Bioremediation of Petroleum Hydrocarbon Contaminated Soil

by

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ABSTRACT

The bioremediation of petroleum contaminated soil in large-scale treatment units was studied in conjunction with Sybron Chemicals Inc., Salem, VA. The soil had been previously contaminated and was spiked with additional petroleum. Water with different characteristics was circulated through the soil in order to evaluate the effect of nutrient enhanced treatment without and with addition of two inoculation materials - Sybron's ABR Hydrocarbon Degraders and *Rhodococcus sp.* - on the rate of hydrocarbon degradation. Treatment units without nutrients and introduced organisms served as controls. Total petroleum hydrocarbon concentrations (TPH) were monitored using two alternative analytical methods, infrared spectrophotometry and gas chromatography. The results of the field study and different laboratory experiments, a radiotracer flask assay, static soil microcosms, and soil columns were compared. While nutrient addition did enhance biodegradation, the addition of autochthonous organisms was not found to accelerate hydrocarbon degradation rates in the previously contaminated soil. A significant decline of surface tension in the circulated water after inoculation with *Rhodococcus*, was thought to be due to microbial production of surfactants, but did not increase TPH degradation. The radiotracer technique and microcosm study confirmed these

results. The soil column study indicated that the rapid degradation of soluble and slower degradation of less soluble hydrocarbons occurred in two subsequent phases with approximately zero order rates. Typical degradation rates for the more soluble or degradable petroleum hydrocarbons were approximately 40 ppm/week and for the less soluble and degradable compounds 10 ppm/week. Microcosms were found to successfully predict the degradation rates of the soluble hydrocarbons, while the soil columns simulated degradation of the less soluble hydrocarbons best. The analysis of soil extracts for petroleum hydrocarbon concentrations with infrared spectrophotometry was found to be defective.

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1. INTRODUCTION

The transport and utilization of hydrocarbons involves the risk of spills and subsequent contamination of soil and groundwater. The most widely used technique for clean-up, pumping, treating and reinjecting the water, has often been found to be unsuccessful in reducing contaminant concentrations to desired levels. The removal of the soil through excavation and off-site disposal or further treatment is an expensive alternative to pump and treat and must meet strict regulations. Tighter federal and state regulations on the management and disposal of hazardous waste, including petroleum contaminated soils, point to the need for more effective and less costly treatment methods (Thayer 1991).

Innovative alternatives for soil remediation involving in situ treatment are being developed and refined. Bioremediation, one of these technologies developed over the last decade, uses microorganisms to degrade petroleum hydrocarbons and represents a low-cost alternative to physicochemical and thermal treatment techniques. A wide range of organic compounds are biodegradable. However the application of in situ bioremediation in field situations is complex and the process is extremely sensitive to environmental conditions (Thayer, 1991).

Biodegradation of organic wastes occurs naturally in the

environment. Companies like Sybron Chemicals Inc. offer bioremediation services, special fertilizers and microbial inoculation materials to speed degradation rates and degrade resistant organic compounds. Whether the biodegradation of organic compounds can be enhanced by these means, is much disputed. This study was designed in conjunction with Sybron Chemicals Inc. to evaluate the effectiveness of different techniques for enhancing the biological degradation of hydrocarbons in soils. The specific objectives were:

1. to compare the effect of nutrient addition, the addition of Sybron's ABR Hydrocarbon Degrading organisms and the addition of a *Rhodococcus sp.* inoculum on hydrocarbon degradation by a microbial population in a large scale soil system;
2. to evaluate the effect of microbial surfactant production by the organism *Rhodococcus* on hydrocarbon degradation.
3. to evaluate the usefulness of a radiorespirometric flask assay, a radiotracer technique using ^{14}C-hexadecane, as a measure of the hydrocarbon degradation potential of soil microorganisms;
4. to compare microcosm data with field data;
5. to compare several methods for the determination of the total petroleum hydrocarbon (TPH) content of contaminated soils.

2 LITERATURE REVIEW

2.1 Petroleum

Crude petroleum consists of a mixture of hydrocarbons and hydrocarbon derivatives, as well as impurities such as sulfur, oxygen and nitrogen. Hydrocarbons are molecules which consist of carbon and hydrogen atoms (Schmidt, 1985).

Hydrocarbons can be classified into one of four groups: paraffins, naphtenes, olefins and aromatics. Paraffins are saturated hydrocarbon molecules, containing only single atomic bonds. Normal paraffins are straight chain molecules, iso-paraffins are branched. Naphtenes, also called cycloparaffins, have a ringlike structure. Olefins are unsaturated hydrocarbons, containing double and triple bonds between atoms, and are therefore the most reactive group of hydrocarbons. Aromatics are hydrocarbon molecules, which contain at least one benzene ring in their structure (Novak, 1992).

The hydrocarbon compounds and derivatives range from gaseous, liquid to solid compounds. These different fractions of crude petroleum can be separated by distillation, extraction or adsorption. Some petroleum products are liquefied petroleum gas, gasoline, kerosene, fuel oil, lubricating oil, grease, wax and bitumen.

Fuel oil includes a variety of substances that can be burned to produce heat. Fuel oils are classified into five grades, 1, 2, 4, 5 and 6, depending on their viscosity. No. 1 and 2 fuel oils, which are called distillate oils, vaporize at relatively low temperatures (149 - 343 °C) under atmospheric pressure and have a very low viscosity. No. 4 and 5 include a range of fuel oils, which are still relatively low in viscosity. No. 6 is the heaviest and most viscous grade of fuel oil. At room temperature it is a black, gummy and semifluid material. No. 6 fuel oil is the residue from crude oil after the extraction of lighter fractions such as gasoline, naphtha, kerosene and distillate. It consists of hydrocarbons with 18 and more carbon atoms. Since contaminants such as water and sediment are concentrated in the residual products of the refining process heavy oils including No. 6 may have a water and sediment content of up to 2 %. Heavy fuel oils also contain gum, resins, asphaltenes, tar and waxes (Schmidt, 1985). Typical properties of no. 6 fuel oil are listed in Table 1.

TABLE 1: Typical properties of No. 6 fuel oil (adapted from Schmidt, P., 1985)

Specifications	Analysis of No. Oil
Specific Gravity	0.97 (0.93 - 1.01)
Kinematic Viscosity, cSt(50°C)	455 (307 - 572)
Flash Point, °C open cup	130
Pour Point, °C	18
BTU/gal	150
Conradson Carbon, %	9.5 (3.9 - 16.3)
Sulfur, %	1.0 (0.85 - 2.5)
Ash, %	0.04 (0.02 - 0.1)
Water and sediment, %	0.4 (0.20 - 2.0)

2.2 Petroleum Contaminated Soil

Petroleum contaminated soil represents a multiphase subsurface system with an aqueous phase, a free or separate contaminant phase, a gas phase and a solid porous medium, the soil. Soils vary in texture, moisture, organic matter and mineral content, pH, and microbial population. The transport and fate of the hydrocarbon contaminants in a the soil system depends on several interacting mechanisms, which include solubilization and transport with the water phase, volatilization, retention on the soil due to adsorption, incorporation into microbial biomass, and biodegradation. The relative importance of these mechanisms depends on the chemical and physical properties of the contaminant and the characteristics of the soil (Canter *et al.*, 1987).

Volatilization occurs in the unsaturated zone, where the more volatile petroleum compounds partition into the gas phase. The vapor phase migrates through the soil and is eventually lost from the soil system (Canter *et al.*, 1987).

Adsorption refers to the partitioning of compounds between the water and solid phase and has been found to be strongly correlated to the total organic carbon (TOC) and surface area of the soil material. Adsorption is thought to be related to the organic matter content of the soil due to hydrophobic bonding of non-polar organics. Surface sorption of organic

molecules appears to be significant in soils with high clay content. This may be due to the high surface area and charge on the clay minerals. Soil sorption is thought to be a dominant factor in the subsurface transport and fate of organics according to several studies cited by Canter *et al.* (1987). Soil adsorption retards the contaminant transport in the aqueous phase and has also been found to decrease the emission of volatile organic carbons (VOC). Organics in the adsorbed phase are not accessible to biodegradation (Canter *et al.*, 1987).

Soluble hydrocarbon compounds partition between the free and aqueous phase. In the water phase the compounds are subject to convective and dispersive transport as well as microbial degradation.

2.3 Soil and Groundwater Remediation

Petroleum hydrocarbon contaminants in soil are a potential source of groundwater contamination. More than 50% of the U.S. population utilizes groundwater as a drinking water supply. Therefore the presence of chemical pollutants in groundwater represents a serious public health concern (Robinson *et al.*, 1990).

