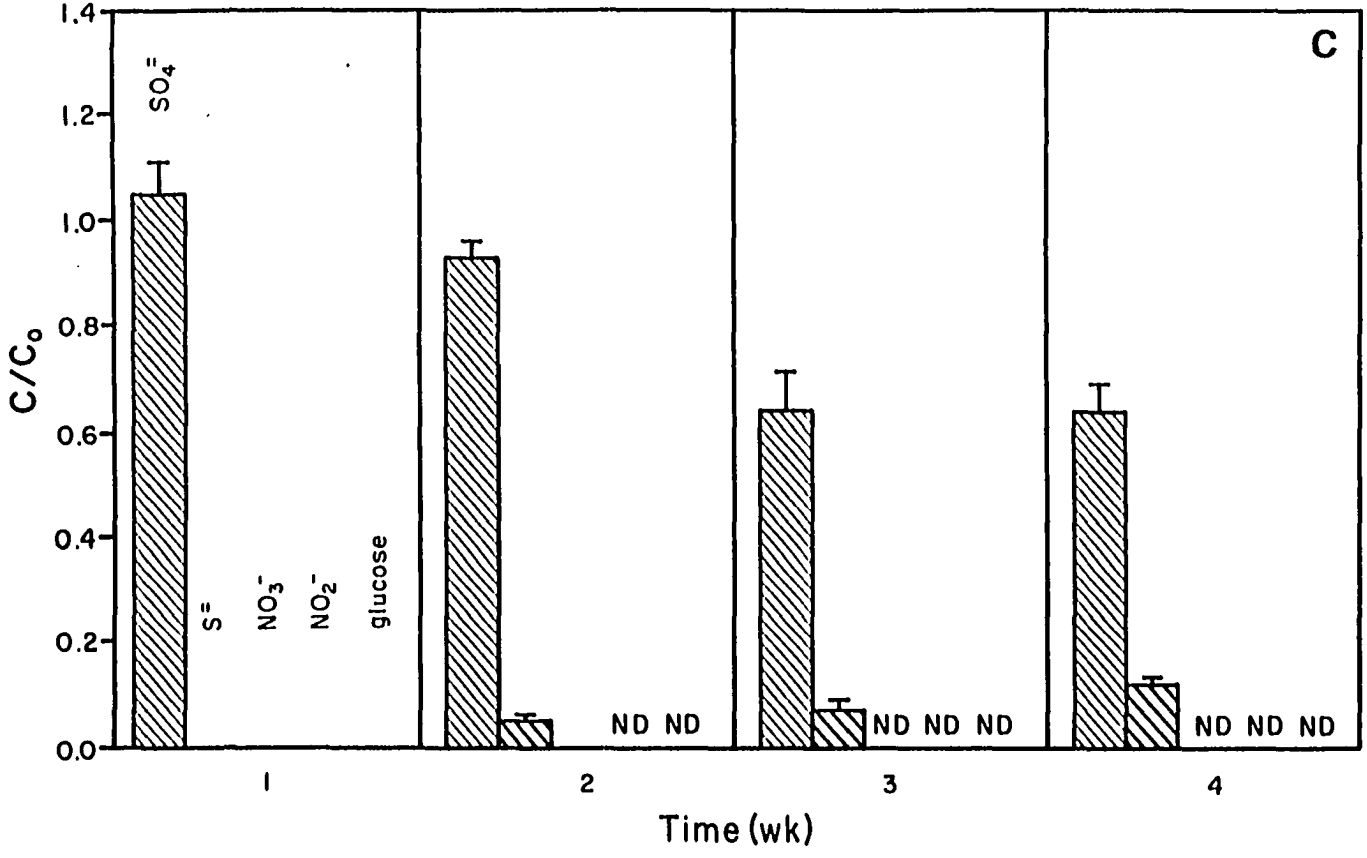
S=
NO3-
NO2-
glucose
ND
ND ND ND
ND ND ND
1
2
3
4
Time (wk)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C/Co
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1.2
1.0
0.8
0.6
0.4
0.2
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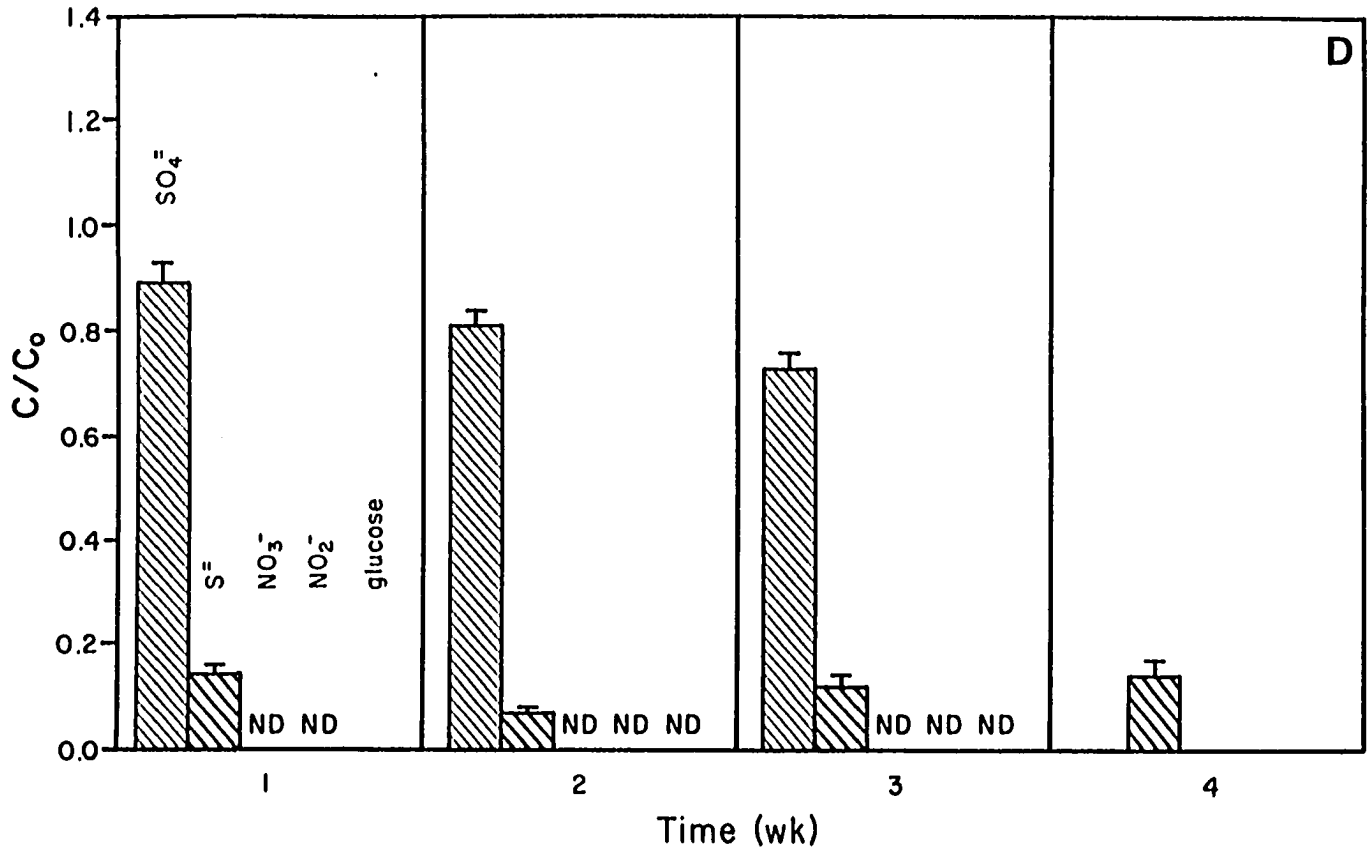
S=
NO3-
NO2-
glucose
ND ND
ND ND ND
ND ND ND
1
2
3
4
Time (wk)


