BIODEGRADATION OF VACUUM PUMP OIL BY NATURALLY OCCURRING BACTERIA

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ABSTRACT

Bacteria are able to degrade any type of hydrocarbon, given the right conditions and enough time. Indeed, many bacteria have been isolated that use toxic hydrocarbons as their only carbon and energy source. This study examines the biodegradation of vacuum pump oil by bacteria. Bacteria that can use vacuum pump oil as their sole carbon and energy source were isolated from soil collected near a waste oil farming site and a fuel oil depot on the Savannah River Plant, near Aiken, South Carolina. Degradation rates of vacuum pump oil were determined by measuring the amount of carbon dioxide produced by the bacteria in a controlled microcosm. Both high concentrations and low concentrations of vacuum pump oil were inhibitory to vacuum pump oil degradation. Phosphorus and nitrogen were found to be significant limiting factors to the rate of vacuum pump oil degradation in the microcosms. Iron, a common co-factor in hydrocarbon degradation, had no measurable effect on the rate of vacuum pump oil degradation. High concentrations of nitrogen and phosphorus combined, were found to have a greater stimulatory effect on vacuum pump oil degradation then either one alone. Hydrogen peroxide, an oxygen source, at very low concentrations had the greatest stimulatory effect on vacuum pump oil degradation of any of the nutrients tested. The degradation of vacuum pump oil by bacteria in microcosms shows great promise for being a controllable and efficient method for eliminating this common laboratory waste.

INTRODUCTION

In the last century man has introduced into the environment many compounds that are not normally encountered by microorganisms. Many of these compounds are recalcitrant, since they are not readily degraded by microorganisms under ambient conditions. ZoBell was one of the first scientists to review the action of microorganisms on hydrocarbons, he realized that microorganisms were able to use a variety of complex hydrocarbons as their only source of energy and carbon (1).

There are many factors which can influence the degradation rate of hydrocarbons and petroleum. The quality of the hydrocarbon content has an effect on the degradability of the compounds. Horowitz *et al.* (8) and Haines and Alexander (7) found that low molecular weight n-alkanes (ranging from 0 to 42 carbons) are more rapidly degraded than branched alkanes and cycloalkanes. It has also been demonstrated that the microbial degradation rate decreases from the n-paraffins to iso-paraffins and cyclic paraffins to aromatic and heterocyclic fractions (2).

The degradation of hydrocarbons can occur in a wide range of temperatures. Degradation occurring below 0°C has been reported by ZoBell (18) and Traxler (15), while Klug and Markovetz (10), and Mateles *et al.* (12) reported hydrocarbon degradation at 70°C. Several nutrients are also considered important for bacteria to maintain themselves. Nirogen and phosphorus levels in the media are considered growth limiting factors (4, 13, 15) in the degradation of oil. Iron is also a co-factor in the breakdown of oil (5).

Since most laboratories have vacuum pumps, large quantities of vacuum pump oil are generated daily at large research institutions. This study looks at the feasibility of developing a bioreactor that could breakdown vacuum pump oil. Soils from petroleum contaminated sites were used as a source for bacterial isolates that could degrade vacuum pump oil. The effect of limiting nutrients (phosphate, nitrate, oxygen, and iron) and the concentration of vacuum pump oil on the bacterial degradation of vacuum pump oil was tested for different isolates, in controlled batch type microcosms.

MATERIALS AND METHODS

Bioreactors. Biometer flasks, 250 ml Erlenmeyer sidearm flasks with ascarite towers were used as reaction vessels (Bellco Company, Vineland, NJ) (Fig. 1). Since CO_2 and water are the products of the complete degradation of