The use of innovative treatment technologies for hazardous

wastes such as petroleum contaminated soil is, to a large extent, regulatory driven. Several federal environmental regulations for waste minimization and treatment have been enacted since the mid-1970s. These include the Resource Conservation and Recovery Act (RCRA) of 1976. RCRA defines hazardous waste as a subset of solid waste, exhibiting one of four characteristics: ignitability, reactivity, corrosivity, or toxicity. Allowable concentrations have been specified for 39 hazardous chemicals, six pesticides, eight metals and 25 organics, which include petroleum hydrocarbon constituents such as benzene, toluene and xylene. RCRA regulates on-going waste production, however also contains regulations on corrective action for hazardous waste sites. As many as 4000 sites may require remediation under the corrective action side of RCRA. The Comprehensive Environmental Response, Compensation & Liability Act (CERCLA) of 1980, also known as Superfund, is targeted directly at the remediation of old waste sites and lists about 1200 sites for cleanup (Bakst, 1991, Thayer, 1991).

The most widely used technology for groundwater remediation involves pumping, treatment and reinjection of the water. Although effective in retaining the contamination the technique has failed to reduce contaminant concentrations by more than 50% in any Superfund site according to a recent study, focused on VOC contaminants. Secondary sources of

contamination, chemicals trapped in the soil often continue to pollute the water. Estimates for pump-and-treat time frames range between 100 and 1000 years, and costs for clean-up of identified Superfund sites may be as high as $1 trillion (Reid, 1992).

Physical treatment methods, e.g. soil washing, adsorption, filtration, or solvent extraction can separate contaminants, which then require additional treatment. Chemical treatment can be applied to various waste materials, but may create hazardous by-products. Thermal treatment methods such as incineration are effective in completely destroying the waste and reducing its volume. However, emissions and ash residues may require treatment, the process is very expensive, and the public generally opposes this method (Thayer, 1991).

Bioremediation, which utilizes microorganisms to degrade the contaminants, is one innovative alternative to traditional treatment options. It is not effective against many corrosive, reactive, radioactive substances, or heavy metals. However bioremediation does present a viable, cost-effective treatment for organic chemicals, such as petroleum hydrocarbons (Thayer, 1991).

Bioremediation includes treatment in bioreactors, land treatment, and in situ treatment. Bioreactors are reaction vessels, typically with a diffuser system to supply oxygen, which provide total control over the process conditions - pH,

temperature, and availability of nutrients. Reactor configurations include fixed film, plug flow, fluidized bed, sequencing batch and soil slurry reactors, as well as conventional waste water treatment processes such as activated sludge, trickling filters and lagoons. Land treatment involves the application of contaminated soil to the soil surface and subsequent tilling, nutrient addition and watering. A drainage system and impermeable liner are usually required by regulations and represent a substantial part of the total project costs. For both bioreactors and land treatment the soil usually must be excavated. In many cases, especially where large quantities of soil are involved it is not possible or economical to excavate the soil for off-site treatment. (King *et al.*, 1992).

In situ bioremediation often requires the extraction and recirculation of groundwater. A solution, containing nutrients, oxygen and sometimes a microbial Inoculum is added through injection wells. After recovery of the solution from a production well it can be recirculated or treated and disposed at the surface (Thomas and Ward, 1989).

The most appropriate bioremediation technology is chosen on the basis of three major factors a) cost, b) time and c) regulatory aspects (King et al., 1992).

Compared to traditional land treatment, a modern in situ biological treatment system requires less surface area, has

lower operating costs, and leads to a quicker reduction of contaminant concentrations to acceptable levels, often in less than 10 weeks. Combined with options such as soil vapor extraction or surface biotreatment, in situ bioremediation is almost always the most cost effective method for soil and groundwater remediation (Hildebrandt and Wilson, 1990).

2.4 Biodegradation of Petroleum Hydrocarbons

Microbial degradation usually involves the stepwise oxidation of hydrocarbons to their corresponding alcohol, aldehyde and fatty acid. The fatty acids are further degraded to smaller fragments that are either utilized by the microorganisms or ultimately mineralized to CO_2. (Bhandari, 1992).

Biodegradation occurs naturally in the environment, but it is a very slow process. The rate of hydrocarbon degradation depends on several factors: a) the availability of the substrate to the microorganisms, b) the availability of nutrients, c) the availability of an electron acceptor, generally oxygen d) the quantity and activity of the microorganisms. Temperature, moisture content, Ph, organic matter and clay content of the soil are other important factors (Tramier and Sirvins, 1983).

The availability of substrate to the organisms is a function of the dissolution rate of the hydrocarbon components into the water. Detergents can be applied to increase their solubility (van den Berg *et al.*, 1988). In soils with a high clay and organic matter content, the rate of hydrocarbon desorption from the soil surface can limit degradation (Bhandari, 1992).

Nutrient availability is seen as a major limiting factor by some researchers. Nitrogen, phosphorous, sulfur and a number of trace minerals are required for microbial metabolism. Fertilizers, containing nitrogen and phosphorous at the correct ratio, have been successfully used to enhance biological degradation of organic compounds in laboratory and field situations. The provision of inorganic nutrients poses several problems. Ammonium ions may bind to mineral surfaces by cation exchange reactions and phosphates may precipitate as insoluble salts, which reduces soil permeability (Morgan and Watkinson, 1992).

An electron acceptor, generally oxygen, is required for microbial metabolism of petroleum hydrocarbons. Aerobic biological degradation in the subsurface is frequently limited by the transport of oxygen to the microorganisms in the zone of contamination. The availability of dissolved oxygen (DO) is further restricted by the limited aqueous oxygen solubility, the slow re-aeration rate of the groundwater in the saturated

zone, and the biological oxygen demand (BOD) of the microbial metabolism (Taylor and Jaffe, 1991, Pardieck *et al.*, 1992). Oxygen can be supplied in the form of air, pure oxygen and hydrogen peroxide. Air and oxygen can only provide 8 and 40 mg/L of dissolved oxygen/L respectively. Hydrogen peroxide is infinitely soluble, but is toxic at concentrations above 100 ppm (Thomas and Ward, 1989).

Another method to promote biodegradation of organic contaminants in soils is by the addition of autochthonous microorganisms selected for their ability to metabolize the compounds in question. The literature reports both success and failure of this method. Inoculation with an autochthonous soil population increased hydrocarbon decomposition in a laboratory study by Vecchioli *et al.* A mixed inoculum of a soil population obtained from an active petrochemical waste treatment site was used. The soil was heavily contaminated with 10% w/w of aromatic hydrocarbons. The degradation over the observed time span was 22 % higher with inoculation than without. Between fertilized and non-fertilized samples without inoculation no difference was observed (Vecchioli *et al.*, 1990). Under field conditions however the addition of organisms has not been proven to increase biodegradation. Definite proof of microbial degradation in itself is difficult to establish. Indigenous bacteria capable of metabolizing hydrocarbons are abundant at sites subject to oil spills.

Introduced organisms face parasitism, predatism and competition with indigenous microorganisms, which are acclimated to the environment and the contaminant (Thayer, 1991, Tramier and Sirvins, 1983).

2.5 Microbial Surfactant Production

Surfactants are molecules with two or more structural parts, one that is polar or hydrophilic and one that is non-polar or hydrophobic. Surfactants accumulate at phase boundaries, with their hydrophilic moiety oriented toward the more polar phase and their hydrophobic moiety oriented toward the less polar phase. This so called surface active characteristic lowers the surface tension of water (Edwards *et al.*, 1991).

In a subsurface system hydrocarbon contaminants adsorb to surfaces and may be retained through capillary forces in the pores as an immiscible, separate phase. Surfactants are thought to be capable of removing the hydrocarbons from the soil surface and mobilizing hydrocarbons from this immiscible separate phase. The rate of hydrocarbon solubilization also plays an important role in the uptake of hydrocarbons to the cells. The addition of surfactants may therefore accelerate hydrocarbon degradation (Goclik *et al.*, 1990, Oberbremer *et*

al., 1990).

Biosurfactants are surface-active agents that are microbially produced. Six major groups of biosurfactants exist: hydroxilated and crosslinked fatty acid, glycolipids, lipopolysaccharides, lipoprotein-lipopeptides, phospholipids and the cell surface itself (Parra *et al.*, 1990).

Biosurfactants, like synthetic surfactants, are effective in emulsifying insoluble organic substances. This characteristic makes biosurfactants applicable for the biological treatment of petroleum contaminated soils. Compared to synthetic surfactants they offer the advantage of being biodegradable (Falatko and Novak, 1992).