D
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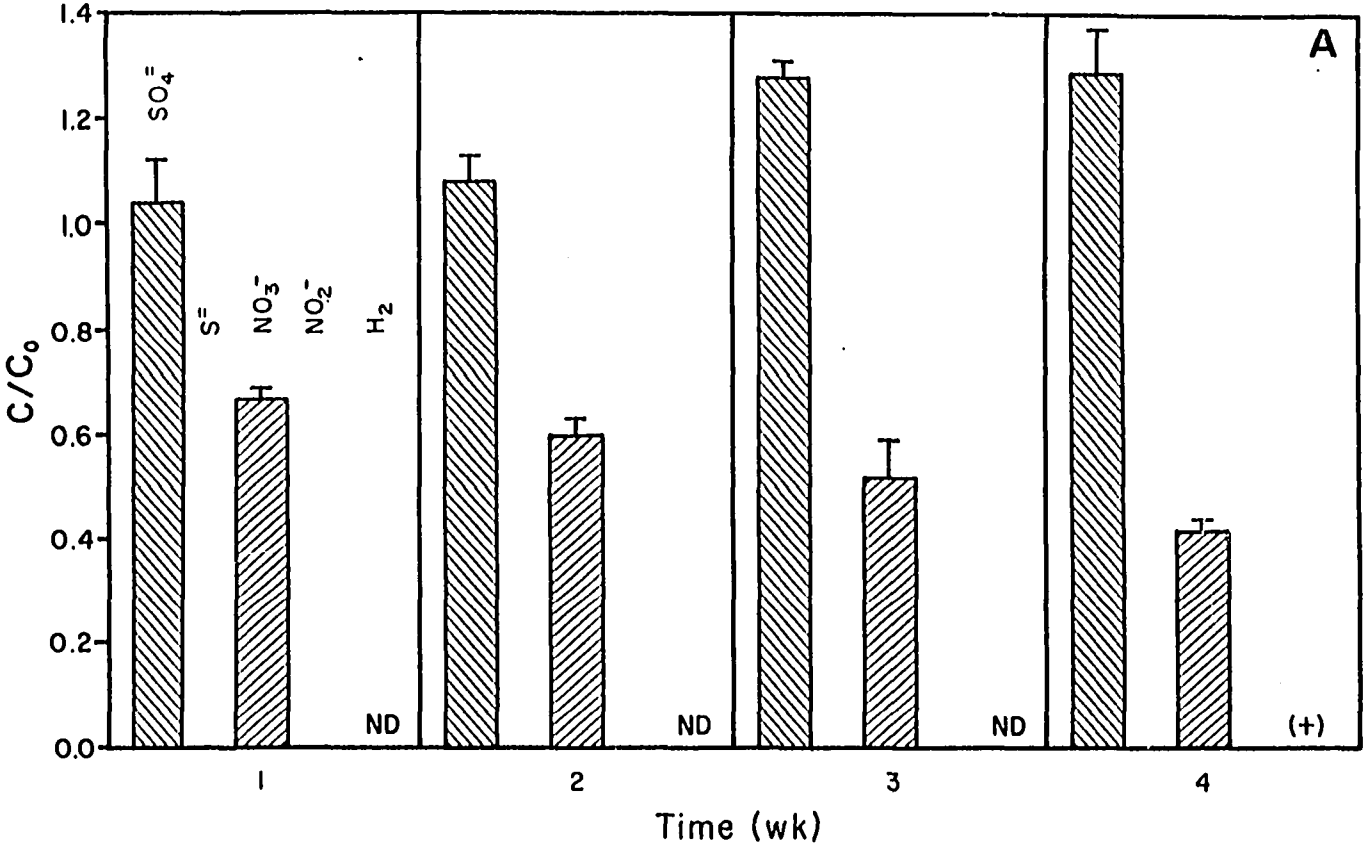
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C/C₀
SO₄⁼
S⁼
NO₃⁻
NO₂⁻
glucose
ND ND
ND ND ND
ND ND ND
1
2
3
4
Time (wk)