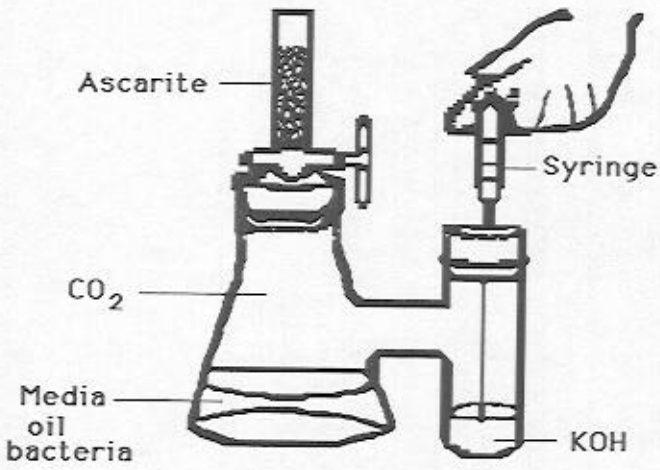

Figure 1. Biometer flask schematic.

vacuum pump oil, the amount of CO_2 produced is proportional to the amount of vacuum pump oil degraded. The amount of CO_2 released from the flask over a given period of time was measured by placing KOH in the sidearm to trap CO_2. The amount of CO_2 trapped by the KOH-containing side arm was measured by acid titration.

The flasks contained bacteria growing in minimal salts media with vacuum pump oil as the only source of carbon and energy. The sidearm contained 10 ml 0.1 N KOH to absorb the CO_2 produced. The KOH recovered from the side arm was immediately fixed with 1 ml saturated BaCl and titrated with 0.05N HCl. Unreacted KOH was fixed and titrated as above and used as the initial concentration. The results from the experiments were subtracted from this initial amount and multiplied by 25 to obtain the number of μmoles of CO_2 being produced, all procedures are as described by Atlas (1). Negative controls were used in all experiments and were the same as experimental conditions, except that 0.1% formalin was added to kill the bacteria and maintain sterility.

Media preparation. Throughout the entire study minimal salt media was used: yeast extract (0.5 g), NH_4Cl (0.250 g), Na_2HPO_4 (0.5 g), NaH_2PO_4 (0.5 g), $MgSO_4$ (0.5 g) per one liter of distilled water. All of the media was autoclaved for each of the experiments.

Bacteria isolation from the soil. Soil samples were taken from the Waste Oil Study Site (17) and the Central Shops Fuel Depot, Savannah River Plant, near Aiken, South Carolina. Soil samples (1 g) were mixed with 10 ml of minimal salt media, and 1 ml of vacuum pump oil. The mixture was agitated with a vortex mixer and incubated at 25°C. After the mixture became turbid, usually several days later, it was streaked onto a 1% vacuum pump oil agar culture plate (same media as minimal salts above except that it also contained 5% agar). After 24 h, distinct colonies were then restreaked onto four more oil agar culture plates. The isolated bacteria were then either used immediately or lypholized and stored for later use.

Bacteria identification. All isolates were gram stained and further screened for phenotypic characteristics using the API Rapid NFT assay strips (Analytab Products, Plainview, NY).

Experimental protocols. To test for effects of vacuum pump oil concentration on vacuum pump oil biodegradation, minimal salt media (MSM), 100 ml was put into each biometer flask with 2 ml of bacteria 88-5.4 suspended in MSM. Isolate 88-5.4 is a soil isolate from the Waste Oil Study Site that uses vacuum pump oil as its sole carbon and energy source. A log series of vacuum pump oil concentrations (0.1X, 1X, and 10X) were tested by adding 0.1, 1, or 10 ml of vacuum pump oil to one of the flasks, 1 ml of vacuum pump oil was also placed in one flask with 0.1 ml of 37% formaldehyde. This last flask served as a killed control.

To test for differences in biodegradation rates between isolates, biometer flasks were set up with 1 ml vacuum pump oil, 100 ml of MSM, and 2 ml of the bacteria to be tested suspended in MSM. *Acinetobacter calcoaceticus* (a type culture, ATCC 19606, known to have hydrocarbon degrading potential), 88-5.4 (a soil isolate from the Waste Oil Study Site), CBF 33 (a soil isolate from trichloroethylene contaminated area), TOL-1 (toluene degrading, *Escherichia coli*), 88-11.1 (a sandy soil isolate from the Waste Oil Study Site), 88-12.1

(isolated from the tar balls at the Waste Oil Study Site), 88-10.1 (a clay soil isolate from the Waste Oil Study Site), and 88-13.1 (a soil isolate from the Central Shops Diesel Fuel Storage Depot), were all compared to each other and with a killed control.

