THE BIO-REACTOR PROJECT, 1993/1994:

LABORATORY TESTS TO ENHANCE THE BIODEGRADATION OF DIESEL INVERT MUD RESIDUES (DIMR)

by

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EXECUTIVE SUMMARY

The Bio-Reactor Project is a five year research project intended to refine the bioremediation process and to apply it to a variety of nonhazardous industrial wastes which contain excessive amounts of nontreatable salts and high levels of refined or unrefined hydrocarbons. The initial two years of the program were devoted to project planning, design and construction of the field unit and testing treatment procedures with the first waste, that being brine and crude oil contaminated topsoil. A diesel invert mud residue (DIMR) was chosen as the second oilfield waste to be tested for potential bioremediation clean-up utilizing the Bio-Reactor technology. Conversely, this complex waste served to test potential improvements in the bioremediation scheme and evaluate the roles of cultivation, forced aeration and aggregate size in the field unit. The purpose of the laboratory testing was to determine if DIMR was a good subject for bioremediation and to then establish the operating criteria of the field unit. The general protocol for lab testing was (1) a basic chemical characterization; (2) to fix the physical factors and create a soil-like medium (accomplished by AEC); (3) to remove water soluble untreatable materials, i.e., brine salts, by leaching; (4) to determine if the material would support heterotrophic microbial activity; and (5) to enhance the degradation rates of hydrocarbons by environmental manipulation.

Characteristics of the DIMR used in the laboratory tests were: TPH 50,000 to 70,000 mg kg^{-1}, EC_{SAT} 37 dS m^{-1}, EC_{SAT} after leaching 1 - 2 dS m^{-1}, pH 7.4, $CaCO_3$ equivalent 6.9%, extractable NO_3, NH_4 and PO_4 < 1 $\mu g\ g^{-1}$, clay 33%, silt 45% and sand 22%. All of the material was aggregated by AEC to create a soil-like medium and provide a system of macropores and micropores to facilitate air and water movement. Salts were removed by saturation leaching, the material partially dried to about 20% water content and stored at 3°C until used.

To access laboratory treatment effectiveness small samples were incubated for 2 to 4 months under controlled conditions and periodic measurements were made of rates of CO_2 evolution. These measurements were assumed to quantitatively reflect general microbial activity which was ultimately dependent upon hydrocarbons as sources of carbon and energy, i.e., hydrocarbon mineralization. These decomposition end-product estimates were backed up with measurements of extractable hydrocarbons at the beginning and end of the tests. Other routine measuarements included NO_3-N, NH_4-N, pH and electrical conductivity (EC).

The leached and aggregated DIMR waste supplemented with N and P demonstrated classic respiration (CO_2 efflux) patterns for complex substrates; a brief high level of activity lasting 1 week followed by a long period (months) of gradually decreasing activity. This is interpreted as rapid growth of

opportunistic fungi and bacteria on low molecular weight hydrocarbons followed by the slow degradation of the more recalcitrant substrates.

The pattern of respiratory activity was similar to that seen for Waste 1 (crude oil) but rates for the DIMR were more rapid throughout the measurement period than the rates for the unrefined waste. However, respiration rates were very similar to those measured previously for two additional DIMR sources and thus may represent baseline decomposition data for this waste type.

Estimates of microbial biomass (living fungi and bacteria that respond metabolically to added glucose) reached maximum values within a week after starting the bioremediation treatment and gradually and steadily decreased thereafter. The quantity of microbial biomass was similar in magnitude or somewhat greater to that found in agricultural soils, i.e., biomass estimates did not distinguish a material with massive hydrocarbon contamination from normal soils. Direct low magnification (25x) observations of microbial growth paralleled the respiration pattern. Depending upon specific enviromental conditions, massive development of fungal mycelium and/or bacterial colonies on aggregate surfaces occurred in the first 1 - 2 weeks and no new growth was observed thereafter. Microbial growth by *Penicillium* or orange bacteria was extensive enough in the initial period that it was often clearly observable with the unaided eye.

Fertilization with N and P in the laboratory was routinely done by adding the nutrients at the initiation of a test and making no further addtions. When medium levels of nutrients (400 μg N g^{-1} 80 μg P g^{-1}) were added, all the KCl extractable NO_3-N disappeared within a week, that is during the rapid growth/decomposition stage. However, respiration and presumably hydrocarbon decomposition, continued at what has come to be recognized as the maximum rate. Additions of N or P after the initial flush of activity had minimal effects on respiration and had negative effects after two months of incubation. The addition of glucose to the bioremediating DIMR might have been expected to expose hidden N or P deficiencies by making additional demands on the available N pool but measurements of substrate induced respiration (SIR) did not confirm this. Glucose stimulated respiration was not N or P limited despite the near-absence of extractable NO_3 or NH_4, i.e., nonextractable N was sufficient to support maximum decay rates.

Further tests on fertilizer types and levels indicated the optimum quantity of N required for an initial one-time loading for DIMR containing 5-7% hydrocarbons was between 600 and 800 μg N g^{-1}. There was little difference amoung the sources of N be they NO_3-N, urea in a soluble complete fertilizer (Plant Prod 28:14:14) or soluble fertilizers teamed with slow release fertilizer (Osmocote 17:9:13). Thus, in terms of effectiveness in promoting the decomposition of hydrocarbons, the N species appears to be unimportant

aside from the potential of N losses in gaseous phase or leachates. The rapidity of the immobilization of mineral N by the decomposers infers that losses from the system (Bio-Reactor) would only be important during the first few days of treatment. It is critical to select the appropriate N form to minimize leaching losses of NO_3, denitrification losses of NO_3 or gaseous losses of NH_3 from NH_4-N or urea if high pH values are expected. The laboratory studies show it is important to provide a large quantity of N at start-up which will be rapidly immobilized in the biomass and internally recycled, providing adequate N to indefinitely support maximum degradation rates.

Early in the project it was recognized that any material treated in the Bio-Reactor must possess soil-like properties including water stable aggregates. Macropores were necessary to provide pathways for mass water movement and drainage to maintain aerobic conditions, and micropores were essential to store water and allow for diffusion of soluble hydrocarbons. A test was conducted to determine the effect of aggregate size on hydrocarbon degradation on both treated Waste 1 and fresh DIMR. CO_2 evolution was greatest from small aggregates (<2mm) and least on large (>4mm) aggregates over an 8 week period. However, residual hydrocarbon analyses indicated that aggregate size had little effect on hydrocarbon degradation. Further testing is required to resolve these conflicting results but it is proposed that aggregate size may be influential when activity is high and diffusion rates critical and aggregate size unimportant as bioavailability becomes the limiting factor.

Related to aggregate size and porosity is the quality of the gaseous environment and its effect on hydrocarbon degradation. When air was forced through DIMR at a rate that would replace the pore volume 100 times per hour, estimates of CO_2 efflux were slightly less than from passively aerated DIMR, indicating that passive aeration was adequate to support maximum activity. However once again the hydrocarbon analyses indicated otherwise; forced aeration resulted in a 14% greater disappearance of extractable hydrocarbons. These differences could have been due to carbonate equilibrium shifts or the physical removal of volatile hydrocarbons; none-the-less, passive aeration did support near-maximum degradation rates in small samples.

The main laboratory experiment conducted with regard to operating conditions of the Bio-Reactor involved the use of previously treated waste as inoculum combined with elevated temperatures. Waste 1 which had been treated in the Bio-Reactor in a cell heated to 30 - 35°C for 11 months was added to DIMR to enrich the high temperature microflora. Inoculation in conjunction with elevating the temperature to 30 or 35°C from 22°C resulted in large increases in CO_2 efflux. Increasing the temperature to 35°C without inoculation resulted in the lowest CO_2 efflux, indicating that the DIMR lacked oil degrading microorganisms that functioned efficiently at elevated

temperatures. Residual hydrocarbon analysis confirmed the CO_2 results. Eight weeks incubation at 30°C combined with inoculation resulted in a 1.8-fold decrease in hydrocarbons compared to 22°C and incubation at 35° resulted in a 2.3-fold increase. Nitrogen consumption also followed the same pattern with the highest demand in inoculated DIMR incubated at 35°. In the laboratory setting, it was clear that utilization of treated wastes in combination with high temperatures could profoundly enhance degradation rates.

Following the successful trial of inoculating with weathered Waste 1, other inoculation or species and metabolic enrichment trials were conducted. A variety of soils were added to DIMR, incubated at 22 and 35°C and hydrocarbon disappearance determined. The soils included forest floor, grassland, pasture, anthill and Waste 1 processed at 37° in the laboratory. Addition of the natural soils had no effect or suppressed hydrocarbon degradation at both temperatures. However, once again the addition of Waste 1 resulted in a strong stimulation of the degradation of hydrocarbons in DIMR. Trials were also made of two commercial inoculants under different fertilizer and inoculation regimes but no increases in CO_2 efflux could be detected and the trials were discontinued.

Methodology for determining CO_2 efflux rates was re-evaluated after obtaining anomolous data for well-weathered hydrocarbon waste. Previous tests had indicated similar results could be obtained by either measurement with a continuous flow of CO_2-free air through the samples or build-up of CO_2 in sealed containers and subsequent headspace analysis. However, it became apparent the method did strongly affect the rate estimates in slow respiring, alkaline, carbonate-containing wastes or soils. The continous flow method resulted in 2 - 5-fold overestimates of CO_2 rates as compared to headspace analysis. Headspace analysis also had problems unless gaseous CO_2 and carbonates in the test materials were at equilibrium. In extreme cases continous flow gave strong positive CO_2 efflux values while headspace gave negative values due to CO_2 adsoprtion. All of these problems are thought to be related to carbonate equilibria and release or sorption of CO_2 by nonbiological processes. It was concluded that continuous flow measurements may be satisfactory for high activity materials above pH 7 which contain carbonates but the method is likely to result in gross overestimates in low activity regimes. Headspace analysis is preferred but the selection of the measurement interval is critical as the system must be in CO_2-carbonate equilibrium.

In summary, laboratory tests indicated that DIMR could be successfully treated in the Bio-Reactor and the recommended conditions were as follows:

- Salinity: reduce to 1 - 4 dS m^{-1}.
- Nitrogen: initial loading at 600 - 800 μg N g^{-1} as either NO_3-N or NH_4-N with no further additions.

- Phosphorus: standard 10 - 20% of N.
- Temperature: 30 to 35°C throughout the year.
- Inoculation: essential at elevated temperature, use 1% or more of treated Waste 1.
- Aeration: passive may be sufficient in shallow beds.
- Aggregation: size probably unimportant below 10 mm.
- Leaching: not necessary after salt removal.
- Anticipated hydrocarbon reduction: reduction from 10% to less than 2% within 4 to 6 months.

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1. INTRODUCTION TO THE SECOND YEAR OF EXPERIMENTATION

Bioremediation is a family of processes (in situ, ex situ solid, ex situ slurry) that utilize microorganisms to treat, detoxify and eliminate some of the large volumes of hydrocarbon wastes produced by the oil and gas industry. Other nonbiologically-based alternative processes are also available to treat wastes but none can promise so benign a solution to reducing degradable waste products. Major attractive features of bioremediation are the low tech, low capital requirements with resulting low treatment costs and the potential to scale-up with a minimum of engineering adjustments to handle large volumes of contaminated materials.

Carried to the ultimate, bioremediation would reduce unwanted hydrocarbons to water, CO_2 and biomass. However, research is required to thoroughly understand the underlying principals and limitations (e.g. rate limiting steps, Hamar 1993) such that the process is confidently reliable, i.e., it will consistently work effectively with a variety of wastes, and residuals and quasi-stable intermediate products (e.g. soil organic matter) will find niches in the environment, be it landfills or landscapes.

Within the scope of this project, the wastes proposed for treatment were many small volumes contaminated with hydrocarbons and salts (e.g. flare pit sludges) rather than a few very large volume wastes (e.g. decommissioned refineries). As a consequence, the process must be flexible, adaptable and require minimal pretreatment evaluations, avoiding extensive, expensive treatability or feasibility studies. Conceptual characteristics of the process will then include:

- simple treatability protocols;
- abilities to handle complex wastes composed of diverse unknown organic and inorganic compounds;
- ability to stabilize wastes and intermediate decomposition products to prevent transport to other receivers, especially ground and surface waters;
- detoxification of the wastes but not complete mineralization.

With regard to the last point, it should be recognized that two endpoints for waste treatment can be defined and used. One is risk-based, the other technology-based and it is highly unlikely that bioremediation can achieve the latter residue standards as it would be defined by incineration (Day 1993). However, biological treatment may well meet risk-based residue levels and thus ecotoxicological testing would become an integral part of the process to answer the question when enough treatment is enough. The method of choice for treatment of particular hydrocarbon wastes then

might be bioremediation over incineration in a trade-off of low cost for long treatment time using risk-based clean-up criteria. An example of this will be shown for the wastes currently being treated in the Bio-Reactor and Bio-Pile in this project.

In a broad sense, the laboratory studies conform to the following protocol.

- Physical factor "fix" - Oily wastes are often in very poor physical condition and this must be remedied by aggregation and stabilization to create a soil-like material with a continuum of micro- and macro-pores that is conducive to biological activity.

- Life support tests - Toxicity should not be so excessive that normal heterotrophic activity, as demonstrated by CO_2 production from organic matter mineralization, is nil. This may be accompanied by additions of N and P and a diverse microflora which may be near-absent in nonsoil based material (e.g. pit linings).

- Removal of water soluble untreatable material - In the case of the Bio-Reactor materials this refers to salts and requires a stabilized physical structure and leaching.

- Degradation activity enhancement - Once stabilized, the microbiologically non-toxic, low salinity material can be tested to determine what environmental factors can be modified to optimize the degradations rates of the hydrocarbons to then further reduce toxicity and eliminate unstable and decomposable contaminants. Factors to be considered here are temperature, and water and gas relations (CO_2 and O_2), inorganic nutrients, substrate availability and the value of manipulating the quality and quantity of the microflora.

The laboratory studies reported here deal largely with the latter point - enhancement of activity - and the factors selected for study were based upon the first years experimentation, both in the lab and field with the crude oil/topsoil waste (Danielson 1993; Johnson et al. 1994; Johnson and Danielson 1994). Key findings from the studies on the crude oil/topsoil waste included:

different bullets to be used here

- The oily waste could be processed and aggregated to produce material with excellent, stable physical structure.

- Salts could be easily leached out, collected and disposed of while maintaining the structural integrity of the waste.

- Light hydrocarbons were rapidly mineralized after charging the system with N fertilizer.

- Utilization of waste heat converted the Bio-Reactor into a 12 month-a-year operating facility.

- Increasing temperature above ambient resulted in increased oil degradation rates in the lab, but increases were less apparent in the field except under winter conditions.

- Internal nutrient cycling (N) in the latter stage of processing (>4 weeks, slow phase) was sufficient to

preclude the need for repeated fertilizer applications and minimized N losses.

- Passive aeration was sufficient to optimize gas relations (CO_2 out, O_2 in) in shallow (15 cm) treatment beds.

- Losses of volatile hydrocarbons were minimal, even when forced aeration was used.

- After one years of treatment about half the hydrocarbons had disappeared (4.3% → 2.5%), but additional time was required to reach desired stability and nontoxicity criteria.

- The native topsoil microflora ably degraded the less recalcitrant fractions of the oil.

- A large fraction of the oil resisted degradation, perhaps due to inherent recalcitrance, low bioavailability or the absence of effective microorganisms.

The second year of work began dawned with the selection of a new waste - Diesel Invert Mud Residues (DIMR); design and construction of a new field treatment facility - Bio-Pile; and development of new field equipment - aggregator and precision tiller. The second waste, DIMR, was slated to undergo lab treatability studies and then to be used in the Bio-Reactor to test long-held assumptions on the necessity for tillage and/or forced aeration to promote biodegradation. The Bio-Pile was a simplified version of the Bio-Reactor, intended to receive and further treat Waste 1 in a 1 m depth bed with provisions for aeration, heating and irrigation. The two new field equipment items were a high volume aggregator and a noncompaction rototiller. Field tests were to be conducted on the value of aggregation as well as the aeration treatments. Laboratory testing would be done to define the operating conditions of the Bio-Reactor containing DIMR and to determine if the recalcitrant crude oil fraction of Waste 1 could be altered in the Bio-Pile.

The objectives of the laboratory treatability/enhancement work in 1993/1994 were as follows:

1. To determine the potential of treating DIMR by bioremediation to reduce hydrocarbon content and eliminate toxicity.

2. To determine the response of the microflora to additions of N at various stages of hydrocarbon decomposition and to clarify fertilizer requirements for DIMR and weathered crude oil.

3. To determine the effects of forced aeration versus passive aeration on hydrocarbon decomposition and mineralization.

4. To determine if aggregate size would affect hydrocarbon degradation and mineralization.

5. To determine the optimum temperature for the degradation of DIMR and to determine if temperature-adapted microbial populations would affect degradation.

6. To determine if the addition of either soil containing species-rich microbial populations or commercially available inocula would alter rates of hydrocarbon mineralization.

7. To determine if leaching of DIMR or crude oil Waste 1 during the slow phase of decomposition would affect mineralization rates.

8. To obtain estimates of microbial biomass of DIMR during the early stages of decomposition and to establish if microbial efficiencies change as substrates weather.

9. To examine critically the methodology for determining CO_2 efflux rates by the continuous flow and headspace methods.

2. GENERAL METHODS AND MATERIALS

2.1 Chemical and analytical methods

The methods used to characterize the chemical characteristics of the DIMR were largely those used on Waste 1 (Danielson 1993) and are as follows.

- Soil pH = 1:2 soil:water, w/w
- Soil $EC_{1:2}$ = 1:2 soil:water extract, conductivity meter
- Soil EC_{SAT} = Estimated by 2 x $EC_{1:2}$
- Loss on ignition = 400°C for 24 h
- Calcium carbonate equivalent = acetic acid method (Moore et al. 1987)
- Total C = Leco C analyzer
- Total N = H_2SO_4 + H_2O_2 digestion, Technicon autoanalyzer
- NH_4-N = 2M KCl extraction, Technicon autoanalyzer
- NO_3-N = 2 M KCl extraction, Technicon autoanalyzer
- PO_4-P = 0.03 N NH_4F + 0.03 N H_2SO_4 extractant, Technicon analyzer
- Total Petrolelum Hydrocarbons (TPH, DIMR Waste 2) = Performed by AEC; methylene chloride extraction, quantification against a diesel standard by gas chromatography
- Oil content (Waste 1) = Performed by AEC, toluene extraction, gravimetric quantification

2.2 Incubation and measurement of CO_2 efflux

The rate and amount of CO_2 produced from incubating samples of the wastes were used as estimates of hydrocarbon mineralization and degradation. In that the subsoil was probably very low in organic matter (no uncontaminated samples were available to determine actual background levels), CO_2 efflux was a reasonable estimate of mineralization. However, if rates were very low, the native $CaCO_3$ may have resulted in large errors due to the production (or sorption) of chemical CO_2. This factor is discussed in detail in Part 9.

Sixty grams dry weight equivalent of waste samples were sieved through a 2 or 4 mm sieve (unless noted otherwise), placed in plastic bags, fertilizer and other additives added if called for, moisture adjusted to 20 - 22% and mixed thoroughly. The moist samples were then placed in 4 x 25 cm glass incubation tubes, the sample held in place with foam stoppers and

incubated in plastic boxes at the appropriate temperature (22°C unless noted otherwise). Measurements of CO_2 efflux were made by placing the tubes on an automatic switching device (maximum 18 tubes) in which CO_2-free air was passed through the samples continuously and each sample was on the measurement cell for 3 min of each hour and the CO_2 concentration measured with an IRGA. Flow rates through the tubes were about 210 ml min^{-1} and the rate measurements were usually taken after 4 h when efflux rates were nearly stable. Total incubation times generally ranged from 8 to 16 weeks; moisture contents were kept at initial levels by periodic weighing the tared samples and replenishing lost water.

A second method for measuring CO_2 production in the temperature experiments was by headspace analysis (Danielson 1993). Samples were prepared as above, placed in 2 quart (1690 ml) Gem jars and incubated with lids with an open 5 mm diameter tube. For the CO_2 production measurements the lid was replaced with another lid with a rubber septum and the jar sealed. An air sample was removed, injected into the IRGA and the jars incubated at the appropriate temperature for about 1 h when a second sample was withdrawn. CO_2 production was calculated by the difference in CO_2 concentration between the two sample times (0 and hour 1). The effects of using other measurement periods are given in Part 9.

2.3 Characteristics of DIMR

The TPH of the DIMR as sampled from the rig storage tank at the Nevis Bio-Reactor field site exceeded 10% and the electrical conductivity was 37 dS m^{-1} (Table 2.3-1). The clay loam textured material was heavily contaminated and contained virtually no available or reserve nutrients. Nonhydrocarbon organic matter was not determined but appeared to be very low in keeping with the subsoil-drilling waste origin of the solid phase.