The addition of purified glycolipid biosurfactants was found to enhance hydrocarbon degradation in fixed bed soil columns and increase the extent of mineralization (Müller-Hurtig *et al.*, 1989). A significant increase of the velocity and efficiency of degradation was also seen in another model system containing soil and a hydrocarbon mixture. The length of adaptation during the degradation of different hydrocarbon compounds was shortened. Ultimately the added glycolipid biosurfactants were degraded. Both studies reported non-oxygen limitation as a precondition (Oberbremer *et al.*, 1990).

Biosurfactant production requires the growth of microorganisms on an organic substrate with limited solubility. Microorganisms found to produce biosurfactants

include strains of *Pseudomonas*, *Rhodococcus*, *Nocardia*, *Athrobacter*, *Rhodococcus sp.* grown on n-alkanes was found to produce glycolipid biosurfactants in several studies (Ramsay *et al.*, Kim *et al.*, 1990). Under growth conditions *Rhodococcus erythropolis* produces trehalosedicoryno-mycolates, which are cell-bound surfactants. An extracellular biosurfactant, trehalose-2,2',3,4-tetraester, is produced under conditions of nitrogen limitation, temperature and pH-shift (Kim *et al.*, 1990).

In principle, the addition of biosurfactant producing microorganisms may have the same effect as the addition of purified biosurfactants. Using *R. erthropolis* as a starter culture the rate of hydrocarbon degradation by an original soil population in a stirred reactor was found to increase. Compared to the addition of a purified glycolipid surfactant, trehalosedicorynomycolate, however the increase in degradation was small. The rate increase appeared to be due to the degradation by *R. erythrolpolis* itself, which was found to have a short adaptation phase. Trehalose-dicorynomycolates are cell-associated biosurfactants and may not be available for the soil bacteria. In order to replace the use of purified agents through the addition of microorganisms, bacteria or conditions that produce extra cellular biosurfactants are required (Goclik *et al.*, 1990).

2.6 Biodegradation Potential

Petroleum hydrocarbons interact with the soil, air and water in the pores. Abiotic factors can lead to the loss of contaminants through volatilization, chemical degradation, redistribution, and adsorption to surfaces (Thayer, 1991). To obtain a strict mass balance under field conditions and prove in situ biodegradation is therefore very difficult. A ^{14}C-radiotracer method can be used to prove the potential for biological degradation (Madsen, 1991).

Atlas (1979) described a laboratory flask assay for the measurement of the hydrocarbon degradation potential of water and sediment samples. Different ^{14}C-radiolabelled compounds and activity levels were used. Duplicate determinations were recommended. Poisoned controls were provided by adding concentrated hydrochloric acid to the flasks. The total CO_2 evolution during a six week incubation with and without weekly measurements showed no significant difference. This indicated that no oxygen limitation or CO_2 loss occurred.

A similar radiotracer method was used by Langschwager (1985) to evaluate the degradation potential of indigenous microbial soil populations. The degradation of methanol and tertiary butanol was compared, and results corresponded with oxygen uptake and microcosm studies. Instead of 25 mL flasks used in his experiments, Langschwager recommended larger

volume to avoid possible oxygen limitation.

Madsen (1991) cited several researchers, who have found that the degradation activity of microbial populations from disturbed or displaced samples was quantitatively, even qualitatively different from activity measurements in the field. A laboratory flask assay allows the direct measurement of microbial degradation activity, but the artificial conditions impose a significant error on the measurement.

2.7 Microcosms

Microcosms are laboratory model ecosystems, designed to simulate the processes and interactions in larger systems in order to evaluate the transport and fate of chemical pollutants. Microcosms can be used to study surface water, terrestrial and subsurface systems (Canter *et al.*, 1987)

Several types of microcosms are used to study subsurface degradation. Glass columns containing soil, through which a flow of water can be created, simulate conditions in an aquifer. Simpler, static soil/water microcosms consist of teflon-lined screw-capped test tubes. They are easier to set up and maintain and less expensive. However no flow can be simulated (White, 1986).

Pritchard described several operational criteria for microcosm studies:

1. a component of a real world system is studied in a laboratory test under standard conditions of light, temperature, humidity, aeration, pH etc.
2. the microcosms should be calibrated with the environmental system to be studied, and the effects of scale and containerization should be considered.
3. a microcosm study should be considered as a means of verification, representing a tool in the research rather than the research object itself (Pritchard, 1982).

Advantages of microcosms are that they allow replication and the use of controls due to their small size. Microcosm systems can be pertubed in various ways and the imports and exports to the system can be controlled. They offer a cost and time-effective alternative to field studies. Disadvantages of microcosms are that the containerization imposes structural and functional changes related to the system design rather than the original environment of the sample. Due to the vessel wall surface laboratory microcosms have unnaturally high surface-to-volume ratios (Pritchard, 1982).

2.8 TPH Analysis

Regulations mandating clean-up and permits for the closure of waste sites specify allowable contaminant concentrations. In the case of petroleum hydrocarbon contaminated soil certain compounds such as benzene, toluene and xylene are regulated, but also the total petroleum hydrocarbon (TPH) concentration, usually at a level of 100 ppm. Different analytical methods are used to determine TPH concentrations (Bakst, 1991).

Laboratory soil analysis for TPH generally involves extraction of the sample with solvents such as carbon disulfide, hexane, freon or methylene chloride, and analysis of the extracts by infrared spectrophotometry (IR), gas chromatography (GC) or gas chromatography/mass spectroscopy (GC/MS). A variety of soil analysis methods are used. These include standard methods developed by the EPA or methods developed individually by each company or institute. Freon or methylene chloride are most widely used as solvents (Moreton, 1991).

Similar to USEPA Method 418.1, Standard Methods for the Examination of Water and Wastewater (1980) described an oil and grease extraction method for water samples using trichloro-fluoroethane (freon) as solvent. This method was modified and used by Sybron Chemicals Inc. for TPH analysis in soils. Total petroleum hydrocarbons are defined by this

method. All compounds extracted by freon and not removed by subsequent filtration through silica gel are regarded as hydrocarbons. It should be noted that freon dissolves other organic substances apart from oil and grease. Compounds contained in heavier oil residuals may not be extractable by this solvent. In addition the more polar hydrocarbons such as complex aromatics and hydrocarbon derivatives of chlorine, sulfur and nitrogen may also be adsorbed by the silica gel (Standard Methods for the Examination of Wastewater, 1980). In freon extracts of groundwater a good recovery of volatile aromatic compounds such as benzene, toluene, ethyl benzene and xylenes, but less efficient recovery of polar compounds was found (Miller and Schmidt, 1991).

The extraction of some semi-volatile compounds may require the use of methylene chloride (MeCl) as a solvent. In comparison to freon, MeCl was found to be less selective and also extracted surfactants and other compounds with polar functional groups. Some methods such as the California method require the reduction of the MeCl solvent, which results in the loss of volatiles and some semi-volatiles (Miller and Schmidt, 1991).

A comparison between different TPH extraction methods was conducted at the Virginia Polytechnic Institute and State University (VPI&SU), a freon extraction similar to Standard Methods, and two MeCl extraction methods. The latter are the

California method, and a method used at VPI&SU, which will be referred to as MeCl/GC method. TPH concentrations in the extracts were determined with a capillary gas chromatograph (GC). Extractions from a clean soil, contaminated with a known quantity of petroleum, resulted in an recovery of 55 to 65% for both the California and MeCl/GC method and about 35% for the freon extraction method. Extractions from a contaminated soil, obtained from the field, resulted in a significantly higher TPH using the California method as compared to the other methods. While the MeCl/GC method was recommended for soils containing a significant amount of volatile compounds, the California method was preferred for heavier compounds (Novak, 1992).

The TPH concentration in the extract can also be analyzed through infrared spectrophotometry (IR). Since this method is more time and cost-effective it allows the collection of a larger number of samples. The method works best for fuels in the medium weight range such as jet fuels, diesel kerosene, and No. 2 fuel oil (Kasper *et al.*, 1991). However any compound recovered by the oil and grease extraction other than hydrocarbons and fatty acids can interfere with the IR measurement (Standard Methods for the Examination of Wastewater, 1980).

3 METHODS AND MATERIALS

3.1 Experimental Approach

Biological remediation of petroleum contaminated soil was studied in a large-scale experiment by Sybron Chemicals Inc. in cooperation with the Virginia Polytechnic Institute and State University (VPI&SU). The soil was obtained from a parking lot on the Sybron Inc. site in Salem, Virginia. To this previously contaminated soil additional petroleum was added. The soil was treated in large-scale treatment vessels (55 gal drums), through which water containing nutrients was circulated. Nutrient enhanced biodegradation by different microbial populations was examined: indigenous microorganisms, Sybron's ABR Hydrocarbon Degrader blend and a *Rhodococcus* strain. Treatment units without nutrient addition served as controls.