Nitrogen effects on vacuum pump oil biodegradation were measured by setting up biometer flasks with 1 ml vacuum pump oil, 100 ml of modified MSM, and 2 ml of bacteria suspended in MSM. The MSM was modified so that it contained 0.1X, 1X, and 10X of the major nitrogen containing component, NH_4Cl. Killed controls were as described above. Phosphorus effects were measured as for nitrogen, except that the MSM was modified to contain 0.1X, 1X, and 10X of the major phosphorus containing components, Na_2HPO_4 and NaH_2PO_4. A killed control was included as before. A 3% solution of $FeCl_3$ was used to test for the effect of iron on vacuum pump oil degradation. Biometer flasks were set up as for nitrogen and phosphorus studies described above, except that the MSM was unmodified. Flasks were then supplemented with 0.01 or 0.1 ml of $FeCl_3$, or left unsupplemented, *i.e.*, 1X, 10X, 0X concentrations of $FeCl_3$. Killed controls were used as before. Synergistic effects of nitrogen and phosphorus on vacuum pump oil biodegradation were tested by setting up flasks with 1 ml vacuum pump oil, 100 ml of modified MSM, and 2 ml of bacteria suspended in MSM. The MSM was modified by having 10X of the major phosphorus components and 10X of the major nitrogen component or by containing 1X of both of these. Killed controls were used as described previously. Isolate 88-5.4 was used for all nitrogen, phosphorus, and iron experiments. Isolate 88-11.2 was also used in the synergistic experiment.

The effects of oxygen concentration on vacuum pump oil biodegradation were tested by adding different amounts of H_2O_2. Each biometer flask contained 100 ml MSM, 1 ml of vacuum pump oil, and 2 ml bacteria solution (88-5.4 or 88-11.2). Flasks were than supplemented with 0.01 ml or 0.1 ml of 1% H_2O_2, or unsupplemented, *i.e.*, 1X, 10X, 0X concentrations H_2O_2. Killed controls were used as before.

RESULTS

The bacteria isolates from the sandy soil area (88-11.1) in the Waste Oil Study Site and the Diesel Fuel Storage Depot (88-13.1) were gram negative, while the tar ball (88-12.1) and clay soil (88-10.1) isolates from the Waste Oil Study Site were gram positive. Isolate 88-11.1 had the 97.9% probability of being *Agrobacterium radiobacter* out of the 59 possible. The 88-13.1 isolate had a 99.9% probability of being *Pseudomonas cepacia*, out of the 59 possible species, the other isolates were unidentifiable by API-NFT.

The biometer flask with 0.1X VPO (vacuum pump oil) degraded the oil at the highest level initially (Fig. 2); however, it did not continue the trend and started declining on the second day. The flask with 1X VPO, though slower at the start, steadily increased and, then declined. The flask with 10X VPO gave the lowest production of CO_2. Over all the flask with the 1X VPO did the best, it kept a steadier and higher CO_2 production rate.

The 88-5.4 isolate at first had the highest CO_2 production rate, but by the third

