BACTERIAL DEGRADATION OF DIESEL FUEL USING PURE AND MIXED CULTURES

by

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ABSTRACT

Eight bacteria capable of using diesel fuel as the sole carbon source were isolated from both a bunker oil-saturated soil sample and from a gasoline-contaminated water sample. The three isolates from the soil were identified as Pseudomonas vesicularis, and two strains of Pseudomonas aeruginosa. The five water isolates were identified as Achromobacter xyloxidans, Achromobacter sp., Aeromonas sp., Pseudomonas sp., and Pseudomonas vesicularis. The components of diesel fuel degraded by the isolates over a three week period were determined by capillary gas chromatography. The growth curves of the isolates in 1.5% yeast extract and in basal salts medium with diesel as carbon source were obtained. The generation times of each isolate in 1.5% yeast extract and in basal salts medium with diesel as carbon source were calculated and the significant differences in generations times were determined. Two sets of mixed cultures were used, one consisting of the three soil isolates and the other of the five water isolates, to determine the effect of mixed cultures on diesel over a three week period. The mixed cultures were found to be more efficient than the pure cultures at reducing the total number of fractions of diesel.

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CHAPTER I

INTRODUCTION

It has been estimated that approximately 0.08% to 0.4% (i.e., 1.7 to 8.8 metric tons) of the world's annual production of petroleum ends up polluting the oceans. An equal percentage contributes to terrestrial pollution (3), of which a considerable amount can seep through the soil and contaminate the groundwater aquifers. Petroleum hydrocarbons only have to reach the groundwater aquifers in very low concentrations to taint the water supplies; concentrations of 1 to 5 ppm hydrocarbons give a foul taste and odor to the water (4). Although it is estimated that only about 1% of the world's groundwater is contaminated, most of this contamination is concentrated in urban areas (16). Since approximately 30% of the people in the U.S. depend on groundwater aquifers for their water supplies, this could be a considerable problem (16).

One current approach to cleaning up terrestrial oil spills is to make use of the petroleum-degrading abilities of microorganisms that occur naturally in the contaminated soils. Microbial degradation of petroleum has been extensively documented in the literature (1, 2, 3, 4, 5, 6, 7, 11, 15, 17, 19). Both soil and marine bacteria have been shown to possess the ability to utilize petroleum hydrocarbons for growth. Investigators have demonstrated that species of Pseudomonas, Flavobacterium, Achromobacter, Bacillus, Nocardia, Alcaligenes and Acinetobacter can degrade petroleum hydrocarbons during growth (6, 7, 8, 17). In most cases, these bacteria were isolated from sites contaminated with petroleum products. This indicates that bacterial populations capable of degrading petroleum are present in contaminated soil and marine environments. Therefore, it is possible to exploit them to facilitate the removal of petroleum contaminants from these environments.

On the surface, the oil which remains after a physical cleanup can be removed by chemical means, i.e., through photooxidation and evaporation, and by enhancing biodegradation through fertilization, pH control and tillage (3, 4).

In the soil, petroleum hydrocarbons pose a special problem since evaporation is limited, photooxidation does not occur, and biodegradation by naturally present bacteria normally is a slow process (3, 4). Hence, pollutants can persist in the soil for years. The easily recovered portion of the oil or diesel from a contaminated site may be reclaimed by techniques currently being used for cleanup of subsurface petroleum contamination, such as water injection and recovery well systems. However, these result in an incomplete removal of the contaminant due to the strong adsorption of hydrocarbons onto soil particles (3). Biodegradation of the pollutants would seem to be a better solution for cleanup of subsurface contamination because bacteria are capable of degrading hydrocarbons with the aid of degradative enzymes coded for by plasmids (3), even in environments with low mineral concentrations (3, 4).

Using naturally occuring aerobic bacteria already present at the site of contamination for cleanup is very attractive, but the process has its limitations due to limited oxygen availability (1, 3, 4). It is known that the first step in attacking intact hydrocarbons almost always requires the action of oxygenases, and hence free oxygen is crucial for starting biodegradation (3). Very little to no biodegradation occurs in anoxic environments (3, 4). Two new methods for increasing oxygen supply to the bacteria which have had some success are injection of oxygen-saturated water and injection of water enriched with hydrogen peroxide (3).

Other factors limiting biodegradation are nitrogen and phosphorus availability, and the toxicity of the pollutant (3, 4). Biodegradability of petroleum hydrocarbons depends

primarily on chemical structure, but is also influenced by toxicity and the physical state of the compound (4, 10). For example, n-alkanes, n-alkylaromatic and aromatic compounds of the C_{10}-C_{22} range are the least toxic to microorganisms and are the most degradable, whereas the same types of compounds in the C_5-C_9 range have high solvent-type membrane toxicity and are biodegradable only in very low concentrations (3, 4, 10). Also, branched alkanes and cycloalkanes are less biodegradable since branching creates tertiary and quarternary carbon atoms that hinder ß-oxidation (4).

In order to enhance biodegradation at a contaminated site it is useful to determine which organisms are present at the site. For complex petroleum pollutants such as diesel, it is helpful to determine which components of the pollutant are degraded by which organisms and the rates at which they are degraded. This has useful predictive value and can be used to determine which components of the diesel are likely to be degraded by the resident microflora at the contaminated site. The bacteria capable of degrading components of diesel fuel can be isolated from contaminated soil and screened to determine their ability to grow using diesel fuel as the sole carbon source. This study assessed the ability of pure cultures and mixed bacterial populations isolated from sites contaminated with hydrocarbons to degrade diesel fuel in shake cultures. The growth dynamics of the pure cultures while growing on diesel fuel as the sole carbon source were also determined. The information obtained in this project will be used in conjunction with new methods that are currently being examined to increase oxygen concentrations at the pollutant-soil interface.

CHAPTER II

MATERIALS AND METHODS

Methods

Isolation of Bacteria from Soil Sample

A soil sample was obtained from the site of the old physical plant at Texas Tech University where underground storage tanks had leaked bunker oil into the soil. The sample was collected at the time the tanks were removed, from a site approximately 6 feet below ground surface. Bunker oil consists of fractions of petroleum that are similiar to the heavier components found in diesel fuel, and hence bacteria capable of degrading those components should have been present in the soil. One gram of the soil was serially diluted using 9.0 ml sterile saline blanks, then 0.1 ml of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated out on tryptic soy agar plates and incubated at 25°C for 48 hours. Isolated colonies were subcultured onto tryptic soy agar plates to check for purity. Three colony types were obtained. These were maintained on tryptic soy agar plates for further study and identification.

Enrichment of the Soil Sample

The soil sample was further treated in order to isolate bacteria which had remained undetected using tryptic soy agar. Fifteen grams of soil were placed into a 1.0 liter flask containing 600 ml of a sterile basal salts solution consisting of the following: Solution A was made up of $(NH_4)_2SO_4$ (1.2 gm), $CaCl_2.2H_2O$ (0.1 gm), $MgSO_4.7H_2O$ (0.1 gm), ferric citrate (0.0002 gm) and 1000 ml distilled water. Solution B consisted of K_2HPO_4 (0.2 gm), KH_2PO_4 (0.1 gm) and 200 ml distilled water. Solutions A and B were sterilized separately and mixed just prior to use. The soil was stirred for 2 hours each day

for two months using a Corning PC-353 magnetic stirrer to select for bacteria that would be able to grow using the bunker oil present in the soil (8). At the end of the period, 25 ml samples of the supernatant fluid were filtered through 0.45 micron Metricel membrane filters. The filters were placed on absorbent pads soaked with 2.0 ml of sterile tryptic soy broth contained in 60x15 mm petri dishes and incubated at 25°C for 48 hours. Isolated colonies were subcultured onto tryptic soy agar plates to check for purity. Pure colonies were plated onto tryptic soy agar for maintenance. Three additional colony types were obtained in this manner.

Isolation of Bacteria from Water Sample

A water sample from 8 feet below ground surface was obtained from a site of gasoline contamination in Fort Worth, Texas (Texaco, Well No. MW-11). Fifteen 25 ml samples of the water were filtered through 0.45 micron Metricel membrane filters. The filters were placed on absorbent pads soaked with 2.0 ml of sterile tryptic soy broth contained in 60x15 mm petri dishes and incubated at 25°C for 48 hours. Isolated colonies were subcultured onto tryptic soy agar plates to check for purity. Twelve colony types were obtained and were plated onto tryptic soy agar plates for maintenance and identification.

Determination of the Isolates Ability to Grow Using Diesel as the Carbon Source

Diesel fuel obtained from Texaco was filter sterilized using a 0.2 micron filter and used as the sole carbon source to determine the ability of the eighteen isolates to grow using diesel . Mid-log phase cultures were used as the inoculum for these experiments. The mid-log phase of each isolate growing in 1.5% yeast extract was determined by

inoculating each isolate into a cuvette containing 5.0 ml of sterile 1.5% yeast extract and incubating at 25°C. The optical density of each isolate was measured at 600 nm every 30 minutes for 18 hours using a Spectronic 20. Optical density was plotted against time in hours to obtain growth curves for each isolate (4). Mid log phase occurred approximately eleven hours after inoculation.

Inoculum for the sole carbon source studies was obtained by growing each isolate to mid-log phase in 10.0 ml of sterile 1.5% yeast extract. The culture was centrifuged at 3000 rpm for 30 minutes. The supernatant fluid was discarded and the pellet resuspended in 10.0 ml of sterile 0.85% saline. The cells were once again centrifuged at 3000 rpm for 30 minutes, and the pellet was resuspended in 10.0 ml of sterile 0.85% saline. A loopful of each culture was inoculated into separate 125 ml flasks containing 60 ml of basal salts solution with 0.5 ml filter sterilized diesel as the carbon source. Each culture was also inoculated into separate 125 ml flasks containing 60 ml of basal salts solution without any diesel. These flasks were used as controls. The flasks were incubated at 25°C on an environmental shaker for 2 weeks at 250 rpm. A visible increase of turbidity in a flask was used as an indication of an isolate's ability to grow using diesel fuel as the carbon source. Three of the six isolates obtained from the soil sample and five of the twelve isolates from the water sample demonstrated growth in the flasks containing diesel fuel as evidenced by the visible increase in turbidity when compared to the controls.

Identification of the Isolates Capable of Degrading Diesel Fuel

The eight isolates were identified on the basis of their morphology, gram staining reactions, biochemical tests and fatty acid profiles (13).

Determination of the Components of Diesel Degraded by the Isolates During Growth

Filter-sterilized diesel fuel was analyzed by capillary gas chromatography to determine which components were present prior to growth of the bacteria. Non-sterilized diesel fuel was also analyzed to determine whether any components of the diesel were lost during sterilization.

All gas chromatograph analyses were carried out using a 30m x 0.25 mm fused silica column with He as the carrier gas at a flow rate of 1.0 ml/min in a Hewlett-Packard 5790A series gas chromatograph equiped with a flame ionization detector. H_2 gas at a flow rate of 52.0 ml/min and air at a velocity of 30.0 ml/min were used in the flame ionization detector. After sample injection, the oven temperature of the gas chromatograph was held at 85°C for 4 minutes and then increased at a rate of 6°C/min to 270°C and held for 5 minutes. The temperatures of the injection and detector ports were 300°C. Sample size used for injection was 0.75 µl.

A Hewlett-Packard 3390 Integrator was used for plotting the chromatograms. The integrator was programmed with the following parameters:

ATT^ = 1.0

CHT SP = 0.4 cm/min

PK WD = 0.4

THRSH = 1.0

AR REJ = 50

INTG 2 TIME 0.5

STOP TIME 40

The gas chromatograph was used for qualitative, not quantative analysis of the diesel. The absence of a peak was used as an isolate's ability to degrade that component of the diesel; partial degradation of components could not be assessed by this method.