In order to compare different analytical methods for the determination of TPH, extractions of hydrocarbons from a number of soil samples were carried out with two different solvents, trichloro-trifluoroethane (freon) and methylene chloride (MeCl). The TPH analysis of MeCl extracts was performed by gas chromatography (GC), of the freon extracts by infrared spectrophotometry as well as GC.

The biodegradation potential of indigenous and added

microorganisms was determined using radiorespirometry, a ^{14}C-radio tracer method. The surface tension of the circulated water as an indication of biosurfactant production was monitored.

In a lab-scale study, microcosms were used to verify the effect of different treatment methods on biological degradation of petroleum contaminants in a more controlled environment. Biodegradation with an acclimated and non-acclimated microbial soil population were compared.

The field experiment was simulated in the laboratory with soil columns in order to give conclusive evidence of biodegradation. In order to reduce the effect of soil heterogeneity and uneven distribution of the petroleum contaminants as a cause of measurement variations, the initial conditions were to be as homogenous as possible. The replication of the system, allowing multiple sampling, was designed to investigate the degree of variation of the degradation process.

3.2 Soil Preparation

3.2.1 Sybron Field Study

The soil used in the field study, a clay loam, was obtained from on-site at Sybron Chemicals Inc., Salem, VA. The site had been used for the repair of trucks, and a number of uncharacterized hydrocarbon spills had occurred over several years. The soil was already contaminated by petroleum, and it was expected that soil microorganisms present in the soil were acclimated to the contaminant. The characteristics of the soil are listed in Table A-1.

The soil was prepared by shaking it through a 1/2 inch mesh screen. No. 6 fuel oil was added to the soil in a mortar mixer to increase TPH levels to approximately 1000 ppm. The relatively heavy fuel oil was chosen to minimize volatilization in the experiment.

Soil from the Sybron site will be referred to as Sybron soil. Soil containing only the initial weathered contamination will be referred to as unspiked Sybron soil. Soil with additional petroleum contamination, prepared for the field experiment, will be referred to as spiked Sybron soil.

3.2.2 Microcosm Study

The spiked soil from the Sybron field study was used without further preparation in an initial microcosm study at the VPI&SU laboratory.

Two different soils were used in a later microcosm study, the unspiked Sybron soil and a clay loam obtained from VPI&SU campus near the Hillcrest dormitory. While the Sybron soil was expected to be acclimated to petroleum contamination, Hillcrest soil was thought to be unacclimated.

The soils were screened with a 0.066 inch mesh screen and contaminated to a level of approximately 2000 ppm with No. 6 fuel oil. The soils were then placed into glass bottles and mixed in an agitation apparatus for four hours.

The soils will be referred to as screened Sybron and Hillcrest soil. The characteristics of the screened Sybron and Hillcrest soil are listed in Tables A-2 and A-3.

3.2.3 Soil Column Study

Unspiked Sybron soil was air dried and screened through a 0.066 inch mesh screen. One kg of the soil was left unspiked. One and one half kg of soil were contaminated with No. 6 fuel oil.

Because of its viscosity, an even distribution of the fuel oil in the soil had been difficult to obtain in the microcosm study. In order to achieve a homogenous contamination in the soil columns, the soil was therefore contaminated with the fuel oil dissolved in methyl-tertiary-butyl ether (MTBE). The soil was spread out under the fume hood for two hours, and the highly volatile MTBE was allowed to evaporate. As MTBE might have had a toxic effect on soil microorganisms, the soil was then mixed with 300 g of uncontaminated soil to reseed it.

Unspiked and spiked soil were allowed to mix in an agitation apparatus for two hours.

3.3 Soil Characterization

3.3.1 Soil pH

Soil pH was measured according to Methods of Soil Analysis (1982). Five g of air dried soil were placed into a 50 mL beaker. Five mL of distilled water were added. The suspension was mixed thoroughly for five seconds and then allowed to stand for 10 min. The pH was read on a standardized pH-meter. The pH of Sybron and Hillcrest soil is presented in Table A-4.

3.3.2 Bacterial Enumeration

Indigenous microbial populations in the soils were enumerated by direct plate counts with Standard Methods Agar (SMA), Tryptic Soy Agar (TSA) and Soil Extract Agar (SE). The plates were incubated in the dark at a constant temperature of 20 °C for one week. Microbial populations for the Sybron and Hillcrest soil are presented in Tables A-5 and A-6.

3.4 Inoculum

Two different Sybron products were used in the experiments, the ABR Hydrocarbon Degrader blew and a *Rhodococcus* strain. The ABR Hydrocarbon Degrader blend consisted of *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Escherichia* strains. *Pseudomonas* included six different species, which specifically degrade benzene, toluene, xylene, naphthalene, anthracite and diesel compounds.

Ten g of Sybron's ABR Hydrocarbon degrader blend were added to 100 mL of buffered water and placed 30 min onto a shaker table. The *Rhodococcus sp.* culture was placed 24 hours into 500 mL of Standard Methods nutrient broth. Both suspensions were centrifuged at 10,000 rpm for 10 min. After decanting the liquid, the microorganisms were resuspended into a smaller volume of phosphate buffered water.

The ABR Hydrocarbon degrader and *Rhodococcus* inoculum were enumerated with plate counts using Standard Methods Agar (SMA). From the bacterial counts of the inoculum the population density of introduced organisms per g of soil in the two microcosm studies was calculated. The results are presented in Table 2.

TABLE 2: Microcosms - Bacterial counts of ABR Hydrocarbon degraders and *Rhodococcus sp.* per g of soil.

	Counts (E6 cfu/g of soil)	
Microcosms w/	ABR HCD	*Rhodococcus*
spiked Sybron soil	1.0	1.2
screened Sybron/Hillcrest soil	2.0	0.5

3.5 Biological Treatment

3.5.1 Sybron Field Study

In situ bioremediation of petroleum contaminated soil was simulated in large-scale treatment vessels at the Sybron Chemicals Inc. laboratory, Salem, VA.

A bioremediation unit, shown in Figure 1, consisted of a 55 gallon plastic drum. Approximately 400 lbs of contaminated Sybron soil were placed into each drum, which was filled to a depth of about 27 inches. Cole Palmer peristaltic pumps were used to circulate water through the drums. The water was sprayed through the crossbar and collected in a ring pipe in the bottom of the drums. Holes with 1/4 inch diameter in the crossbar allowed steady recirculation of water, nutrients and inoculum. The ring pipe, equipped with larger holes was covered with a nylon hose to prevent soil from passing into the tubing and through the pump.

Different treatment methods with regard to the addition of nutrients and autochthonous organisms were used in the Sybron field experiment. Duplicate units were provided for each treatment. Nutrients were supplied by the addition of 470 mg diammonium phosphate (DAP)/kg of soil in all but the control units. Two of Sybron's products, the ABR Hydrocarbon Degrader blend and a *Rhodococcus* strain, were used as inoculum for biotreatment with introduced organisms and added to the