Figure 11. Effect of hydrogen and different concentrations of nitrate on sulfide production in 10% sewage sludge (+); hydrogen detectable in headspace; (-), headspace contained a vacuum, no attempt to measure hydrogen. (11A) 58.8 mM NO_3^-; (11B) 19.6 mM NO_3^-; (11C) 5.88 mM NO_3^-; (11D) no nitrate; (see Figure 9 for additional comments).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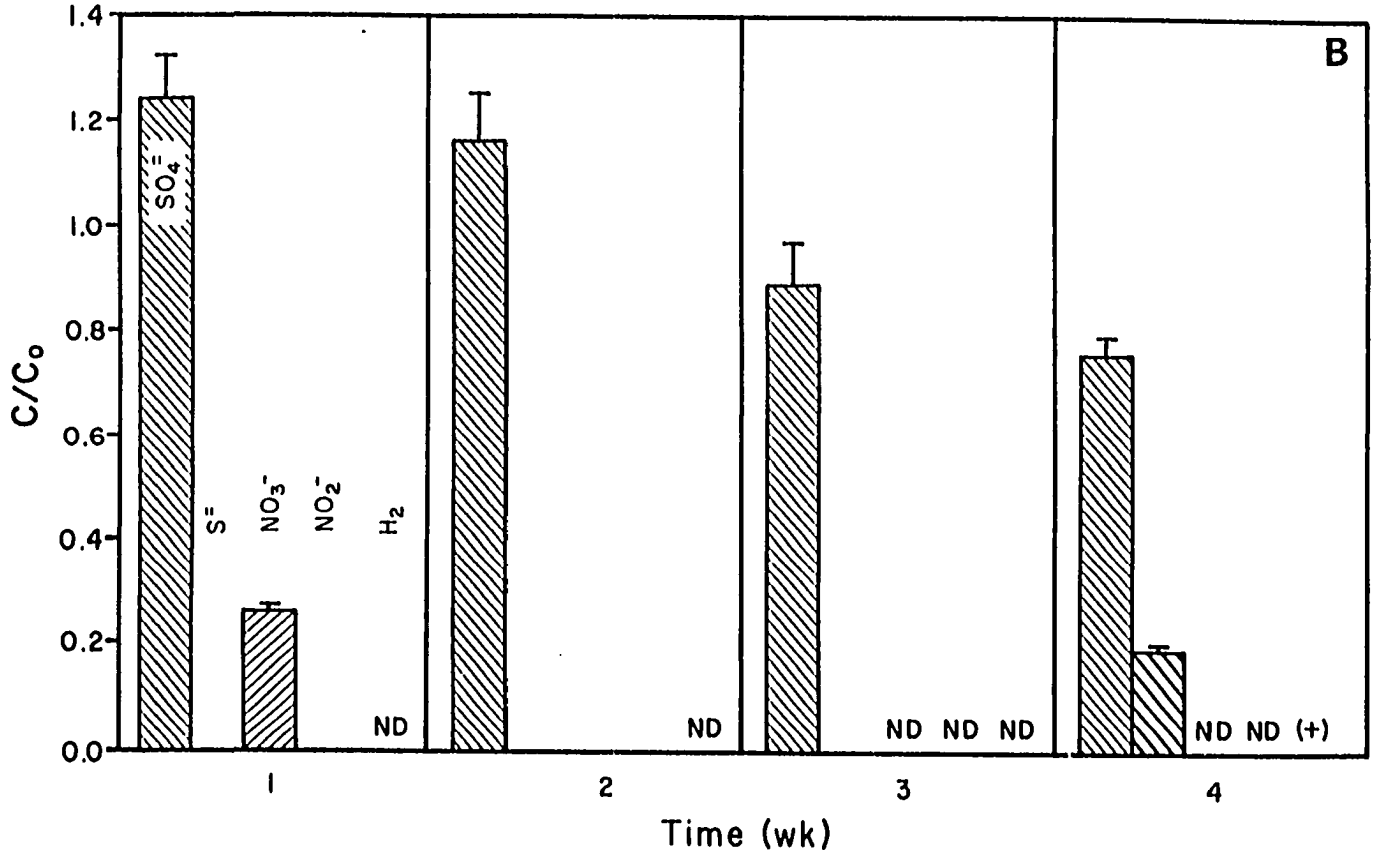
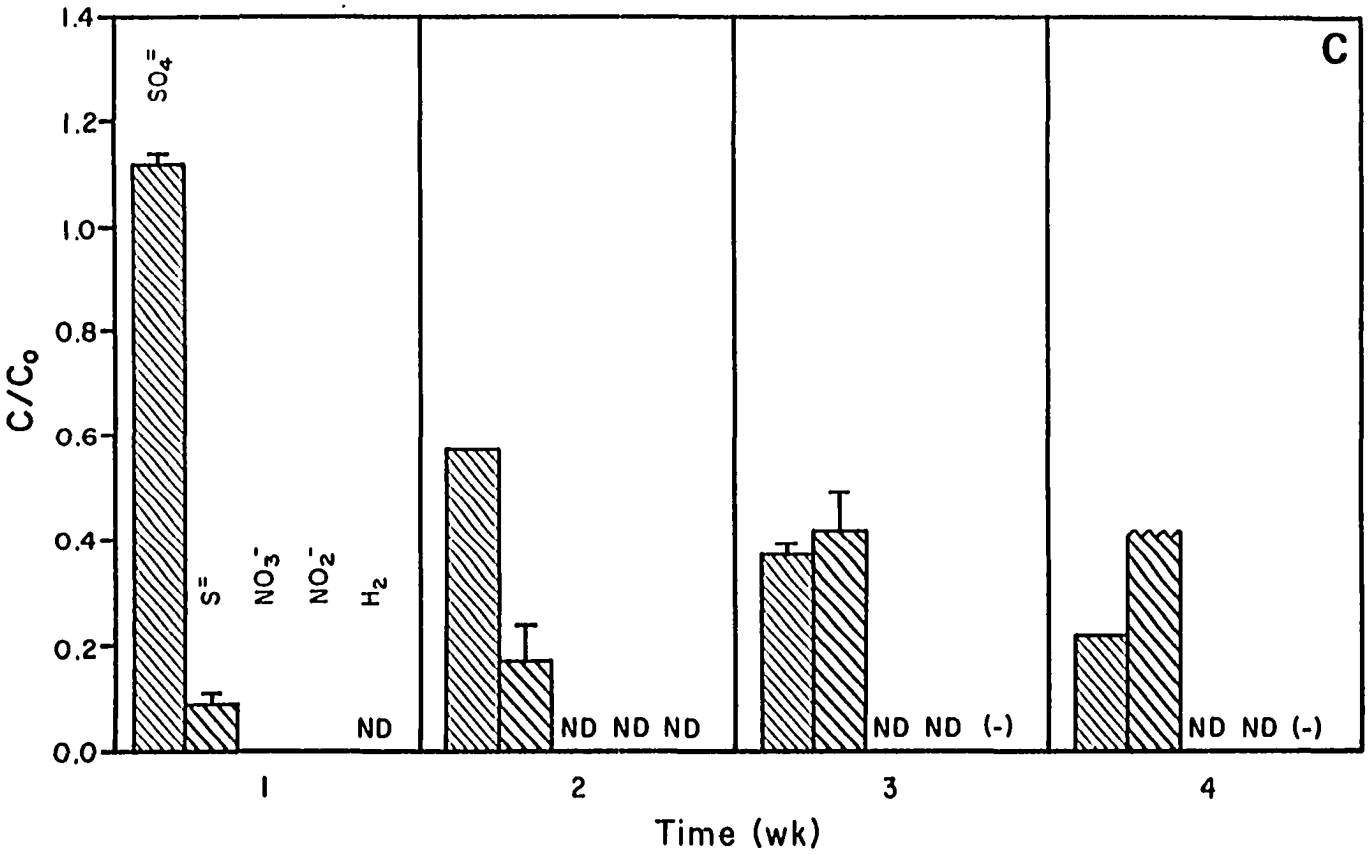
C/C0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SO4=
S=
NO3-
NO2-
H2
ND
ND
ND
(+)
1
2
3
4
Time (wk)