To determine which components of the diesel were degraded during bacterial growth, each of the eight isolates was inoculated into separate 125 ml flasks containing 60 ml of basal salts solution with 0.5 ml filter sterilized diesel. There were three replicates of each isolate. Two uninoculated flasks were used as controls to determine which components of the diesel were lost due to evaporation or photooxidation. There also was a possibility that diesel might stick to the walls of the flasks, and some components could be lost due to this phenomenon. However, if any component had an affinity for glass and was not recovered for gas chromatography, it would not have been recovered from either the flasks containing bacteria or from the control flasks. Hence, that component would not be considered to have been degraded due to bacterial action. The flasks were incubated at 25°C on an environmental shaker for three weeks at 250 rpm. Three weeks were used as opposed to the two weeks used for the growth study to allow adequate time for complete degradation of components. After three weeks, the residual diesel was recovered by centrifuging the cultures at 3000 rpm for 40 minutes. The top layer of the supernatant fluid which consisted of the residual fuel was removed using sterile Pasteur pipets and placed into vials. The flasks which contained the cultures Pseudomonas aeruginosa (strain 1) and Pseudomonas aeruginosa (strain 2) had little residual diesel and hence 300 µl of hexane was added to these two flasks to have enough volume for gas chromatography. The residual diesel was analyzed by gas chromatography.

Determination of Generation Times of Isolates

To obtain generation times of the isolates grown in both 1.5% yeast extract and diesel fuel as the carbon source, each isolate was grown to mid-log phase in 10.0 ml of 1.5% yeast extract at 25°C. The cells were collected by centrifuging at 3000 rpm for 30 minutes, washed twice with 0.85% sterile saline and resuspended in 10.0 ml of sterile saline. A loopful of each isolate was then inoculated into one flask containing 60 ml of sterile 1.5% yeast extract and one flask containing 60 ml of basal salts solution with 0.5% filter sterilized diesel fuel as the carbon source. The flasks were incubated at 25°C on an environmental incubator and shaken at 250 rpm. Growth curves of the pure cultures growing in a basal salts medium with 0.5 ml diesel as the carbon source and in 1.5% yeast extract were determined by removing 1.0 ml samples of the culture every 2 hours from the 1.5% yeast extract, and every 4 hours from the basal salts salts solution with diesel. These were serially diluted using 9.0 ml sterile saline blanks. One ml aliquots of the appropriate dilutions were plated in tryptic soy agar using the pour plate technique. The plates were poured thin to allow oxygen diffusion so that the strict aerobes would grow. For a few samples, the counts obtained were compared to those obtained by the spread plate method to ensure that the pour plate method did not result in lowered counts for the strict aerobes. The t-test was used for compare the means. The means were not significantly different at alpha = 0.05. There were five replicates of each isolate in each medium. The plates were incubated at 25°C for 48 hours and then counted. The log of viable cells was plotted against time and the generation time of each isolate growing while using diesel fuel and in 1.5% yeast extract was obtained (4). The generation time data was analyzed for homogeneity of variances using Bartlett's test (20). Since the variances were heterogenous, the data were log-transformed and analyzed by a two-way ANOVA (20), using the program Statworks to determine whether there were significant

differences between the cultures grown in the two media. Scheffe's interval test (18) was used to determine the individual significant differences in mean generation times. Soil and water isolates were treated separately. This experiment was performed once, with 5 replicates as indicated above.

Effect of Mixed Populations on Diesel Fuel

To study the effect of mixed populations on diesel fuel, two sets of mixed populations were prepared. Set A consisted of equal proportions of the three isolates obtained from the soil sample, and set B was made up of equal proportions of the five isolates obtained from the water sample. Each isolate was grown to a concentration of approximately $1x10^7$cells/ml in 50 ml of 1.5% yeast extract, and the cells were collected by centrifugation at 3000 rpm for 30 minutes. The supernatant fluid was discarded, the cells washed with sterile 0.85% saline, and centrifuged again. The cells were resuspended in 50 ml of sterile 0.85% saline and 2.0 ml aliquots of the three isolates from the soil sample were mixed to form set A, and 2.0 ml aliquots of the five isolates from the water sample were mixed to form set B. Fifty µl samples were used for inoculation of the flasks to ensure that the initial inoculum in each flask would be approximately $5x10^5$ cells/ml. At this concentration there should have been enough cells of each isolate so that no single isolate could have had an immediate competitive advantage over the other isolates. Fourteen 125 ml flasks containing 60 ml of sterile basal salts medium with 0.5 ml diesel fuel were inoculated with either 50 µl of bacterial set A or B. One ml aliquots of the medium were removed from each flask at the initiation of the study, diluted to 10^{-3}, and the dilutions were plated out to determine the initial concentration of cells in each set. Three uninoculated flasks were used as controls. The flasks were incubated at 25°C in an environmental shaker at 250 rpm for three weeks. At the end of the period 1.0 ml aliquots

were removed from each flask, serially diluted, and the 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated out using the pour plate technique to determine the final concentration of cells in the two mixed populations. No attempt was made to determine the concentration of the individual isolates. The cells were then collected by centrifugation at 3000 rpm for 30 minutes to separate the cells from the residual diesel and the top layer of the supernatant fluid which contained residual diesel fuel and medium was removed using sterile Pasteur pipets and placed in vials. Three hundred µl of n-hexane were added to each vial to obtain enough volume for gas chromatographic analysis. The n-hexane and diesel mixtures were then transferred to new vials to separate them from the medium, and subjected to gas chromatographic analysis to determine which components had been degraded. Since hexane is an excellent solvent and diesel readily dissolves in it, there was little chance that any components would be lost due to diesel sticking to the sides of the vials. The experiment was repeated using three replicates, and the results of the two experiments were pooled for analysis.

Since not all components were completely degraded in all replicates, a 95% confidence interval for proportions (20) was used to determine the number of replicates in which a component would have to be completely degraded for the results to be statistically the same as the component being completely degraded in all replicates.

Figure 1 is a summary of the experiments performed and the number of replicates in each experiment.

Experiment	Number of samples and replicates
Isolation of bacteria from soil sample	1 soil sample.
Enrichment of soil sample	Fifteen, 25 ml samples.
Isolation of bacteria from water sample	Fifteen, 25 ml samples.
Growth of isolates using diesel as carbon source	1 flask per isolate + 1 control per isolate.
Determination of components of diesel degraded by pure cultures	3 replicates per isolate + 2 control flasks.
Determination of generation times	5 replicates of each isolate in each medium.
Effect of mixed populations on diesel control	10 replicates of each mixed culture set + 3 flasks.

Figure 1. Matrix showing the experiments performed and the number of replicates in each experiment.

CHAPTER III

RESULTS

Taxa

Results of biochemical tests confirmed the three soil isolates as two strains of Pseudomonas aeruginosa and a strain of Pseudomonas vesicularis (Table 1). The five isolates from the water sample were identified as Achromobacter xyloxidans, Achromobacter species, Aeromonas species, Pseudomonas species, and Pseudomonas vesicularis (Table 2) (13). The Pseudomonas species was narrowed down to three choices, Pseudomonas cepacia, Pseudomonas diminuta and Pseudomonas putida.

Effects of Filter-Sterilizing Diesel

Comparing non-sterilized diesel and filter-sterilized diesel (Figure 2) reveals a few differences between their chromatograms. While two of the lighter fractions were lost during filter sterilization (Figure 3), the rest of the fractions were present in both non-sterile and filter-sterilized diesel. A list of all the fractions detected in both sterile and non-sterile diesel fuel is found in Figure 3. The list was generated by reading the column retention time of each peak on the chromatogram of the sterile diesel and comparing it with the corresponding peak on the chromatogram of the non-sterile diesel. Each retention time corresponds to the time needed for a component of the diesel to elute out of the column. The lighter the component the earlier it elutes and the heavier components have the longer column retention times.

Effect of Pure Cultures on Diesel

Chromatograms of the pooled results from the five control flasks (Figure 4) indicated that ten components of the diesel with column retention times of 2.51, 2.65, 2.85, 2.98,

3.10, 3.45, 3.77, 4.15, 4.32, 4.92 and 5.54 were lost from each of the five replicates (Figure 5). These losses were not due to microbial activity and may have been caused by evaporation or photooxidation. Components with retention times of 6.02 and 20.04 also were lost in four of the five replicates, and one component with a retention time of 9.61 was lost in two in two of the five replicates. Chromatograms of the diesel fuel acted upon by individual pure cultures of bacteria revealed that each bacterium acted only on specific components of the diesel. Table 3 lists by column retention times the components that were degraded by each of the soil sample isolates. The term "degraded" indicates that the component is absent in the chromatogram. As mentioned in the methods section this was a qualitative study based on the absence of a component and partially degraded peaks were not used as an indication of degradation. Pseudomonas aeruginosa (strain 1) degraded nine components while Pseudomonas aeruginosa (strain 2) and Pseudomonas vesicularis degraded seven and eight components respectively (Figure 6).

Table 4 lists the components of diesel which were degraded by each of the water sample isolates. Pseudomonas sp. and Pseudomonas vesicularis completely degraded eight components each, Achromobacter xyloxidans degraded seven components and the Achromobacter and Aeromonas species degraded six components each (Figure 7). Although this was a qualitative study to determine which components were degraded pure and mixed cultures, the approximate amount of diesel that was degraded or lost due to evaporation was estimated by measuring the residual diesel using graduated vials. The control flasks showed a reduction of approximately 30% in the diesel fuel after three weeks.The flasks containing the two strains of Pseudomonas vesicularis, Achromobacter xyloxidans and Achromobacter sp. had a reduction of approximately 40% of the diesel. Pseudomonas aeruginosa (strain 1) had only 30% of the diesel remaining, and Pseudomonas aeruginosa (strain 2) had about 40% of residual diesel.

When the mean generation times of the soil sample isolates (Table 5) grown in 1.5% yeast extract were compared to those in the basal salts medium with 0.5 ml of diesel using a two-way ANOVA, significance was found between isolates ($p < 0.001$)[1], between the two media ($p < 0.001$), and also in the interaction between isolates and media ($p < 0.001$). The results of Scheffe's interval test (Table 5) which was used to determine the significant differences in the mean generation times for an experimentwise 0.05 level of significance showed that the mean generation time of Pseudomonas vesicularis in basal salts medium with 0.5 ml diesel was not significantly different from the mean generation times of the two strains of Pseudomonas aeruginosa in 1.5% yeast extract, indicating that P. vesicularis had a growth rate while using diesel that was statistically equal to the growth of the two strains of P. aeruginosa in 1.5% yeast extract. The mean generation times of the two strains of P. aeruginosa in 1.5% yeast extract were not significantly different from each other, but they were significantly different in basal salts medium with diesel as the carbon source, and thus provided a basis for differentiating the two isolates as different strains of P. aeruginosa.

When the mean generation times of the water sample isolates (Table 6) grown in 1.5% yeast extract were compared to those in the basal salts medium with 0.5 ml of diesel using a two-way ANOVA, significance was found between isolates ($p < 0.001$), between the two media ($p < 0.001$), and also in the interaction between isolates and media ($p < 0.001$). The results of Scheffe's interval test (Table 6), which was used to determine the significant differences in the mean generation times for an experimentwise 0.05 level of

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[1]Alpha is the level of significance used for a test. An alpha of 0.05 was used for all tests. "p" is the maximum alpha for which the null hypothesis is rejected. For example, the p value of 0.110 in Tables 8 and 9 indicates that there is an 11% chance of the event being a random ocurence.

significance, revealed no significant differences in the mean generation times of the Aeromonas sp. and those of Achromobacter xyloxidans and Pseudomonas vesicularis in 1.5% yeast extract. The mean generation times of Achromobacter sp. did not differ significantly from those of the Pseudomonas sp. in either media.

Growth Curves

Growth curves of the soil sample isolates in 1.5% yeast extract and in basal salts medium with 0.5 ml sterile diesel (Figure 8) revealed that growth while using diesel was slower than while growing in 1.5% yeast extract. Each isolate exhibited more gradual log phases and prolonged stationary phases when diesel was the carbon source.