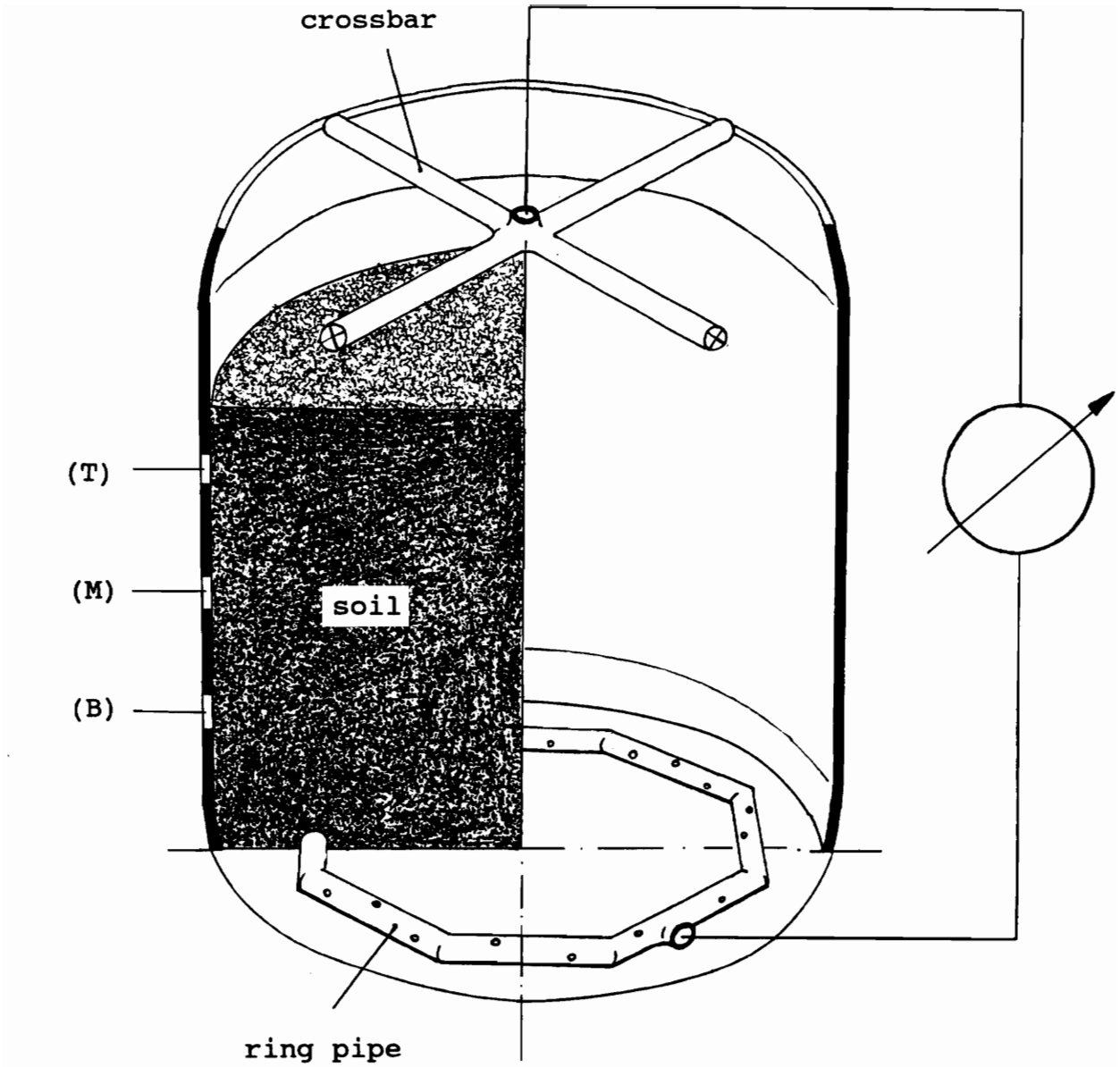

Figure 1: Soil bioremediation treatment unit.

circulated water initially, at week 3 and 10. The treatment methods as they will be referred to, the corresponding treatment units, and the variable parameters are listed in Table 3.

Sybron's research lab personnel collected soil samples weekly from all eight treatment units to obtain total petroleum hydrocarbon (TPH) concentrations and bacterial counts. Soil samples were collected from three different depths of the soil bed, 4 inch, 12 inch and 20 inch, through holes provided at the top (T), middle (M) and bottom (B) of the drums respectively (Fig. 1).

Soil samples were collected every two weeks by VPI&SU personnel from the middle (M) of drums 1 to 4 for TPH and biodegradation potential analysis. An iron pipe was inserted into the vessel, from which the soil was extruded with a plunger into sterilized flasks. Circulated water, dripping from the cross bar, was collected to be analyzed for surface tension. Sterile procedures were followed.

TABLE 3: Sybron field experiment - Unit designations by Sybron and matrix of variable parameters for different treatment units.

Treatment	Unit designation	Nutrients	Inoculum
Controls	a, 1	none	none
N + P	b, 2	N, P	none
ABR HCD	c, 3	N, P	ABR Hydrocarbon Degraders
Rhod.	d, 4	N, P	*Rhodococcus sp.*

3.5.2 Microcosm Study

Static microcosm were used to monitor the concentrations of TPH in different soils. An initial microcosm study used the spiked Sybron soil from the Sybron field experiment. A later study used screened Sybron and Hillcrest soil, which had been contaminated in the lab.

The microcosms, shown in Figure 2, consisted of culture test tubes (10 mL) with teflon lined screw caps from Fischer Scientific. Five g of soil were placed into the tubes, using sterile procedures. Approximately 6.5 mL of distilled water with four different characteristics were added. Table 3 lists the treatments as they will be referred to and the variable parameters. Nutrients were provided through the addition of 360 mg of diammonium phosphate (DAP)/Liter in all but the control microcosms. Autoclaved controls were prepared by autoclaving the microcosms for 30 min. at 125 °C.

The microcosms were incubated in the dark and at a constant temperature of 20° C. Two μL of hydrogen peroxide 30% were added every two weeks to provide oxygen. The microcosm contents were gently agitated with a rotary mixer (Fischer brand touch-mixer). The amount of hydrogen peroxide, equivalent to 92 ppm, was below a reported toxicity level of 100 ppm (Thomas and Ward, 1989).

TABLE 4: Microcosms - Matrix of variable parameters for different treatments.

Treatment	Nutrients	Inoculum
autoclaved controls	none	(autoclaved)
controls	none	none
N+P	N, P	none
ABR HCD	N, P	ABR Hydrocarbon Degraders
Rhod.	N, P	*Rhodococcus sp.*

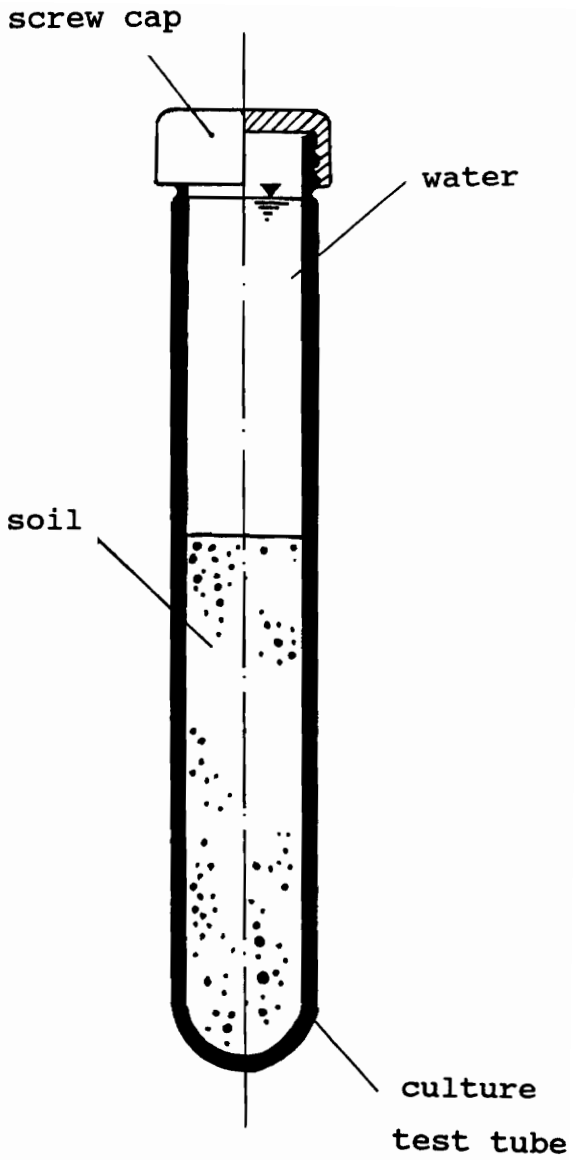

Figure 2: Soil microcosm.

For TPH analysis two microcosms were sacrificed every two to six weeks to obtain one composite soil sample of each treatment. During initial weeks sterile procedures were used to collect a number of samples for the determination of biodegradation potential.

3.5.3 Soil Column Study

The soil column study was to model the reactions occurring in the large scale treatment units of the field experiment. Nutrient enhanced treatment (N+P) of the unspiked and spiked Sybron soil was performed in multiple glass columns.

A soil column, shown in Figure 3, consisted of a 12 inch glass tube with a 1 inch diameter. The bottom of the column was blocked with a screen, glass wool and coarse gravel. Eight soil columns with uncontaminated and 17 soil columns with contaminated soil were prepared. Approximately 100 g of soil were placed into each glass column. About 20 mL of distilled water, containing 360 mg/L of DAP, were recirculated through each column every day. The liquid was poured by hand and allowed to drip into a beaker under the columns. Evaporation of water was compensated by regularly adding distilled water to the beaker, keeping the volume at 40 mL.