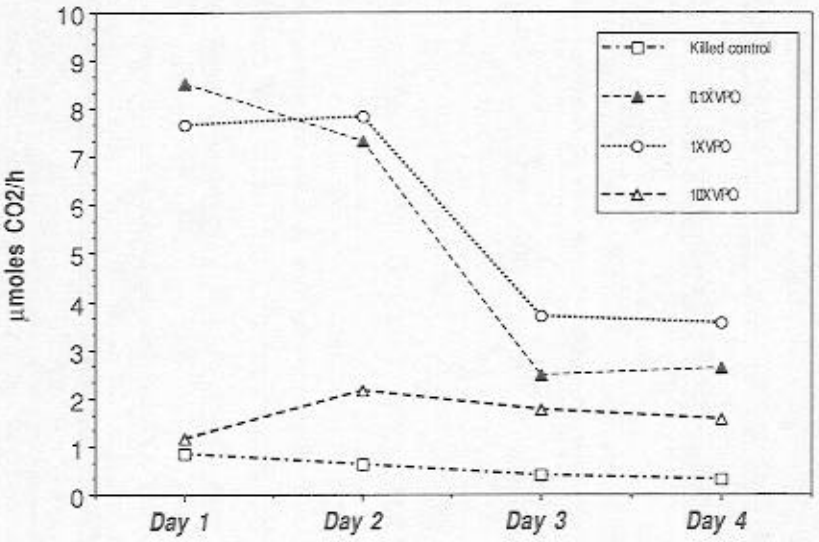

Figure 2. Effect of vacuum pump oil concentration on degradation rate (CO_2 production/h) compared at 24 h intervals for 4 days at 0.1X, 1X, and 10X concentrations of vacuum pump oil, killed control was formalin fixed with 1X vacuum pump oil, all tests were with isolate 88-5.4.

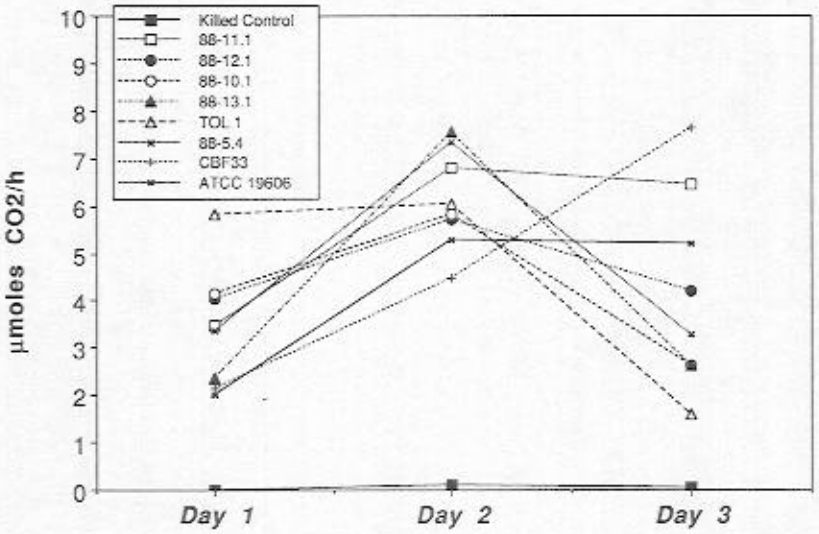

Figure 3. Effect of bacterial strains on degradation rate (CO_2 production/h) compared at 24 h intervals for 4 days with 1X concentrations of phosphorus, nitrogen, and vacuum pump oil, only bacterial strains were varied, killed control was formalin fixed.

day the CBF 33 bacteria had increased and surpassed the rate of 88-5.4 (Fig. 3). In another experiment, initially TOL 1 had the highest CO_2 production, but by day 2, 88-13.1 had the highest production rate (Fig. 3). The averages are from highest to lowest: TOL 1, 88-5.4, 88-11.1, 88-13.1, 88-10.1, and 88-12.1.

Initially the flask with the 0.1X phosphorus level had the highest CO_2 production; however, it started declining the second day (Fig. 4A). While the 0.1X