Growth curves of the soil sample isolates in 1.5% yeast extract and in basal salts medium with 0.5 ml sterile diesel (Figure 9) also revealed that growth while using diesel was slower than while growing in 1.5% yeast extract. Each isolate demonstrated longer lag and log phases in the presence of diesel. Aeromonas species and Pseudomonas species showed diauxic type growth.

Effect of Mixed Cultures on Diesel

When mixtures of bacteria were tested for their ability to degrade filter-sterilized diesel, the following results were obtained. Gas chromatograms of diesel fuel acted upon by the mixed culture set A (Figure 10) revealed that not every component was degraded in each sample. Table 7 lists the components based on the number of samples in which they present or absent in the ten samples. Components with retention times of 9.61 and 27.25 were degraded in all samples. Components with retention times of 25.94, 26.11, 27.38 and 34.48 were not degraded in all replicate samples but the number of replicates

showing utilization was not statistically different from one[2] , indicating that the disappearance of these components was not due to chance. Six components were present in all replicates. The disappearance of eight components among the replicates was not significantly different from zero, indicating that these fractions were not preferentially utilized and that their disappearance from the sample was random. The number of samples in which the other fourteen components were degraded was significantly different from degradation in all samples. The mean initial inoculum for all flasks was $269x10^3 \pm 24x10^3$ cells/ml and the final cell concentration was $47x10^5 \pm 22x10^5$.

Gas chromatograms of diesel acted upon by mixed culture set B (Figure 11) also revealed that not every component was degraded in each sample. The list of components (Table 8) based on their presence or absence in a sample revealed that components with retention times of 9.61 and 27.25 were degraded in all samples. Only one component with a retention time of 27.38 occurred with a frequency that was statistically equal to zero, i.e., degradation in all samples. Thirteen components were found to be present in all samples. Five fractions were degraded in several replicates but their frequency of occurrence was not significantly different from zero, indicating that their absence might be due to chance and not selected utilization. The number of samples in which the other fourteen components were absent was significantly different from absence in all samples. The mean initial inoculum for all flasks was $279x10^3 \pm 17.8x10^3$ and the final cell concentration was found to be $116.8x10^6 \pm 41x10^6$.

With both sets of mixed cultures the chromatograms had flatter baselines, indicating that the amount of diesel in the sample was lower than in the residual diesel recovered from the pure cultures. The peaks of the components also were smaller, indicating that the

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2 "One" refers to absence of a component from all samples. "Zero" refers to presence of component in all samples.

amount of that component in the residual diesel was lower than in the original sample. The flatter baselines could also have been due to the dilution of the diesel by adding 300 µl of hexane, i.e., the sample which was injected into the gas chromatograph contained more solvent and very little diesel. However, it should be remembered that hexane was added to the residual diesel because there was very little of the diesel remaining for analysis.

The action of mixed cultures on the diesel fuel resulted in considerably more degradation of the diesel than with any of the pure cultures. At the end of the three-week incubation period with mixed culture set A, only trace amounts of diesel were visible in the flask. After the residual diesel had been extracted with 300 µl of hexane, it was observed that the total volume of the n-hexane and diesel mixtue was just slightly greater than 300 µl, while the control flasks had approximately 300 µl to 400 µl of residual diesel. In the flasks containing mixed culture set A, approximately 90% to 95% of the diesel was lost after three weeks and thus, mixed culture set A was found to be very efficient at degrading diesel fuel. After treating diesel with mixed culture set B for three weeks the results were more varied. In three of the flasks, degradation of diesel similar to that with set A occured, while with the other seven flasks considerably less amounts of the diesel were degraded and the volume of the n-hexane (300 µl) and diesel mixtures ranged between 350 µl to 450 µl. In the flasks containing mixed culture set B aproximately 85% to 90% of the diesel was utilized.

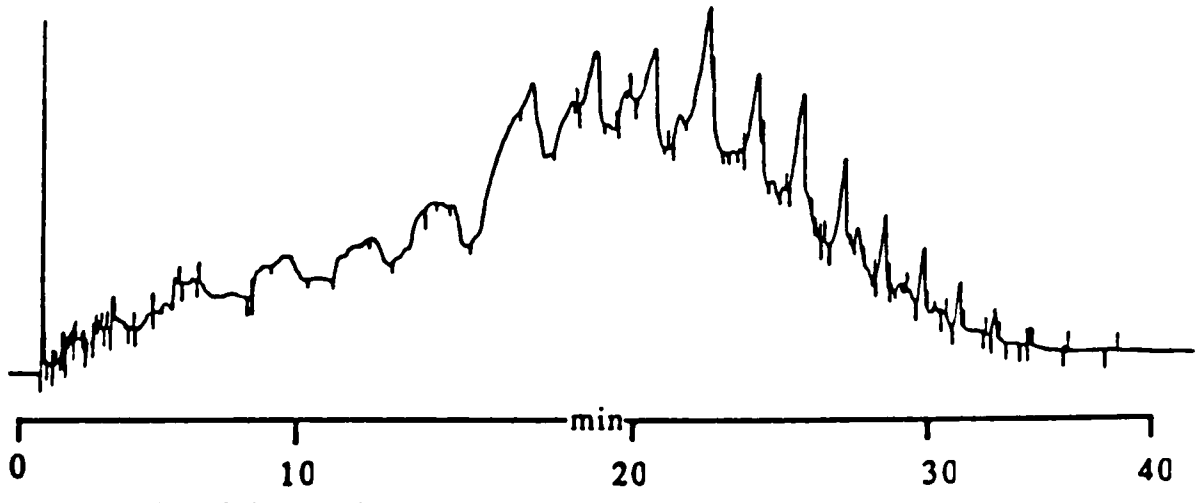
Table 1. Identification of bacterial isolates obtained from oil contaminated soil capable of utilizing diesel fuel for growth.

Test	Isolate # 3	Isolate LS*	Isolate LS"
Morphology	rods	rods	rods
Gram staining reaction	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Growth on MacConkey agar	+	+	+
Indole	-	-	-
Nitrate reduction	-	+	+
Nitrite reduction	-	+	+
Growth at 42°C	-	+	+
Pigment production	-	Blue-green	Blue-green
Motility	+	+	+
Urea	-	+	+
OF Glucose	+	+	+
OF Xylose	+	+	+
OF Mannitol	-	+	+
OF Lactose	-	-	-
OF Sucrose	-	-	-
OF Maltose	+	-	-
Identification	Pseudomonas vesicularis	Pseudomonas aeruginosa	Pseudomonas aeruginosa

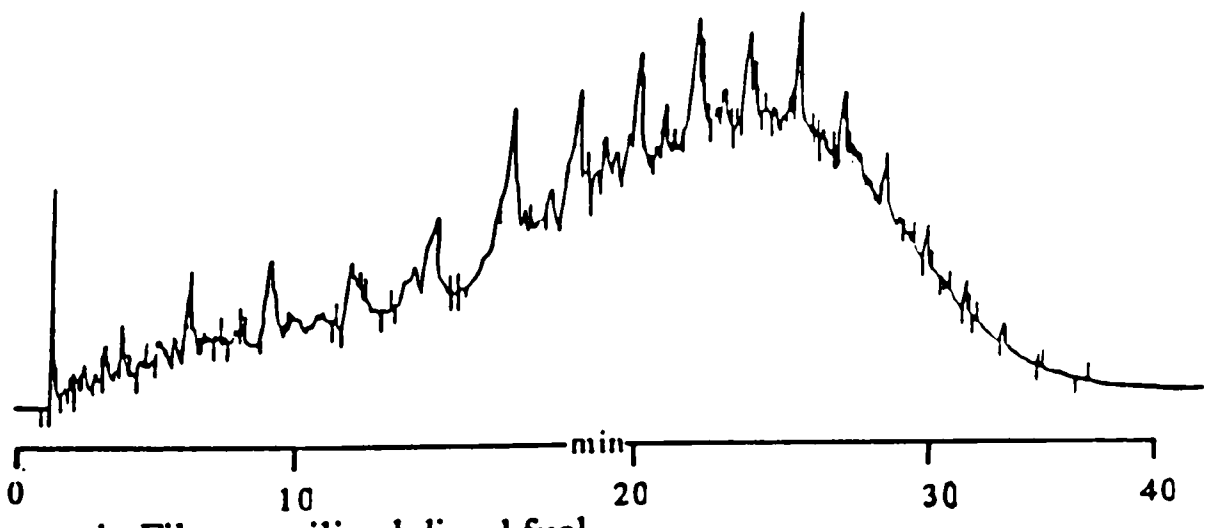
Table 2. Identification of bacterial isolates obtained from gasoline contaminated water capable of utilizing diesel fuel for growth.

Test	Isolate # 5	8	12	14	15
Morphology	rods	rods	rods	rods	rods
Gram staining reaction	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Growth on MacConkey agar	+	+	+	+	+
Indole	-	-	+	-	-
Nitrate reduction	+	+	+	+	-
Nitrite reduction	+	+	-	+	-
Motility	+	+	+	+	+
Urea	-	+	-	-	-
Pigment	-	-	-	-	-
OF Glucose	+	+	+/+	-	+
OF Xylose	+	+	-	NA	+
OF Mannitol	-	-	-	NA	-
OF Lactose	-	-	-	NA	-
OF Sucrose	-	-	-	NA	-
OF Maltose	-	-	+	NA	-
PR Lactose	NA	NA	NA	-	NA
PR Mannitol	NA	NA	NA	+	NA
PR Inositol	NA	NA	NA	-	NA
Identification	Achromobacter xyloxidans	Achromobacter sp.	Aeromonas sp.	Pseudomonas sp.	Pseudomonas vesicularis

+/+ = oxidative/fermentative
NA = Not applicable



a. Diesel fuel prior to filter-sterilization.



b. Filter-sterilized diesel fuel.

Figure 2. Chromatograms of diesel fuel before and after filter-sterilization.

Column retention times of component (min)						
2.06	3.45	6.02	14.44	20.42	23.75	26.48
2.41*	3.77	6.50	17.00	20.83	23.95	27.25
2.51	3.85*	9.14	17.40	21.20	24.82	27.38
2.65	4.15	9.61	18.26	21.73	25.00	27.99
2.85	4.32	11.71	19.20	22.05	25.61	30.77
2.98	4.92	12.00	19.80	23.12	25.94	32.06
3.10	5.54	13.77	20.04	23.25	26.11	33.28
						34.48

Figure 3. Column retention times of components found in both non-sterile diesel and in filter-sterilized diesel[a].

[a] = Column retention times of non-sterile and sterile diesel fuel differed slightly. The column retention times of sterile diesel are presented.

* = These peaks were not present in sterile diesel. The column retention times of non-sterile diesel are given.