2.4 Characteristics of Waste 1, crude oil/topsoil, treated for 1 year

Detailed characteristics of the treated Waste 1 can be found in Johnson et al. (1994). As received in the lab, the $EC_{1:2}$ = 1.9 dS m^{-1}, pH = 7.1, oil content = 2.9%, NO_3-N = 313 μg g^{-1}, NH_4-N = nil, PO_4-P = 103 μg g^{-1}, $CaCO_3$ equivalent = 2.5% and respiration = 3 μg CO_2-C g^{-1} h^{-1}. Thus nutrient levels were high, substantial oil remained, and microbial activity was low.

Table 2.3-1. Characteristics of Diesel Invert Mud Residues (DIMR) in subsoil prior to any treatments and after aggregation and leaching for laboratory experiments.

Total Petroleum Hydrocarbons (TPH) prior to treatments	108,000 mg kg^{-1}
TPH of lab samples	50000-70000 mg kg^{-1}
EC_{SAT} prior to any treatments	37 dS m^{-1}
EC_{SAT} after leaching	1-2 dS m^{-1}
$pH_{1:2\ (H2O)}$	7.4
Calcium carbonate equivalent	6.9%
Loss on ignition	6.8%
Total carbon	3.9%
Total N	0.05%
Extractable NO_3-N + NH_4-N	1 $\mu g\ g^{-1}$
Extractable PO_4-P	$<1\ \mu g\ g^{-1}$
C/N ratio	78
Clay	33%
Silt	45%
Sand	22%

Note: Further information on DIMR can be found in Johnson et al. (1994). Differences in field and lab TPH is attributed to losses while handling and leaching. Analytical methods can be found in Danielson (1993).

3. TREATABILITY AND MICROBIOLOGY OF DIMR

3.1 Introduction

Oil field wastes are usually difficult to chemically characterize as their histories are often unknown and the inputs to pits and sumps are loosely controlled resulting in extremely complex materials. It is thus uncertain if the wastes can be treated effectively by biological degradation without actual laboratory trials due to the possible presence of toxic materials, possible lack of readily decomposable components or other unfavorable chemical constituents. Based on the lab and field studies with Waste 1, crude oil in topsoil, simple incubation trials in the lab can serve as accurate predictors of the rates of oil disappearance under field conditions in the Bio-Reactor.

As a first step in evaluating the usefulness of bioremediation in treating DIMR to reduce hydrocarbon content and ecosystem toxicity, degradation of hydrocarbons was monitored in the laboratory by measuring CO_2 efflux (i.e. mineralization). As the supporting matrix was subsoil pit material, it was assumed that little native organic matter was present and that microbial activity was dependent upon hydrocarbons for carbon and energy sources. In addition, the response to N and P was determined during the initial 8 weeks of treatments as an indicator of mineral nutrient requirements and deficiencies. Microbial response to a readily metabolized C substrate, glucose, and microbial biomass changes were also measured as indicators of total microbial population sizes and functional compositional changes as the hydrocarbons weathered.

3.2 Experimental conditions

The DIMR was aggregated by the AEC without the use of any amendments and salts were leached from the material by saturating the material from below in buckets with 250 μm mesh stainless screen replacing the bottom of the buckets. The material was saturated and drained three times, partially dried (approx. 12 h) in a hood to a workable moisture content (approx. 20%), placed in plastic bags in plastic buckets and stored at 3°C. For the two parts of this experiment, the incubation tubes (70 g tube^{-1}) were prepared as in Part 2.2 with additions of $Ca(NO_3)_2$ (400 μg N g^{-1}) and KH_2PO_4 (80 μg P g^{-1}) and incubated at 22°C.

In the first part, 6 tubes were incubated for 8 weeks with periodic measurements of CO_2 efflux to obtain the general decomposition pattern for the DIMR. In the second part 18 tubes were frozen and at six times three replicates were thawed and incubated so that at the end of 8 weeks a range of ages were simultaneously available for respiration measurements. Twenty

gram subsamples were used to determine basal respiration with and without the addition of 200 μg N g^{-1} and 40 μg P g^{-1}, followed by the addition of 8 mg glucose g^{-1} to determine substrate induced respiration (SIR) and microbial biomass (Anderson and Domsch 1978).

3.3 Results and Discussion

The CO_2 efflux pattern for the DIMR was similar to that found for Waste 1, crude oil in topsoil (Fig. 3.1-1; Fig. 10.1.1 in Danielson 1993). There was a brief 2 to 3 day adaptation period, followed by a period of high activity for about 7 days and then relatively stable low activity for a period exceeding two months. These CO_2 efflux values include both CO_2 from biotic (mineralization) and abiotic (chemical) sources but the abiotic portion probably did not exceed 2 μg $g^{-1}h^{-1}$ for the 2 month period (see Fig. 6.1-2). The rates of CO_2 efflux from this DIMR source was similar to that observed in an earlier experiment on two other DIMR sources, when rates after 8 weeks were 4 - 7 μg CO_2-C g^{-1} h^{-1} (Visser et al. 1987). This test indicated that the environment of the aggregated and leached DIMR was favorable to support bioremediation and that the hydrocarbons were subject to biodegradation.

Direct observation of the incubating DIMR through the walls of the glass tubes showed that after 6 days a species of *Penicillium* was growing and sporulating throughout the material and that microcolonies (200-500 μm diameter) of bacteria were common and visible on the surface of the aggregates. Further visual observations from 2 to 4 weeks showed that fungi failed to develop any more mycelium after the initial burst of activity but that the "pustule-like" bacterial colonies, composed of 2 x 1 μm rods, continued to increase in abundance for at least 2 weeks at which time visible growth increases ceased.

The material that was frozen and withdrawn from the freezer at weekly or biweekly intervals to obtain a time sequence exhibited a similar respiration profile although the initial peak was apparently missed (Table 3-1). Basal respiration (= CO_2 efflux) decreased throughout the 1 to 8 week measurement period. Addition of N and P resulted in a small stimulation of respiration but slightly inhibited respiration at the end of the eighth week.

As would be expected, the addition of glucose increased total respiration (SIR) throughout the 8 week incubation. The addition of N and P increased respiration in the second and third week but had no effect thereafter, and there was a small inhibition after 8 weeks just as with the non-glucose amended DIMR.

As estimated by KCl extraction, the added NO_3-N was very rapidly consumed in the first week, correlating with the initial growth flush. Both NO_3-N and NH_4-N were present in very small quantities after the first week although there was not a N deficiency for microbial activity as indicated by the small response of the microflora to N and P additions. The

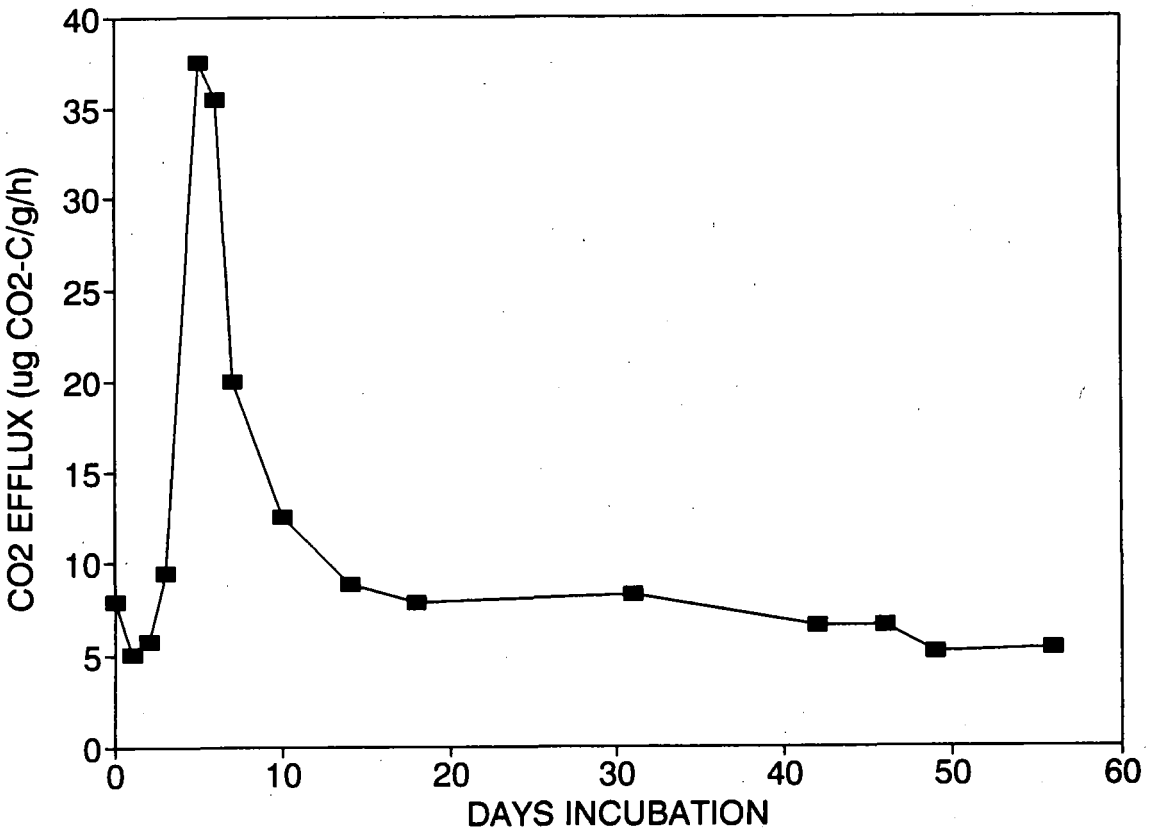

Fig. 3.1-1. Total CO2 efflux pattern of DIMR with 400 ug N g-1 (n=6).

Table 3-1. Basal respiration (BR), substrate induced respiration (SIR), microbial biomass (MB), and KCl extractable NO_3-N and NH_4-N in DIMR incubated for 0 to 8 weeks tested with and without N and P additions (± SD, n = 3).

Incubation time	BR	BR + N & P	SIR	SIR + N & P	MB	MB + N & P	BR/MB	BR/MB + N & P	NO_3-N	NH_4-N
(weeks)	($\mu g\ CO_2$-C $g^{-1}\ h^{-1}$)				(mg C $100g^{-1}$)		(x 10^3)		($\mu g\ g^{-1}$)	
0	3.3 ± 4.6	4.7 ± 5.8	10.7 ± 7.4	10.7 ± 10.2	80 ± 56	80 ± 76	4.1 ± 1.3	5.9 ± 0.8	329 ± 167	2 ± 0.3
1	11.5 ± 0.9	18.2 ± 1.8	24.0 ± 1.6	32.4 ± 1.6	179 ± 12	241 ± 12	6.4 ± 0.4	7.5 ± 0.6	0 ± 0	2 ± 2.0
2	9.2 ± 0.5	12.1 ± 1.4	20.2 ± 1.1	24.8 ± 3.2	151 ± 9	185 ± 24	6.1 ± 0.5	6.6 ± 0.2	0.3 ± 0.3	1 ± 0.2
4	7.6 ± 0.3	8.7 ± 1.6	17.8 ± 1.3	17.4 ± 3.5	133 ± 9	130 ± 26	5.7 ± 0.3	6.7 ± 0.5	0 ± 0	1 ± 1.0
6	6.4 ± 0.2	7.5 ± 0.6	13.3 ± 1.2	14.3 ± 1.3	100 ± 9	107 ± 10	6.5 ± 0.9	7.0 ± 0.1	0 ± 0	5 ± 6.0
8	5.8 ± 0.3	5.0 ± 0.6	11.3 ± 0.7	9.1 ± 1.1	84 ± 5	68 ± 8	7.0 ± 0.6	7.3 ± 0.1	3 ± 2	3 ± 4.0

failure of KCl extractable N to indicate the true nature of microbially available N has been demonstrated in a forest soil (Nordgren 1992). Nordgren estimated that microbially available N was 6 to 18 times greater than indicated by the standard KCl extraction. It is clear that DIMR hydrocarbon mineralization and/or mineralization of hydrocarbon-derived biomass or soil organic matter (SOM) proceeded at very low mineral N levels and neither N nor P were rate limiting.

Estimates of microbial biomass (i.e. living fungi and bacteria that metabolically respond to added glucose) peaked in the first week and decreased to about one-half the maximum value by week eight. The biomass values obtained for the DIMR are of the same magnitude as values obtained for 22 agricultural soils by either the SIR method (range 17-138 mg C 100 g^{-1}) or the fumigation method (range 13 to 163) (Martens 1987). Estimates of biomass are lacking for hydrocarbon-containing soils but it would appear that the total amount of active microbial tissue in such materials is similar or somewhat higher than that in normal mineral soils.

The ratio of basal respiration (BR) to microbial biomass (MB) is an index of the efficiency of C utilization, i.e, amount of C lost as CO_2 per unit of microbial tissue. The ratio could differ due to the composition and physiological state of the microflora as well as to changes in substrate quality and it is thought that fungi are more C-conservative than bacterial with the former losing about 60% of the C as CO_2 in contrast to 90% by the bacteria (Sakamoto and Oba 1994) and thus broad shifts in the taxonomic composition of the microflora might be reflected in changes in biomass efficiency. During the 8 week incubation of the DIMR, the BR/MB ratio remained relatively constant, suggesting that no major changes occurred. The addition of N and P had no or very little effect on the efficiency values indicating that high nutrient levels did not affect C-utilization.

In summary, lab tests indicated that microbial degradation of DIMR followed a two stage activity profile and that if a large quantity of N was added at the beginning of the process, it was self-sustaining albeit at a low level. Whether this level can be increased will depend on what the rate-limiting factors are - inherent substrate recalcitrance (unfavorable biochemistry), bioavailability (substrate accessibility) or bioactivity (including genetic limitations of the indigenous organisms) (Blackburn and Hafker 1993).

4. ASSESSMENT OF FULL TERM (CRADLE TO GRAVE) ENVIRONMENTAL FACTORS

4.1 Aggregate size effects on CO_2 efflux and TPH

4.1.1 *Introduction*

In order for the Bio-Reactor or any similar biological unit to operate successfully to bioremediate hydrocarbon contaminated materials, the matrix must be soil-like in character. Implicit with this maxim is that the material must possess a stable structure. Structure has been defined simply as the arrangement of mineral particles and pores and includes aggregates (Oades 1993). Just as good crop growth is dependent upon aggregates in the 1 to 10 mm range (Tisdall and Oades 1982) the bioremediation process is likely to be dependent upon a similar size range which insures that large macropores are present for periodic mass movement of water and which readily drain to maintain aerobic conditions. In addition, micropores are essential to retain water and to allow diffusion of soluble materials in and out of aggregates.

The microporosity of aggregated materials becomes a two-edged sword in the bioremediation process as micropores are essential for water storage and as diffusion pathways for soluble hydrocarbons but water-filled pores will severely limit both diffusion of O_2 in and CO_2 out of aggregate interiors. Further, the presence of microanoxic sites may be beneficial as it allows both aerobic and anaerobic processes to proceed simultaneously in very close proximity and to complement one another in the degradation of certain organic compounds (Focht 1992). The question of optimum aggregate size for a particular oily waste then must strike a compromise; a value difficult to determine without experimental evidence. The objective of this experiment was to determine the effect of aggregate size on hydrocarbon degradation of fresh DIMR and Waste 1.

4.1.2 *Experimental conditions*

Samples of DIMR and Waste 1 (crude oil/topsoil, stored at 3°C for 6 months) were sorted by sieving into three aggregate sizes, < 2 mm, 2 - 4 mm, and > 4 mm diameter. Three replicates of each fraction were supplemented with $Ca(NO_3)_2$ (400 μg N g^{-1}) and KH_2PO_4 (80 μg P g^{-1}), loaded into respiration tubes, incubated at 22°C and CO_2 efflux monitored for 8 weeks. Following incubation solvent extractable hydrocarbons were determined by AEC and extractable NH_4-N and NO_3-N measured.

4.1.3 *Results and discussion*

Throughout the incubation period the highest rates of respiration occurred in the smallest aggregate size fraction for both wastes (Figs. 4.1-1 and 4.1-2). The differences in

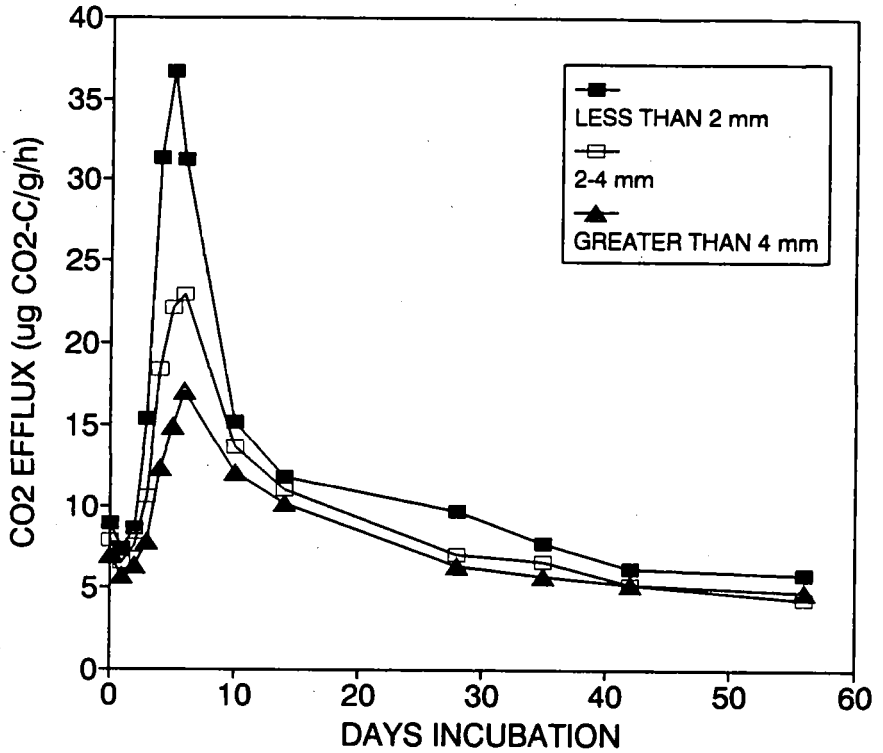

Fig. 4.1-1. Total CO2 efflux from DIMR aggregates in three size classes.

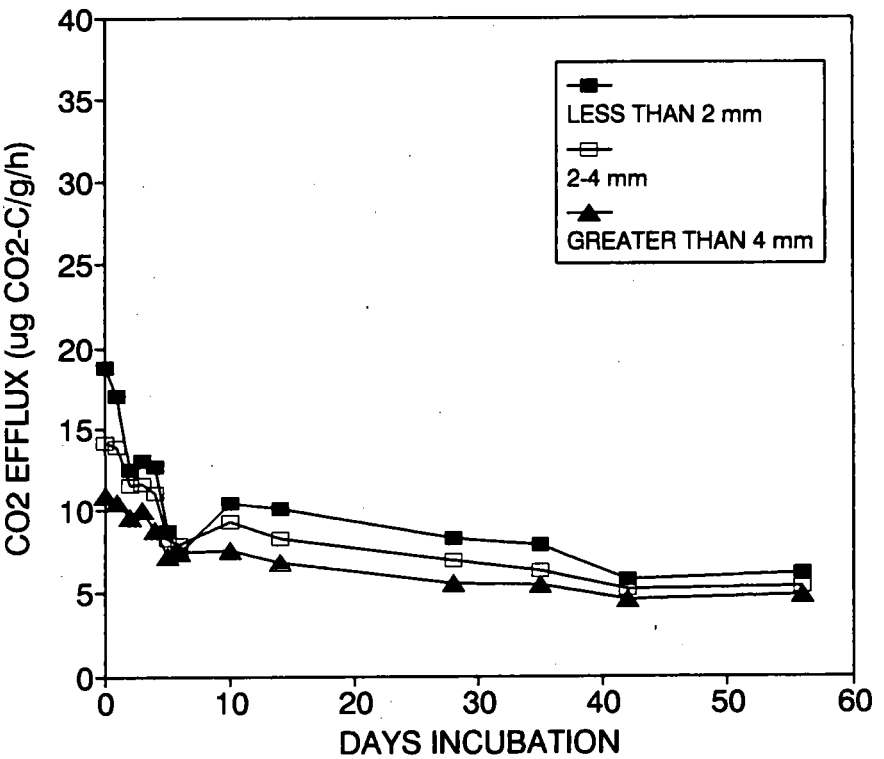

Fig. 4.1-2. Total CO2 efflux from crude oil / top soil aggregates in three size classes.

respiration rates among the three aggregate sizes decreased with time until the differences were minimal after 8 weeks.