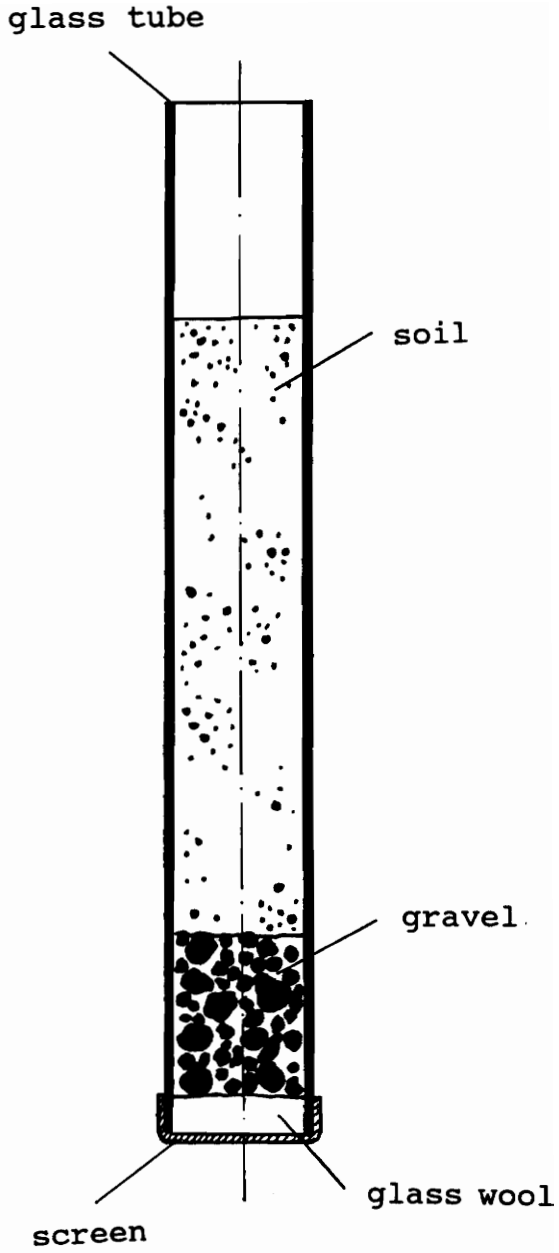

Figure 3: Soil Column.

Soil samples were taken weekly for TPH analysis. At least three samples of contaminated soil from two different columns and two samples of uncontaminated soil were taken. The soil was carefully extruded with a plunger through the bottom of the tube in order to not disturb the matrix of the remaining soil. Ultimately each column was sacrificed.

Surface tension of the circulated liquid was measured at least weekly. Three beakers containing the circulated water were randomly chosen for analysis.

3.6 Analytical Methods

3.6.1 Biodegradation Potential

The biodegradation potential of soil microorganisms was determined with a radiorespirometric flask assay, which will be referred to as ^{14}C-tracer technique. The radiotracer technique, which measured the evolution of radiolabelled CO_2, used ^{14}C-hexadecane as recommended by Atlas (Atlas, 1979). BOD water, prepared according to Standard Methods for the Examination of Waste Water (1980), was used as dilution water (Langschwager, 1985). In one experiment 100 mg of DAP/L were added to the dilution water. The results of this particular experiment will be referred to as nonnutrient-limited degradation potential.

In a preliminary study different quantities of dilution water were found to have an effect on the measured biodegradation activity. One gram of uncontaminated Sybron soil in 10 mL dilution water resulted in a higher CO_2 evolution than the same amount of soil in 50 mL of BOD dilution water. Different quantities of soil did not result in significant differences between the measured degradation activities. The results of the preliminary study are presented in Table B-1.

Capped Erlenmeyer flasks (250 mL), which are shown in Figure 4, with a center well fixed to the screw top served as

reaction vessels during incubation. In accordance with the results of the preliminary study 10 mL of sterilized dilution water and 1.3 g of wet soil, corresponding approximately to 1 g of dry soil, were added to the flask. A drop of No. 6 fuel oil, approximately 40 mg, was added.

Five μL of hexadecane with an activity of approximately 50,000 DPM (0.023 μCi) were added to the flask. To trap the evolving $^{14}CO_2$, 100 μL of 0.1 M potassium hydroxide (KOH) solution were placed into the center well and the flask tightly closed. Triplicate flasks were prepared for each soil sample. Two controls were prepared in the same way as the test flasks, except that no soil was added. The controls were to measure background radioactivity due to volatility and spontaneous decomposition of the compound. The flasks were incubated in the dark at room temperature, about 20°C, and its contents agitated daily by gently shaking the flasks.

The evolution of $^{14}CO_2$ was measured after seven days. The flask contents were acidified by adding 250 μL of hydrochloric acid (HCl), thereby dropping the pH below 2. The flasks were then placed on a shaker table for one hour. The KOH solution was pipetted from the center well into a glass scintillation vial containing 20 mL of Scintiverse scintillation fluid. The vial was shaken vigorously to ensure sample homogeneity and wiped with an ethanol moistened tissue to remove fingerprints and other residues.

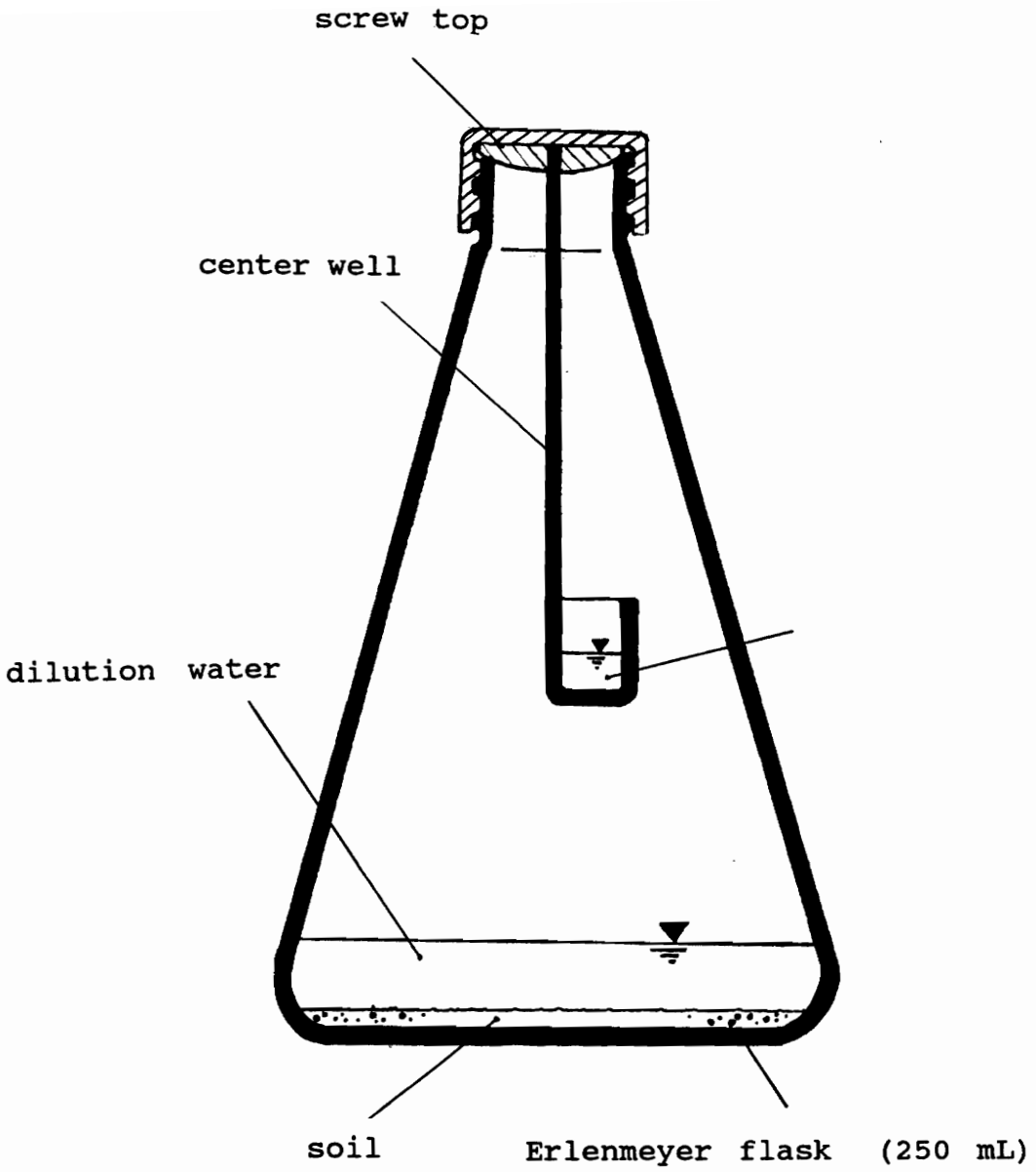

Figure 4: Incubation flask fitted with center well.

Blanks were prepared by adding 20 mL of scintillation fluid to a scintillation vial.

