Effect of Amendments on the Microbial Utilization of Oil Applied to Soil

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Replicate field plots comprising a control, plus oil, plus oil and bacteria, plus oil and fertilizer (urea-phosphate; \(27:27:0\)), and plus oil, bacteria, and fertilizer were monitored over a 308-day period for changes in bacterial and mold numbers. Changes in the chemical composition of the oil applied to the plots was followed by using chromatographic techniques. Application of fertilizer resulted in a stimulation of bacterial numbers and in the rate of utilization of \(n\)-alkane components of the saturate fraction. The application of oil-utilizing bacteria, however, resulted in only a slightly accelerated rate of utilization of \(n\)-alkane components of chain lengths \(C20\) to \(C25\). The isoprenoids, phytane and pristane, were still present in gas-liquid chromatography profiles after digestion of the \(n\)-alkane components of the saturate fraction. Those plots which received fertilizer showed an accelerated rate of recovery of native vegetation.

Oil spills, whether on water or soil, do disappear (6), but very little is known about what can be done to accelerate this process. Recent work by Reisfeld et al. (5) and Atlas and Bartha (1) indicated that the disappearance of oil from sea water could be accelerated by the addition of deficient nutrients such as nitrogen or phosphorus, or both. Suggestions have also been made (4, 7) for microbial seeding of spills since bacteria and fungi are the only biological species which have the metabolic capability of utilizing petroleum carbon for cell synthesis. There is, however, very little information in the literature evaluating the effect of such treatments on the acceleration of the utilization of oil spilled under natural conditions.

Crude oil is essentially a mixture of carbon and hydrogen, and thus spills will result in an imbalance in the carbon-nitrogen ratio at the spill site. For bacteria to grow efficiently, they require about 10 parts carbon to 1 part nitrogen. If the ratio is greater, e.g. 100:1 or 1,000:1, growth of the bacteria and utilization of carbon source(s) will be retarded. In addition to there being a nitrogen deficiency in oil-soaked soil, other nutrients such as phosphorus may become growth-rate limiting. Therefore, in the experiments described in this paper, urea-phosphate, a fertilizer, was added to oil spilled on soil, thus correcting both deficiencies in one application.

A survey of soils (unpublished observations) from the northwest area of Canada for the presence of oil-utilizing microorganisms indicated that not all soils have an indigenous population capable of utilizing oil. Thus, oil spills were also inoculated with oil-utilizing bacteria with and without a concurrent application of the urea-phosphate amendment.

The experimental site chosen was in the Swan Hills area of north central Alberta, which represents a major oil-producing center in this province. The soils are of low fertility, are in a frozen state for approximately 5 to 6 months of the year, and are representative of soil and climatic conditions existing in the production and pipeline transport areas of this province and western Canada.

MATERIALS AND METHODS

Field sites. The plots in the Swan Hills area were placed on an overgrown, unused strip. Four replicate plots of each treatment, i.e., control, control plus oil, plus oil and bacteria, plus oil and fertilizer, plus oil, bacteria, and fertilizer, were placed in a random manner. The composition of the oil used in the spill is presented in Table 1. This crude petroleum was obtained from the Shell Oil Co. and is representative of producing wells in this area. The oil was applied in mid-July 1972 by sprinkling from perforated cans at a rate of 60 liters of crude oil per 9 square meters of soil, and it completely covered the surface of the plots with a thin layer of oil. Fertilizer, urea-phosphate (nitrogen-P\(_2\)O\(_5\)-potassium, \(27:27:0\)) was applied simultaneously to eight plots at a rate of 60 g of nitrogen per m\(^2\) (equivalent to 600 kg of nitrogen per hectare). A mixed culture of bacteria capable of utilizing an oil of similar quality (Norman Wells crude oil; chemical composition in Table 1) was also at this time applied
to eight plots, four of which had received a fertilizer treatment. The cells were grown on a rotary shaker (300 rpm, 1-inch [about 2.5 cm] eccentricity) at 25 °C in 2-liter Erlenmeyer flasks containing 1 liter of basal salts medium (2) and 1 ml of Norman Wells crude oil as sole carbon source. The cells were recovered by centrifugation after 96 h of growth, washed, and resuspended in tap water at 4 °C. This suspension was diluted to a concentration such that application of 6 liters of suspension per plot yielded an application rate of 10⁸ bacterial cells per cm². The mesophilic bacterial population used for bacterial seeding was composed of the following genera: Flavobacterium and Cytophaga sp. (41%), Pseudomonas sp. (34%), Xanthomonas sp. (10%), Alcaligenes sp. (9%) and Arthrobacter (5%).