The CO_2 efflux pattern matched the visible development of the microflora on aggregate surfaces. Some hyphal growth was visible on all treatments after 3 days and maximum development had occurred after 8 days. All DIMR aggregates were heavily colonized by a *Penicillium* species but most obvious was the massive formation of orange pustulate bacterial microcolonies on all aggregate surfaces. Development was so extensive that the orange color was visible to the unaided eye. Further growth was not noted but both *Penicillium* and the bacteria remained on the surfaces throughout the incubation period. Mycelial development on the crude oil/topsoil material was much less extensive and no bacterial colonies were observed.

The losses of CO_2 reported in Figs. 4.1-1 and 4.1-2 represent both CO_2 from abiotic and biotic sources. If estimates of abiotic CO_2 are subtracted from total CO_2 efflux, the amount of CO_2 evolved was about 36% less for the large fraction than for the less than 2 mm fraction for both materials (Table 4.2-1). When adjustments are made for differences in hydrocarbon content of the different size fractions (see Table 4.1-2), estimates of the total hydrocarbons mineralized are 23% more in the fine fraction of DIMR and 33% more for the fine crude oil fraction than in the coarse fraction. The utilization of NO_3-N in the DIMR also indicates that more degradation occurred in the small aggregates than in the larger fractions (Table 4.1-1).

In contrast to the CO_2 data, hydrocarbon losses as estimated by solvent extraction indicated that there was little effect of aggregate size or that perhaps more hydrocarbons were lost from the larger aggregates (Table 4.1-2). However, there was considerable variability in the data and any conclusions from these data are tenuous. It would appear that aggregate size had little effect on the disappearance of DIMR but that CO_2 efflux was affected by size. CO_2 and TPH data for the crude oil is conflicting and cannot be resolved, especially as the data indicates that nearly all the carbon that disappeared could be accounted for in the CO_2, i.e, no volatiles or biomass - very unlikely.

The large quantities of bacteria on the surface of DIMR aggregates suggests that the surface was a prime site for hydrocarbon degradation. This seems reasonable, as on the aggregate surfaces compared their interiors, CO_2 would be low and O_2 concentrations high at least when total microbial activity is high, i.e., in the first 2 weeks.

With regard as to what are the factors limiting decay rates, it is proposed that diffusion distances and therefore aggregate size is very influential when activity is high. In a water column over sediments the aerobic layer may only be 10 μm thick when bacterial densities are high (Focht 1992) as they obviously were in the DIMR and thus aerobic activity restricted to the surface. As a

Table 4.1-1. Total CO_2-C, biotic CO_2-C, and KCl extractable N in DIMR and crude oil/topsoil aggregates incubated for 8 weeks.[1]

Material	Aggregate size	Total CO_2-C evolved	Total % of <2 mm fraction	Biotic CO_2-C evolved	Biotic % of <2 mm fraction	HC-C lost as CO_2-C	NO_3-N	NH_4-N
	(mm)	(mg g^{-1})		(mg g^{-1})		(%)	($\mu g\ g^{-1}$ ± SD)	
DIMR	<2	14.9 ± 0.4	100	12.6	100	27	1 ± 1	5 ± 4
DIMR	2-4	11.8 ± 0.3	79	9.5	75	26	35 ± 14	2 ± 1
DIMR	>4	10.4 ± 0.2	70	8.1	64	22	76 ± 23	3 ± 1
Crude	<2	11.6 ± 0.7	100	8.6	100	21	115 ± 14	2 ± 1
Crude	2-4	10.0 ± 0.3	86	7.0	81	17	147 ± 17	4 ± 2
Crude	>4	8.4 ± 0.5	72	5.4	63	14	90 ± 34	3 ± 1

[1] Abiotic DIMR assumed to be a total of 2.3 mg CO_2-C g^{-1} (Part 6.1), and abiotic CO_2 from crude oil/topsoil assumed to be 3.0 mg (Part 10.1 in Danielson 1993).

Table 4.1-2. Hydrocarbon contents of three aggregate sizes of crude oil/topsoil and DIMR before and after 8 weeks incubation.

Material	Aggregate Size (mm)	Initial TPH (% ± SD)	Final TPH (%)	TPH Loss (%)
DIMR/subsoil	<2	5.1 ± 0.5	3.1 ± 0.5	38 ± 10
DIMR/subsoil	2-4	4.4 ± 0.5	2.3 ± 0.1	47 ± 2
DIMR/subsoil	>4	4.3 ± 0.7	2.5 ± 0.1	43 ± 4
Crude Oil/topsoil	<2	4.8 ± 0.2	3.9 ± 0.1	18 ± 1
Crude Oil/topsoil	2-4	5.0 ± 0.3	3.8 ± 0.2	25 ± 5
Crude Oil/topsoil	>4	4.8 ± 0.4	3.6 ± 0.1	26 ± 2

consequence, water-soluble C-substrates must be transported to the surface via diffusion and this diffusion rate may be the limiting factor.

As activity decreases and as highly soluble hydrocarbons are consumed, gas relations within aggregates would improve and the aerobic zone would enlarge toward the centre of the aggregates. Diffusion rates would assume less importance and desorption rates of slightly soluble, high molecular weight hydrophobic compounds would become the limiting factor. In a slurry system composed of microaggregates desorption rates were shown to be rate limiting, not the bioactivity (Rijnaarts et al. 1990). Further, Weissenfel et al. (1992) cleverly showed that bioavailability (desorption) clearly limited degradation of PAH's. No degradation of PAH's occurred in contaminated soil but once the PAH's were solvent extracted and reintroduced unchanged chemically, 80% of PAH's were rapidly degraded.

If these proposals are true then aggregate size would become less influential as hydrocarbon degradation proceeds and bioavailability sensu Blackburn and Hafker (1993) and not bioactivity limit degradation in the slow second stage. In the short term small aggregate size had a positive influence on the rate of CO_2 efflux just as short term (24 day) positive effects were found by Mott et al. (1990) on preformed aggregates coated (i.e., surface application, not inside) with oil. In the latter study differences in respiration rates with different sized aggregates were attributed to surface area differences, however these results are not necessarily applicable to aggregates with oil uniformly distributed inside or to long incubations.

Ultrasonic disruption of oily aggregates produced very similar CO_2 efflux patterns as was found with the DIMR (Rasiah et al. 1992). The greater the energy used to break up the aggregates, the greater the respiration rate, the differences being attributed to increased bioavailability. The prolonged effect (85 days) would suggest that desorption rates were increased as well as possibly diffusion distances were decreased (aggregate sizes not reported).

In summary, smaller aggregates of either DIMR or crude oil produced higher rates of respiration than did larger aggregates over an 8 week period during which bacteria and fungi developed profusely on aggregate surfaces. However, residual hydrocarbon analyses failed to confirm that aggregate size affected rates of oil disappearance. The apparent discrepancy between these two estimates remains to be resolved. None the less, the CO_2 rate differences were real and attempts to explain them are based upon diffusion rate limitations when microbial activity is high and desorption rates once high solubility C-sources have been exhausted which leads to nil effects of aggregate sizes as oil weathers.

4.2 Effect of fertilizer type on DIMR mineralization and degradation

4.2.1 *Introduction*

Nitrogen, and to a lesser degree phosphorus, is unquestionably one of the critical environmental variables that can and must be managed to optimize degradation rates of oily wastes. Unlike plant materials, the most abundant decomposition substrates, in which the C and energy sources are accompanied by a host of mineral nutrients, hydrocarbons are devoid of any nutrients and these must be provided in the substrate, soil or geologic material, or supplemented by intent or decomposition times will measure in millenia. Despite the recognized importance of N and P, applications of fertilizer to bioremediation processes are usually made with no regard for actual microbiological demands but rather according some inflexible formula such as hydrocarbon-C/fertilizer-N ratios. Repeated applications may be made again on faulty premises, such as assumed deficiencies or inappropriate soil tests.

Unlike plants, in which nutrient deficiencies can be seen and measured using fertilizer trials and crop yields, microorganisms do not reveal deficiencies or needs with such congenial ease. Biomass measurements of plants are a straightforward but biomass measurements of the microflora are difficult, time consuming, usually indirect and fraught with uncertainty. Thus respiration is the method of choice as responses to nutrients can be rapidly determined over a long period of time. Nonetheless, few experimental tests have been conducted to determine N demands in hydrocarbon dominated systems.

In previous tests with crude oil/topsoil wastes no differences were found in respiration responses to NO_3-N versus NH_4-N and an initial loading of 400 to 800 μg N g^{-1} resulted in maximum oil mineralization rates (Danielson 1993). Additional fertilizer supplements usually had no effect or inhibited respiration. However, there was a suggestion that a soluble complete fertilizer might have additional benefits. Further, it has been proposed that slow release fertilizers might be superior to soluble fertilizers. The objective of this experiment was to evaluate, by means of monitoring soil respiration, the effects of fertilizer levels and types on DIMR degradation utilizing a single preincubation application.

4.2.2 *Experimental conditions*

General conditions were 22°C, 20.4% moisture, sample size 60 g O.D.W. and three replicates per treatment. The four fertilizers were (1) $Ca(NO_3)_2$ + KH_2PO_4 (N = 400 and 800 μg g^{-1}, P = 80 and 160 μg g^{-1}); (2) Plant Prod 28:14:14 (400 and 800 μg N g^{-1}); (3) number 1 + Osmocote Microprill 17:9:13 (each at 400 μg N g^{-1}); and (4) number 2 + Osmocote (each at 400 μg N g^{-1}). The N source of the Plant Prod was 75% urea, 14% nitrate and 10% ammonium. Nitrogen in Osmocote is 9.3% NH_4-N and 7.7% NO_3-N and the release rate was 70 - 90 days. All fertilizers were mixed into the DIMR with no further additions.

4.2.3 *Results and discussion*

The pattern of CO_2 efflux of from DIMR for all fertilizer types was

similar with the initial week at a high rate and a relatively slow and steady rate thereafter (Fig. 4.2-1). The 800 μg N g^{-1} level of Plant Prod 28:14:14 resulted in the highest rate of CO_2 efflux for the first two weeks but after that time the use of a complete fertilizer had no advantage over simple N and P sources. At the end of 8 weeks respiration rates were similar in all treatments.

Cumulative CO_2 efflux was consistently highest with 800 μg N g^{-1} Plant Prod and lowest with the 400 level of the same fertilizer (Fig. 4.2-2). The other two fertilizer types were intermediate with no differences between 400 and 800 μg N g^{-1} as nitrate or between slow release and Plant Prod or nitrate. The reason for the poor performance of the 400 N Plant Prod may be due to losses of N as NH_3 after urea hydrolysis and microenvironmental increases in pH. Large losses of NH_3 can occur when urea is applied to the soil surface or in excessive amounts (Wild 1988).

To determine if adding Plant Prod increased bulk pH, rates of 400 and 800 μg N g^{-1} were added to DIMR and the pH measured after 4h, 1, 2, 3 and 4 days. After 1 day the pH had increased from 7.5 - 7.6 to 8.1 and by day 4 it had fallen to 7.2. DIMR with no fertilizer added rose to 7.9 and remained at that pH. Although direct losses of NH_3 were not measured it is possible that NH_3 losses occurred and a sufficient quantity was lost to produce a deficiency at the 400 μg N level but not at the 800 μg N level.

Extractable N measurements at the end of the incubation period showed that about 500 μg N g^{-1} was the maximum amount consumed when NO_3 or NO_3 + Osmocote was used (Table 4.2-1). The small quantity of N remaining with the 800 μg N Plant Prod again suggests gaseous losses of N.

Visible microbial development was similar on all treatments and was not extensive. Small amounts of fungal mycelium were present in all tubes and by the end of two weeks, orange colonies consisting of bacterial rods were present in modest quantities on the surface of the aggregates in all tubes.

Methylene chloride extractable residual hydrocarbons were lowest in DIMR fertilized with 800 μg N Plant Prod and highest with the 400 μg N Plant Prod, reflecting the CO_2 data (Table 4.2-1). As in other experiments, the relationship between CO_2 efflux and TPH was sometimes not as closely linked as might be expected. In the two Osmocote treatments CO_2 efflux values were very close indicating similar mineralization rates but the TPH data suggested very different results for the two fertilizers. Whether the differences are due to experimental error or are real remains to be resolved.

In general these results suggest that the form of N has little effect on hydrocarbon mineralization as estimated by CO_2 efflux. Further, there appears to be no advantage to adding very high levels of N and P, at least in closed systems. Additional fertilizer may be beneficial if leaching

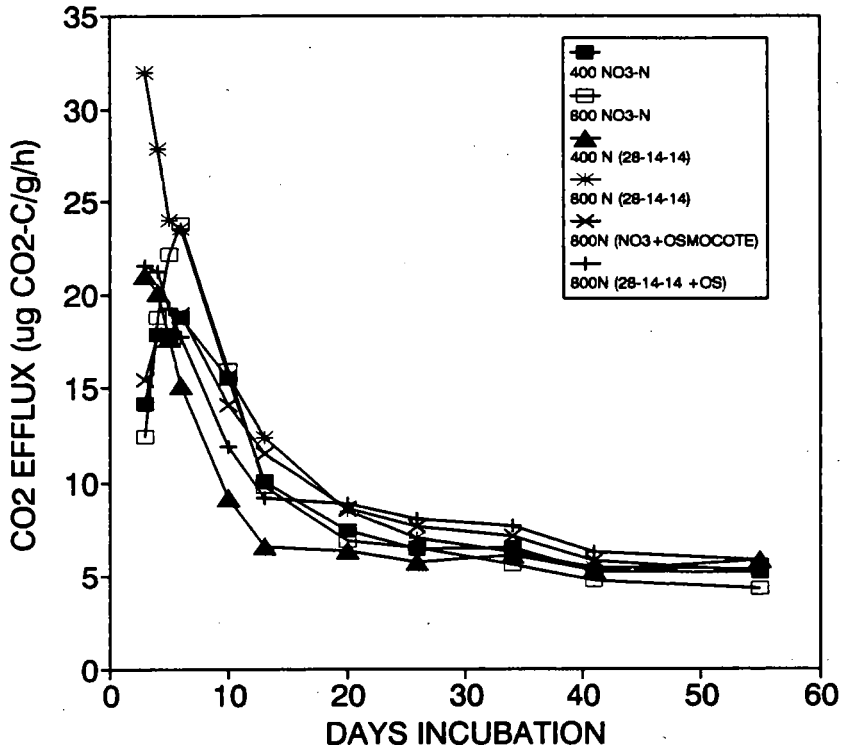

Fig. 4.2-1. CO2 efflux of DIMR with 400 or 800 ug N g-1 added.

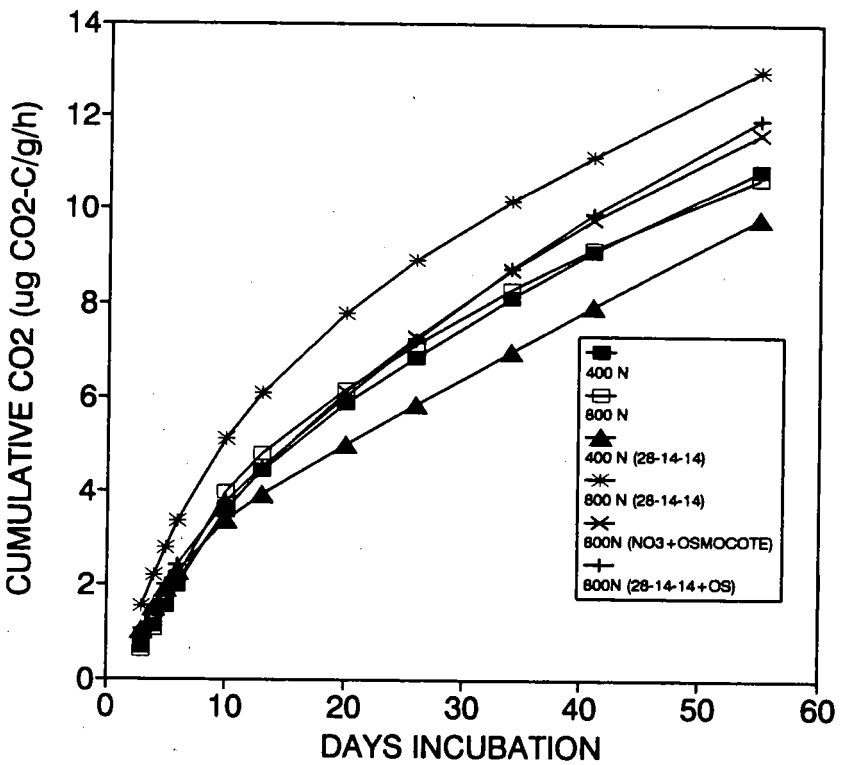

Fig. 4.2-2. Cumulative CO2 efflux of DIMR with 400 or 800 ug N g-1 added.

Table 4.2-1. Effect of $Ca(NO_3)_2$, Plant Prod 28:14:14, and Osmocote slow release fertilizer on degradation of DIMR.

Fertilizer	N added ($\mu g\ g^{-1}$)	Final NO_3-N ($\mu g\ g^{-1}$)	Final NH_4-N ($\mu g\ g^{-1}$)	Total CO_2-C efflux ($mg\ g^{-1}$)	Final TPH (%)	Final pH	Final EC ($dS\ m^{-1}$)
$Ca(NO_3)_2$	400	1 ± 1	4 ± 3	10.8 ± 0.1	4.2 ± 0.5	7.5	0.8
$Ca(NO_3)_2$	800	290 ± 42	5 ± 2	10.7 ± 0.5	4.1 ± 0.1	7.4	1.5
28:14:14	400	0	4 ± 1	9.8 ± 0.3	4.7 ± 0.3	7.4	0.7
28:14:14	800	11 ± 6	10 ± 7	13.0 ± 0.6	3.7 ± 0	7.4	0.8
$Ca(NO_3)_2$ + Osmo[1]	800	141 ± 38	62 ± 6	11.6 ± 0.6	4.6 ± 0.3	7.3	1.3
28:14:14 + Osmo[1]	800	79 ± 7	49 ± 8	11.9 ± 0.6	3.9 ± 0.2	7.3	1.1

[1] One half of N added in soluble form and one half in slow release form.

or gaseous losses are encountered; however, from Part 3 it is clear that N is very rapidly immobilized by the microflora and thus substantial losses could only occur within a few days of application.

Other laboratory tests have given apparently conflicting results even with much lower hydrocarbon contents. Rasiah et al. (1991) found increases in CO_2 efflux from a waste containing 0.75% hydrocarbons (and 40% carbonates) up to 3500 μg N g^{-1} and, in addition, NO_3-N outperformed urea and NH_4NO_3. In another study, Rasiah et al. (1992) made four repeated applications of 200 μg N g^{-1} and again found CO_2 response differed amoung N sources; even significantly different CO_2 efflux was reported amoung $Ca(NO_3)_2$, $NaNO_3$ and KNO_3 although moderate amounts of NO_3-N and NH_4-N were present in all treatments at the end of the 4 week incubation. No such subtle sensitivity to N forms has been noted in any studies with Waste 1 or DIMR and in fact N species performed almost identically with Waste 1 (Danielson 1993).

Unless remediation systems are operated under microbially selective conditions (e.g. high temperatures) which might possibly discriminate against NO_3-N utilizers, or leakage or gaseous losses (denitrification or as NH_3 if pH high) are anticipated, the N species appears to be unimportant. Further, there appears to be a maximum loading of N beyond which N fertilizers will be wasted. In the DIMR system containing about 6% hydrocarbons the limit was about 500 μg N g^{-1} or a C/N ratio of 100:1.

4.3 Effect of forced aeration versus passive aeration on DIMR degradation

4.3.1 *Introduction*

It is almost axiomatic to state that it is necessary to maintain aerobic conditions in bioremediation units to achieve reasonable treatment rates. However, it is not at all obvious what measures must be taken to maintain suitable gas relations or in the case of CO_2 concentrations, what the suitable gas relations are. Often times only O_2 concentrations are considered and the possible inhibitory effects of CO_2 are ignored, even though relatively low concentrations of CO_2 may have physiological consequences whereas O_2 levels must be severely depleted (1% of the partial pressure of O_2 in the atmosphere) to have any demonstrable effects (Wild 1988, p. 307-308).

It is certain that high concentrations (>10%) of CO_2 are strongly inhibitory to many species of bacteria and this phenomenon has been exploited by the food preservation industry (Daniels et al. 1985). However, the effects of low CO_2 concentrations on microbial activity has been less studied but may be of importance in bioremediation systems. Apparently, different soil populations can differ considerably in sensitivity to CO_2 as Macfadyen (1973) showed that aerobic respiration was strongly inhibited in different soils at CO_2 levels as low as 0.25% or 1.2% but others were tolerant to 5 to 10% levels. Data from Koizuma et al. (1991) suggest

ever greater sensitivity to CO_2 with bacterial activity inhibited below 0.1%. Although O_2 concentrations as low as 1% are often considered to be nonlimiting, Devinney and Islander (1989) concluded via modelling techniques that O_2 levels in land farms could often be limiting and that daily tillage was necessary to maintain aerobic conditions. They did not consider CO_2 effects on microbial activity.