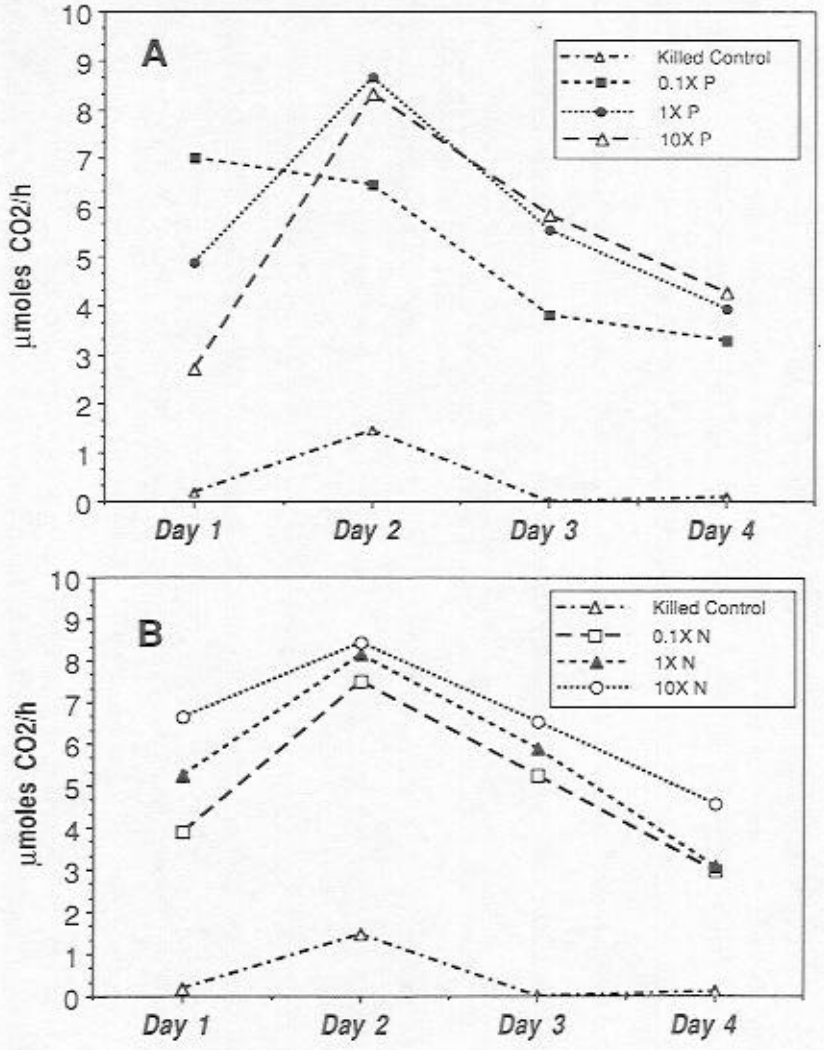

Figure 4. Effects of phosphorus and nitrogen on degradation rate (CO_2 production/h) compared at 24 h intervals for 4 days at **A***: 0.1X, 1X, and 10X concentrations of phosphorus and* **B***: 0.1X. 1X, and 10X concentrations of nitrogen, killed control was formalin fixed, all tests were with isolate 88-5.4.*

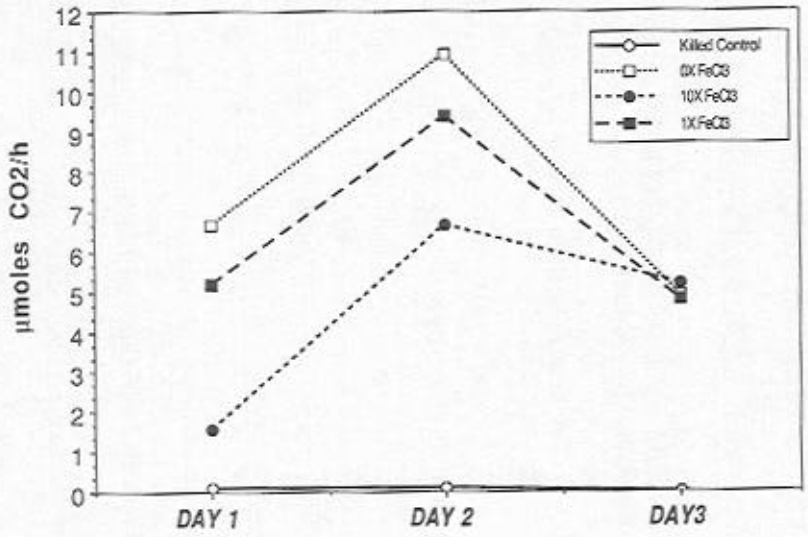

Figure 5. Effect of iron on degradation rate (CO_2 production/h) compared at 24 h intervals for 3 days at 0X, 1X, and 10X concentrations of $FeCl_3$, killed control was formalin fixed, all tests were with isolate 88-5.4.