Soil samples (total weight approximately 500 g) taken periodically and analyzed for total bacterial and fungal counts. When such samples were obtained from plots which received an oil treatment, the oil was extracted and its chemical composition was determined by chromatographic techniques (2). Thus, this experimental protocol allowed the statistical analysis of the effect of treatments on the microbial population and on the utilization of the applied oil.

Microbiological methods. Changes in microbial numbers were monitored by using a spread plate count technique. Plate count agar (Difco) was used for the enumeration of bacteria, and malt extract agar (Difco) adjusted to pH 4.5 was used for molds. Quintuplicate plates of each dilution were incubated at 21 °C for 6 days before counting.

The bacteria which comprised the mixed population were classified to the generic level on the basis of the following tests: Gram reaction, presence and position of flagella (determined by electron microscopy); oxidation and/or fermentation of sugars with and without acid and/or gas production; catalase and oxidase activity. Isolates which were classified as Pseudomonas were streaked on Pseudomonas F and P agar (Difco) and observed for fluorescein or pyocyanin production, respectively.

Chemical methods. The chromatographic techniques used for the analysis of crude oil was as described in our previous paper (2). These techniques resolve crude oil into asphaltene, saturate, aromatic, and the polar nitrogen-sulfur-oxygen-containing organic fractions. The saturate fraction was further resolved by using gas chromatography as reported previously (2).

Recovery of oil from soil. Approximately 100 g of a soil sample was extracted four times with 100-ml portions of n-pentane, and the extracts were combined and evaporated to dryness in a fume hood at room temperature. The dry residue was redissolved in 4- to 25-ml portions of benzene, and, after pooling the benzene fraction, non-hydrocarbon material present was allowed to settle out. A portion of the benzene-soluble fraction was evaporated to dryness, weighted, resuspended in n-pentane, and analyzed by liquid and gas chromatographic procedures (2).

Statistical analysis. Before statistical analysis, all data were subjected to the Nalimov test (3), which rejected data outside of the confidence limit of 95%. The means and t tests were made between sets, each possessing a minimum of four observations. All statistical analyses were performed on a minimum of four observations. All statistical analyses were performed by an IBM 360 computer.

RESULTS

The gas-liquid chromatography (GLC) profile of the n-saturate fraction of the oil before and immediately after application to soil is presented in Fig. 1. The mean pH values for these plots 12, 66, and 308 days after treatment ranged from pH 4.8 to 5.9. Statistical analysis of these data indicated that there were no significant differences between plots as a result of amendment application.

Changes in the bacterial count observed 12, 66, and 308 days after treatment are presented in Table 2. These data show that there was a statistically significant increase in bacterial numbers within 12 days of the application of oil when fertilizer had been applied to the plot as well. The stimulation of bacterial numbers decreased within 66 days of treatment, and by 308 days the values for those plots that had received fertilizer, with or without bacteria, were similar. Statistical analysis of these results (Table 2) indicated a consistent significant difference at the 95% confidence level and occasionally at the 99% confidence level when the effects of the fertilizer amendments on bacterial counts were compared with those values obtained from the control oil plots.

Changes in the mold counts are presented in Table 3, and statistical analyses indicated that the application of amendments was without effect on this parameter at the 95% confidence level.
The chemical composition of the oil recovered from the plots 12 days after treatment is presented in Table 4. Statistical analysis of these data showed that the application of amendments was without effect on the chemical composition of the oil recovered from the plots. The data in Tables 5 and 6 suggest that the oil recovered 66 and 308 days after treatment from plots which received the fertilizer amendment have a reduced n-saturate content. Statistical analysis of this reduction in n-saturate content of the recovered oil indicated a significant reduction (at both the 95 and 99% confidence levels) 66 and 308 days after treatment in those plots which had received a fertilizer treatment.

A comparison of the n-saturate profiles 12 days after the oil had been applied to the soil (Fig. 2) shows that the application of fertilizer resulted in a slightly accelerated rate of n-saturate utilization. The change in profile is reflected in a utilization of the middle chain-length (i.e., C18 to C29) n-saturate components. The data in Fig. 3 show that the fertilizer application resulted in a complete disappearance of the n-saturate components, with the exception of the branch-chain isoprenoids, phytane and pristane, 66 days after treatment. These data also suggest a stimulatory effect by the application of the bacteria at this time which is reflected in an increased utilization of the C20 to C25 chain-length n-saturate compounds. These results are confirmed in Fig. 4, where a comparison of the GLC profiles of the n-saturate fraction 308 days after application of amendments shows the accelerated utilization of the n-saturate.