Although the effects of gas relations on microbial activity of hydrocarbon degrading microorganisms is always considered to be important, critical assessments of this factor are lacking. It was not the objective of this study to resolve the intricacies of aeration but rather to make a gross assessment of the effects of constant forced aeration versus a passive system involving only gaseous diffusion.

4.3.2 *Experimental conditions*

Respiration was measured in glass tubes with sieved DIMR (<2mm initial TPH = 7.1 ± 0.8%) at 20% moisture and with the addition of Plant Prod 28:14:14 fertilizer at 800 μg N g^{-1}. Nine tubes were constantly aerated at a rate of about 3 Lh^{-1} per tube of humidified air and 9 tubes were incubated on the lab bench. After 4 weeks, four tubes were harvested for chemical analysis and the remaining five tubes were harvested after 8 weeks. Replicates for respiration were nine for 4 weeks and five thereafter.

4.3.3 *Results and discussion*

Rates of CO_2 efflux for the two aeration regimes were very similar throughout the 8 weeks incubation period and followed the familiar brief fast/long slow rate pattern (Fig. 4.3-1). Cumulative CO_2 patterns indicated that more CO_2 was released from the passive tubes than from the constantly aerated tubes (Fig. 3.2-2). This small difference could be an artifact of the measurement technique in which the tubes are on the automatic switching device for 4 h before measurement and CO_2-free air is passing through the tubes to remove accumulated gases. If equilibrium is not reached in this time, which may be the case with the calcareous material, it will result in small overestimates of respiration.

Estimates of TPH after 8 week incubation indicated that more hydrocarbons had disappeared under forced aeration conditions than under passive aeration (Table 4.3-1). There was a 68% decrease in hydrocarbons in the aerated DIMR and 59% in the diffusion aeration DIMR. This further suggests that the CO_2 efflux from the calcareous waste was not an ~~all-together~~ accurate estimate of TPH mineralization although additional volatile hydrocarbons could have been lost from the aerated DIMR.

All of the extractable NO_3-N and most of the NH_4-N was consumed before 4 weeks had elasped. The near complete disappearance of mineral N supplied as Plant Prod 28:14:14 agrees with the previous N fertilizer test (Part 4.2). Fungi were not observed growing on the aggregates but as in previous tests, orange bacteria clouded all water films and were the

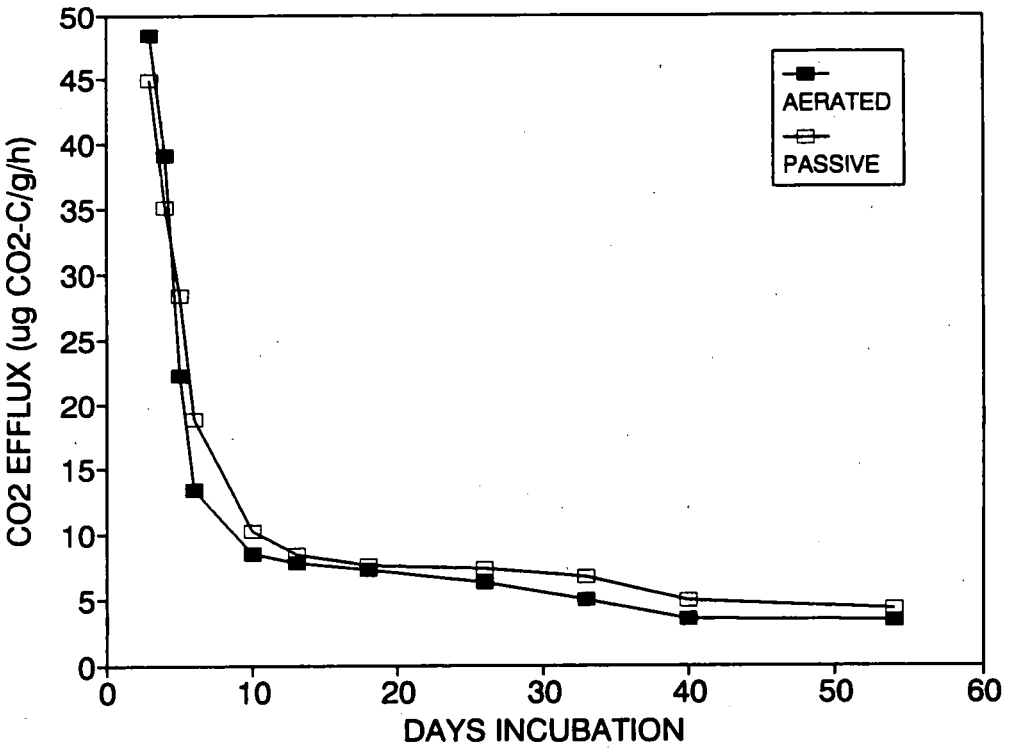

Fig. 4.3-1. CO2 efflux from DIMR (+ 800 ug N g-1) with forced or passive aeration.

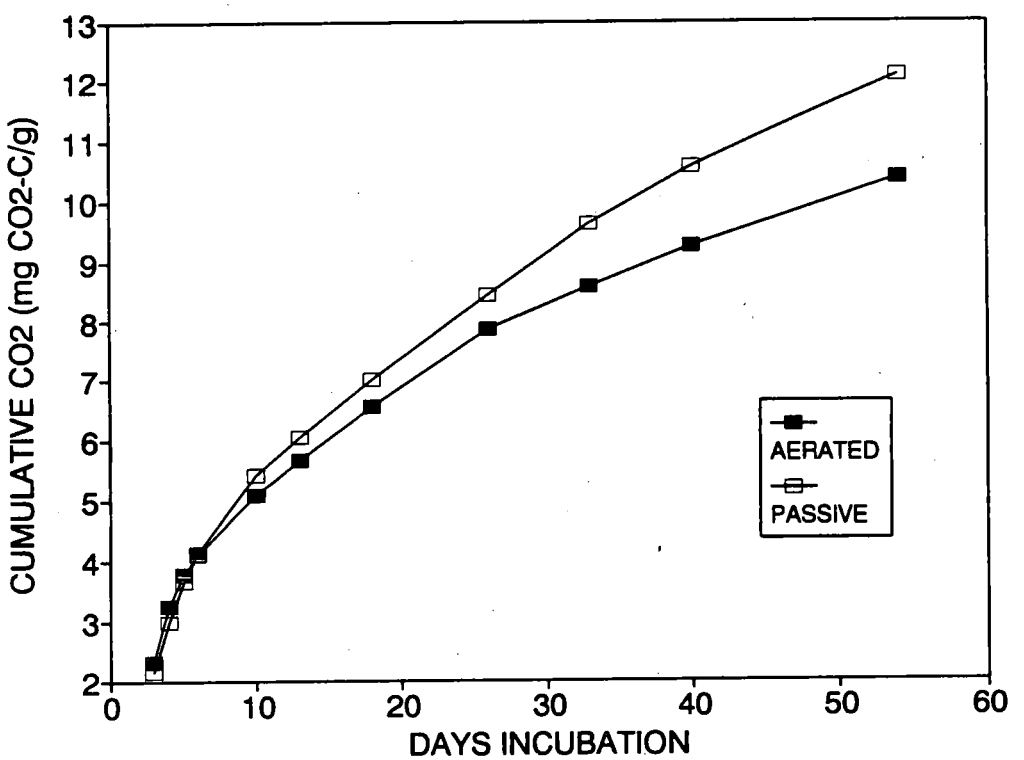

Fig. 4.3-2. Cumulative CO2 efflux from DIMR (+ 800 ug N g-1) with forced or passive aeration.

Table 4.3-1. Total CO_2 losses, TPH and DIMR chemistry of DIMR constantly aerated or passively aerated for 8 weeks (initial TPH 7.1 ± 0.8%).

Treatment	Incubation (weeks)	CO_2-C loss (mg g^{-1})	TPH (%)	NO_3-N ($\mu g\ g^{-1}$)	NH_4-N ($\mu g\ g^{-1}$)	pH	EC (dS m^{-1})
Aerated	4	7.9 ± 0.4	-	<0.02	0.5 ± 0.3	7.2 ± 0.02	0.5 ± 0.03
Aerated	8	10.4 ± 0.4	2.3 ± 0.06	<0.02	0.4 ± 0.6	7.4 ± 0.04	0.6 ± 0.06
Passive	4	8.4 ± 0.1	-	<0.02	2.4 ± 2.0	7.2 ± 0.03	0.6 ± 0.01
Passive	8	12.1 ± 0.2	2.9 ± 0.04	<0.02	0.3 ± 0.4	7.4 ± 0.03	0.6 ± 0.03

apparent prime decomposers.

TPH decreased substantially whether the DIMR was forcibly aerated or not indicating the gaseous environment, while perhaps not optimum, was satisfactory in both systems. This situation would probably apply to surface materials in the Bio-Reactor but the question of whether the gaseous environment would limit degradation in deep, metabolically active wastes remains to be answered by methods other than those used here.

4.4 Temperature effects on DIMR degradation and the sourdough approach to microbial enrichment

4.4.1 *Introduction*

Temperature is perhaps the most obvious variable to manipulate to change rates of biological processes. Refrigeration removes heat to slow processes and protect vulnerable organic products from the grief of decomposition whereas composting or fermentation processes will add controlled amounts of heat to speed the destruction of organics. The addition of heat to the Bio-Reactor has permitted the bioremediation process to proceed year long, an obvious advantage to the treatment scheme. However, heating above summer ambient is still a contentious issue. Laboratory tests indicated that optimum rates for degradation of crude oil in topsoil was between 30 and 35°C but results from the Bio-Reactor did not indicate an unequivocal temperature effect (Danielson 1993; Johnson et al. 1994).

Due to the promise of rate increases with increasing temperature, an additional test of temperature was planned for the DIMR. However, one possible major difference between the crude oil/waste and DIMR was the microbial potential of the two materials. The crude oil was in the topsoil and would be expected to harbor a rich and metabolically diverse microflora whereas the DIMR was subsoil based and no doubt microbially species-poor. In that a majority of soil and subsoil microflora have maximum temperatures for growth near 30°C, it was possible that the DIMR could lack organisms which could grow at elevated temperatures (e.g. 35°C) and at the same time effectively degrade hydrocarbons and utilize NO_3 as a N source.

In order to enrich the metabolic potential it was proposed that previously treated Waste 1 be used as a "starter or seed" for the treatment of DIMR. This type of inoculum had the advantage of being readily available and could easily be incorporated into the DIMR during the aggregation process. If successful, the use of a "sourdough starter" could be repeated endlessly and might result in an increasingly effective microbial population which was essentially cost-free and would increase the reliability and predictiveness of the Bio-Reactor or similar field units.

The objectives of this study were (1) to determine if increasing the

temperature above summer ambient would increase degradation rates of DIMR; (2) to determine if adding a microbially adapted starter soil (sourdough) would affect decomposition rates; and (3) to monitor N relations in the different starter/temperature treatments.

4.4.2 *Experimental conditions*

The technique used for CO_2 efflux was headspace analysis (Part 2) in 2 quart Gem jars with 80 g O.D.W. DIMR adjusted to 21% moisture content with 800 μg N g^{-1} and 160 μg P g^{-1} supplied as $Ca(NO_3)_2$ and KH_2PO_4. Four replicates of each treatment were used at each of three temperatures, 22, 30 and 35°C. Inoculum in the form of 1% Waste 1, crude oil on topsoil, taken from heated Cell 5 (30-35°C) in the Bio-Reactor after 12 months treatment was added to DIMR prepared as above to give a total of 24 jars (3 temperatures x 2 inoculation treatments x 4 replicates).

An identical set of jars was prepared for destructive sampling for substrate induced respiration (SIR) and for periodic extractable N determinations. After 4 weeks incubation the jars were sampled, and SIR (8 mg glucose g^{-1}) determined with without the addition of 200 μg N g^{-1} as $Ca(NO_3)_2$ and 80 μg P g^{-1} as KH_2PO_4. SIR was determined at 22°C for all three temperature treatments rather than at the incubation temperature due to technical restraints.

4.4.3 *Results and discussion*

There was a strong interaction between inoculation with treated Waste 1 and incubation temperature as indicated by CO_2 efflux (Fig. 4.4-1). Respiration from uninoculated DIMR was initially lowest at the highest (35°C) temperature but after the first two weeks incubation, rates of CO_2 efflux among the uninoculated treatments were similar. This suggests that the DIMR did not contain organisms well adapted to growth at high temperature.

Inoculation with Waste 1 had virtually no effect at 22° but resulted in a strong increase in CO_2 production at 30 and 35°; the most total CO_2 evolved occurred in DIMR inoculated and incubated at 30 and 35°C (Fig. 4.4-2). At the end of 8 weeks the highest respiration rate at each temperature was with the inoculated treatments, and greatest total CO_2 evolved was also with these treatments, suggesting that over the long term inoculation may be beneficial at all temperatures but is especially useful at above-ambient temperatures.

The CO_2 efflux data clearly indicated a positive effect of both inoculation with treated Waste 1 and with elevating the temperature. Estimates of methylene chloride extractable hydrocarbons (TPH) confirmed those results (Table 4.4-1). The greatest decreases in TPH occurred at 30 and 35°C with inoculated DIMR. Loss of hydrocarbons without inoculation was the same regardless of incubation temperature and amounted to one-half the losses of inoculated DIMR at 35°C.

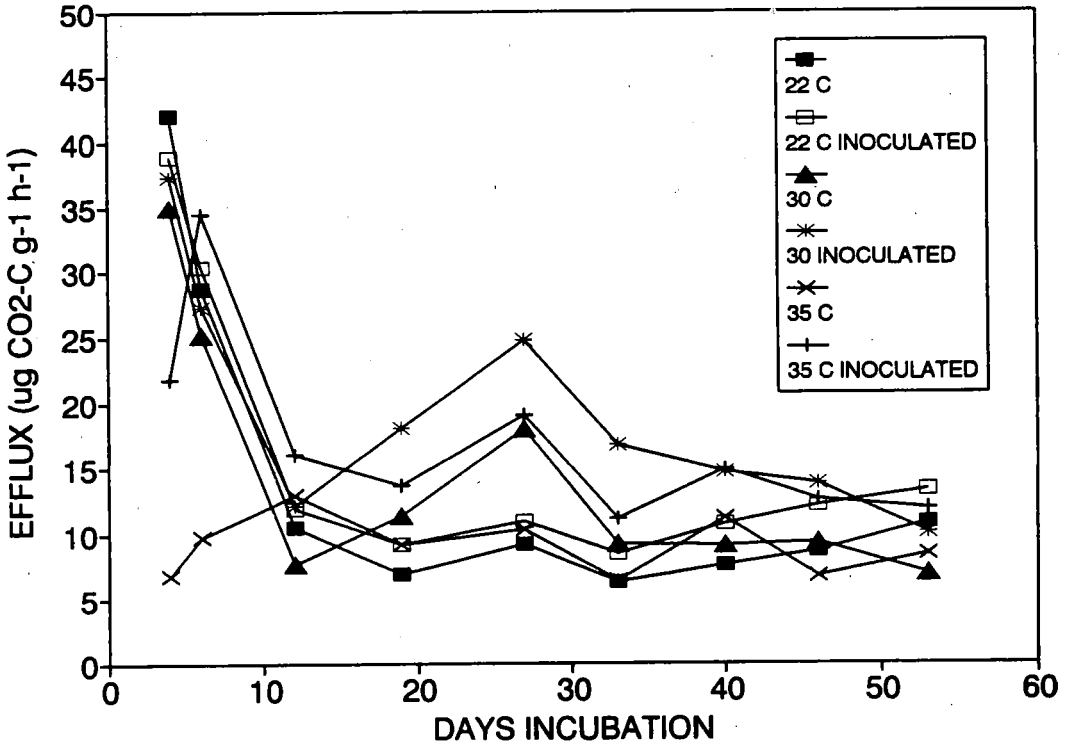

Fig. 4.4-1. Effect of temperature and inoculation with 1% Waste 1 on CO2 efflux from DIMR.

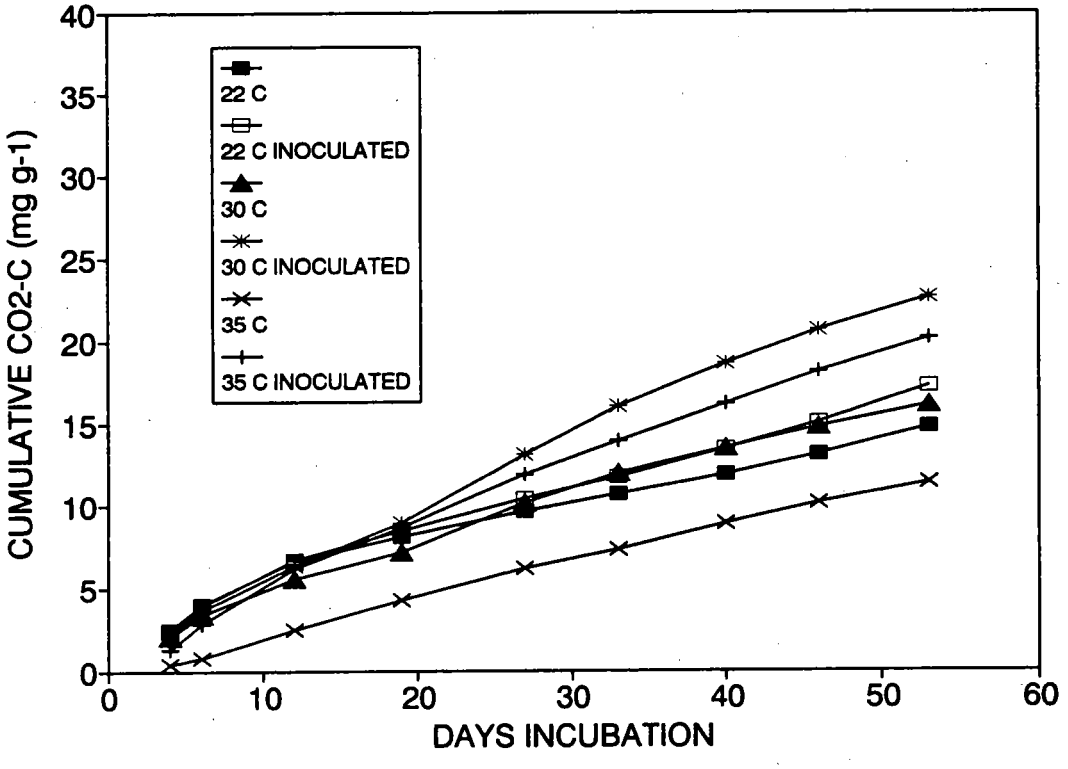

Fig. 4.4-2. Effect of temperature and inoculation with 1% Waste 1 on cumulative CO2 efflux from DIMR.

Table 4.4-1. Total CO_2-C loss and TPH of DIMR after 8 weeks incubation at 22, 30 or 35°C with and without Waste 1 inoculum; initial TPH = 5.3 ± 0.5%.

Temperature (°C)	Waste 1 Inoculum	CO_2-C loss (mg g^{-1})	Final TPH (%)	Decrease in TPH (%)
22	-	14.8±0.3	3.7±0.4	30
22	+	17.2±0.6	3.9±0.2	26
30	-	16.1±2.0	3.8±0.1	28
30	+	22.6±1.1	2.8±0.2	47
35	-	11.4±0.8	3.8±0.2	28
35	+	20.2±0.7	2.1±0.1	60

The consumption or loss of mineral N also reflected the same pattern as CO_2 efflux. After 2, 4 and 8 weeks of incubation, nitrate levels were consistently lower in inoculated treatments at all temperatures than in uninoculated DIMR (Fig. 4.4-3; Table 4.4-2). Nitrate concentrations decreased with time in all treatments except uninoculated DIMR at 22°. Nitrate consumed in uninoculated treatments accounted for 400 to 600 $\mu g\ N\ g^{-1}$, values similar to those found in Part 4.2. However, nearly all the extractable nitrate was lost from the inoculated treatments which could be due to higher N demands, less efficient use of N, or less likely, denitrification losses. Extractable ammonium was negligible except at 35°C early in the incubation period.

Nitrate levels were high in all treatments at 4 weeks and thus it is hardly suprising that the addition of more nitrate did not enhance CO_2 efflux (Table 4.4-3). There was either no effect or a slight decrease in mineralization rates. Even with the addition of glucose which increased N demands, there was little or no indication of N deficiencies.

The increase in SIR in inoculated DIMR in comparison to uninoculated DIMR indicates that inoculation introduced a more responsive and probably more species diverse population of decomposers. However, comparison of respiration rates at 22° and those at the actual incubation temperatures show that SIR biomass estimates must be done at the appropriate temperature and not simply at one which is convenient. Modifications to the technique used here are necessary to accomplish that requirement.