B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C/Co
SO4=
S=
NO3-
NO2-
H2
ND
ND
ND ND ND
ND ND (+)
1
2
3
4
Time (wk)


C
1.4
1.2
1.0
0.8
0.6
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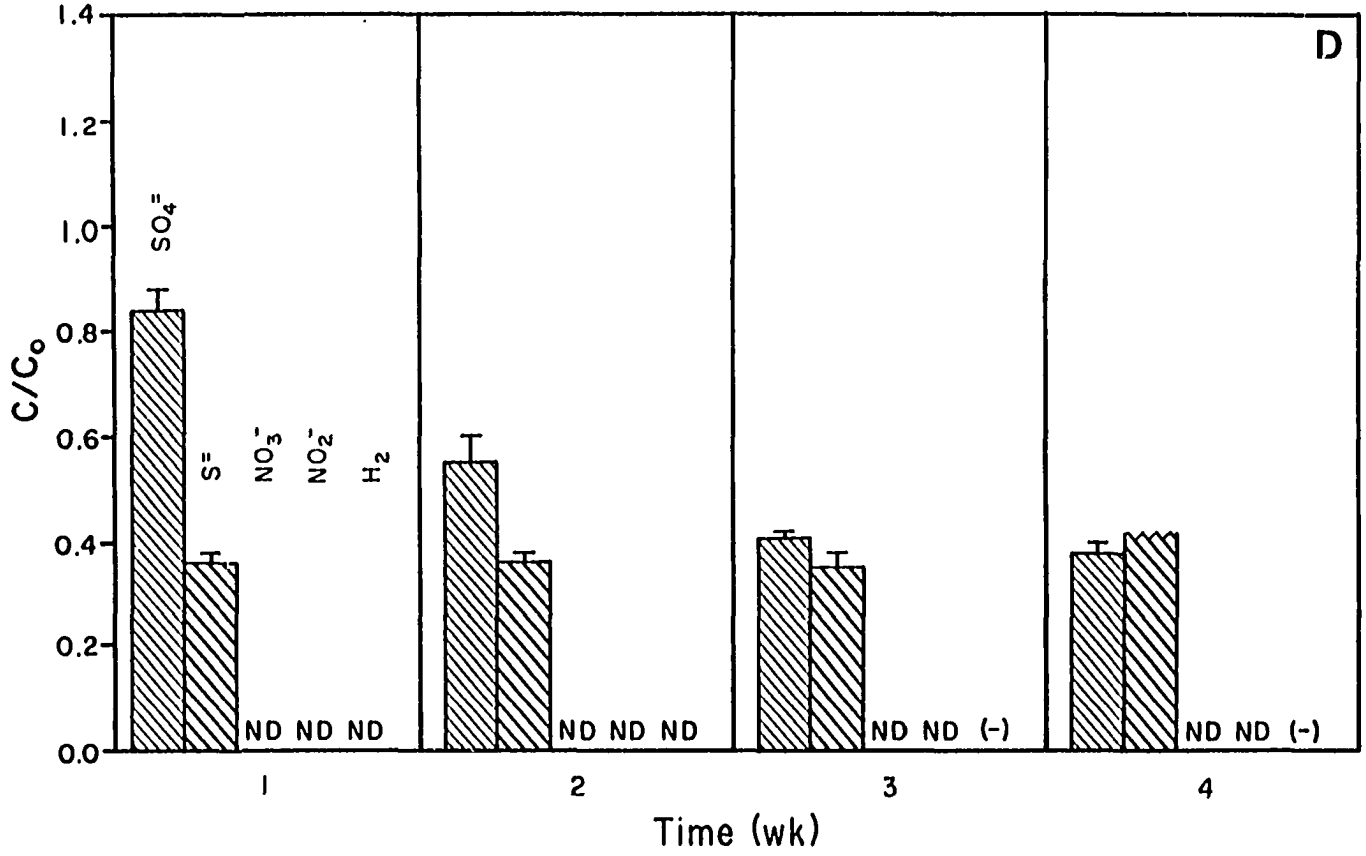
C/Co
SO4=
S=
NO3-
NO2-
H2
ND
ND ND ND
ND ND (-)
ND ND (-)
1
2
3
4
Time (wk)


D
1.4
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C/Co
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NO3-
NO2-
H2
ND ND ND
ND ND ND
ND ND (-)
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1
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Time (wk)

period. However, color changes were observed with bottles containing sewage sludge amended with acetate and nitrate when vented via sterile filters to the atmosphere inside the glove-box. These bottles contained either 1 mM dithiothreitol or cysteine-sulfide as reductant. This indicates that oxidation can occur even in open systems with no gas build-up.