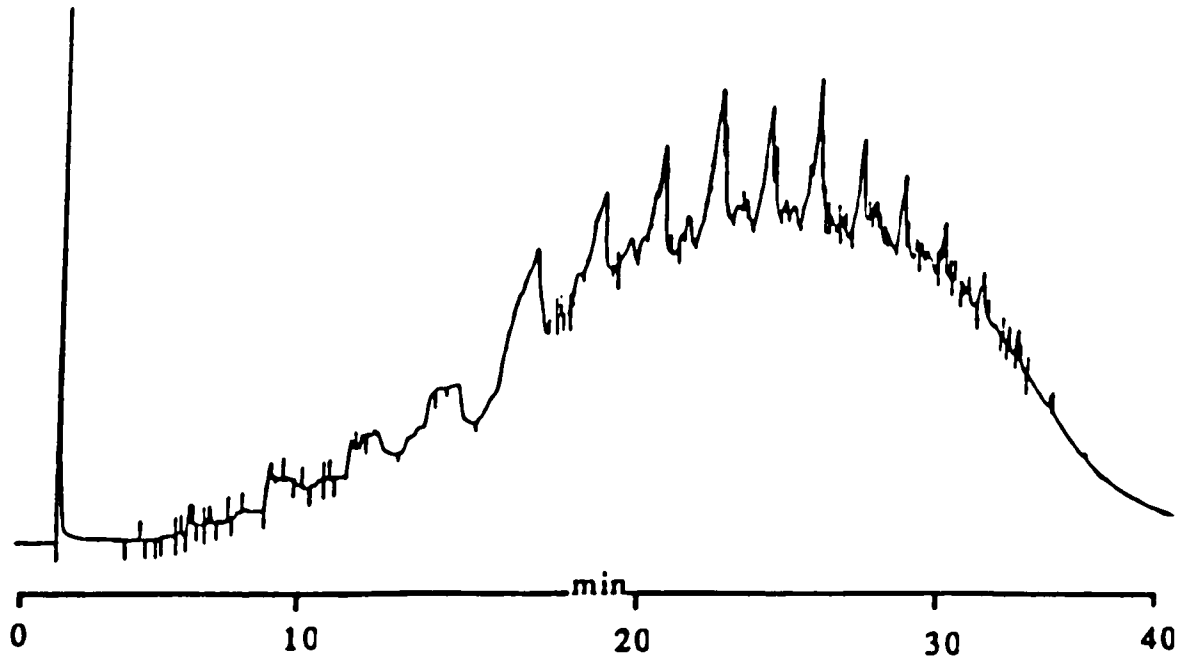


Figure 4. Chromatogram of diesel fuel from a control treatment.

Column retention times of component (min)
2.06
2.51
2.65
2.85
2.98
3.10
3.45
3.77
4.15
4.32
4.92
5.54
6.02 (4/5)
9.61 (2/5)
20.04 (4/5)

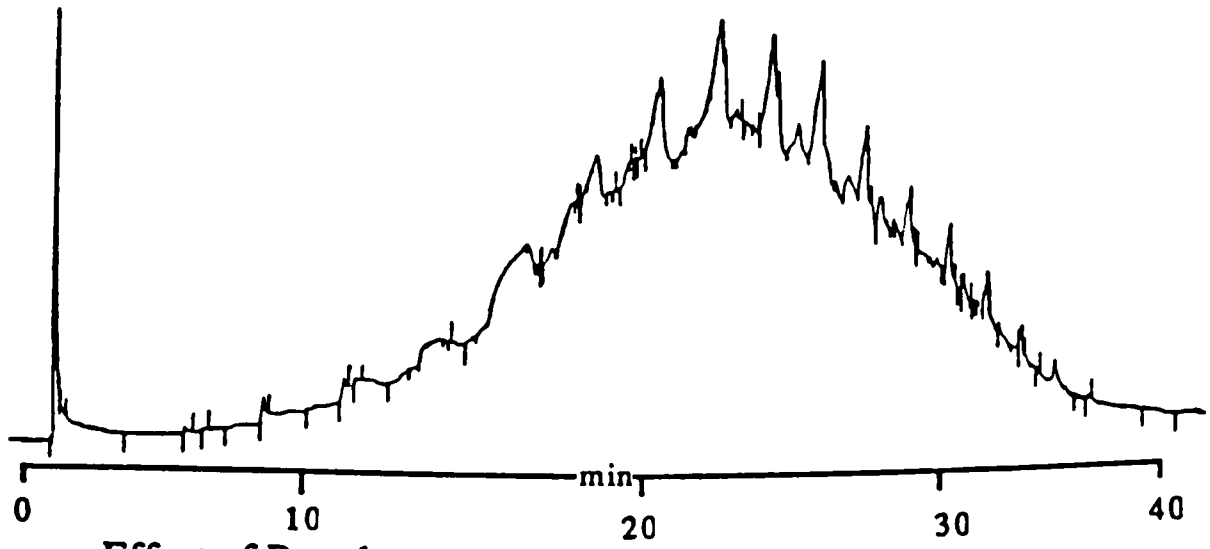
Figure 5. Column retention times of components lost due to evaporation and/or photooxidation. (n=5)

(4/5), and(2/5) represent the number of samples in which the components were lost.

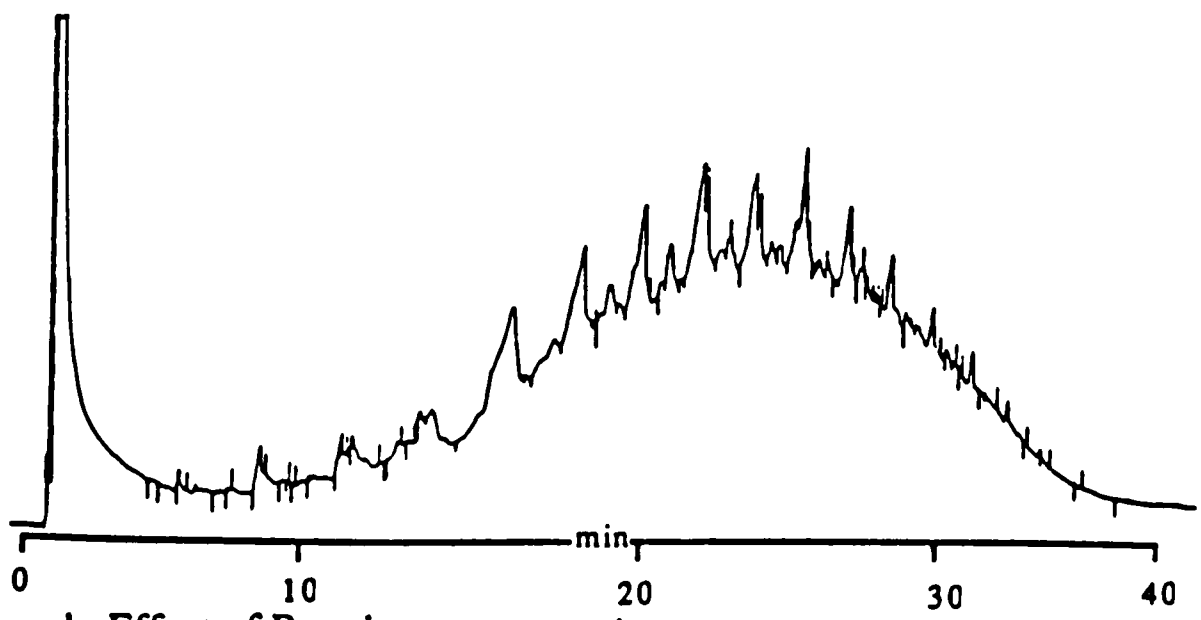
Table 3. Column retention times of components of diesel fuel completely degraded by the three strains of bacteria isolated from the soil sample.

Pseudomonas vesicularis (min)	Pseudomonas aeruginosa (strain 1) (min)	Pseudomonas aeruginosa (strain 2) (min)
9.61	6.50	9.61
17.40	9.61	17.40
20.42(2/3)	13.77(1/3)	19.80
21.73	17.40(2/3)	20.83(2/3)
25.00	19.80	27.25
25.94	20.42	27.38
26.11	23.75	34.48(1/3)
27.25	25.61	
	27.38	

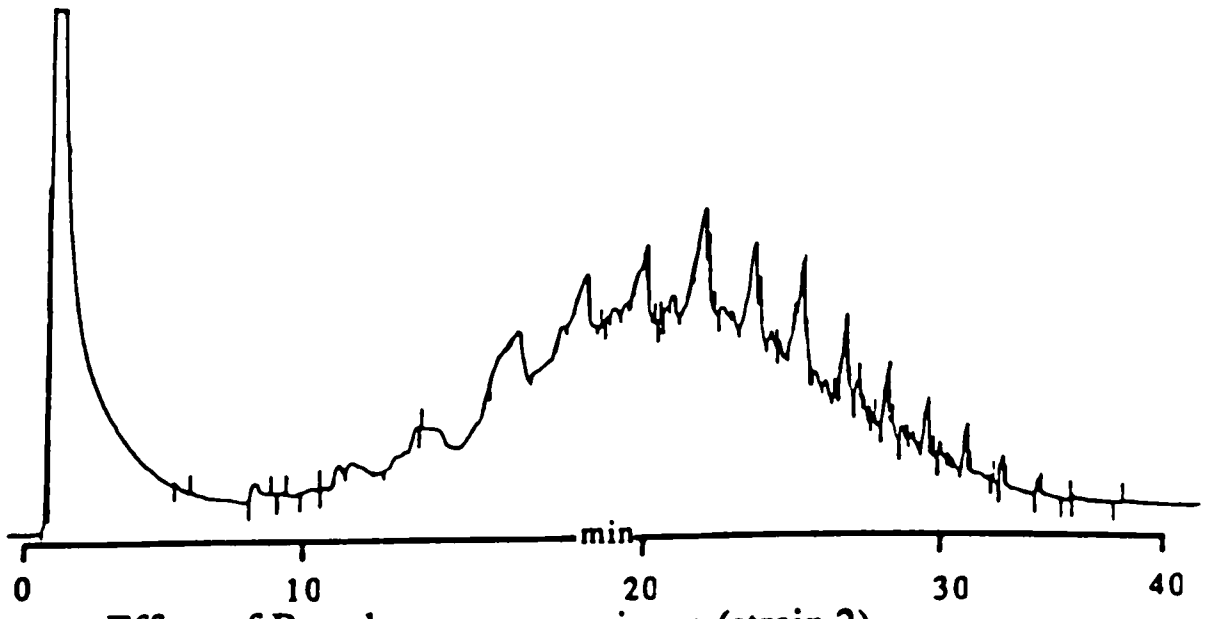
(1/3) = Degraded in one out of three replicates.
(2/3) = Degraded in two out of three replicates.



a. Effect of Pseudomonas vesicularis.



b. Effect of Pseudomonas aeruginosa (strain 1).



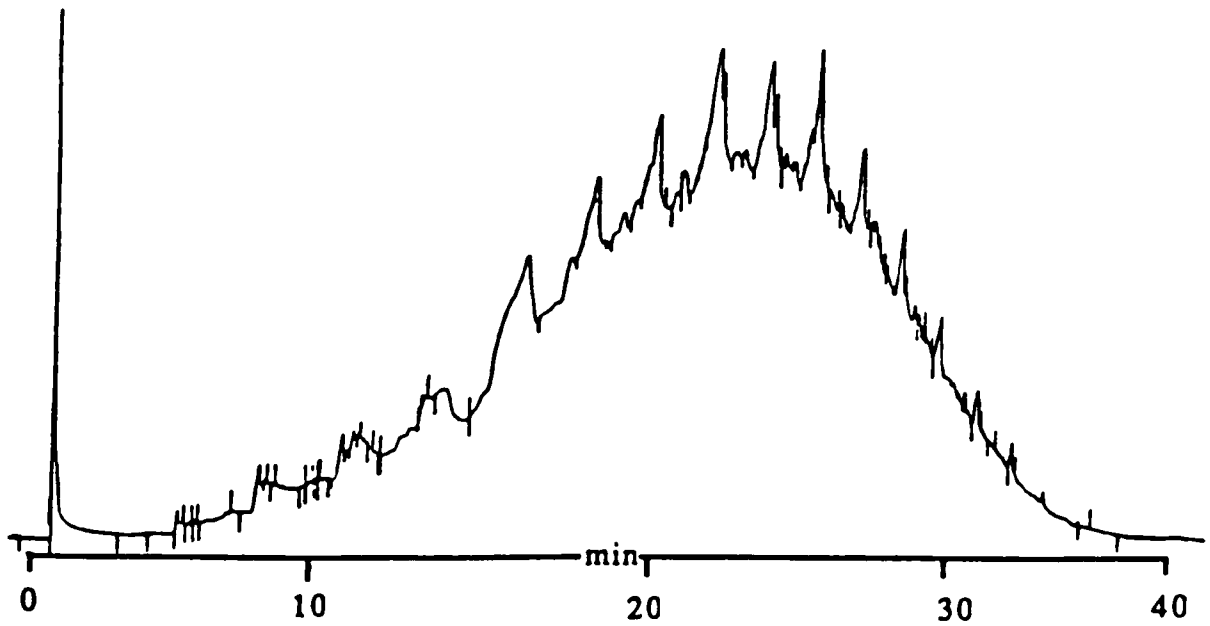
c. Effect of Pseudomonas aeruginosa (strain 2).