P degradation rate was declining, the 1X P and 10X P were increasing, but the highest P concentration never reached the highest level of the 1X phosphorus (Fig. 4A). The flask with the highest concentration of nitrogen had the highest level of CO_2 production (Fig. 4B). The flask that was not supplemented with $FeCl_3$ had the highest CO_2 production rate, the 1X $FeCl_3$ was close, while the 10X had the lowest of the three (Fig. 5).

The results show the 10X phosphorus and nitrogen and 88-5.4 to have the highest production peak on the second day, while the flask with the 10X phosphorus and nitrogen had the highest level on the third day (Fig. 6). In the 88-11.2 flasks the 1X phosphorus and nitrogen flasks had the highest average, but the 10X phosphorus and nitrogen was not more than a fraction behind. The 1X phosphorus and nitrogen flask had the highest peak, but the 10X phosphorus and nitrogen did not decline as rapidly and its level of CO_2 production remained high (Fig. 6). In the 88-5.4 flasks the 10X phosphorus and nitrogen flask had the highest peak and average (Fig. 6).

The two 1X H_2O_2 flasks had the highest CO_2 production rate of all the flasks; however, the difference in production rate between the two H_2O_2 flasks was not great. During the second day the CO_2 production peaked (Fig. 7).

DISCUSSION

The bacteria found are typical for soil bacteria isolated in petroleum contaminated soil (3); however, since the Rapid NFT assay system was developed for a

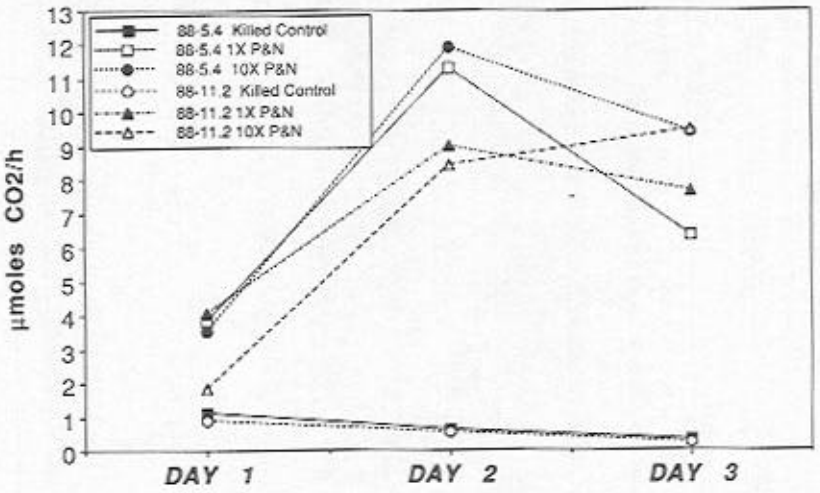

Figure 6. Effect of P+N on degradation rate (CO_2 production/h) compared at 24 h intervals for 3 days at 1X and 10X concentrations of phosphorus and nitrogen, killed control was formalin fixed, tests were compared with isolate 88-5.4 and 88-11.2.