Enumeration of microbial populations in sewage sludge before nitrate was added showed that sulfate-reducing bacteria comprised 2.0 - 5.6 x 10^7 cfu·g^{-1} of residue (Table 9). Initial sulfide concentrations in sludge were 0.93, 4.6, and 7.3 mg·l^{-1} for sewage sludge sampled on three different occasions, and total residue ranged from 0.8 to 2.3%. Total numbers of cultivatable bacteria in sewage were 5.0 x 10^8 and 6.3 x 10^9 g^{-1} of residue when acetate or glucose plates were used, respectively. The NOB were present at roughly 10-20% of the population of non-NOB when acetate or glucose plates were used, whereas the number of NOB growing in presence of hydrogen, as estimated by MPN analysis, was significantly lower.

After 17.5 weeks, the bottles amended with 58.8 mM NO_3^-, 15.0 mM acetate, and 20.0 mM sulfate remained oxidized (pink). At this time bottle B (Fig. 12) was evacuated and the headspace purged with O_2-free nitrogen (1.22 atm) three consecutive times. The same treatment was applied to another bottle (A) but, in addition, the medium was re-reduced to the colorless state of resazurin with the addition of a 2.0% (v/v) cysteine-sulfide solution, while bottle C was the untreated control. Figure 12 shows the concentration of sulfate, sulfide, NO_3^- /NO_2^- and acetate in each bottle after 4, 17.5, and 21 weeks. Sufficient amounts of sulfate and acetate remained during this period to support biogenic sulfide production; however, the levels of NO_3^- and NO_2^- did not decrease. After 21 weeks, no sulfide was detected in any of these bottles. In fact, within a few days

Table 9. Enumeration of nitrate and sulfate-reducing populations in sewage sludge growing on acetate, glucose, or hydrogen.

Nitrate	Acetate		Glucose		Hydrogen
mM	NOB[a]	non-NOB[a]	NOB[a]	non-NOB[a]	NOB[a]
58.8	1.5 (0.28)[b]	7.7 (1.8)[b]	3.5 (0.7)[b]	20.0 (9.9)[b]	7.4 (1.7-31.3)[c]
19.6	1.6 (0.78)	10.1 (0.42)	13.5 (2.1)	47.5 (3.5)	14.4 (4.0-5.14)
5.88	1.4 (0.99)	11.3 (0.78)	10.5 (0.7)	53.0 (9.9)	0.36 (.09-1.4)
0	11.5 (0.21)		50.5 (2.1)		ND
SRB	0.93 (.05)[b]		0.16 (.03)[b]		$\geq$1.0[d]
Total residue (%)	2.3		0.8		1.8
Initial Sulfide ($mg \cdot l^{-1}$)	0.93		4.6		7.3

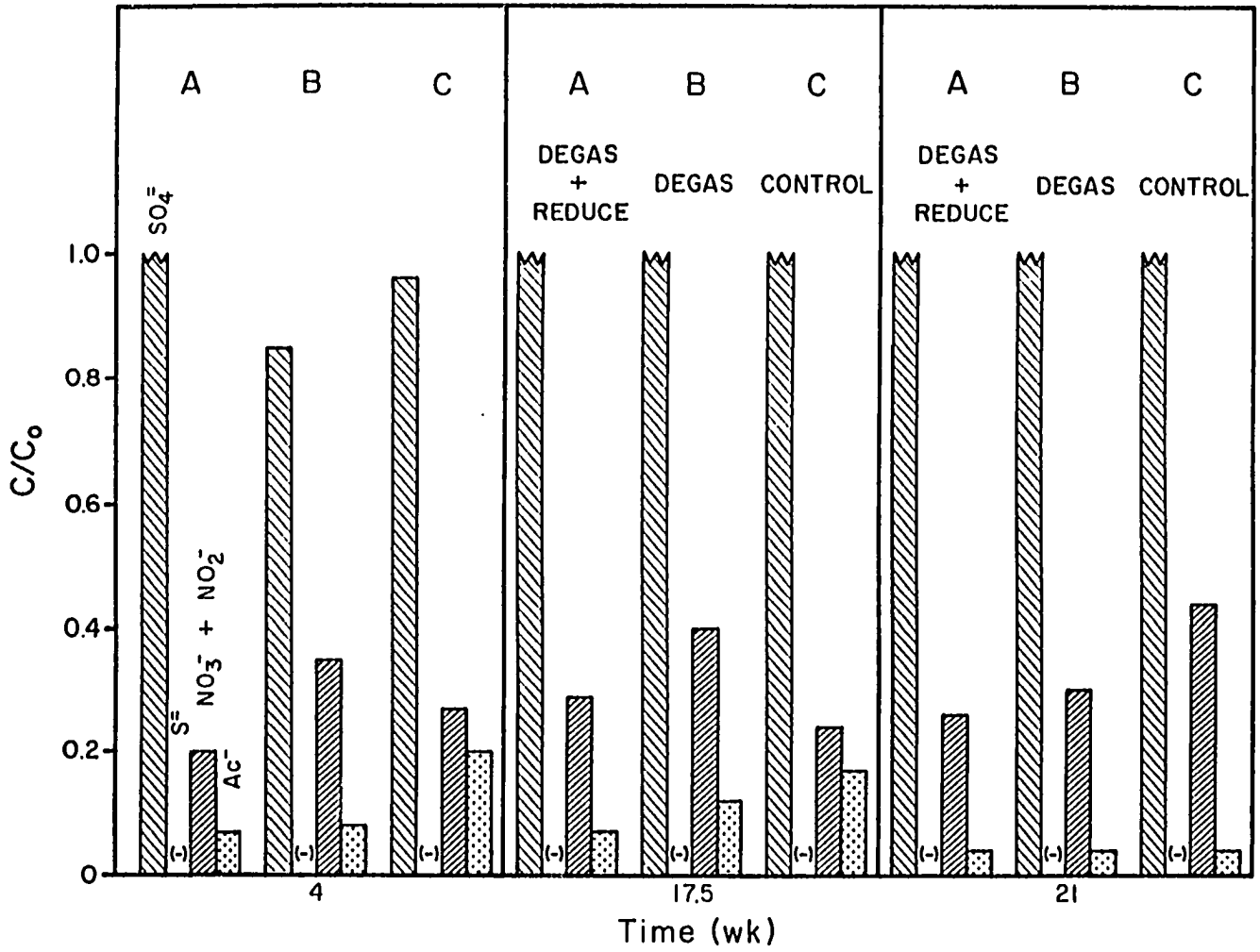
[a] NOB, nitrous oxide-producing bacteria; SRB, sulfate-reducing bacteria; ND, not determined.