Figure 6. Chromatograms of diesel fuel acted upon by the three soil isolates.

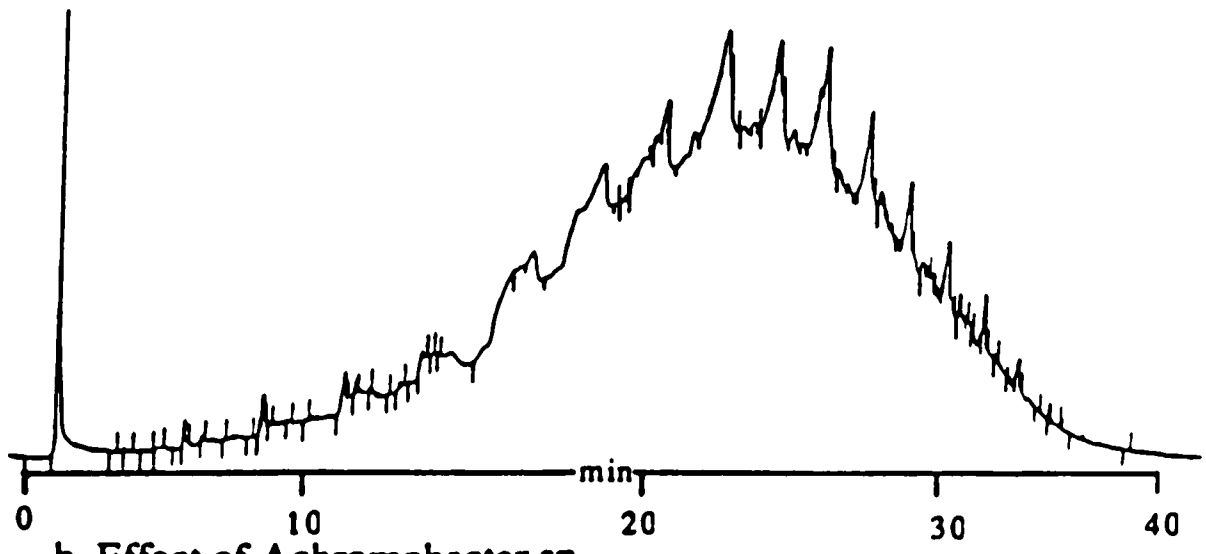
Table 4. Column retention times of components of diesel fuel completely degraded by the five strains of bacteria isolated from gasoline contaminated water.

Achromobacter xyloxidans (min)	Achromobacter species (min)	Aeromonas species (min)	Pseudomonas species (min)	Pseudomonas vesicularis (min)
9.61	9.61	9.61	9.61	9.61
13.77(1/3)	18.26(1/3)	17.40	17.40	17.40
17.40(2/3)	22.05	22.05	18.26	19.80
18.26	23.25(2/3)	23.75	19.80	20.04
20.42(2/3)	23.75	23.95(2/3)	20.42	23.75
20.83	23.95	27.38	23.75	25.61
22.05			23.95	25.94(2/3)
			25.61(1/3)	27.38

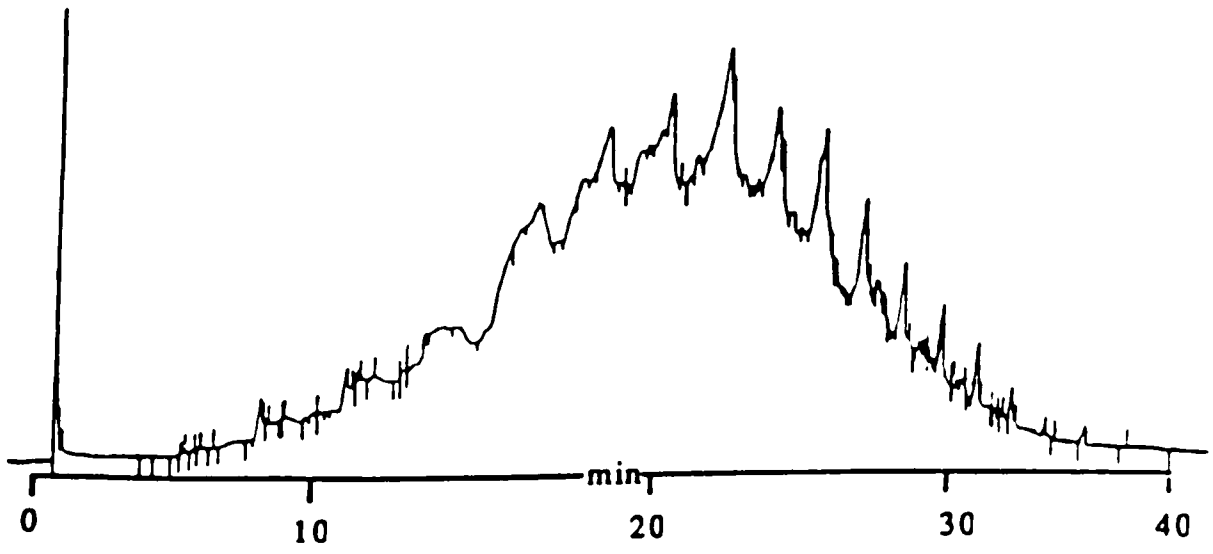
(1/3) = Degraded in one out of three replicates.
(2/3) = Degraded in two out of three replicates.



a. Effect of Achromobacter xyloxidans

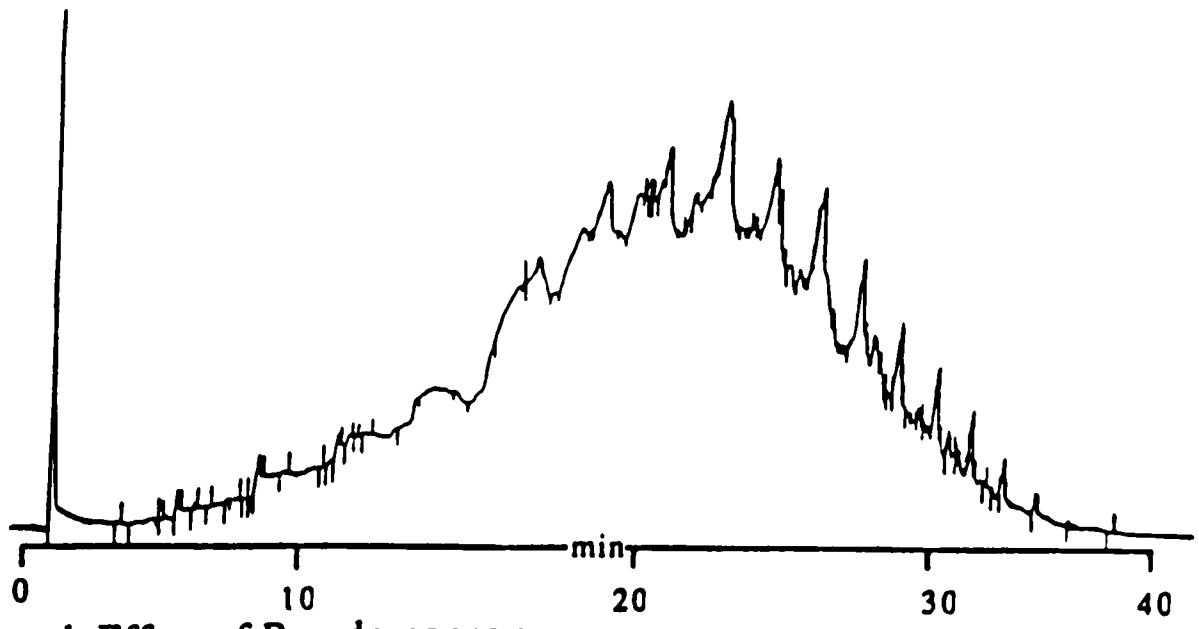


b. Effect of Achromobacter sp.

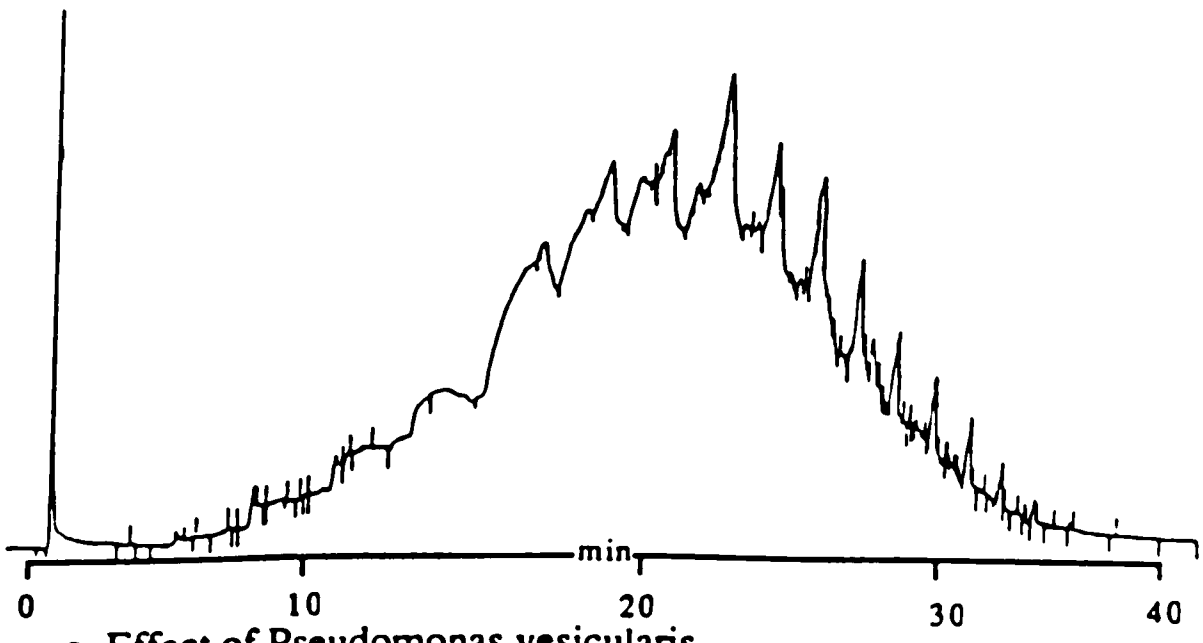


c. Effect of Aeromonas sp.

Figure 7. Chromatograms of diesel fuel acted upon by the five water isolates.



d. Effect of Pseudomonas sp.



e. Effect of Pseudomonas vesicularis

Figure 7. Continued.

Table 5. Mean generation times in hours for soil sample isolates[a] growing on yeast extract and basal salts medium with 0.5 ml diesel.

	Medium	
	1.5% Yeast Extract (Mean ± S.D.)	Basal salts medium + 0.5 ml diesel (Mean ± S.D.)
Pseudomonas vesicularis	0.6[b] ± 0.07	1.4[c] ± 0.2
Pseudomonas aeruginosa (strain 1)	1.7[c] ± 0.1	2.9[d] ± 0.3
Pseudomonas aeruginosa (strain 2)	1.6[c] ± 0.2	5.3[e] ± 0.6

[a] = Raw data are presented. The data were coded by multiplying by 10 and then log transformed to meet the assumptions of the anova.