Observations on visible growth of microorganisms confirm that temperature selected for certain organisms. At 22° and 30° in both inoculated and uninoculated treatments, aggregate surfaces were covered with orange, pustulate colonies of small rod-shaped bacteria. No fungi were observed growing at these two temperatures except sparse growth of a *Penicillium* in the inoculated/30° treatment.

At 35°C no orange or other bacteria were observed on aggregate surfaces. However, *Penicillium* mycelium developed in all the 35° treatments. Microscopic mounts of DIMR incubated at 35° did reveal abundant bacterial rods but they did not form macroscopic colonies.

In summary, temperature increases combined with treated waste, profoundly affected CO_2 losses, N consumption and the quantity of residual hydrocarbons. When temperatures are maintained above ambient (~ 20-25°) and the material to be treated is likely to be species-poor, it would appear to be essential to use the sourdough approach and introduce temperature adapted hydrocarbon decomposers.

The slow rate of nitrate consumption at 35° in inoculated DIMR also suggests that native organisms could not collectively (1) utilize hydrocarbons as C and energy sources; (2) grow at 35°; and (3) utilize the nitrate form of N. Thus it may be necessary to add or develop a

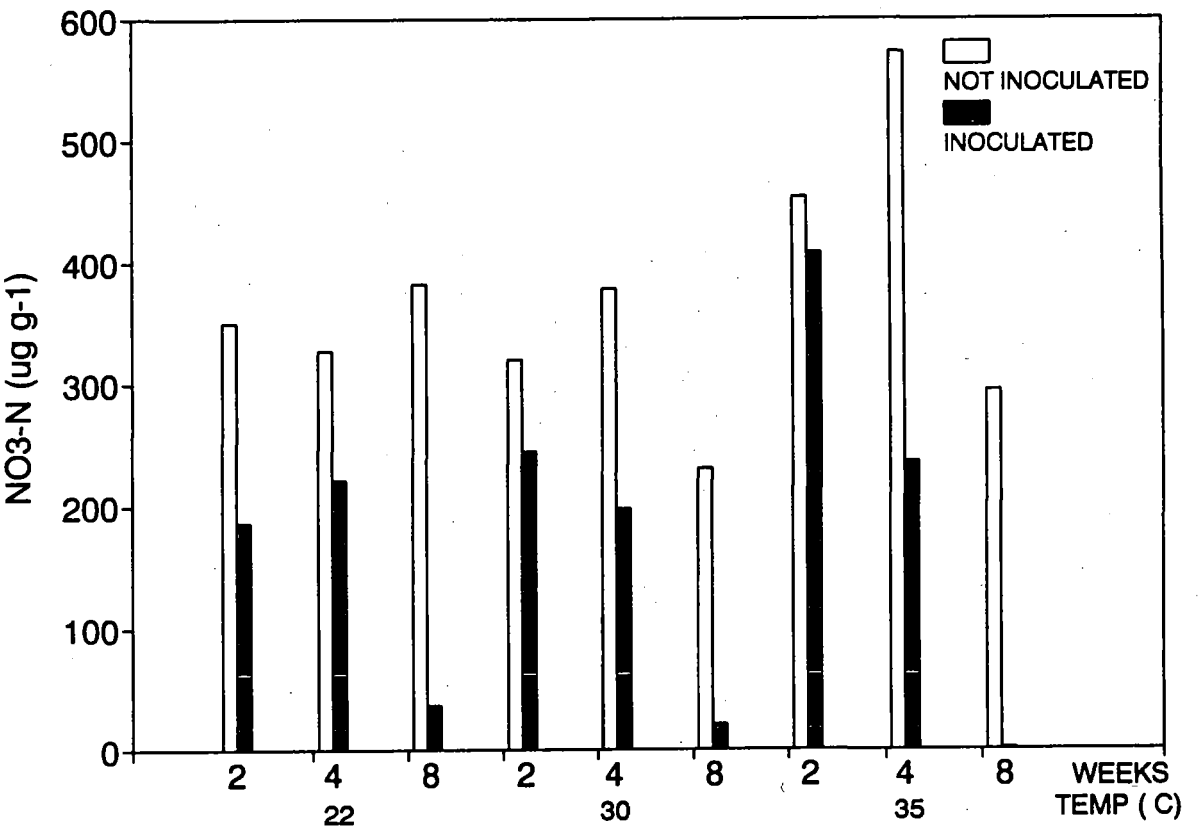

Fig. 4.4-3. NO3-N in DIMR incubated at 22, 30, and 35 C and inoculated or not with Waste 1 and incubated for 2, 4, and 8 weeks.

Table 4.4-2. Mineral N in DIMR incubated at 22,30 and 35°C with and without 1% Waste 1 inoculum.

Temperature (°C)	Waste 1 Inoculum	NO_3-N ($\mu g\ g^{-1}$)			NH_4-N ($\mu g\ g^{-1}$)		
		Incubation Time (weeks)					
		2	4	8	2	4	8
22	-	350±44	327±56	382±53	1	3	1
22	+	186±42	221±36	37±23	1	0	1
30	-	319±37	378±39	230±67	0	0	1
30	+	244±15	198±85	20±30	0	1	2
35	-	453±82	572±101	295±112	4	7	7
35	+	408±52	236±59	1±1	47	8	4

Table 4.4-3. Extractable NO_3-N and NH_4-N and basal respiration (CO_2 efflux) and substrate induced respiration (SIR, glucose) with and without added N (200 μg N g^{-1}) of DIMR incubated for 4 weeks with and without 1% Waste 1 at three temperatures.

Temp.	Inoculum	NO_3-N	NH_4-N	Basal Resp. (22°C) -N	Basal Resp. (22°C) +N	SIR (22°C) - N	SIR (22°C) +N	Efflux measured at 22, 30 or 35°C
(°C)		(μg g^{-1})		(μg CO_2-C g^{-1} h^{-1})		(μg CO_2-C g^{-1} h^{-1})		(μg CO_2-C g^{-1} h^{-1})
22	-	326±56	3±3	9.5±0.2	9.1±0.6	13.7±1.0	16.4±1.4	9.2±0.8
22	+	221±36	<1	12.3±0.7	12.6±0.6	22.2±1.4	23.6±0.4	11.0±1.1
30	-	378±39	0	11.6±0.9	11.3±1.4	14.3±2.0	15.2±1.5	17.9±2.6
30	+	198±85	<1	12.4±0.6	11.3±1.0	21.3±2.2	20.2±2.8	24.8±3.0
35	-	572±101	7±5	9.1±2.4	7.4±2.3	10.5±2.1	8.3±2.3	10.3±2.6
35	+	236±59	8±2	7.3±0.8	7.3±0.9	11.7±0.4	12.7±0.8	19.0±1.9

Note: Basal respiration and SIR measured at 22°C.

specialized microflora and the easiest and cost-effective method is the sourdough approach.

5. INOCULATION OR SEEDING OF DIMR

5.1 Use of soil to increase microbial metabolic diversity

5.1.1 *Introduction*

Although it is recognized that bacteria and fungi capable of degrading hydrocarbons exist in virtually all terrestrial and aquatic environments, some materials contaminated with oily wastes may be species-poor. As thousands of hydrocarbon compounds may occur in a single contaminant sources and that new compounds are formed during degradation, it is also recognized that a consortia or broad spectrum population of microorganisms may be required to efficiently and completely degrade an oily waste (Hamar 1993). Populations may possibly be enriched by adding specially selected organisms, genetically engineered bacteria or a natural source containing diverse, but unknown, microorganisms. From a practical and economic standpoint the method of choice might be to use natural materials rich in heterotrophic organisms to increase taxonomic, metabolic and ecological diversity.

A possible candidate for the poor diversity role is the DIMR waste which is subsoil based and very low in native organic matter (i.e., microbial substrates). In the previous section (Part 4.4), seeding with previously treated Waste 1 resulted in higher CO_2 efflux rates and greater losses of TPH, especially at above ambient temperatures. It was the purpose of this study to determine if adding a variety of soils to the DIMR waste would alter the mineralization or disappearance of the diesel hydrocarbons at 22 and 35°C.

5.1.2 *Experimental conditions*

In the first experiment, DIMR with a TPH content of 7.1 ± 0.8% was amended with Plant Prod 28:14:14 fertilizer at 800 μg N g^{-1} and incubated in glass tubes. One uninoculated set was incubated at 22° and one set at 35° was inoculated with a combination of 1% forest floor F horizon from a lodgepole pine stand and 1% black chernozem soil from the Porcupine Hills of Alberta. Four replicates were harvested after 4 weeks and five replicates at the end of 8 weeks for TPH and soil analyses. Respiration was not measured.

In the second experiment, DIMR with 5.8 ± 0.2% TPH was amended with Plant Prod 28:14:14 at 800 μg N g^{-1}. The soil additions were (1) none; (2) Porcupine Hills native grassland soil; (3) 2% "soil" from ant hills in the foothills (naturally heated sites); (4) pasture soil from the foothills; and (5) Waste 1 which had been incubated at 37°C for 8 weeks in the laboratory (Danielson 1993, Part 9.3). Three replicates of each soil treatment were incubated at

either 22 or 35°C for 16 weeks. Respiration measurements were made only on DIMR incubated at 22°.

5.1.3 *Results and discussion*

Seeding the DIMR with a combination of forest and grassland soil had little or no effect on the disappearance of extractable hydrocarbons (Table 5.1-1). About 54% of the hydrocarbons were lost in the 8 week incubation period at 35°. Somewhat greater hydrocarbon losses occurred at the lower temperature and extractable N levels also decreased more rapidly at 22° than at 35°. It would appear that the microflora native to the DIMR was best adapted to 22° and that soil additions did not introduce organisms capable of growth on hydrocarbons at 35°. Also notable was the slow utilization of NO_3-N at 35°. Of the 800 μg N g^{-1} introduced in the fertilizer only 117 μg g^{-1} was as nitrate and most of this remained after 4 weeks, indicating a preferential use of NH_4-N over NO_3-N at 35°. Further study of N relations at elevated temperatures may be necessary if N cycling is not the same as at lower soil temperatures (e.g. NO_3-N utilization and nitrification).

At 22° all water films contained orange bacteria and no fungal mycelium was observed. In contrast, the bacteria at 35° were all colourless for the first 2 to 3 weeks and orange colonies were only occasionally seen on aggregates after 4 weeks, indicating that different bacteria were functioning at the two temperatures. No fungi were observed in the 35° tubes and there was no visible effect of soil inoculations.

In the second experiment total respiration of CO_2 was unaffected by adding four soils as inoculum to the DIMR (Table 5.1-2). The final TPH after incubation at 22°C was also unaffacted or somewhat higher in inoculated DIMR as compared to uninoculated DIMR. At 35°, adding field soils had no effect on extractable hydrocarbons but the addition of treated Waste 1 resulted in a strong reduction in TPH. The value of 1.6% was the lowest value obtained in any laboratory experiment (77% loss) and confirms the results in Part 4.4 that the addition of a temperature adapted microflora will speed the disappearance of hydrocarbons.

Visible microbial growth was the same in all the 22° treatments. Fungal development was minimal and all moisture films were orange with the growth of bacteria after 2 weeks. At 35° no fungi were observed and there was not any apparent effect of inoculation. Water films were densely colonized with colourless bacteria and few orange pustules of bacteria were present on aggregates. At the end of the 16 week incubation period no extractable NO_3-N was present and NH_4-N was less than 2 μg N g^{-1}.

5.2 Trials with commercial inoculants

5.2.1 *Introduction*

The idea of adding a few grams of selected microbes to a large mass of highly contaminated material and that those microbes will render the

Table 5.1-1. Effects of incubating DIMR at 22° and 35°C and inoculating with 1% pine F horizon plus 1% grassland soil and incubating at 35°C on TPH and DIMR chemistry (initial TPH = 7.1 ± 0.8%).

Treatment	Incubation (weeks)	TPH (%)	NO_3-N ($\mu g\ g^{-1}$)	NH_4-N ($\mu g\ g^{-1}$)	pH	EC ($dS\ m^{-1}$)
22° Uninoculated	4	-	<0.02	2.4±2.0	7.2±0.03	0.6±0.01
22° Uninoculated	8	2.9±0.04	<0.02	0.3±0.4	7.4±0.03	0.6±0.01
35° Uninoculated	4	-	102±13	1.5±0.4	7.2±0.04	0.8±0.04
35° Uninoculated	8	3.3±0.1	16±19	0.3±0.3	7.5±0.04	0.8±0.07
35° Inoculated	4	-	83±14	7.9±7.0	7.2±0.01	0.8±0.09
35° Inoculated	8	3.3±0.2	47±28	0.5±1.0	7.4±0.05	0.9±0.11

Table 5.1-2. Loss of TPH from DIMR incubated for 16 weeks at 22°C or 35°C following the addition of 2% topsoil.

Soil Inoculum	Temp. (°C)	CO_2 Loss (mg g^{-1})	Final TPH (%)
None	22	20.2 ± 0.5	2.3 ± 0.1
Grassland	22	21.4 ± 0.6	3.4 ± 0.2
Ant Hill	22	20.8 ± 1.1	3.5 ± 0.2
Pasture	22	21.6 ± 0.5	2.9 ± 0.3
Waste 1 - 37°	22	20.2 ± 1.3	2.5 ± 0.2
None	35	ND	2.9 ± 0.2
Grassland	35	ND	2.9 ± 0.2
Ant Hill	35	ND	2.9 ± 0.1
Pasture	35	ND	2.8 ± 0.1
Waste 1 - 37°	35	ND	1.6 ± 0.1

Note: Initial TPH = 5.8 ± 0.2%. Waste 1 inoculum previously incubated at 37°C for 8 weeks. At the end of 16 weeks the pH of all treatments was between 7.2 and 7.4, extractable NO_3-N was nil and extractable NH_4-N was 1 to 2 μg g^{-1}.

oily contaminants harmless or completely mineralize them, is indeed a seductive one. Based upon this premise, an entire industry has recently developed to produce microbial inoculants to be used to search out and destroy an enormous variety of organic compounds, foremost of which are hydrocarbons. Not unlike some treatments for extreme medical disorders, most microbial inoculant treatments have not undergone critical scientific evaluation. However, the anecdotal evidence promises so much that operators confronted with massive problems succumb to the urge to try methods that are inexpensive, so easy to apply and perhaps a bit magical.

Recent tests with commercial inoculants, either in marine or terrestrial systems, for enhancing degradation of hydrocarbons have resulted in at best, ambiguous results (Atlas 1991). Even under laboratory conditions in liquid cultures where competition from indigenous microorganisms is minimized, inoculants may perform poorly. Dott et al. (1989) found that uncontrolled populations in activated sewage sludge outperformed a battery of selected bacteria in cultures with fuel oil as a C-source. Under both laboratory and field conditions of the Exxon Valdez oilspill, it was found that commercial inoculants were ineffective and indigenous organisms were credited with degrading all the oil that was degraded in the tests (Venosa et al. 1993 a,b).

In a literature review prepared for the Ontario Ministry of the Environment, Major and Cox (1992) were unable to document a single successful application of inoculants. However inoculation of a creosote contaminated soil with a lignin-degrading fungus resulted in a substantial increase in the degradation of PAHs, illustrating that inoculation may work if situations and organisms are properly matched (Davis et al. 1993).

Regardless of the numerous past reports of noneffects, it was felt necessary to test a few inoculants with the DIMR/subsoil material, as this material might lack effective oil degraders and benefit from the introduction of alien organisms.

5.2.2 *Experimental conditions*

Carbon dioxide efflux conducted in the usual way in glass tubes was used as the nondestructive method to determine inoculation effects. No TPH analyses were made on any treated material. Formulations of two products were added to the DIMR in two separate experiments. Treatments with the KBC product were (three replicates per treatment):

(1) Inoculate time 0/Live/400N + 80P ($Ca(NO_3)_2$ + KH_2PO_4)

(2) Inoculate time 0/Dead/400N + 80P ($Ca(NO_3)_2$ + KH_2PO_4)

(3) Inoculate time 0/Live/Osmocote 400 μg N g^{-1}

(4) Inoculate time 0/Dead/ Osmocote 400 μg N g^{-1}

(5) Inoculate 4 weeks/Live/400N + 80P

(6) Inoculate 4 weeks/Dead/400 N + 80P

The inoculum was a mixed culture in starch and bran which was added at a 1% rate. Autoclaved inoculum was added as control.

A fresh suspension of 7 g inoculum in 35 ml water, shaken for 5 min and allowed to stand for 3 h so the bran and starch settled out was used for treatments 5 and 6. Four milliliters of fresh or autoclaved suspension was added to each of six tubes. After 70 days incubation, suspensions of live and dead inoculum was added to their respective treatments. Ten grams inoculum was suspended in 30 mL water and shaken for 9 days to propagate the bacteria, the starch allowed to settle for 4 h and 2 mL of live or autoclaved suspension used to reinoculate the DIMR.

A 1:1 mixture of Munox 512 and 112 formulations, white powders consisting of bacteria and unknown insoluble material, was used in the second test. The mixture was added at a rate of 1 mg g^{-1} to fresh DIMR and DIMR which had been incubated in the lab for 120 days to provide both high and low activity substrates. The treatments were:

(1) Fresh DIMR/Live/800 μg N g^{-1} as 28:14:14

(2) Fresh DIMR/Nil/800 μg N g^{-1} as 28:14:14

(3) Old DIMR/Live/200 μg N g^{-1} as 28:14:14

(4) Old DIMR/Nil/200 μg N g^{-1} as 28:14:14

At day 35 all tubes were reinoculated with live or dead (autoclaved) bacteria at a rate of 10 mg g^{-1}. This rate is about 100 times the recommended application rate.

5.2.3 *Results and discussion*

The starch included in the inoculum resulted in a brief rapid respiration rate with both live and dead inoculum and soluble fertilizer but only a small initial peak with Osmocote indicating a mineral nutrient deficiency (Fig. 5.2-1). A small starch peak also occurred when inoculum was added at day 31. Respiration rates otherwise were similar and no differences in activity could be attributed to introduced inoculum at any date (Fig. 5.2-1) or in the total CO_2 evolved (Fig. 5.2-2). The addition of new inoculum on day 70 did not result in any increase in respiration in any of the three paired treatments.

The second type of inoculum did not increase respiration on either fresh or weathered DIMR (Figs. 5.2-3, 5.2-4). A second addition of inoculum at 100x the recommended rate had no effect.

In summary, the introduction of commercial inoculants had no effect on respiration rates of DIMR and it appeared that the indigenous organisms were capable of degrading the hydrocarbons even though the

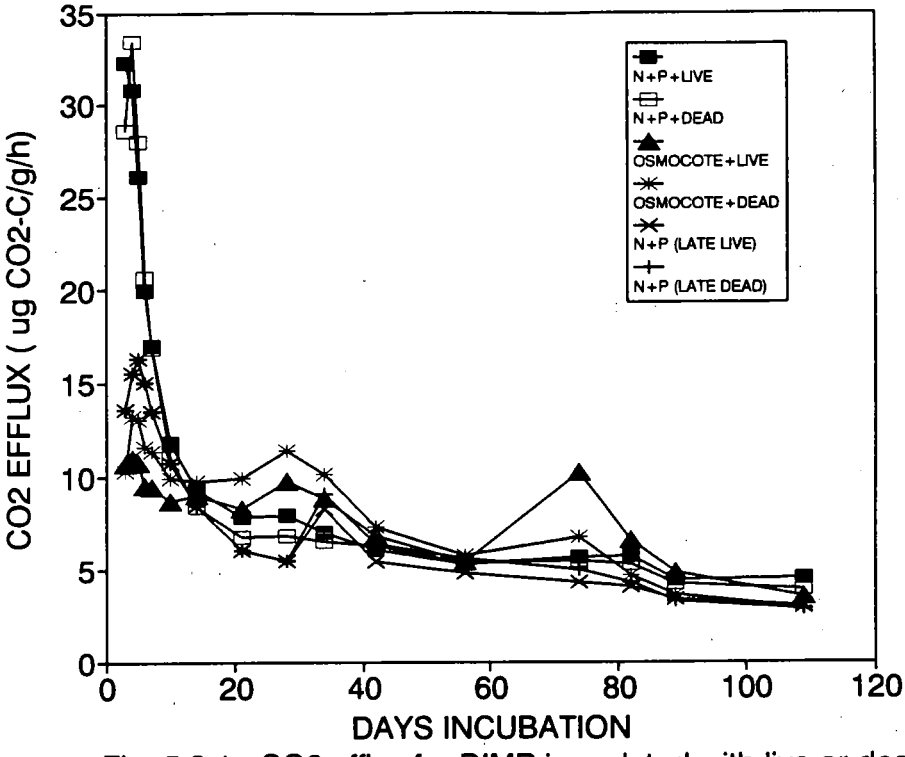

Fig. 5.2-1. CO2 efflux for DIMR inoculated with live or dead KBC (1%) at time 0 or day 31.