[b] $cfu \cdot ml^{-1} \times 10^6$; ( ), ± standard error.

[c] MPN $\times 10^4$; ( ), 95% Confidence intervals

[d] MPN $\times 10^6$

Figure 12. The effect of gas purging and re-reduction of oxidized media containing 10% (v/v) sewage sludge after 17.5 and 21 wks incubation. All bottles initially contained 20 mM sulfate, 15 mM acetate, and 58.8 mM nitrate. Sulfate, acetate as well as nitrate and nitrite nitrogen are represented as fractions of their initial concentration (Co). No sulfide (-) was detectable throughout the period sampled. The concentrations after 4 wks incubation are the same as those for the three replicates in Figure 9A. Jagged lines on top of bars means that actual measured concentrations were above these values. Gas-purging was done with O_2-free nitrogen that was pressurized (1.22 atm) and then evacuated three consecutive times. Re-reduction was accomplished by the addition of cysteine-sulfide (see Materials and Methods).


A
B
C
A
B
C
A
B
C
DEGAS + REDUCE
DEGAS
CONTROL
DEGAS + REDUCE
DEGAS
CONTROL
$SO_4^=$
$S^=$
NO_3^- + NO_2^-
Ac^-
(-)
C/Co
1.0
0.8
0.6
0.4
0.2
0
4
17.5
21
Time (wk)

after reduction of the medium in bottle A, the medium was reoxidized (pink). The pink coloration originated from the sediment/liquid interface indicative of biogenic oxidation. Examination of the samples revealed that NO_3^-/NO_2^- decreased in bottles A and B but increased in the control (C). Acetate levels were lower in all bottles after 21 weeks incubation. Analysis of headspace gas indicated the presence of 5.6% and 6.4% nitrous oxide in bottle B and A, respectively, while 19.3% nitrous oxide was detected in the untreated control. Table 10 shows the concentration of nitrous oxide in the aqueous phase of the bottles calculated by using the Ostwald coefficients. A small amount of oxygen (less than 1%) was found in bottles incubating for long periods of time. However, no detectable O_2 was detectable in bottles 1A and 1B that had been re-evacuated and purged with O_2-free nitrogen at the end of 17.5 weeks incubation. Significant amounts of nitrous oxide were also found in bottles originally amended with glucose or hydrogen as the energy source. After 21 weeks, bottles A and B were again treated as above, but these bottles became oxidized within a few days and no sulfide was detected.

Results of bacterial enumeration of both nitrous oxide producing, as well as, sulfate-reducing populations in acetate amended bottles after 17.5 weeks incubation revealed that as many as $10^5 - 10^6$ cfu$\cdot g^{-1}$ of residue of NOB were still detectable in all bottles (A-C). However, no detectable (less than 10 cfu$\cdot ml^{-1}$ or 435 cfu$\cdot g^{-1}$ of residue) sulfate reducers were found. Microscopic examination of cells from bottles oxidized for long periods revealed a morphological predominance of spirillum-like organisms.

Table 10. Gas composition of bottles containing 10% (v/v) sewage sludge amended with 58.8 mM nitrate and 20 mM sulfate after prolonged incubation periods.

Bottle	Incubation time (weeks)	Substrate	N_2O (% total gas)	Soluble N_2O (mM)	Other gases detected[b]
1A			6.4	1.0	none
1B	25[a]	Acetate	5.6	0.9	CO_2
1C			19.3	1.5	CH_4, CO_2, O_2
2A			5.6	0.5	CH_4, CO_2, O_2
2B	21	Glucose	10.1	1.0	CH_4, CO_2, O_2
2C			12.8	1.2	CH_4, CO_2, O_2
3A			-[c]	ND[c]	ND
3B	9	Hydrogen	-	ND	ND
3C			9.6	ND	ND

[a]At 17.5 weeks and at 21 weeks both 1A and 1B were evacuated and purged with O_2-free N_2 and bottle 1A was re-reduced with cysteine-sulfide to the colorless redox state of resazurin. Bottle 1C was an untreated control.

[b]Oxygen concentration was always less than 1.0% of total gas.

[c]-, negative pressure measured; ND, Not determined.

CHAPTER XV

DISCUSSION

The results support the contention of Poduska and Anderson (32) that the ORP of an anaerobic habitat is increased as a result of the action of nitrate-reducing populations. However, I attribute the cause for this ORP increase to be a result of accumulation of N_2O or NO intermediates, instead of simply the reduction in soluble organics. Furthermore, this increase in ORP is at least partially responsible for the absence of biogenic sulfide during times when the resazurin was oxidized, since biological sulfide production is unfavorable at ORP above -100 mV (33). It is not certain in all cases of nitrate amendment whether the absence of biogenic sulfide is a direct or indirect effect of ORP since in bottles where ORP was consistently high for long periods of time no SRB were detectable, however numerous NOB were still viable, implying that death of SRB and not increased ORP may be responsible for lack of sulfide.