The radioactivity of the trapped $^{14}CO_2$, was determined with a Beckman LS-230 Liquid Scintillation System. The scintillation counter was set to calculate the External Standard-Channels Ratio (ESR). Ten minute counts on channel B, which monitored the wide ^{14}C energy window, were recorded as counts per minute (CPM). The counting efficiency as a function of ESR was obtained from a quench curve. Corrected disintegrations per minute (DPM) were calculated by dividing the difference between CPM of each sample and the blank by the counting efficiency:

$$DPM = \frac{(CPM \text{ of sample}) - (CPM \text{ of blank})}{\text{counting efficiency}}$$

The DPM of the control flasks were subtracted from the DPM of the soil sample. The arbitrary number recorded indicated the biological degradation potential of the soil population. The average of the three replicate flasks was recorded as biodegradation activity (DPM).

3.7.3 TPH Determination by Methylene Chloride Extraction and Gas Chromatography

Soil samples from Sybron's field experiment, VPI&SU's microcosm and soil column study were analyzed for TPH at the VPI&SU laboratory, using a method which will be referred to as MeCl/GC.

The soil samples were air dried and ground in a mortar. Five g of soil were placed into a 40 mL amber vial and 20 mL of methylene chloride (MeCl) added. The vials were placed onto a shaker table and agitated for 24 hours, then refrigerated until the soil had settled. One mL of the extract and 0.5 mL of internal standard solution, containing 1,4-dichlorobenzene (DCB) (180 mg DCB/L MeCl), were pipetted into a 1.5 mL vial.

External standards were prepared from a mixture of petroleum hydrocarbons, including fuel oil, diesel, kerosene, petroleum distillate, crude oil and mineral oil. One mL of external standard solution and 0.5 mL of internal standard solution were transferred into a vial in the same way as the extracts. Standard concentrations of 55, 110, 274 and 550 ppm were obtained.

For the determination of TPH concentration, a Hewlett Packard 5890 gas chromatograph (GC), equipped with a flame ionization detector (FID) was used. A J&W Scientific fused silica capillary column (DB-1) with a 0.25 micron film

thickness, 0.32 mm internal diameter and 30 m length was installed. The column head pressure was kept at 105 kPa. The carrier gas was helium (He). Split flow was used and the total flow set to 40 mL/min and septum purge to 5.4 mL/min. The temperature program used had an initial temperature of 60°C for 4 minutes, a temperature rise of 7°C/minute and a final temperature of 325°C for 8.15 minutes. The total run time was 50 minutes. The injected sample size was 2 μL.

To obtain a standard curve the external standards were run on the GC and chromatograms obtained. The response factor for the external standards was calculated as the ratio of peak area of TPH to peak area of the internal standard:

Response factor = (TPH Area) / (DCB Area)

For low TPH concentrations the standard curve was found to be nonlinear. Therefore the function of response factor R versus the external standard concentration C

$$R = a \times C^n$$

was analyzed for its order n, using a linear fit to a log-log plot on QuattroPro. With a linear fit to R vs C^n the coefficient a of the function was determined.

The TPH concentration of a sample was determined by

calculating R and the corresponding TPH concentration from the standard curve. Adjusting for dilution, the TPH concentration in the soil was calculated in the following way:

$$\text{TPH (ppm)} = (1.5) \times C\ (\text{mg/L}) \times \frac{20\ \text{mL}}{S\ (\text{g})} \times \frac{\text{L}}{1000\ \text{mL}} \times \frac{1000\ \text{g}}{\text{kg}}$$

or

$$\text{TPH (ppm)} = C \times 30 / S\ (\text{g}),$$

where C is the TPH concentration obtained from the standard curve, and S is the dry weight of the extracted soil sample.

3.6.4 TPH Determination by Freon Extraction and Gas Chromatography

The TPH measurements carried out by the Sybron research lab used a modified version of Standard Method's oil and grease extraction and infrared spectrophotometry. The same extraction method together with GC analysis was used at VPI&SU with a number of stored samples from Sybron's field experiment to verify the results.

A sample size of 5 g instead of 2 g of soil in the Sybron method was used. Anhydrous sodium sulfate was added to dry the soil and mixed until of a granular and easily flowing

consistency. The mixture was transferred to a separatory funnel, 1L, with TFE stopcock. 10 mL of trichloro-trifluoroethane (freon) were added and shaken for 2 min. The solvent was drained through a funnel containing solvent-moistened filter paper and about 1/2 inch layer of silica gel to remove the fatty acid or grease fraction. This procedure was repeated two more times with a total of 3 x 10 mL of solvent. The volume of the extract was made up to 20 mL.

For analysis on the GC, the extract was treated and analyzed in the same way as MeCl extracts. The moisture content of the soil was determined. The obtained TPH concentration was corrected for moisture content by dividing it through the solids fraction of the soil.

3.6.5 Surface Tension

Surface tension was measured using a Fischer Scientific surface tensiometer Model 20, using the de Nouy method. The samples were analyzed within 24 hours after sampling and at a temperature of about 20°C. Distilled water, which has a surface tension of 72 dynes/cm at 20°C was used to calibrate the instrument.

Approximately 40 mL of the supernatant of the liquid sample were transferred into the surface tension measurement

cup and then agitated on a rotary mixer to ensure an even dispersion of the surfactants in the sample. Three surface tension measurements were conducted per sample. The measurement cup was rinsed with distilled water and wiped dry between measuring different samples.

4 RESULTS AND DISCUSSION

In this section the results of the joint bioremediation project by Sybron and VPI&SU and the laboratory experiments by VPI&SU are presented and discussed. It is therefore subdivided into the three major parts of the study, the bioremediation field experiment, the microcosm study and the soil column study. The two alternative procedures for TPH analysis are discussed with regard to the results of the field and laboratory experiments in a fourth subsection. The different field and laboratory experiments are compared and their usefulness for predicting in situ biodegradation evaluated in the final part of this section. The data determined by VPI&SU is presented in Appendix C.

4.1 Sybron Bioremediation Field Study

The pilot scale bioremediation project was conducted in several large scale treatment vessels for a total of 29 weeks. TPH degradation was monitored by both Sybron and VPI&SU using freon extraction/IR spectrophotometry and MeCl extraction/GC analysis, respectively. In addition, VPI&SU measured biodegradation activity of the microbial soil populations and surface tension of the circulated water, while Sybron

conducted microbial enumerations. Both Sybron's and VPI&SU's results for the field experiment are presented and discussed in this section.

4.1.1 TPH Degradation measured by Freon Extraction/IR Spectrophotometry

The total petroleum hydrocarbon (TPH) concentrations in all treatment units were determined weekly by Sybron, using the freon extraction/IR spectrophotometry method. The TPH concentrations are based on the definition of hydrocarbons by Standard Methods for the Examination of Waste Water (1980). Average TPH concentrations of different treatment units over time are presented in Figure 5. The calculated concentrations represent the average of duplicate units, each with samples from three different depths.

The same trend of TPH over time occurred for each treatment method. An initial drop of TPH was seen at week 1, followed by an increase, then another drop up to week 6. The TPH increased until week 10 or 12 then declined to week 18, when some samples had reached target treatment level of 100 ppm. After week 18 the TPH rose steadily to the end of the study at week 29, when TPH levels were found nearly equal to initial values.