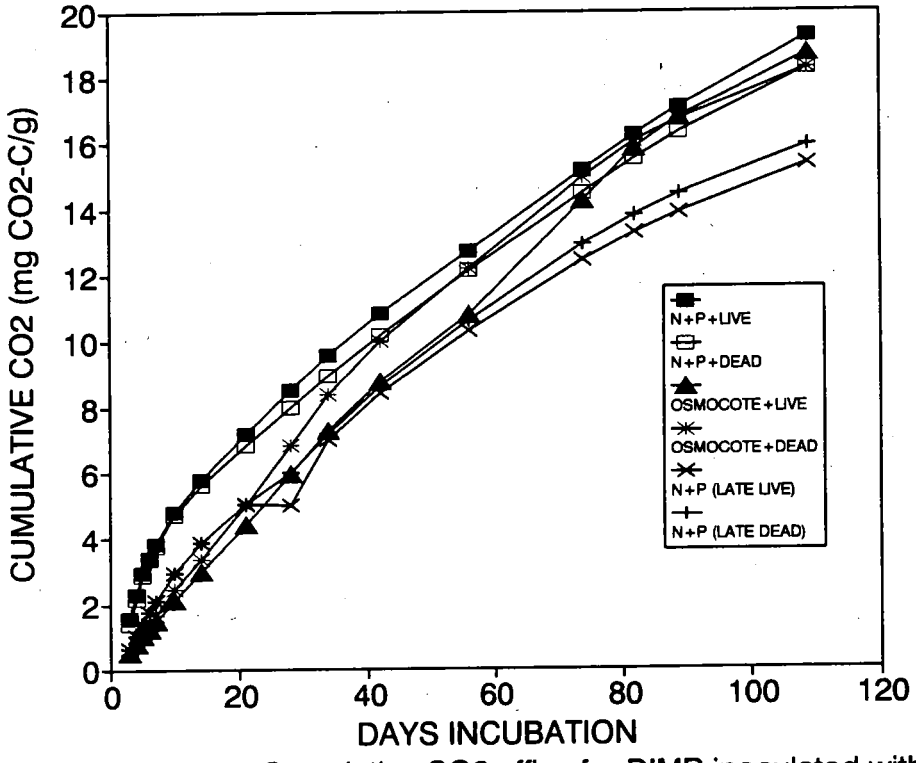

Fig. 5.2-2. Cumulative CO2 efflux for DIMR inoculated with KBC (1%) at time 0 or day 31.

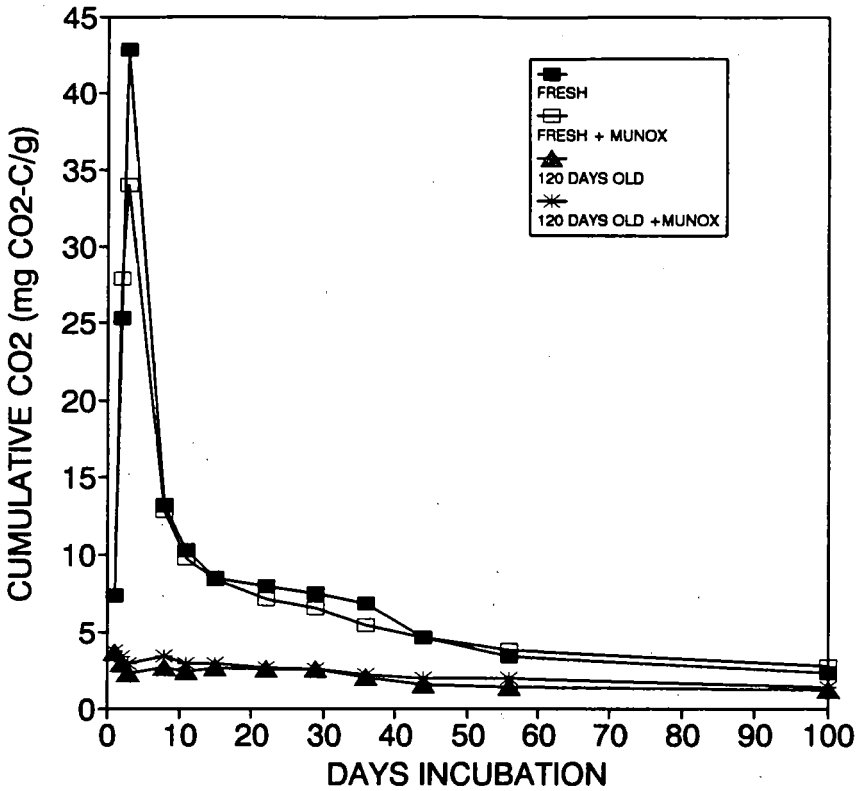

Fig. 5.2-3. CO2 efflux for fresh and aged DIMR inoculated with Munox inoculum on day 0 and reinoculated on day 35.

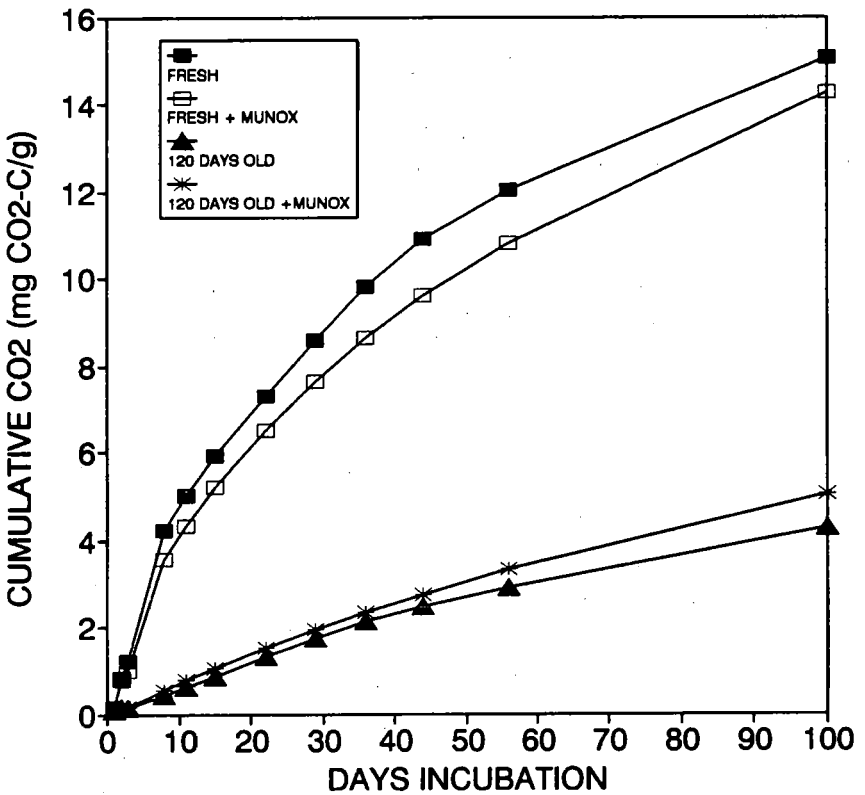

Fig. 5.2-4. Cumulative CO2 efflux for fresh and aged DIMR inoculated with Munox inoculum on day 0 and reinoculated on day 35.

flora was probably species-poor. It cannot be determined if any of the introduced organisms became established, but it is likely that they were unable to surmount the ecological dam formed by the native flora.

6. ATTEMPTS TO BOOST SECOND-STAGE CO_2 EFFLUX RATES

6.1 *Introduction*

The decomposition or respiration patterns of both Wastes 1 and 2, crude oil/topsoil and DIMR/subsoil, were very similar in all laboratory experiments. There was a burst of activity after N fertilizers were added which lasted for 1 - 2 weeks, followed by a low rate of respiration which very slowly decreased and continued for as long as the incubation lasted. This pattern no doubt also occurs in the field and the slow, grinding away pattern continued for Waste 1 in excess of a year and will probably continue for years to come. It is this slow, long-lasting phase that must be changed if decomposition rates are to be increased. Possible rate-limiting factors were listed in the previous report and included substrate intractability, diffusion limitations, ineffective microflora, mineral deficiencies and toxic end product accumulation (Danielson 1993).

The purpose of the studies reported here were to determine if simple manipulation of incubating DIMR would result in increased respiration (hydrocarbon mineralization). Factors to be addressed included mineral nutrient deficiencies, toxins and water soluble inhibitors.

6.2 *Experimental conditions*

Three separate tests on DIMR and one on Waste 1 (crude oil/topsoil) were made to attempt to increase CO_2 efflux of material incubated in glass tubes at 22°. In the first test 15, 60 g aliquots of DIMR with 400 μg N g^{-1} ($Ca(NO_3)_2$) and 80 μg P g^{-1} (KH_2PO_4) were incubated for 24 days and then removed from the tubes and to three replicates was added (1) 5% activated charcoal, (2) 200 μg N g^{-1}, (3) charcoal + N, and (4) nil. All were mixed, returned to the tubes and incubation continued for a total of 60 days. An additional 3 tubes with DIMR + 2% $HgCl_2$ served to estimate abiotic CO_2 production.

In the second test, 15 tubes prepared as above were incubated for 40 days and (1) left undisturbed, (2) leached with water, (3) leached with $Ca(NO_3)_2$, 2000 μg N g^{-1}, (3) leached with Plant Prod 28:14:14, 2000 μg N g^{-1}, and (4) Plant Prod plus Tween 40 surfactant (3 drops per liter). The high concentration of N was used to reestablish a soil N level of 400 μg N g^{-1} after drainage (24 mg N per 60 g soil, 12 g water retained). Leaching was done *in situ* by standing the open ended tubes with foam stoppers containing the DIMR in the beakers of each leachate. Straws were inserted alongside the top plug to allow air to escape and the tubes were soaked in a saturated condition for 30 min, placed on Buchner funnels and excess leachate drawn off. This was repeated once more and the CO_2 measured for an additional 85 days (125 days total).

The third test involved leaching

of materials collected from the Bio-Reactor and the Bio-Pile in the summer of 1993. Samples of Waste 1 were taken from Cell 5 (heated, aerated and 30 cm depth) and from the Bio-Pile soon after all treatments had been mixed and placed in the unit (July, after 12 months treatment). Samples of DIMR from aggregated and nonaggregated cells which had been heated, aerated and cultivated were taken one month after treatments had been initiated.

Laboratory treatments for all four field treatments were (1) none, (2) leaching twice with water by submersion, and (3) leaching and the addition of 200 μg N g^{-1} ($Ca(NO_3)_2$ and 40 μg P g^{-1} (KH_2PO_4). Respiration was then measured at 22°C on duplicate samples of each treatment and initial soil chemical characteristics determined. No analyses for TPH were performed.

6.3 *Results and discussion*

The addition of $HgCl_2$ to the DIMR indicates that the contribution of abiotic CO_2 to total CO_2 efflux remained constant at about 2 μg CO_2-C g^{-1} h^{-1} throughout the 8 week incubation period (Fig. 6-1). During the rapid phase of mineralization this quantity was relative insignificant but as biotic respiration rates decreased the abiotic CO_2 contribution rose to about 50% of the total. Clearly after 6 - 8 weeks total CO_2 efflux overestimates biological activity to large degree. However, abiotic CO_2 is probably also overestimated as $HgCl_2$ acidifies the DIMR, resulting in increased CO_2 from carbonates. In addition, the method of venting the samples with CO_2-free air during the measurement period (Part 2.2) would also shift the aqueous carbonate equilibrium in favor of gaseous CO_2 (Sparling and West 1990). Thus the estimates of abiotic CO_2 represent maximum values and are overestimated but, nonetheless, should not be ignored when biological activity is low (see further discussion in Part 9).

As would be expected, removal of the DIMR from the tubes and mixing it resulted in increased respiration for about 1 week (Fig. 6-2). The addition of charcoal, NO_3-N, or both charcoal and NO_3-N had no additional effect, either positive or negative, to the disturbance CO_2 flush. Aggregates treated with charcoal were completely coated and black and the charcoal could have adsorbed CO_2 as well as organics (Part 9) but if so, the total effect was nil and did not indicate any relief from toxic factors.

The general respiration pattern for the *in situ* leaching test was typical for the DIMR and activity was relatively low when samples were leached after 40 days incubation (Fig. 6-3). Leaching with water had a very small, short term positive effect on CO_2 efflux but the magnitude of the effect did not indicted that soluble toxic byproducts had accumulated during incubation. Leaching with either $Ca(NO_3)_2$ or a complete fertilizer also resulted in insignificant effects. The slight stimulation of CO_2 release by the Plant Prod fertilizer can probably be attributed to the release of CO_2 as urea was enzymatically decomposed to form NH_3 and CO_2

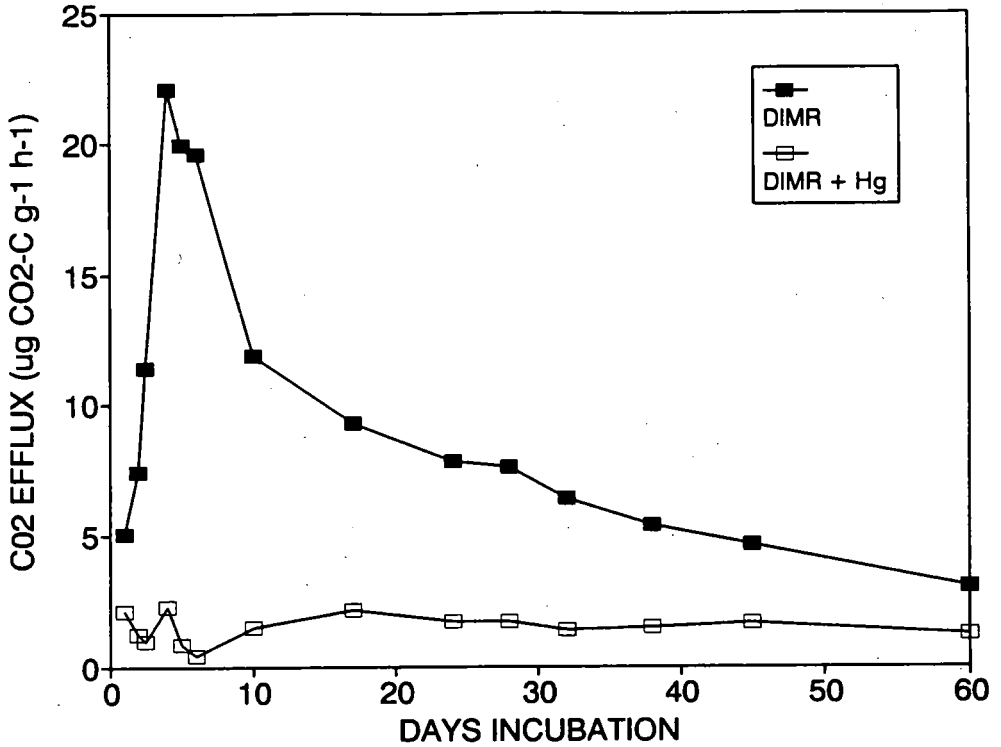

Fig. 6-1. Total CO2 efflus from DIMR with and without 2% HgCl2.

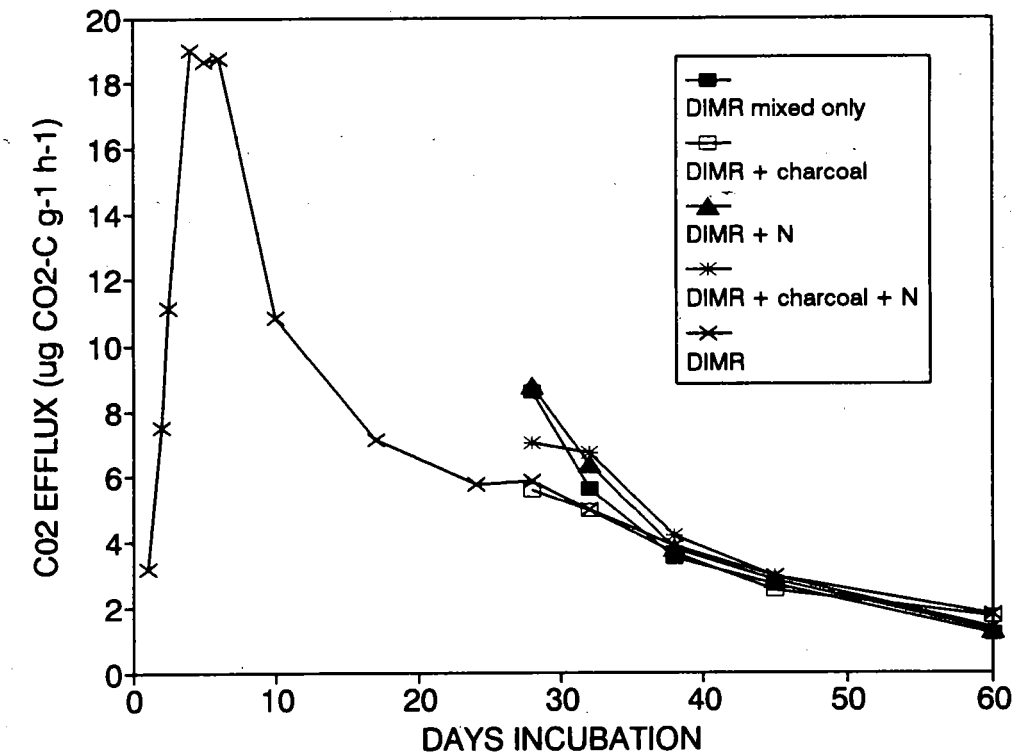

Fig. 6-2. Biotic CO2 efflux from DIMR incubated for 24 days and then mixed and charcoal (5%) and/or 200 ug N g-1 added.

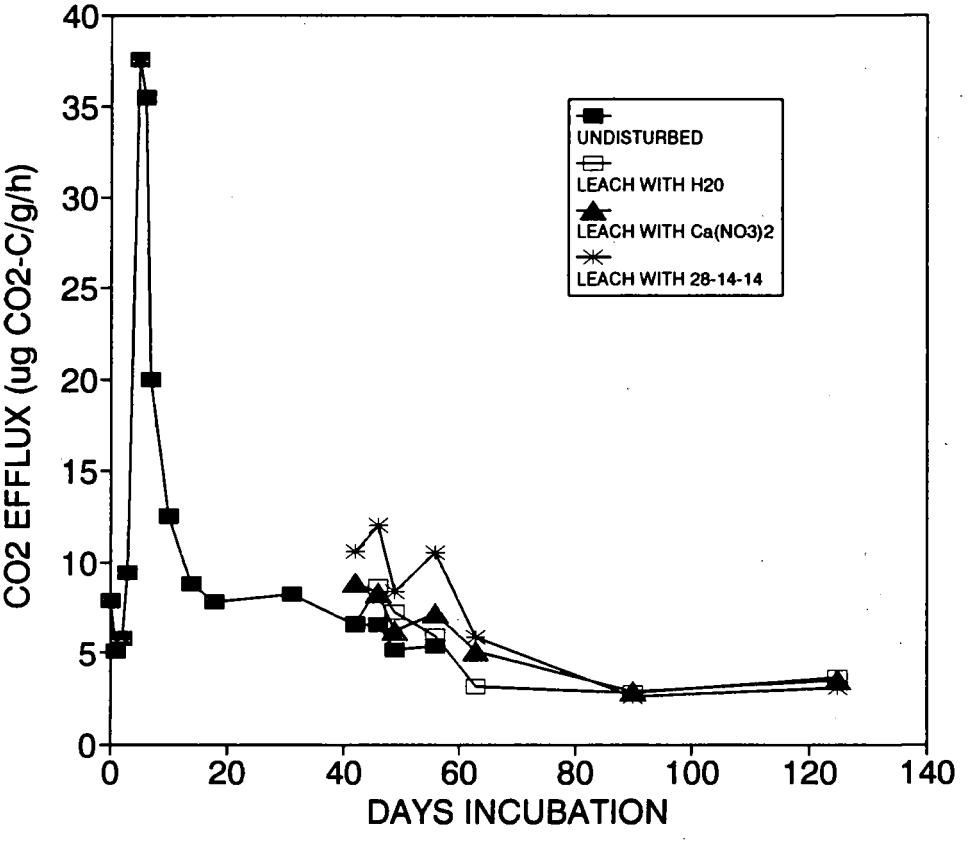

Fig. 6-3. CO2 efflux pattern from DIMR with 400 ug N g-1 undisturbed or leached in situ after 40 days.

(Alexander 1977). Urea constitutes 75% of the N in the Plant Prod fertilizer. The addition of a surfactant with the fertilizer had no effect whatsoever on respiration (data not presented). In all treatments no fungi were observed to grow after leaching and the predominant bacteria were orange rods which were present in all water films.