### Table 2. Bacterial count of Swan Hills plots

| Treatment          | Bacterial count \( \times 10^6/g \) after* |
|--------------------|------------------------------------------|
|                    | 12 days | 66 days | 308 days |
| Control            | 13.5    | 56.7    | 41.7     |
| Plus oil           | 59.5    | 281.5   | 60.1     |
| Plus oil and bacteria | 77.0   | 312.5   | 47.5     |
| Plus oil and urea-phosphate | 5,162.5* | 574.5*  | 680.0*   |
| Plus oil and bacteria and urea-phosphate | 3,753.3* | 1,710.0* | 525.0*   |

*All counts are the mean values from quadruplicate plot counts. Each plot count was executed in quintuplicate as described in Materials and Methods. A significantly different from the oil treatment values at the 95% confidence level.

### Table 3. Mold count of Swan Hills plots

| Treatment                  | Mold count (Molds \( \times 10^6/g \)) after* |
|----------------------------|-----------------------------------------------|
|                            | 12 days | 66 days | 308 days |
| Control                    | 205.5   | 53.0    | 121.3    |
| Plus oil                   | 47.8    | 182.9   | 87.8     |
| Plus oil and bacteria      | 124.2   | 131.3   | 79.5     |
| Plus oil and fertilizer    | 29.5    | 107.0   | 145.5    |
| Plus oil and bacteria and fertilizer | 26.8 | 59.4    | 90.0     |

*All counts are the mean values from quadruplicate plot counts. Each plot count was executed in quintuplicate as described in Materials and Methods.

### Table 4. Chemical composition of Swan Hills oil after 12 days in contact with soil plus various amendments

|                         | Composition of oil (%)*                  |
|-------------------------|-----------------------------------------|
| Crude oil fraction      | Barrel | Soil | + Bacteria | + Fertilizer | Bacteria + Fertilizer |
| Asphaltene, soluble     | 3.00   | 1.00 | 1.29       | 1.48         | 1.62                   |
| Asphaltene, insoluble   | 0.24   | 5.94 | 3.96       | 4.55         | 4.70                   |
| Saturates               | 62.10  | 62.75| 62.88      | 59.12        | 59.89                  |
| Aromatics               | 23.20  | 21.18| 21.70      | 23.72        | 24.13                  |
| NSO, soluble            | 5.60   | 6.40 | 6.23       | 6.72         | 7.15                   |
| NSO, insoluble          | 5.70   | 3.65 | 5.37       | 4.28         | 4.32                   |

*Average of values from four replicate plots.
*NSO, nitrogen-sulfur-oxygen-containing organic compounds.
TABLE 5. Chemical composition of Swan Hills oil after 66 days in contact with soil plus various amendments

| Crude oil fraction | Composition of oil (%)a | + Bacteria | + Fertilizer | + Bacteria + Fertilizer |
|--------------------|-------------------------|------------|-------------|------------------------|
|                     | Barrel | Soil |       |           |                        |                        |
| Asphaltenes, soluble | 3.00   | 2.52 | 2.77  | 4.34      | 2.99                   |
| Asphaltenes, insoluble | 0.24   | 5.88 | 6.49  | 10.81     | 9.00                   |
| Saturates           | 62.10  | 61.45| 60.78 | 46.32*    | 46.23*                 |
| Aromatics           | 23.20  | 22.62| 20.19 | 23.23     | 25.58                  |
| NSO, soluble        | 5.60   | 7.09 | 7.68  | 10.43     | 10.27                  |
| NSO, insoluble      | 5.70   | 2.12 | 5.76  | 4.88      | 5.93                   |

a Average of values from four replicate plots.
* Significantly different from the unamended oil treatment values at both the 95 and 99% confidence levels.