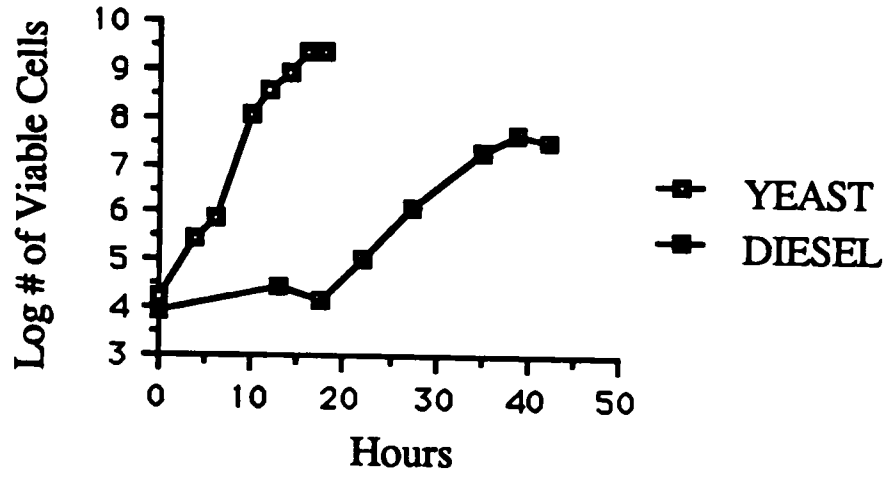
b, c, d, e = Means superscripted differently differ at p = 0.05 as determined by Scheffe's Intervals.

Table 6. Mean generation times in hours for bacterial isolates[a] obtained from gasoline contaminated water, growing on yeast extract and basal salts medium with 0.5 ml diesel.

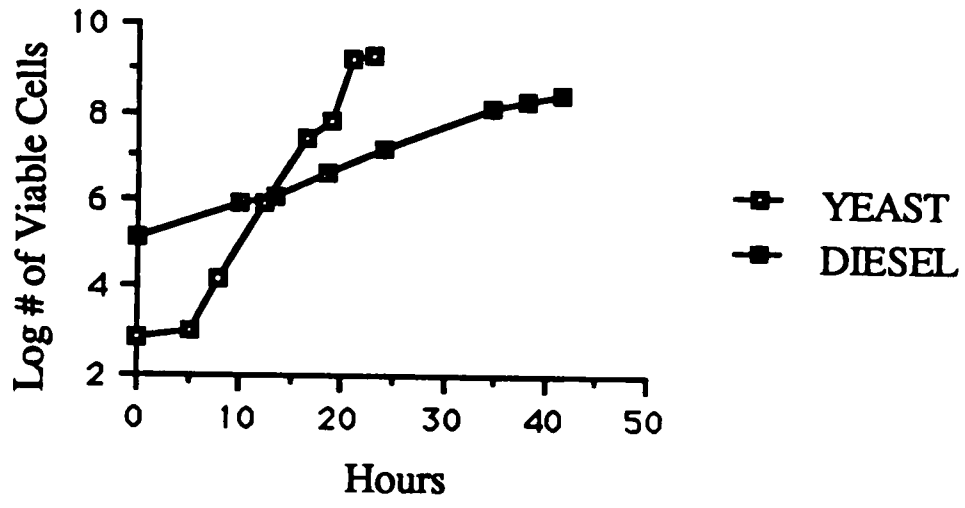
	Medium	
	1.5% Yeast Extract (Mean ± S.D.)	Basal salts medium + 0.5 ml diesel (Mean ± S.D.)
Achromobacter xyloxidans	$0.6^{bc} \pm 0.03$	$1.4^{e} \pm 0.2$
Achromobacter sp.	$0.6^{bc} \pm 0.03$	$2.2^{g} \pm 0.5$
Aeromonas sp.	$0.7^{cd} \pm 0.06$	$1.4^{e} \pm 0.2$
Pseudomonas sp.	$0.5^{b} \pm 0.06$	$1.8^{fg} \pm 0.1$
Pseudomonas vesicularis	$0.9^{d} \pm 0.03$	$1.5^{ef} \pm 0.2$

a = Raw data are presented. The data were coded by multiplying by 10 and then log transformed to meet the assumptions of the anova.

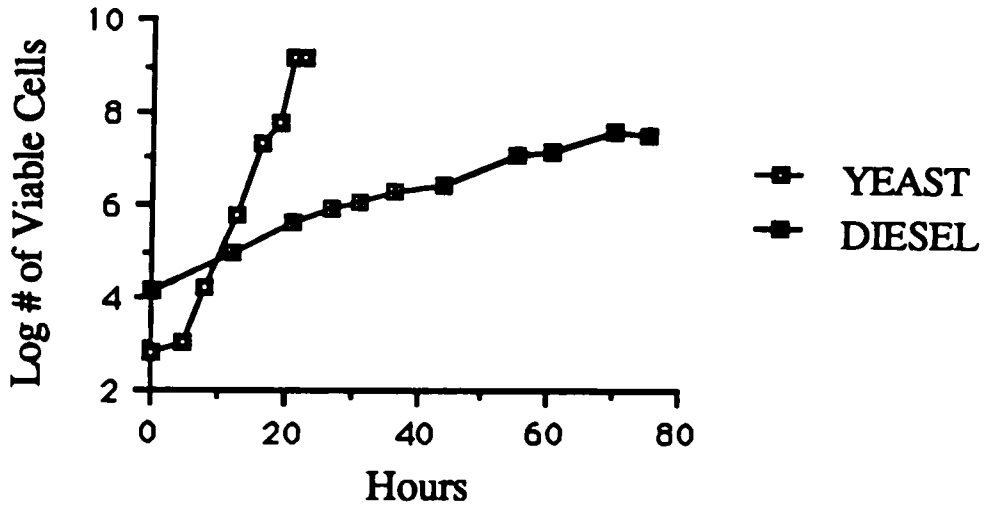
b, c, d, e, f, g = Means superscripted differently differ at p = 0.05, as determined by Scheffe's Intervals.



a. Growth of Pseudomonas vesicularis.

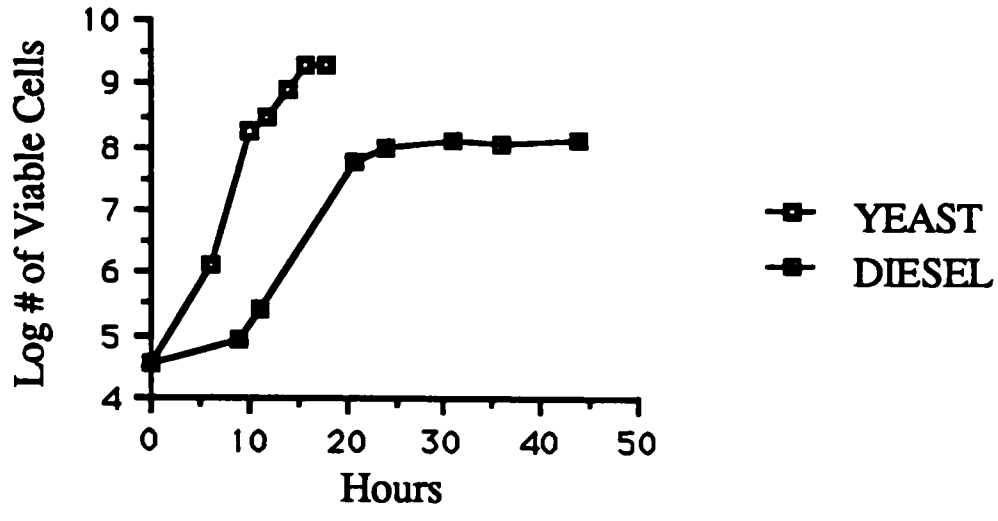


b. Growth of Pseudomonas aeruginosa (strain 1)

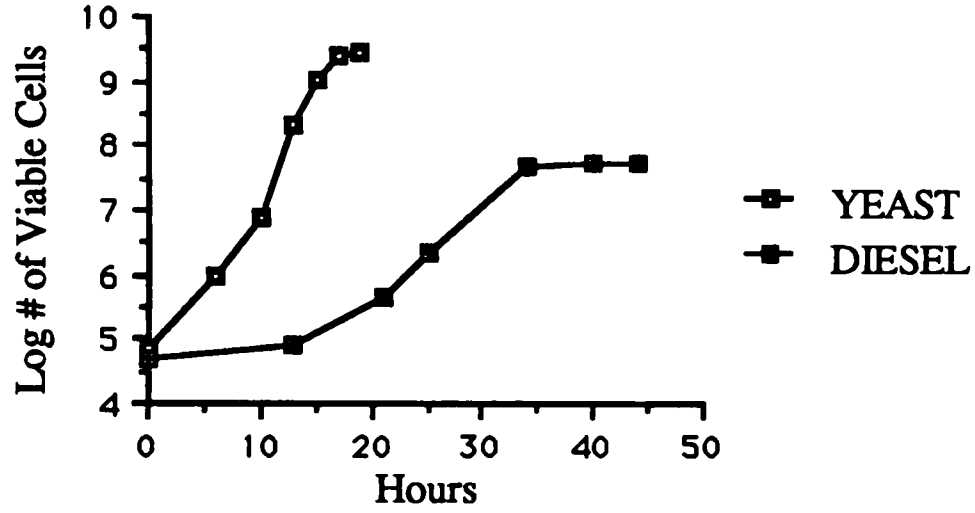


c. Growth of Pseudomonas aeruginosa (strain 2)

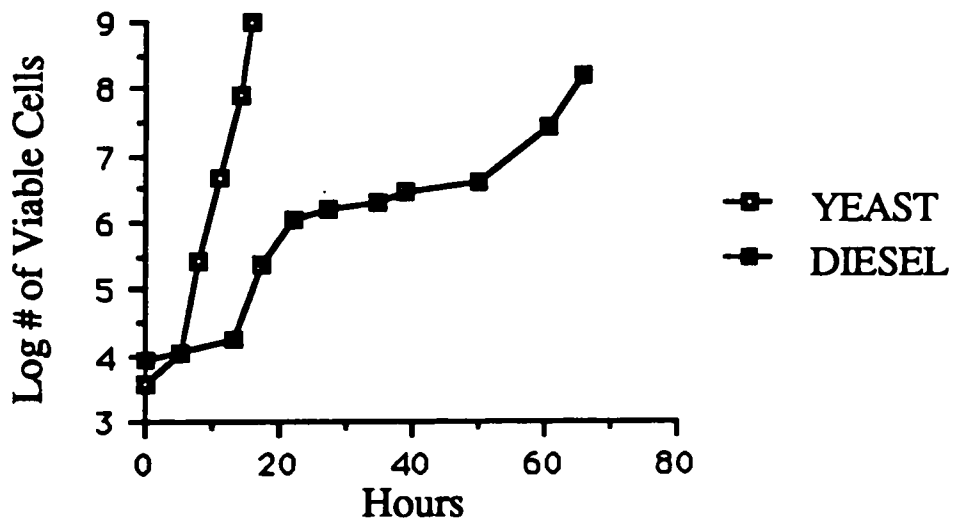
Figure 8. Growth of the three soil isolates in 1.5% yeast extract and in basal salts medium with 0.5 ml diesel.



a. Growth of Achromobacter xyloxidans.

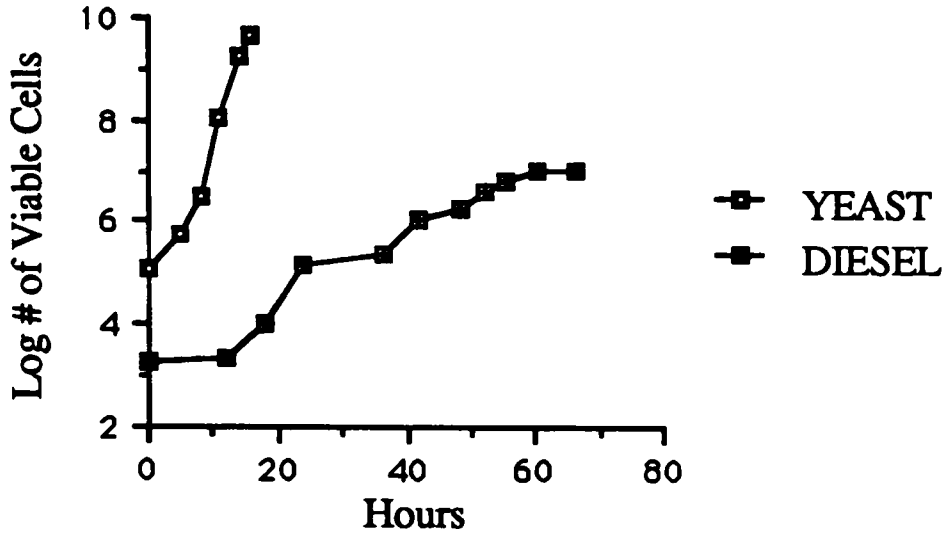


b. Growth of Achromobacter sp.