One-month-old DIMR from the Bio-Reactor had high levels of mineral N and moderately high electrical conductivities (Table 6-1). Leaching effectively removed NO_3-N and reduced conductivities to ideal levels and adding N restored NO_3-N levels to over 200 μg N g^{-1}. Phosphorus was more than adequate as indicated by extractable levels. The addition of N had a short term stimulation on CO_2 efflux in the aggregated DIMR and the opposite effect in the nonaggregated DIMR. After 3 weeks the added N had no effect on DIMR respiration.

Waste 1 was high in NO_3-N and PO_4 and leaching removed most of the NO_3-N (Table 6-2). Neither leaching nor leaching and the addition of N had any effect on DIMR respiration over the 8 week incubation period.

In summary, it was not possible to change respiration rates of oily wastes in the slow phase of degradation by the treatments imposed here. There was no indication that toxic materials were inhibiting microbial activity, nor were there any deficiencies of mineral nutrients. Although laboratory samples are not normally leached, this factor did not appear to affect degradation in any way and thus unleached laboratory systems may accurately simulate field conditions. The apparent immutability of the slow respiration phase may be largely due to inherent substrate recalcitrance or restricted bioavailability. The latter factor, the strong sorption of hydrophobic compounds to organic materials, appears to be a major factor (e.g. Weissenfels et al. 1992; Bouwer and Zehnda 1993) and new management procedures must be developed to overcome the sorption/diffusion constraint on hydrocarbon degradation.

Table 6-1. Effect of leaching 12 month old Waste 1 (July 1993) with water and adding 200 μg N g^{-1} + 40 μg P g^{-1} after leaching on CO_2 efflux at 22°C.

Waste 1 type	Treatment	Initial NO_3-N (μg g^{-1})	Initial NH_4-N (μg g^{-1})	Initial PO_4-P (μg g^{-1})	Initial EC (dS m^{-1})	Days Incubation 7	22	42	57 (μg CO_2-C g^{-1} h^{-1})	Total CO_2-C lost (mg g^{-1})
Mixed	None	313	<1	103	1.9	3.2	2.4	2.4	1.9	3.4
Mixed	Leach	10	<1	94	0.6	3.5	2.7	2.5	1.7	3.7
Mixed	Leach + NP	280	<1	127	1.1	3.4	2.5	2.5	1.8	3.6
Cell 5	None	456	7	180	2.9	--	--	--	--	--
Cell 5	Leach	12	3	182	0.7	2.9	1.9	1.6	1.1	2.7
Cell 5	Leach + NP	274	3	251	1.3	2.7	1.8	1.7	1.1	2.6

Note: Cell 5 sample from Bio-Reactor received elevated temperature and forced aeration treatments; mixed sample from Bio-Pile after all treatments mixed.

Table 6-2. Effect of leaching 12 month old Waste 1 (July 1993) with water and adding 200 μg N g^{-1} + 40 μg P g^{-1} after leaching on CO_2 efflux at 22°C.

Waste 1 type	Treatment	Initial NO_3-N (μg g^{-1})	Initial NH_4-N (μg g^{-1})	Initial PO_4-P (μg g^{-1})	Initial EC (dS m^{-1})	Days Incubation 7	22	42	57 (μg CO_2-C g^{-1} h^{-1})	Total CO_2-C lost (mg g^{-1})
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Mixed	Leach	10	<1	94	0.6	3.5	2.7	2.5	1.7	3.7
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Cell 5	None	456	7	180	2.9	--	--	--	--	--
Cell 5	Leach	12	3	182	0.7	2.9	1.9	1.6	1.1	2.7
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Note: Cell 5 sample from Bio-Reactor received elevated temperature and forced aeration treatments; mixed sample from Bio-Pile after all treatments mixed.

7. NITRIFICATION DURING TREATMENT OF DIMR

7.1 *Introduction*

The choice of either form of mineral nitrogen as fertilizer to support hydrocarbon degradation depends not on biological effectiveness, as both forms support equal amount of biological activity, but rather on conservation of N (Part 4.2). If NH_4-N or urea is applied in large quantities and the pH is high, significant quantities of N may be lost as NH_3. Gaseous losses of NO_3-N may also occur via denitrification in materials rich in organic matter and under conditions of poor aeration (Alexander 1977). Nitrates can also be readily lost in leachates, whereas NH_4-N will be retained on exchange sites of clays or organic matter. If the N conservation choice is for ammonium-N, it should be recognized that in normal soils, NH_4 can be rapidly converted to NO_3 by the nitrification process and the net result may be similar to adding nitrate fertilizer. It was the purpose of this experiment to determine if nitrification occurs in the subsoil material contaminated with DIMR.

7.2 *Experimental conditions*

Two types of DIMR were used, the first was fresh untreated material and the second (aged) was DIMR which had incubated in bulk for 2 months after adding 400 μg N g^{-1} of NO_3-N. Both types had 1% topsoil added during the aggregation process. To both types $(NH_4)_2SO_4$ was added to provide 800 μg N g^{-1} and KH_2PO_4 to provide 160 μg P g^{-1}. In addition, one half of the aged DIMR was amended with 1% chernozem topsoil from a grassland site in the Porcupine Hills. This soil had been previously shown to have a high nitrification capacity and thus insured that nitrifying bacteria were present in the DIMR. The nitrification inhibitor, N-Serve, was added to aliquots of each of the three DIMR materials at a rate of 50 μg g^{-1} (10x normal field rate) to block nitrification. As nitrates can be constantly produced and consumed, the use of N-Serve served as an additional check on the activity of the nitrifiers, i.e., if nitrification was occurring, it would not be expected that N pools would be equal in the plus and minus N-Serve treatments.

The treatments were prepared in triplicate and incubated at 22°C. Periodically samples were taken, extracted with 2M KCl and levels of NH_4-N and NO_3-N determined. As extractable N became very low in the fresh DIMR, an additional 200 μg N g^{-1} as $(NH_4)SO_4$ was added after 60 days.

7.3 *Results and discussion*

In the fresh DIMR there were only very small quantities of NO_3-N present throughout the 103 day sampling period (Table 7-1). A majority of the NH_4 disappeared in the first week and slowly decreased to minimal levels. The addition of N-serve had no effect on either NO_3 or

Table 7-1. Levels of NO_3-N and NH_4-N in DIMR (+1% topsoil) after adding 800 μg N g^{-1} as $(NH_4)_2 SO_4$ at Day 0 and 200 μg N g^{-1} after 60 days to the fresh DIMR.

DIMR used	Chernozem added	N-Serve added	N form	Days incubation 5	10	20	40	103
				N (μg g^{-1})				
Fresh	-	-	NO_3	0	0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Fresh	-	+	NO_3	0	0.1 ± 0.1	0.6 ± 0.6	0.1 ± 0.1	0.1 ± 0.1
Fresh	-	-	NH_4	169 ± 5	86 ± 11	49 ± 20	7 ± 9	4 ± 4
Fresh	-	+	NH_4	204 ± 16	103 ± 189	65 ± 10	4 ± 2	12 ± 10
Aged	-	-	NO_3	273 ± 14	223 ± 19	225 ± 10	260 ± 12	338 ± 50
Aged	-	+	NO_3	299 ± 7	233 ± 12	228 ± 4	271 ± 3	298 ± 53
Aged	-	-	NH_4	621 ± 13	564 ± 14	500 ± 6	430 ± 13	165 ± 7
Aged	-	+	NH_4	600 ± 53	585 ± 14	510 ± 18	453 ± 16	196 ± 58
Aged	+	-	NO_3	354 ± 123	226 ± 6	210 ± 4	256 ± 9	318 ± 17
Aged	+	+	NO_3	290 ± 6	239 ± 3	234 ± 3	262 ± 1	319 ± 3
Aged	+	-	NH_4	637 ± 5	568 ± 8	511 ± 11	417 ± 25	181 ± 55
Aged	+	+	NH_4	647 ± 15	569 ± 6	520 ± 12	446 ± 17	221 ± 27

NH_4 pools.

The addition of chernozem soil or N-serve had no or little effect in the aged DIMR which contained substantial NO_3 from the pretest application. Concentrations of NH_4 decreased throughout the incubation period whereas it appeared that NO_3 levels increased after 3 weeks.

The results of the study proved to be inconclusive with regard to nitrification in oily materials. It appeared that some nitrification was occurring in the aged DIMR but none in the fresh DIMR. The lack of the appearance of NO_3 in the fresh DIMR could have been due to rapid utilization if some NH_4 was nitrified.

The use of N-Serve failed to affect N pools, either because no nitrification occurred or that N-Serve was ineffective as an inhibitor. The same concentration of N-Serve has been shown to effectively block nitrification for at least 18 weeks in sludge amended soils (McClung et al. 1983) and at much lower concentrations in other soils (Chancy and Kamprath 1987). However, Chancy and Kamprath (1987) indicate the effectiveness of N-Serve decreases with increased temperature, moisture, pH and organic matter. This suggests that oily wastes in the Bio-Reactor may be poor candidates for using nitrification inhibitors. Further examinations of N-transformations in oily materials are warranted as large amounts of N fertilizers are required to remediate such materials.

8. DEGRADATION OF WASTE 1 TAKEN FROM THE BIO-PILE

8.1 *Introduction*

Waste 1, crude oil in topsoil, was removed from the Bio-Reactor cells in July 1993, mixed and placed in the Bio-Pile to a depth of 1 m. At that time, Waste 1 still contained about 2.5% hydrocarbons and it was decided further treatment was necessary to reduce the oil content. Treatment options in the Bio-Pile included forced aeration, temperature control, irrigation and fertilization. Laboratory tests were made to determine if fertilization and heating could be used to increase oil degradation.

8.2 *Experimental conditions*

Three tests were made on weathered Waste 1 to set conditions in the Bio-Pile.

(1) $Ca(NO_3)_2$ fertilizer was added to a composite sample of Waste 1 at a rate of 200 μg N g^{-1} and KH_2PO_4 added at a rate of 40 μg P g^{-1}. Triplicate tubes were incubated at 22°C and respiration measured for 8 weeks.

(2) Material from the Bio-Pile was placed in Gem jars, incubated at either 22, 30 or 35°C and CO_2 efflux determined by headspace analysis. No fertilizer was added and six replicates were harvested at 8 weeks and six replicates after 18 weeks for chemical and oil content analyses.

(3) Bio-Pile samples were incubated in a slurry at 30°C in a rotary shaker to determine what might be considered the ultimate rates of degradation. Twenty grams of soil was placed in 40 ml water and four replicates were harvested after 0, 2, 4 and 8 weeks incubation. Harvesting was done by evaporating off most of the water in a plastic sandwich container (approx. 12 h), scraping off the solid residue and placing it in Teflon sealed vials. Dry controls were incubated in open containers at 22°C to determine nonbiological losses.

8.3 *Results and discussion*

The addition of N and P to weathered Waste 1 resulted in decreases in CO_2 efflux for at least 8 weeks (Fig. 8-1). This is not surprising as extractable NO_3 levels exceeded 300 μg N g^{-1} (see Table 8-2) and that previous tests of Waste 1 had indicated that fertilizer additions may well decrease microbial activity (Danielson 1993).

Rates of hydrocarbon mineralization were so slow that changes in CO_2 efflux (or O_2 uptake) are the only reasonable tests that can

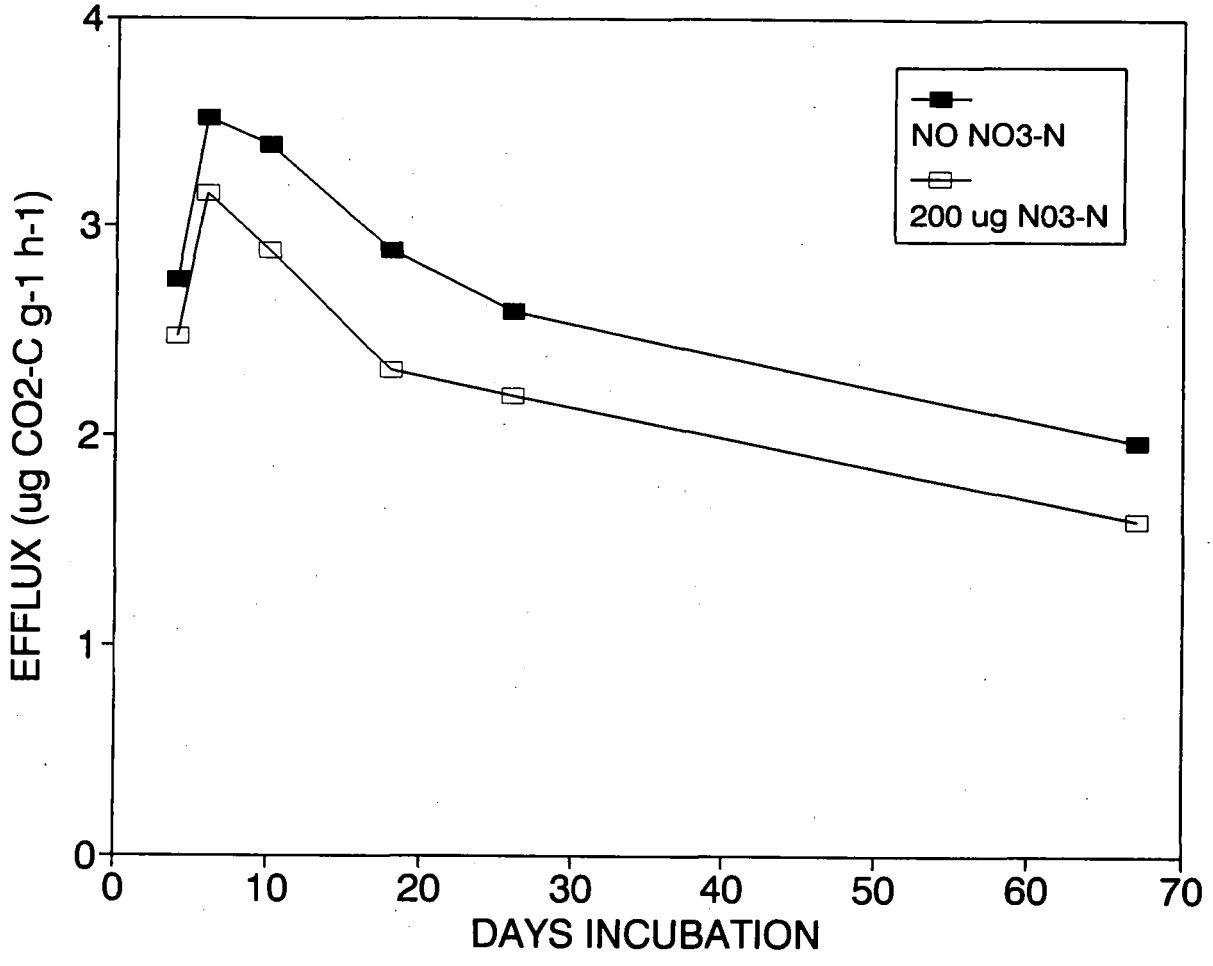

Fig. 8-1. Effect of 200 ug g-1 NO3-N on CO2-C efflux from composite sample of 10 month old Waste 1.

determine if N is limiting degradation rates. It is clear that fertilizer additions should not be made on the basis that "a little is good, more should be better" but rather on a microbial response test such as CO_2 efflux. Excessive fertilization is both wasteful and detrimental to the prime objective - reduction of contaminants.

Temperature did not appear to have any effect on C-mineralization of material from the Bio-Pile (Table 8-1). The estimate of total CO_2 lost over the 8 week period was very similar for all three incubation temperatures. However, further investigation of the headspace analysis indicates that the exact method used here for carbonate-containing waste respiring at low rates may lead to erroneous conclusions (Part 9). Estimates reported in Part 9 indicate that mineralization rates were higher at 30 and 35°C than at 22°C although true biotic rates were about one-fifth those reported in Table 8-1. If these rates are used (0.2 - 0.4 mg C g^{-1} lost in 2 months), then it would require about 4 months for the oil content to decrease 0.1%. This indicates the stability of the hydrocarbons and the very long period required if very low (e.g. 0.1 - 0.5%) hydrocarbon levels are required for treatment endpoints.

Results of the oil analyses substantiate the corrected efflux results that heating to 35°C increases oil degradation as compared to 22°C (Table 8-2). Further, the potential decreases in oil content in the Bio-Pile are quite small and over a 4 month period the maximum decrease to be expected would only be 0.2 to 0.3% (2.5% reduced to 2.2%). Indeed, results from the Bio-Pile show that the oil content was almost constant at about 2.4% (Johnson et al. 1994). During the lab incubation, pH and EC remained constant and NO_3 levels appeared to increase at all temperatures. An increase in NO_3 could be due to biomass mineralization and nitrification, once again indicating the fallacy of fertilizing weathered wastes.

When the same waste was incubated in a slurry in which diffusion factors are largely eliminated, there appeared to be an accelerated rate of oil degradation (Table 8-3). Within 8 weeks waste 1 contained 1.8% oil, substantially less than in the solid phase incubation. However, a similar decrease in oil content was measured in the samples incubated in an air dried condition. This result seems very unlikely and must be retested. It may also be noted that NO_3 levels increased with time as in the solid phase incubation and that poisoning samples with $HgCl_2$ rendered the material unsuitable for oil analyses as the $HgCl_2$ was extracted with the oil and interfered with the gravimetric determination (P. Yeung, pers. comm.).

Table 8-1. Effect of incubation temperature on CO_2-C loss from Waste 1 sampled from the Bio-Pile in July after 12 months treatment in the Bio-Reactor.

Incubation Temp.	Days Incubation				Total CO_2-C Loss
	8	22	44	56	
	(μg CO_2-C g^{-1} h^{-1})				(mg g^{-1})
22	2.2 ± 0.8	1.7 ± 0.7	2.3 ± 0.5	1.8 ± 0.4	3.2 ± 0.4
30	2.5 ± 1.0	2.2 ± 0.4	1.9 ± 0.7	1.8 ± 0.5	2.9 ± 0.3
35	3.9 ± 1.1	3.4 ± 0.6	2.2 ± 0.9	1.5 ± 0.4	3.0 ± 0.8

Note: Rates ± stable at ~2 μg C g^{-1} h^{-1} or 1.4 mg C g^{-1} $month^{-1}$. If it is assumed that CO_2 efflux is steady state (biomass formed = biomass degraded), then 1.4 mg loss represents a 0.17% decrease in HC per month (0.83% C) or a loss of 1% in 6 months.

Table 8-2. Oil content and chemical characteristics of Waste 1 sampled from the Bio-Pile (July, 1993) and incubated for 8 and 18 weeks at 22, 30 and 35°C (n = 6, $\bar{x}$ ± SD).

Temp.	Oil content (%)[1]		pH		EC (dS m^{-1})		NO_3-N ($\mu g\ g^{-1}$)		NH_4-N ($\mu g\ g^{-1}$)	
	Weeks Incubation									
(°C)	8	18	8	18	8	18	8	18	8	18
22	2.5 ± 0.1	2.4 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	285 ± 19	352 ± 40	5 ± 0.5	6 ± 1.2
30	2.3 ± 0.2	2.6 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	397 ± 8	473 ± 20	12 ± 1.0	9 ± 0.6
35	2.4 ± 0.1	2.2 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	405 ± 28	498 ± 20	11 ± 1.9	7 ± 0.9

[1] Initial oil content 2.5 ± 0.4% (n = 7).

Table 8-3. TPH of Waste 1 sampled in July 1993 from the Bio-Pile and incubated in a slurry (20 g soil in 40 ml water) at 30°C in a rotary shaker (n = 8 for initial, n = 4 for other times).

Treatment	Incubation Time (weeks)	TPH ± SD (%)	NO_3-N ($\mu g\ g^{-1}$)	EC ($dS\ m^{-1}$)
Slurry[1]	0	2.5 ± 0.40	274 ± 15	1.8 ± 0.1
Slurry	2	2.3 ± 0.10	362 ± 33	2.4 ± 0.1
Slurry	4	2.8 ± 0.05	375 ± 24	2.6 ± 0.1
Slurry	8	1.8 ± 0.13	424 ± 16	2.9 ± 0.3
Air Dried[2]	4	3.0 ± 0.26	ND	ND
Air Dried	8	1.8 ± 0.13	ND	ND

[1] Slurries harvested by evaporating the water off in a plastic dish until the solids could be scraped off (~12h) and put in Teflon sealed vials, frozen and shipped to AEC for TPH analyses.

[2] Samples allowed to air dry at room temperature at time 0 and stored in open containers.

9. CO_2 EFFLUX AS AN ESTIMATE OF MICROBIAL ACTIVITY IN CARBONATE CONTAINING WASTES

9.1 *Introduction*

Results of a temperature effects test of Waste 1 from the Bio-Pile indicated that the microbial activity did not differ amoung the three temperature regimes (22, 30 and 35°), contrary to what is normally expected (Part 8). The method used to estimate microbial activity (oil and biomass mineralization) was headspace analysis by measuring CO_2 concentrations at time zero (jars sealed) and 1 h later. This technique correlated well with the IRGA continuous flow method in incubation tubes in an earlier experiment (Danielson 1993, Part 9.2).