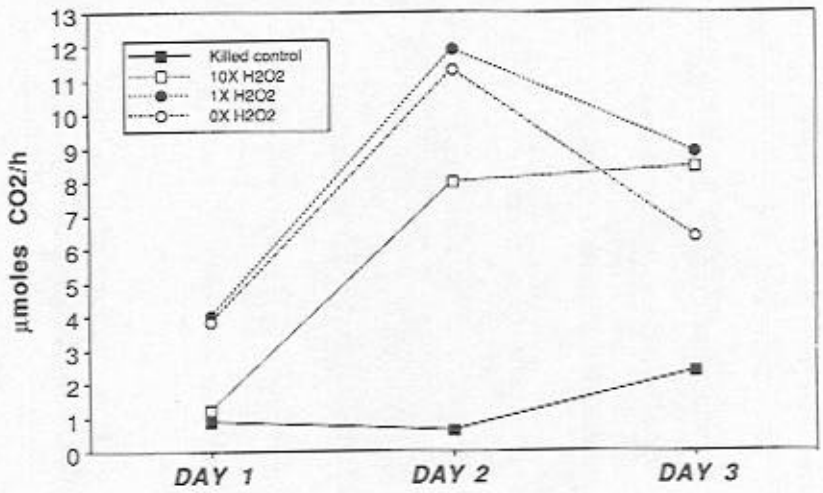

Figure 7. Effect of hydrogen peroxide on degradation rate (CO_2 production/h) compared at 24 h intervals for 3 days at 0X, 1X, and 10X concentrations of H_2O_2, killed control was formalin fixed, all tests were with isolate 88-5.4.

data base of clinical bacteria isolates and not for environmental isolates these data are preliminary at best.

Different amounts of vacuum pump oil effect the rate of degradation by bacteria. The flask with the least VPO, even though it had a higher initial CO_2 production, appeared to run out of vacuum pump oil. The flask with 1X VPO had the highest overall CO_2 production. The highest concentration of VPO used in this study appears to be toxic to the bacteria. These findings compare favorably with those of Bossert and Bartha (3) who also reported that all petroleum compounds are toxic to degraders at some concentration.

All of the bacteria from the two different bacteria comparison tests had different rates of degrading the vacuum pump oil. It is logical that the bacteria would have different rates since these bacteria were probably not even the same species. This study also shows that bacteria which are enriched in other toxic environments may be as able to degrade vacuum pump oil as those enriched in environments by VPO, as seen for CBF 33 an isolate enriched in a trichloroethylene environment. Different bacteria apparently utilize the same processes for degrading different classes of hydrocarbons (14). It is also possible that since bacteria have different processes for the breakdown of vacuum pump oil, one method of breakdown might be more efficient in degrading VPO than another.

Nitrogen was more limiting to these bacteria then phosphorus, indeed, high concentrations of phosphorus decreased VPO degradation. Many scientists (4, 16) have already observed that nitrogen is a limiting factor in the bacteria degradation of hydrocarbons. Had the highest concentration of P been supplemented with more N than the higher concentration of P could be utilized. This was demonstrated by the flasks containing high concentrations of both P and N. The degradation rate in these flasks was higher than the N alone. Analysis of the vacuum pump oil reveals that it contains dithiophosphate as a preservative. This preservative may provide enough phosphorus to account for the inability of phosphorus additions to stimulate degradation. Thus for these microcosms nitrogen is more limiting than phosphorus when vacuum pump oil is the principal carbon source.

Surprisingly no effect was seen by supplementing the medium with iron. Other studies have shown that Fe does have a positive effect on some types of hydrocarbon degradation (5). Since iron is required for decane and hexadecane oxidation (14), and vacuum pump oil contains some of these compounds, the data suggests that sufficient iron is already present.

Oxygen was supplied to the bacteria via H_2O_2 and found to have its greatest stimulatory effect at low concentrations. The toxic effect of hydrogen peroxide at high concentrations was undoubtedly due to its extreme oxidizing and thus toxic nature at high concentrations. A better source of oxygen might have an even greater stimulatory effect on VPO degradation. Other investigators have also shown oxygen to be the single most important nutrient in the degradation of hydrocarbons (3, 4).

The present study demonstrates that naturally occurring bacteria can be isolated from soil that will mineralize vacuum pump oil. The mineralization process is limited mainly by nitrogen and oxygen. Thus by optimizing concentrations of certain nutrients a process could be developed for the complete

degradation of vacuum pump oil to carbon dioxide and water. This study demonstrates the potential for developing bioreactors capable of degrading this and other toxic wastes.

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