Recently, (G. E. Jenneman, A. Montgomery and M. J. McInerney, unpublished results), found that the oxidation of the redox indicator resazurin from its reduced, colorless state to its oxidized, pink state was strongly associated with the accumulation of nitrous oxide produced by nitrate reducers isolated from sewage sludge. Furthermore, when reduced media containing

resazurin as a redox indicator was titrated with either NO or N_2O they were found to oxidize resazurin at concentrations of 0.5 nM and 0.2 mM, respectively. This should not be unexpected since the E'_o of NO/N_2O is +1175 mV and the E'_o of N_2O/N_2 is +1355 mV (40) while the E'_o of resazurin in approximately -42 mV (19). Therefore, the change in the redox indicator from colorless to pink in these experiments is an indication of an increase in redox potential which is unfavorable to biogenic sulfide production.

The yellowish-green pigmentation of the medium observed in bottles prior to and sometimes subsequent to the onset of the pink color is probably a result of nitrite accumulation. If nitrite is autoclaved in the presence of the growth medium containing resazurin and cysteine-sulfide as a reductant, then the yellowish-green color is evident (unpublished results). The white cloudy appearance of the medium is most likely due to cystine production as a result of oxidation of cysteine-sulfide, since no white cloudy appearance was observed using dithiothreitol as the reductant.

Poduska and Anderson (32) found that 7,500 - 15,000 $mg \cdot l^{-1}$ of $NaNO_3$ was necessary to raise the ORP of concentrated waste sludge in a reactor vessel from below -200 mV to over +100 mV after 21 days of incubation. During this time nitrate concentrations were reduced by over 50%, and acetate and propionate concentrations were reduced form 3,353 and 934 $mg \cdot l^{-1}$, respectively, to undetectable levels within a few days after nitrate addition. They further observed that, as long as acetate and propionate levels remained undetectable, the ORP remained positive which led them to believe that soluble organics were required to reduce the ORP. I feel that the decrease in ORP observed was due to a complete reduction of nitrate and the gaseous N-oxide intermediates of denitrification which allowed the subsequent reduction of sulfate to sulfide. This

is supported by our observations that biogenic sulfide did not occur when residual levels of NO_3^- or NO_2^- were observed and gaseous N-oxides were present (see below).

Inhibition of sulfide production by nitrate addition was more pronounced with low concentrations of sludge (i.e. 10% v/v) than with low concentrations of either eutrophic pond sediment or aquifer material, implying that denitrification capacity is greater in sludge than in the other samples. Tiedje et al. (42) found that the capacity of sludge to denitrify (i.e. to produce nitrous oxide) was almost 5 times greater than eutrophic lake sediment and at least 500 times greater than oligotrophic intertidal sediments. They further implied that the ratio of electron donor/electron acceptor in an anaerobic habitat could determine if nitrate was denitrified or whether it was reduced to ammonium (i.e. dissimilatory nitrate reduction). In anaerobic digestor sludge, the electron donor/electron acceptor ratio is high so it would be expected that ammonium would be the major end product of nitrate reduction, since the reduction of nitrate to ammonium would result in utilization of 8 electrons per NO_3^- molecules as opposed to 5 electrons for nitrate reduced to N_2. This presents a possible explanation as to why sulfide production was not inhibited when higher concentrations of sludge were used. With increased sludge concentrations, nitrate would be more limiting since an increase in nitrate utilizing populations would exist and thus ammonium would be the expected end-product of nitrate reduction. Further proof that limiting nitrate leads to ammonium production is supported by several investigators studying the effect of nitrate concentrations on marine (35) and salt marsh sediments (29). Essentially they found that the addition of increasing amounts of nitrate to marine sediments resulted in an increase in the rate of denitrification, whereas the rate of denitrification decreased and rate of ammonium production increased

in the deep marine sediments where it would be expected that nitrate would be limiting. King and Nedwell (23) found that the addition of 2 mM nitrate to an anoxic salt marsh sediment resulted in 83% of the nitrate being converted to nitrous oxide when using the acetylene blockage technique to measure N_2O. Jack et. al (21) found that as little as 2.4 mM NO_3^- could inhibit sulfide production for up to 4 months (longest time tested) when using 10% (v/v) inocula of either sewage sludge or oil filter samples in the presence of various crude oils as sole energy sources. Nitrate was probably not limiting in this system, instead it is more likely that a utilizable energy source was limiting. This resulted in the accumulation of visible gas but no attempt was made to assay N_2O, N_2, or NO. Therefore it can only be presumed in this case that gaseous N-oxides were being produced.

Kinetics could also explain the lack of sulfide inhibition with increasing sludge concentration or decreasing nitrate concentration. The rate of NO and N_2O reduction would be expected to be faster in the presence of increasing sludge size since this would result in increased cell pool size and consequently increased turnover rates of these gaseous N-oxides. As nitrate concentrations decrease this would result in less buildup of gaseous intermediates (i.e. NO or N_2O) since less nitrate-nitrogen would be available for reduction to N_2O. Betlach and Tiedje (6) proposed a kinetic model for denitrification in which both NO and N_2O are maintained at low steady-state concentrations soon after the addition of nitrate; however, relatively low concentrations of nitrate were used compared to levels used in these experiments. Furthermore, NO and N_2O were not completely reduced until after NO_3^- and NO_2^- were depleted. It has been shown that during denitrification ionic N-oxides disappear prior to the gaseous N-oxides (31).

My results further indicate that inhibition of sulfide production can be overcome in both sludge and pond sediment if sufficient sulfate (156 mM) is added even in the presence of 58.8 mM NO_3^-. This implies that an energetic model alone is not sufficient to explain the fate of NO_3^- and $SO_4^=$ in an anaerobic habitat, since high NO_3^- concentrations alone cannot inhibit sulfide production. No attempt was made to follow the fate of NO_3^- in these samples in order to determine if any denitrification occurred prior to, during, or after sulfide production commenced; however, Capone and Bautista (8) stated that denitrification in the marine sediments which they studied was inversely correlated with sulfate reduction. Meyers (28) found that sulfide depressed denitrification but stimulated reduction of nitrate to ammonium.