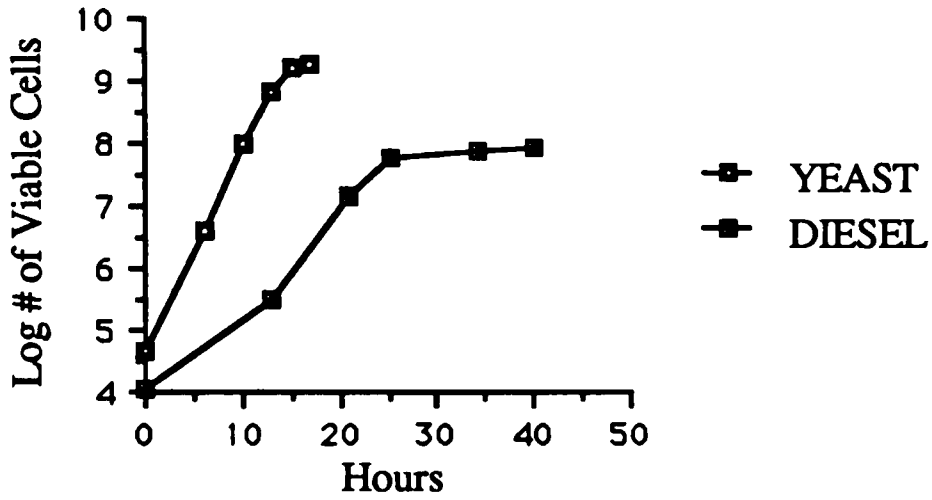


c. Growth of Aeromonas sp.

Figure 9. Growth of the five water isolates in 1.5% yeast extract and in basal salts medium with 0.5 ml diesel.



d. Growth of Pseudomonas sp.



e. Growth of Pseudomonas vesicularis.

Figure 9. Continued.

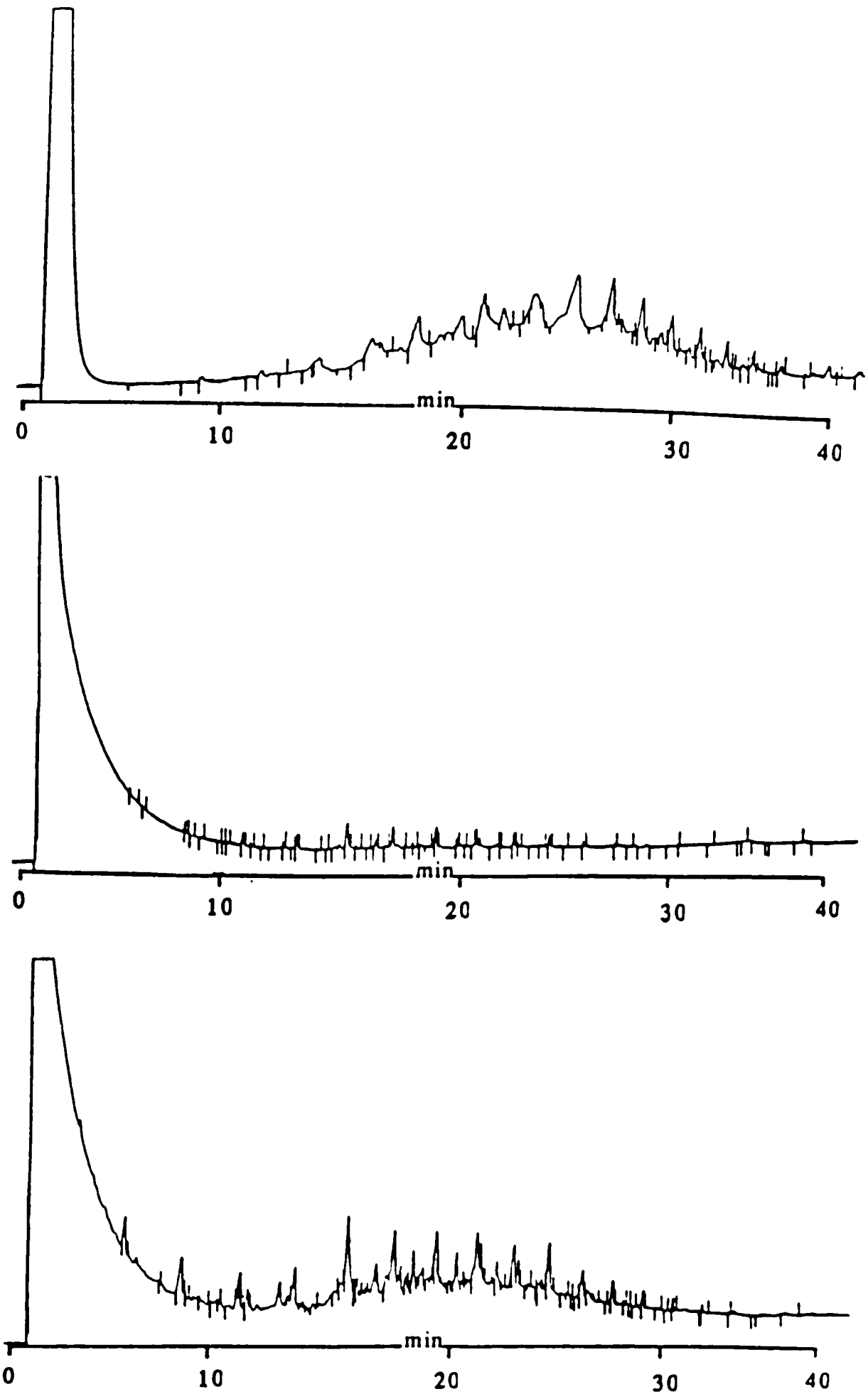
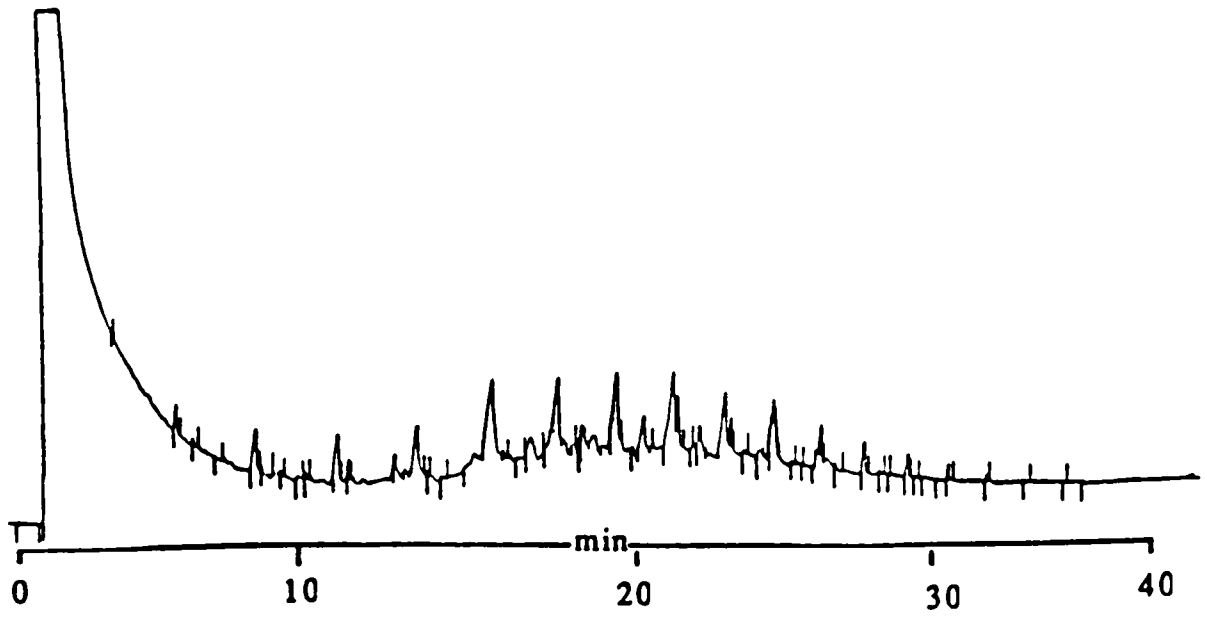


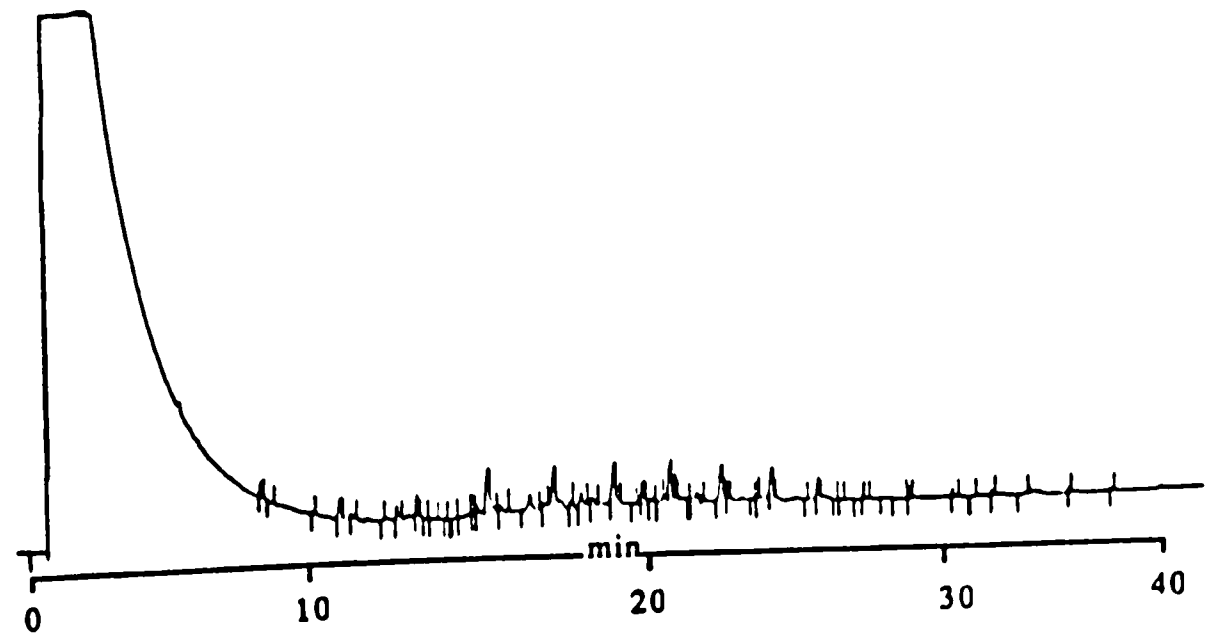
Figure 10. Three representative chromatograms of diesel fuel acted upon by mixed culture set A.

Table 7. List of components[a] based on their presence or absence in chromatograms of diesel fuel acted upon by mixed culture set A[b].