However, the temperature test results aroused suspicions that here may be methodological problems in measuring CO_2 efflux rates in soils or wastes in which the pH is high and free carbonates are present. Indeed, shifts in chemical equilibria or test conditions can profoundly affect CO_2 measurements in high pH soils which can lead to erroneous short term activity estimates (Martens 1987; Sparling and West 1990).

Suspicions regarding faulty estimates of activity were sharpened when rate estimates of CO_2 efflux from Waste 1 (Part 8) were made using different measurement time intervals. A basic assumption of headspace analysis is that the method does not interfere with the microbial activity and that the rate is constant during the measurement period. That is, sealing the jars and allowing CO_2 to build up, oxygen to deplete, and other volatiles to accumulate in the gaseous phase, will not affect either the rate of biological CO_2 production, the respiratory quotient (ratio of CO_2 produced unit O_2 consumed) or alter the carbonate equilibrium (inorganic CO_2 source and sink relationship). Data gathered on measurement intervals at the three temperatures indicated that the basic assumption had been violated.

CO_2 concentrations were determined in the sealed jars for up to 48 h and rate estimates decreased from 2.0 (35°) and 2.7 (22°) μg CO_2-C g^{-1} h^{-1} during the 0 - 1 interval to 0.4 (22°) and 0.7 (35°) μg CO_2-C g^{-1} h^{-1} in the 0 - 48 h interval. Further, there was a positive response to temperature after sealing the jars for 24 h or more whereas in the first hour no effect was measured. Estimates of CO_2 production after 24 h were about 20% of the estimates made in the first hour after sealing the jars. Clearly, the CO_2 efflux rate decreases documented after sealing the jars needed to be explained so that the best estimates of activity could be made. If the rate decreases were real, it was postulated that they could be due to either accumulation of organic volatiles or CO_2, which inhibited activity via product feedback

mechanisms. If the decreases were measurement artifacts, then it was necessary to determine the proper interval for estimating rates that were occurring in unsealed (normal incubation conditions) containers.

Questions to be answered regarding discrepancies in CO_2 rate measurements included the following.

(1) Were organic volatiles from oil released and inhibiting activity in sealed jars?

(2) Were the reduced rates in sealed jars an exclusive property of oily wastes/soils?

(3) How does the continous measurement with CO_2-free air compare with headspace analysis or with continuous flow with CO_2-containing air, i.e., how does the presence of gaseous CO_2 affect apparent CO_2 production?

(4) Will eliminating the carbonates from the waste remove the CO_2 rate anomalies?

(5) What is the best method for estimating microbial activity in high pH materials?

9.2 *Experimental conditions*

9.2.1 When the respiration decrease in sealed jars was first observed, it was thought that organic volatiles originating from the hydrocarbon waste might be responsible. In order to test this, charcoal was placed in the jars to adsorb the volatiles. Replicate jars (3) were prepared with 60 g of Waste 1 from the Bio-Pile and 20 g Sigma activated charcoal (100 - 400 mesh) in a Kleenex suspended above the waste. Jars without charcoal acted as controls and all jars were incubated at 22° and headspace CO_2 estimated in intervals from 1 to 24 h.

9.2.2 To determine if noncontaminated soils behaved in a similar manner to Waste 1, three soils were tested as Waste 1 had been in the jars at 22°. The first soil was forest floor material from a white spruce stand in Saskatchewan composed of FH material with a pH of 6.8. The second was from a pasture in the Rocky Mountain foothills, low in organic matter with a pH of 7.9 and the third was the uncontaminated agricultural topspoil from Erskine (pH 7.1).

9.2.3 In order to measure CO_2 efflux of the same samples by different methods without any disturbance, wastes and soil were incubated in glass respiration tubes. Either continuous flow measurements could be made or the ends of tubes stopped with rubber bungs which had a sampling septum in place and headspace gases analyzed. In this way a continual sequence of measurements were made on replicate samples (3) by switching back and forth between headspace, open tubes and continuous flow with and without CO_2 scrubbing to determine if methodology and/or pretreatment affected CO_2 efflux rate estimates (see Table 9-3 for precise sequence of conditions). The materials tested were Waste 1 from the Bio-Pile (pH 7.5), DIMR from Cell

7 (pH 7.6) (both sampled in November 1993) and the alkaline pasture soil (pH 7.9).

9.2.4 A second series of measurements were made on the DIMR and Waste 1 in tubes as described above. In this case, the materials were acidified to pH 6 with HCl and 2% $HgCl_2$ added to the acidified and nonacidified materials. The sequence of headspace/continous flow measurements are given in Table 9-4. To acidify the materials, 6.7 ml conc. HCl was added to 400 g of Waste 1 and 18.3 ml of HCl to the DIMR. Following the respiration tests, 8 mg glucose/g material was added to the contents of the tubes and respiration response (SIR) measured to see if the HCl and Hg had killed the microflora.

9.3 *Results and discussion*

When the decrease in respiration rates measured in sealed jars was originally observed, it was surmised that one possible cause could be the build-up of inhibitory volatiles from the oily waste. Retesting of Waste 1 confirmed that the rates did decrease as the measurement period was lengthened from 1 h to 24 h (Table 9-1). At this time charcoal was placed in the jars to adsorb volatile organics; however, the charcoal was an effective adsorber of CO_2 and thus microbial activity could not be measured by the CO_2 production method. Other adsorbants were assumed to perform in a similar manner, i.e., adsorb CO_2 as well as other gases, and this approach was abandoned but it did confirm that rates estimates were strongly dependent on the time of measurement.

Noncontaminated soils were tested in the same manner as the oily materials and the two minerals soils behaved in the same way, with CO_2 production rates decreasing after the first hour (Table 9-2). The CO_2 production rate estimates for the forest floor materials remained relatively constant throughout the two day period despite the accumulation of very high levels of CO_2 (excess of 5%) in the jars. Total accumulation of CO_2 was much less with the mineral soils but production estimates decreased one-half after 1 h and about 70 - 80% thereafter. Both of these soils contained free carbonates as evidenced by the vigorous effervescence when HCl was placed on the soils.

It has been suggested that levels of CO_2 in the range from 1000 to 2500 $\mu g\ g^{-1}$ may inhibit soil respiration (Koizumi et al. 1991; Nakadai et al. 1993) but these studies did not take into account the sorption and release of CO_2 from carbonates or other changes in chemical equilibria. It was thus still uncertain whether decreased respiration was a real repression or a methodological artifact. Clearly, some soils, i.e., the high activity forest floor, were immune to these effects whereas carbonate containing mineral soils were susceptible. If the effects were real then different methods for detecting CO_2 efflux should give similar results; conversely, if rates are subject to method effects, rates should vary widely depending on the method.

Table 9-1. Effect of measurement period and the presence of activated charcoal on CO_2 production rate estimates and CO_2 concentrations in sealed jars containing Waste 1 from Bio-Pile.

Treatment	CO_2 Parameter	Measurement time (h)			
		0-1	0-2	0-4	0-24
None	$\mu g\ g^{-1}$	562 ± 36	645 ± 57	770 ± 63	1511 ± 76
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$	1.3 ± 0.1	1.3 ± 0.4	0.8 ± 0.2	0.5 ± 0.1
Charcoal	$\mu g\ g^{-1}$	392 ± 42	297 ± 14	144 ± 23	64 ± 6
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$	0.2 ± 0.2	0	0	0

Note: CO_2 concentration is that at the end of each measurement period and rates are calculated from time 0 to the end of each period.

Table 9-2. Rate of CO_2 production and CO_2 concentration in sealed jars containing clean soils as estimated in different measurement periods.

Soil	CO_2 Parameter	Measurement Period (h)			
		0-1	1-2	4-22	22-46
Forest Floor	$\mu g\ g^{-1}$	2400 ±88	4070 ±92	26000 ±1870	54000 ±2080
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$	15.7 ±0.7	20.1 ±1.7	12.3 ±1.1	13.0 ±0.9
Pasture	$\mu g\ g^{-1}$	590 ±8	630 ±47	900 ±52	1400 ±45
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$	1.0 ±0.1	0.5 ±0.4	0.2 ±0.02	0.2 ±0.02
Erskine	$\mu g\ g^{-1}$	670 ±10	740 ±43	1600 ±133	2400 ±350
	$\mu g\ CO_2\text{-}C\ g^{-1}\ h^{-1}$	1.5 ±0.1	0.8 ±0.4	0.5 ±0.06	0.4 ±0.1

Note: CO_2 concentration is that at the end of each measurement period and rates are calculated for the intervals indicated.

Measurement of CO_2 efflux rates via the continous flow technique in which CO_2-free air was rapidly (approx. 200 ml/min) passed through the sample for 4 h or more gave much higher rate estimates than did the sealed container method for two wastes and the pasture soil (Table 9-3). In that the same samples were measured by both methods the results should have been close unless the background CO_2 levels had a major influence on apparent rates.

In the most extreme case, the pasture soil gave high CO_2 rates in the presence of CO_2-free air and negative or zero rates when resealed and headspace CO_2 measured. This can only be explained by the release of abiotic CO_2 when gaseous CO_2 is low, which creates a CO_2 sink for biologically produced CO_2 once containers are sealed. A long time (>24 h) would be required for equilibrium to be reached and meaningful rate measurements to be made by headspace analysis following air-stripping of CO_2.

Waste 1 was intermediate in response and again the flow method gave high results which created a sink but apparent headspace equilibrium was reached between 2 and 20 h. In this case the correct rate appears to be 0.3 - 0.5 μg CO_2-C g^{-1} h^{-1} and the flow method results in about a 5-fold overestimate due to carbonate equilibrium shifts. The value for continuous ambient flow (approx. 400 μg CO_2 g^{-1}) was intermediate but exact measurement using ambient air are more difficult due to small peak height differences and shifting baselines.

The DIMR was more active than Waste 1 but the flow method still gave values 2-fold higher than the headspace method. Carbonate equilibrium shifts were similar to Waste 1 even though the $CaCO_3$ equivalent values were over twice as high as for Waste 1 (6.9 versus 2.4%). Reliable headspace values were obtained in the first hour, indicating that equilibrium was rapidly established and net efflux was in a steady state. Although it may appear that measuring rates in carbonate-containing soils is a "pick any number" game, this is due to the profound influence of gaseous CO_2 on carbonate equilibria and the different times required to reach steady state.

In an attempt to eliminate the carbonate, the wastes were treated with acid until efferescence ceased and the pH was about 6. $HgCl_2$ was added to determine if biological activity was present after acidification. Rates of CO_2 efflux were similar for acidified samples with and without $HgCl_2$ suggesting the acid had killed the microflora (Table 9-4). When glucose was added to the wastes at the end of the experiment, the lack of a respiratory response indicated this was true (Figs. 9-1, 9-2) and carbonate effect could not be evaluated directly.

Results for nonacidified samples were similar to the previous experiment with headspace values about one-tenth the values for continuous flow for Waste 1. The relatively high values for $HgCl_2$ treated material was probably due the acidifying effect of the mercury as the

Table 9-3. Measurements of CO_2 efflux of Waste 1 from the Bio-Pile, DIMR from the Bio-Reactor Cell 7 and clean pasture soil by continuous flow or headspace analysis.

Treatment/Measurement	CO_2 Efflux (μg CO_2-C g^{-1} h^{-1})		
	Bio-Pile	DIMR	Pasture
Continuous CO_2-free (4h)	2.9	4.4	5.6
(Open for 30 min)	-	-	-
Headspace 0-1h	0	1.5	-0.4
Headspace 1-2h	0	1.7	0
Headspace 2-20h	0.3	1.9	0
(Open tubes and reseal)	-	-	-
Headspace 0-1h	1.0	3.8	0.2
Headspace 1-2h	0.5	2.3	0.1
Continuous CO_2-free (4h)	2.5	4.2	4.4
(Open and reseal)	-	-	-
Headspace 0-1h	0	1.4	-0.5
Headspace 1-2h	0.2	1.3	-0.1
Continuous ambient (18h)	1.2	2.9	0.5
Headspace 0-1h	0.3	1.9	0
Headspace 1-2h	0.2	1.9	0

Note: The same samples were carried through the entire treatment chain beginning at the top of the table and progressing for 3 days to the bottom of the table

Table 9-4. Measurements of CO_2 efflux from treated Waste 1 from Bio-Pile and DIMR Cell 7 from Bio-Reactor with HCl acidification and/or addition of 2% $HgCl_2$.

	CO_2 Efflux (μg CO_2-C G^{-1} h^{-1})					
	CO_2-free Continuous	0-1h Headspace	1-2h Headspace	2-24h Headspace	CO_2-free Continous	Ambient Continous
Bio-Pile	2.5±0.16	0.6±0.08	0.2±0.20	0.2±0.01	2.2±0.1	0.8±0.1
Bio-Pile + Hg	2.1±0.14	0.3±0.06	0.3±0.05	0.1±0.01	1.6±0.1	0.4±0.1
Bio-Pile + Acid	1.0±0.05	0.3±0.06	0.2±0.04	0.1±0.01	0.8±0.3	0.7±0.1
Bio-Pile + Acid + Hg	0.8±0	0.3±0.05	0.3±0.04	0.1±0.02	0.6±0.1	0.4±0.1
DIMR	3.8±0.04	1.2±0.04	0.8±0	0.9±0.06	3.2±0.1	1.9±0.1
DIMR + Hg	2.5±0.02	0.3±0.02	0.1±0.10	0.04±0	1.8±0.1	0.3±0.1
DIMR + Acid	1.0±0.06	0.3±0.11	0.02±0.02	0.06±0	0.8±0.1	0.2±0.1
DIMR + Acid + Hg	1.1±0.45	0.2±0.12	0.06±0.05	0.04±0	0.7±0.1	0.1±0.1

Note: $HgCl_2$ added on day 5, first measurement on day 10 and same samples measured for 3 days from left to right with 30 min open tubes between continuous and headspace measurement.

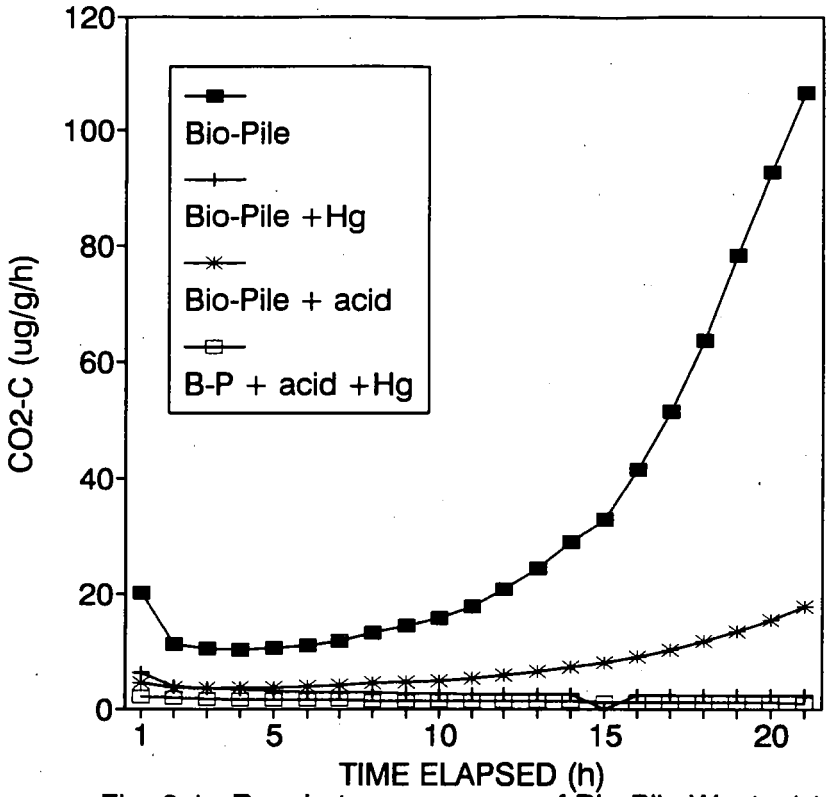

Fig. 9-1. Respiratory response of Bio-Pile Waste 1 to added glucose.

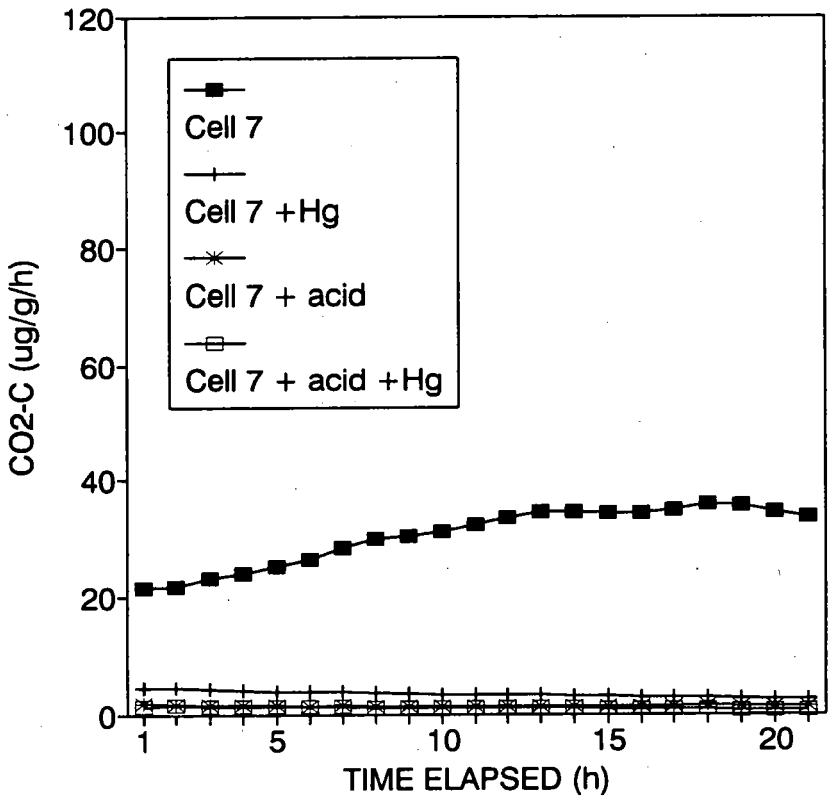

Fig. 9-2. Respiratory response of Bio-Reactor Cell 7 DIMR to added glucose.

pH was reduced from 7.7 to 6.8 at the end of the experiment.

CO_2 values for the DIMR were about 75% lower by headspace analysis than continous flow. The contribution by carbonate-CO_2 was probably less in Waste 1 as the pH in the $HgCl_2$ and nonpoisoned DIMR were both pH 7 and CO_2 from the mercury treatment was minimal.

The problems associated with measuring microbial activity in high pH soils have been recognized previously and are based on chemical phenomena which can be quantified under ideal circumstances. Martens (1987) criticized the use of the headspace procedure for measuring CO_2 efflux from neutral or alkaline soils as he demonstrated that CO_2 was adsorbed by the soil. However, this was under nonequilibrium conditions using a short (30 min) time span. He proposed that either a continuous flow or alkaline adsorption method be used to avoid problems with shifting chemical equilibria. The results found here agree that CO_2 is indeed adsorbed by carbonates but that the CO_2-free continuous flow method causes an opposite shift in the carbonage equilibrium which results in the release of fixed CO_2 and an overestimate of biologically produced CO_2.

Results by Koizumi et al. (1991) and Nakadai et al. (1993) suggested the alkali adsorption method overestimated CO_2 efflux in comparison to the continous flow method but as the pH of the soil they used was not reported, it is impossible to evaluate the role, if any, of changes in carbonate equilibria. Nonetheless, they showed again that the method strongly affected the quantitative results.

Sparling and West (1990) used the CO_2 headspace technique to estimate SIR and evaluated the potential errors caused by nonequilibria between gaseous CO_2 and solution phase CO_2 and confirmed that above pH 6.5 a large proportion of the CO_2 was retained in the solution phase. This resulted in underestimates of microbial activity but as they were adding glucose and making short term measurements, the systems were not in equilibrium. They recommended that the headspace technique be used for respiration measurements, but only on soils below pH 6.5. The amount of water in the sample also strongly affected CO_2 efflux and the less water present, the quicker equilibrium would be established.

For measuring CO_2 efflux as an estimate of total microbial activity in slightly alkaline carbonate-containing wastes, the best method would appear to be headspace analysis. The systems contain low amounts of water and it can be assumed that CO_2 in gaseous and solution phases are in equilibrium or reach equilibrium within a few hours. Continuous flow measurements may be satisfactory for materials with high microbial activity as CO_2 from inorganic sources is a small proportion of total CO_2 efflux. Specifically, the best time interval for headspace measurements of low activity wastes would appear to be from hour one to hour 4 or 22. Equilibrium can be readily verified if

necessary by making successive measurements as done in this series of experiments. Wastes subjected to continous flow of CO_2-free air are never in equilibrium and a 4 h measurement represents a practical compromise.

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