The presence of sulfide could be important in maintaining low ORP in anoxic habitats amended with nitrate. I found as much as 15 $mg \cdot l^{-1}$ sulfide in sewage sludge before nitrate addition but after three days incubation less than 4.4 $mg \cdot l^{-1}$ was detectable. Possibly denitrifiers capable of utilizing sulfide as an electron donor (e.g. Thiobacillus denitrificans) oxidized the sulfide upon nitrate addition or possibly the sulfide was chemically oxidized by the added nitrate, or perhaps sulfide precipitated out of solution with heavy metals.

Also in those cases where prolonged inhibition of sulfide production was observed, it was evident that sulfate remained at or near the level at which it was added and resazurin remained oxidized for periods of at least 5 months. In fact, sulfate was practically unmetabolized during this time until NO_3^- and NO_2^- decreased below detectable levels and the resazurin was re-reduced by the addition of cysteine-sulfide. This indicates that denitrification can have a very profound effect on the fate of sulfate in an anaerobic habitat. If sufficient levels of nitrate are maintained in these sediments sulfate levels could remain high until the redox potential declines or diffusion of sulfate into deeper sediments occurs.

The prolonged oxidation of the medium could indirectly be due to nitrate or the accumulation of nitrite. It has been observed under cetain nutrient conditions that both NO_3^- and NO_2^- can inhibit the reduction of NO and N_2O (7, 14, 30), however Betlach and Tiedje (6) did not observe this inhibition under the conditions which they tested. My results show that when high NO_3^- concentrations were added to sludge, both NO_3^-, and in some instances NO_2^-, were still present in residual amounts after 4 weeks incubation; whereas, in sludge amended with lesser amounts of nitrate no residual NO_3^- or $NO_2^-$was detectable at the onset of sulfide production. Therefore it is possible that residual levels of NO_3^- and NO_2^- are at least partially responsible for the accumulation of gaseous N-oxides. Kucera, et al. (24) have recently found that Paracoccus denitrificans is under feedback redox control in which the build-up of NO_2^- and N_2O can inhibit nitrate reductase by channeling electrons through nitrite and nitrous oxide reductases. This may explain why levels of NO_3^- are still detectable after 5 months of incubation since large amounts of N_2O are present which could inhibit the reduction of nitrate. However, if N_2O reductase is not inhibited by NO_3^- or NO_2^- then it is difficult to explain why such high levels of N_2O were still present after 5 months in the presence of ample electron donor, since bottles vented to an atmosphere of H_2 (electron donor) and N_2 still remained oxidized in the presence of 58.8 mM NO_3^-. It may be that along with redox control exerted by N_2O that prolonged exposure to high ORP inhibits or inactivates the N_2O reductase or its expression. Nitrous oxide reductase has been shown to be very sensitive to oxygen (18). This could explain why N_2O still accumulatd after N_2O was purged from oxidized bottles with O_2-free nitrogen which were then re-reduced with cysteine-sulfide. Another possibility is that the high NO_3^- concentrations select for populations of denitrifiers that produce N_2O as an end product. Microscopic

observation of cells from oxidized media did reveal a predominance of spirillum-like organisms and it is known that Aquaspirillum itersonii produces N_2O as a terminal end-product of denitrification (31). It is also interesting to note that this prolonged oxidation in the presence of nitrate is not only due to build-up of N_2O gas in a closed system but can also take place in bottles that are vented inside a large anaerobic chamber. Therefore, buildup of gaseous N-oxides in the headspace is not a likely factor. Also if 1 mM dithiothreitol is used as a reductant in place of cysteine-sulfide the bottles containing high NO_3^- still exhibit prolonged oxidation. Sorenson, et al. (37) have shown that sulfide can inhibit nitrous oxide reduction. Therefore free sulfide could be a cause of nitrous oxide accumulation in cysteine-sulfide systems, but this does not explain N_2O accumulation when dithiothreitol is used. It was observed that sulfide production did not commence in medium that was oxidized for prolonged periods even if this medium was re-reduced after 17.5 weeks with the addition of cysteine-sulfide. The reason for this was likely due, at least in part, to the absence of SRB. No SRB were detected (<10 cfu$\cdot$ml^{-1}) at this time even though as many as 10^5 cfu$\cdot$ml^{-1} of NOB were found to be present in these bottles after 5 months incubation. Possibly the accumulation of some by-product of denitrification or long term exposure to high ORP may have been toxic to the SRB. Nitric oxide is known to display bacteriostatic effects on certain bacteria (26) while N_2O can be cytotoxic (41), but their effect on SRB is not known.

Therefore, the results suggest that two mechanisms may be responsible for sulfide abatement at high nitrate concentrations. The first effect (direct effect) is an increase in ORP due to build-up of denitrification intermediates (i.e. NO and N_2O) providing an oxidizing environment incapable of supporting sulfide production. The second effect (indirect effect) is the apparent loss of viability of

SRB upon prolonged exposure to the oxidizing condition prompted by N_2O accumulation. The actual cause of this apparent cytotoxic effect is unknown at this time. It is likely that the direct effect is important in the short term but the cytotoxic effect could be more important in explaining prolonged inhibition of sulfide production.

It is evident from the results that the addition of high nitrate concentrations to anaerobic habitats could result in perturbation of the biological cycling of sulfur compounds. In fact, the results may explain the unexpectedly high redox potential (+222 mV) observed by Sorensen (36) in marine sediments amended with nitrate. Also it could explain the lack of sulfide production observed by Balderston and Sieburth (4) who investigated denitrification as a means of reducing nitrate levels in an aquaculture based. It could also explain why strict anaerobes that respire nitrate reduce it to ammonium and not N_2O and N_2 (10, 22, 27, 38).

This work suggests that the use of nitrate in oxygen-limited habitats such as groundwater, petroleum reservoirs for MEOR, and clean-up of wastes can be used without the concommitant production of sulfide, and may be useful in the commercial control of biogenic sulfide production.

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