	Components degraded in some, but not all 10 replicates[c]			
Peaks absent in all 10 replicates	Peaks absent in all 10 replicates	Peaks absent in 8 or 9 replicates[d] $p > 0.110$	Peaks absent in 1 or 2 replicates[e] $p > 0.110$	Peaks absent[f] in 3 to 7 replicates[g] $p < 0.035$
9.61	17.00	25.94	11.71	6.50 (5)
27.25	19.20	26.11	13.77	9.14 (3)
	21.20	27.38	11.44	12.00 (6)
	23.12	34.48	20.04	17.40 (6)
	26.48		22.05	18.26 (3)
	27.99		24.82	19.80 (4)
			30.77	20.42 (4)
			32.06	20.83 (6)
				21.73 (6)
				23.25 (3)
				23.95 (3)
				25.00 (3)
				25.61 (7)
				33.28 (3)

a = Components denoted by their retention times.
b = This included : Pseudomonas vesicularis, Pseudomonas aeruginosa (strain 1) Pseudomonas aeruginosa (strain 2).
c = Based on 10 replicates. Data analyzed by placing 95% confidence interval for proportions.
d = Absence of a peak in 8 or 9 replicates is statistically the same as absence in all replicates.
e = Absence of a peak in 1 or 2 replicates is statistically the same as absence in all replicates.
f = Numbers in parentheses represent the number of samples in which the peak is completely degraded.
g = Absence of a peak in 3 to 7 replicates is significantly different from absence or presence in all replicates.





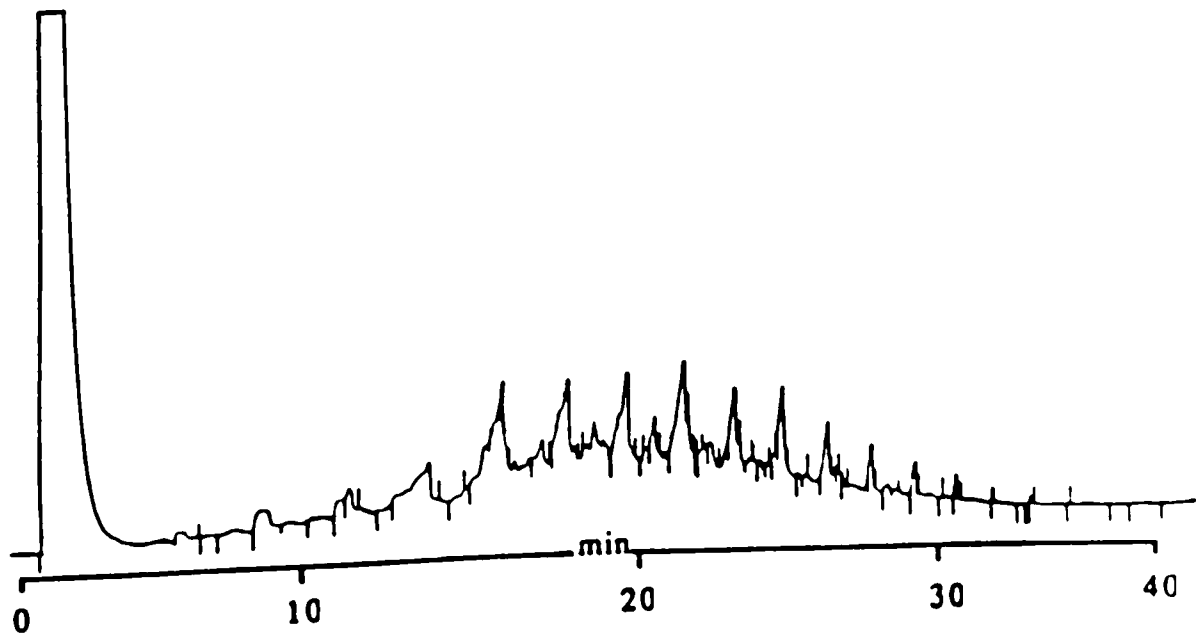


Figure 11. Three representative chromatograms of diesel fuel acted upon by mixed culture set B.

Table 8. List of components[a] based on their presence or absence in chromatograms of diesel fuel acted upon by mixed culture set B[b].

		Components degraded in some, but not all 10 replicates[c]		
Peaks absent in all 10 replicates	Peaks present in all 10 replicates	Peaks absent in 8 or 9 replicates[d] $p > 0.110$	Peaks absent in 1 or 2 replicates[e] $p > 0.110$	Peaks absent[f] in 3 to 7 replicates[g] $p < 0.035$
9.61	11.71	27.38	9.14	6.50 (4)
27.25	17.00		13.77	12.00 (5)
	18.26		14.44	17.40 (7)
	19.20		23.25	19.80 (6)
	21.20		33.28	20.04 (4)
	22.05			20.42 (5)
	23.12			20.83 (4)
	24.82			21.73 (5)
	25.00			23.75 (6)
	26.48			23.95 (4)
	27.99			25.61 (6)
	30.77			25.94 (7)
	32.06			26.11 (5)
				34.48 (7)

a = Components denoted by their retention times.

b = This included : Achromobacter xyloxidans, Achromobacter sp., Aeromonas sp., Pseudomonas sp., and Pseudomonas vesicularis.

c = Based on 10 replicates. Data analyzed by placing 95% confidence interval for proportions.

d = Absence of a peak in 8 or 9 replicates is statistically the same as absence in all replicates.

e = Absence of a peak in 1 or 2 replicates is statistically the same as absence in all replicates.

f = Numbers in parentheses represent the number of replicates in which the peak was completely degraded.

g = Absence of a peak in 3 to 7 replicates is significantly different from absence or presence in all replicates.

CHAPTER IV

DISCUSSION

Bacterial identification revealed that only Pseudomonas vesicularis was common to both sites of hydrocarbon spills from which samples were collected to isolate diesel-degrading bacteria. All of the other isolates with the exception of Aeromonas sp. have been reported in the literature as petroleum degraders (1, 2, 3). Although Aeromonas has not been reported earlier as a petroleum degrader, Vibrio which is closely related to Aeromonas has been shown to degrade hydrocarbons (3).

As revealed in the chromatograms of non-sterilized diesel and filter-sterilized diesel, two of the lighter fractions of diesel were lost during sterilization. However, this loss of components was not critical to the bacterial studies since the entire range of components within which these components fell were lost due to evaporation or photooxidation during the incubation period, and there was no loss of components from the later fractions where most of the bacterial degradation occurred.

The chromatograms of diesel from the control flasks showed that the lighter fractions of the diesel fuel, which had column retention times of less than 7 minutes, were lost due to evaporation. These results were expected since the phenomenon of evaporation in shake-culture studies has been previously documented (1, 8, 9). Diesel which was acted upon by the eight pure cultures as well as the two sets of mixed cultures also showed the same loss of lighter fractions attributable to evaporation and/or photooxidation and not to bacterial action. Interestingly, the peak with a column retention time of 20.04 was found to persist in the diesel acted upon by all isolates with the exception of Pseudomonas vesicularis. It was absent from all three replicates of Pseudomonas vesicularis (soil sample isolate), and it was absent in two of the three replicates of Pseudomonas vesicularis (water sample isolate). This fraction was also absent from the control

treatments. The persistence of the component with the retention time of 20.04 in all isolates with the exception of the two strains of Pseudomonas vesicularis and its loss in the control flasks indicated that the presence of the other bacteria or their metabolites prevented the component from being volatilized.

The chromatograms of the diesel fuel acted upon by individual strains revealed that the fraction with a column retention time of 9.61 was completely degraded by all strains individually, and the component with a column retention time of 17.40 was degraded by all strains with the exception of Achromobacter sp. This indicated that these two components were compounds that may be commonly available to bacteria in petroleum contaminated sites. Other than these two components, the chromatograms showed that certain fractions were degraded by some strains but not by all strains. This was expected since most of the degradative enzymes for the components found in diesel fuel are plasmid encoded (1, 3, 4), and not all strains could be expected to possess all the plasmids or genes necessary for degrading all the components.

Since the components in diesel are carbon sources not usually encountered by the bacteria in nature, the longer lag phases can be explained as the time needed for the bacteria to synthesize the enzymes necessary for utilizing the new carbon source. Since diesel is made up of many different components, the isolates would have had to synthesize new enzymes as the primary components being degraded neared depletion, and this phenomenon should should have resulted in growth curves with more than two lag and log phases. Diauxic growth was clearly seen in the growth curves of the Aeromonas species and the Pseudomonas species, and also in those of both strains of Pseudomonas aeruginosa. Since one of the reasons for diauxy is the presence of two carbon sources and these isolates degraded more than two components of the diesel, it would appear that in log phase the isolates were degrading more than one component of

diesel at the same time. The other bacteria showed smooth log phases, and this could have been due to their ability to degrade more than one compound at a time, i.e., they may have co-induced enzymes for different diesel fractions at the same time. It is also possible that the different components belonging to a specific group of components were attacked by the same enzyme. Thus, the bacterium could attack a number of components at the same time. Another reason which undoubtedly played a role in causing such extended growth curves was that the basal salts medium was a very stringent medium compared to the yeast extract, and hence the generation times of the bacteria while degrading diesel were greater than those in 1.5% yeast extract.

Visible degradation of the diesel fuel, as seen by the formation of stringy diesel globules, occurred when the bacteria reached late log phase to early stationary phase. The action of all eight isolates resulted in the formation of string-like diesel globules, and this denaturation continued throughout the incubation period.

The mean generation times of most of the isolates in the two media were significantly different from each other ($p = 0.05$). This was expected since the isolates grew more rapidly in the rich yeast extract medium than in the stringent medium containing diesel as the carbon source. Most of the isolates showed significant differences in their mean generation times while using diesel, and this also was expected since the isolates degraded different components and hence would have had different rates of enzyme synthesis and hence different generation times.

The significant differences in generation times were not unexpected since the chromatograms of diesel degraded by the three soil sample isolates showed that there were only two components, with column retention times of 9.61 and 17.40, which could be degraded by all three isolates, and only two other components, with column retention times of 19.80 and 27.38, were degraded by both strains of P. aeruginosa. Thus, since

the two strains of P. aeruginosa were degrading different compounds during growth in the basal salts medium with diesel, they had different generation times.

Similarily, only one component, with column retention time of 9.61, was degraded by all isolates from the water sample. Since the isolates degraded different component and belonged to three different genera it was expected that they would show different growth rates while using diesel.

The chromatograms obtained from the diesel fuel acted upon by the mixed culture set A showed that components with column retention times of 9.14, 12.00, 18.26, 23.25, and 33.28 were completely degraded in some of the replicates but were not degraded by any bacteria individually. The components which were always completely degraded by the mixed culture were also completely degraded by some bacteria individually. Several of the components which were completely degraded by individual bacteria were also completely degraded by the mixed culture in some but not all of the replicates. This significant difference from complete degradation in all samples may be attributed to the species interactions which may have prevented complete degradation of those compounds.

Slightly different results were obtained with the mixed culture set B. Components with column retention times of 6.50, 12.00, 21.73, 26.11, and 34.48 were completely degraded in some of the replicates but were not degraded by any bacteria individually. With the exception of components with column retention times of 18.26 and 22.05, all the components which were completely degraded by the individual bacteria were also completely degraded in some of the replicates of the mixed culture treatments, but only two components were degraded in all samples. One component with a column retention time of 27.25 was completely degraded in all the mixed culture replicates but was not degraded by any of the bacteria individually. This indicated that there were positive as

well as negative species interactions since the mixed culture could degrade components that were not degraded by the individual bacteria, but at the same time components which were always degraded by individual bacteria were degraded only in a few replicates by the mixed culture. It should be noted that since the total amount of diesel in the mixed cultures was being drastically reduced from its original volume of 0.5 ml by 85% to 95%, even the components which were always present in all samples of the residual diesel may have been degraded in all replicates to some extent. All of the components that persisted in all the samples of residual diesel were present in large amounts in the original diesel and hence were the ones most likely to persist for longer periods of time.

Mixed culture set A appears to be better at degrading diesel than set B. The bacteria which made up set A were isolated from the soil sample and hence, had been conditioned to degrade heavier fractions. The bacteria which made up set B were isolated from the gasoline-contaminated water and, since gasoline contains lighter fractions of petroleum, probably could not degrade all the heavier fractions found in diesel. Thus, it would appear that the mixed cultures could be used for cleanup of a contaminated site since both sets of mixed cultures were proficient at reducing the total amount of diesel fuel. It would be better to use set A since it can degrade a wider range of fractions. Further research will assess the efficiency with which culture sets A and B degrade diesel in soil columns.

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