TABLE 6. Chemical composition of Swan Hills oil after 308 days in contact with soil plus various amendments

| Crude oil fraction | Composition of oil (%)a | + Bacteria | + Fertilizer | + Bacteria + Fertilizer |
|--------------------|-------------------------|------------|-------------|------------------------|
|                     | Barrel | Soil |       |           |                        |                        |
| Asphaltenes, soluble | 3.00   | 1.35 | 1.52  | 2.01      | 2.58                   |
| Asphaltenes, insoluble | 0.24   | 12.71| 12.42 | 20.69     | 21.31                  |
| Saturates           | 62.10  | 54.74| 56.36 | 39.30*    | 37.91*                 |
| Aromatics           | 23.20  | 17.94| 18.11 | 20.90     | 21.14                  |
| NSO, soluble        | 5.60   | 6.85 | 7.63  | 9.06      | 9.98                   |
| NSO, insoluble      | 5.70   | 6.42 | 4.77  | 8.04      | 7.27                   |

a Average of values from four replicate plots.
* Significantly different from the unamended oil treatment values at both the 95 and 99% confidence levels.

DISCUSSION

The hypothesis that a nutrient-deficient condition can be responsible, in part, for the persistence of oil applied to soil has been substantiated by the data presented in this paper. The application of nitrogen and phosphorus as urea-phosphate resulted not only in a rapid increase in the numbers of bacteria present but also in an accelerated rate of disappearance of the n-saturate fraction of the crude oil applied to the soil. However, the efficacy of the application of oil-utilizing bacteria is still unanswered at this time since the effect reported, i.e., the slightly accelerated utilization of n-saturates of chain lengths C20 to C25, was not observed on all replicates.

The low degree of change observed between the 66- and 308-day samples can be accounted for by the fact that for most of this period the area was in a frozen state. The persistence of the isoprenoids, phytane and pristane, in the GLC profile of those plots where n-saturation utilization occurred (i.e., where fertilizer was applied)
suggests that psychrophilic conditions prevail in the soils in this area. It has been noted (2) that these compounds are more recalcitrant to microbial attack under psychrophilic than under mesophilic conditions.

The slight difference in the chemical composition of the oil recovered from soil and that obtained from the barrel was probably a result of our extraction procedure. However, the procedure, at least for the major components of oil (i.e., saturates and aromatics) gave very highly reproducible results (Fig. 1). Initial studies were performed comparing n-pentane, benzene, methanol, and methylene chloride as extractives for recovering oil from soil. All of these solvents readily recovered the n-saturate fraction of oil applied to soil, but only n-pentane did this without removing residual soil material which subsequently interfered with our analytical procedures. For example, the material extracted from the soil by the other solvents was subsequently extracted into the benzene fraction and thus effected the further purification of the oil.

The increase in the insoluble fractions when fertilizer had been applied to the plots (for example, the insoluble asphaltenes) suggests that transformation of oil components is taking place along with the assimilation of the n-saturate fraction. Changes in polarity of components result from the introduction of oxygen into the various components. Since our liquid chromatographic separation procedure is based on polarity, this change would shift these compounds into more polar fractions. At this time, however, it is not possible to account for the increase in total asphaltenes which were detected in the oil recovered from the plots 308 days after treatment.

The lack of stimulation of the mold population as determined by our plate count technique is possibly an artifact resulting from our procedure, or more likely it reflects the inability of molds to successfully compete with bacteria under the nonrestrictive environmental conditions found in our plots.

The slight increase in the rate of oil utilization by the application of bacteria to the spills could be a result of too low a level of application or to the inability of these bacteria to survive under the natural field conditions existing in the Swan Hills area. However, considering that

**FIG. 3.** Gas chromatographic profiles of the saturate fraction of the Swan Hills oil 66 days after contact with the soil and various amendments.

**FIG. 4.** Gas chromatographic profiles of the saturate fraction of the Swan Hills oil 308 days after contact with the soil and various amendments.
the bacterial content of these soils is 10-fold higher than what we applied, the failure to obtain a clear-cut effect is probably a result of too low a level of application. The partial utilization of \( n \)-saturates observed 308 days after application in those plots where oil alone was applied indicates that some component(s) of the indigenous flora present in the Swan Hills soils has the capability of utilizing crude oil.

Visual observation of the rate of recovery of plant growth indicates an accelerated rate of recovery for those plots which received fertilizer. Thus, an increase in bacterial numbers can be correlated with an increased rate of disappearance of the \( n \)-saturate fraction component of crude oil, and this latter observation can be correlated with an increased rate of recovery of vegetation. All of these effects can be directly related to the application of fertilizer (urea-phosphate) to oil-soaked plots.

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