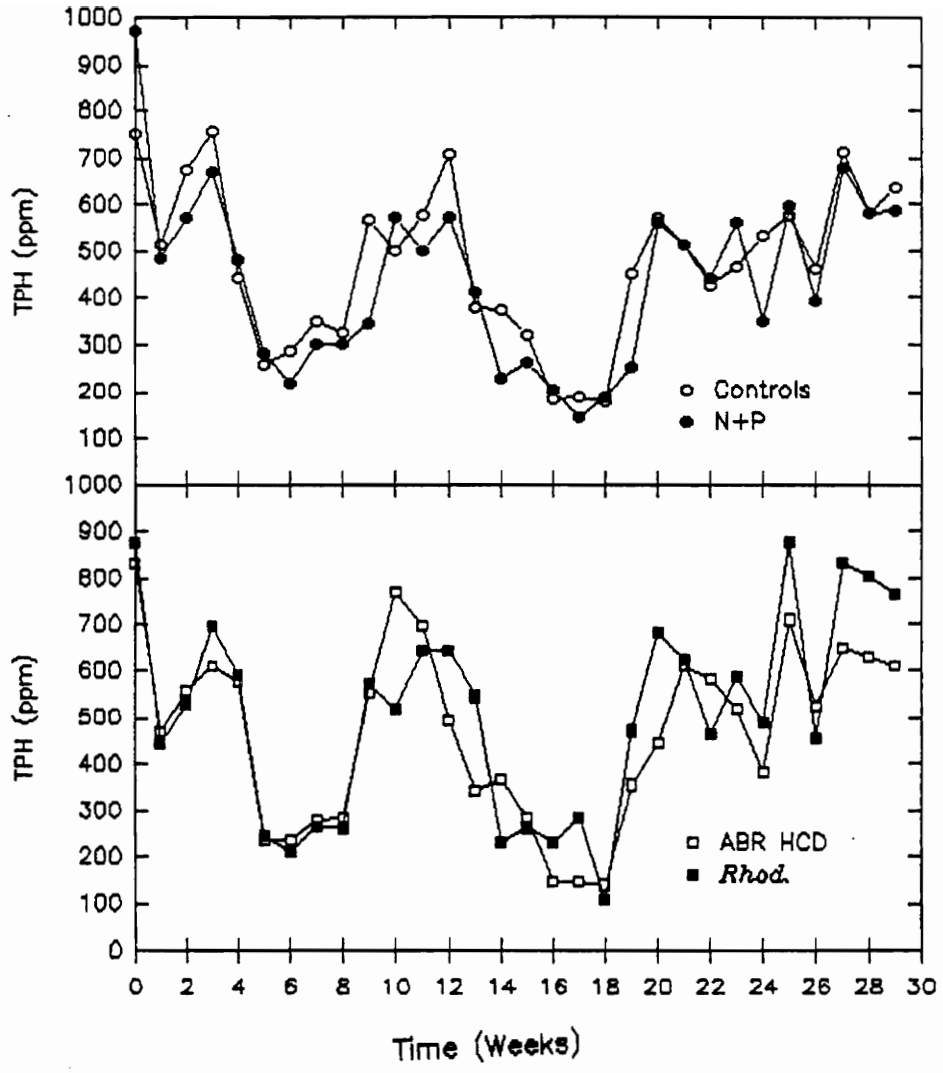

Figure 5: Sybron field experiment - average TPH concentration vs time, measured by freon/IR method, in units without nutrient addition (controls), and nutrient enhanced treatment units without organism addition (N+P), addition of ABR Hydrocarbon Degrader blend (ABR HCD), and *Rhodococcus sp.* inoculum (*Rhod.*).

The TPH trends of samples from different depths of an individual treatment unit, *Rhod.* unit 4, are presented in Figure 6. Samples from top, middle and bottom are denoted T, M and B respectively. Considerable deviation in TPH was observed. The TPH of the three soil samples from different depths does, however, qualitatively follow the same trend, which is reflected in the average TPH concentrations. This suggests that the observed average TPH fluctuations are not caused by stray measurements.

The results seem to indicate a failure of the bioremediation process, or at least a failure to prove biodegradation. The observed TPH concentration trend does not show degradation, but may instead reflect a different phenomenon. In order to explain the variation of the data in Figure 5 and 6, three possible explanations were evaluated. These are:

1. Biological degradation of TPH, if it occurred, may have been masked by some superimposed phenomena, e.g. adsorption-desorption processes.

Desorption due to microbial surfactant production may occur, but does not represent a satisfactory explanation for the continuing fluctuations and the magnitude of TPH variations for a period of 29 weeks. For mass balance reasons, particularly under the assumption that some degradation of the petroleum hydrocarbons occurred, it is impossible that the

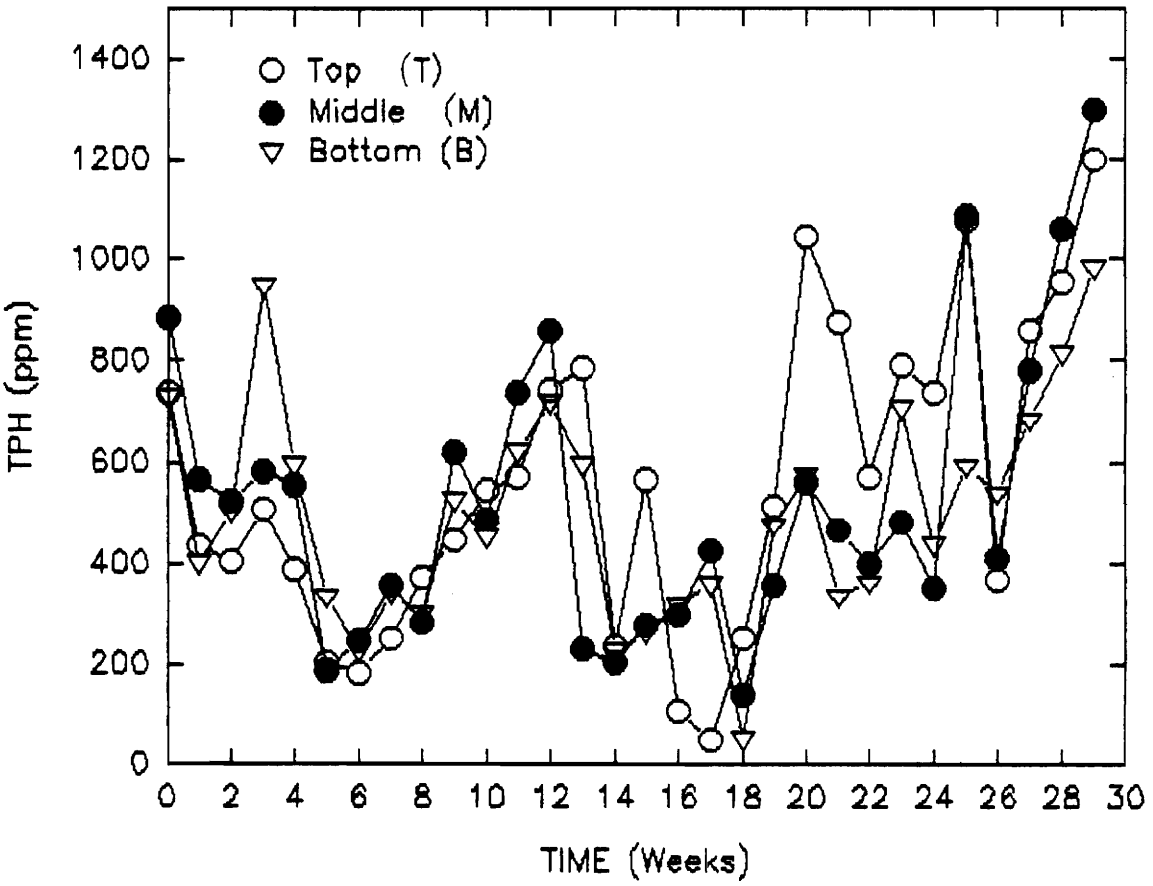

Figure 6: Sybron field experiment - TPH concentration vs time, measured by the freon/IR method, in the nutrient enhanced and *Rhodococcus sp.* inoculated treatment unit 4 (*Rhod.*). Soil samples originated from various depths, the bottom (B), middle (M) and top (T) of the treatment unit.

final concentrations were as high or higher as initial concentrations.

2. Random measurement errors occurred due to soil heterogeneity, uneven distribution of the contaminant, varying extraction efficiency and instrument errors.

Because of the large sample population analyzed these measurement errors can however not account for the magnitude of variation of average TPH. In addition random errors do not explain the uniform TPH trend displayed.

3. Systematic measurement errors may be due to the extraction method or the analysis by IR spectrophotometry.

Substances other than petroleum hydrocarbons, humic substances or microbially produced surfactants, may have been extracted by the solvent and may interfere with the analysis by IR spectrophotometry. The uniform trend of TPH concentrations can be explained best by the occurrence of systematic measurement errors.

For the above reasons and based on further investigations presented later in this study, which confirmed the occurrence of systematic measurement errors, the TPH measurements by the freon/IR method were regarded as invalid.

4.1.2 TPH Degradation measured by the MeCl Extraction/GC Method

In response to the unusual TPH results by Sybron additional measurements of TPH were conducted at VPI&SU after week 6 using methylene chloride extraction and GC analysis. For each treatment, units 1, 2, 3, and 4, one soil sample from the middle (M) of the treatment vessel was analyzed. The results are presented in Figure 7.

The trends observed were a slow TPH decline from between 600 ppm and 900 ppm at week 10 to between 100 and 200 ppm at week 29 for all treatment units with nutrient addition (N+P, ABR HCD, and *Rhod*.). The TPH of the nutrient controls was reduced to approximately 400 ppm at week 29. Assuming a zero order degradation rate, the TPH decline can be quantified by a linear regression of the data. The corresponding degradation rates for the treatment units are 13 ppm/week (controls), 33 ppm/week (N+P), 23 ppm/week (ABR HCD) and 27 ppm/week (*Rhod*.).

The results in Figure 7 indicate that steady biodegradation occurred, in contrast to the measurements by the freon/IR method (Fig. 5). Neither the very low TPH concentrations at week 16 and 18 nor the high values at week 10, 20, and 29, measured by the freon/IR method, could be verified by the MeCl/GC method.

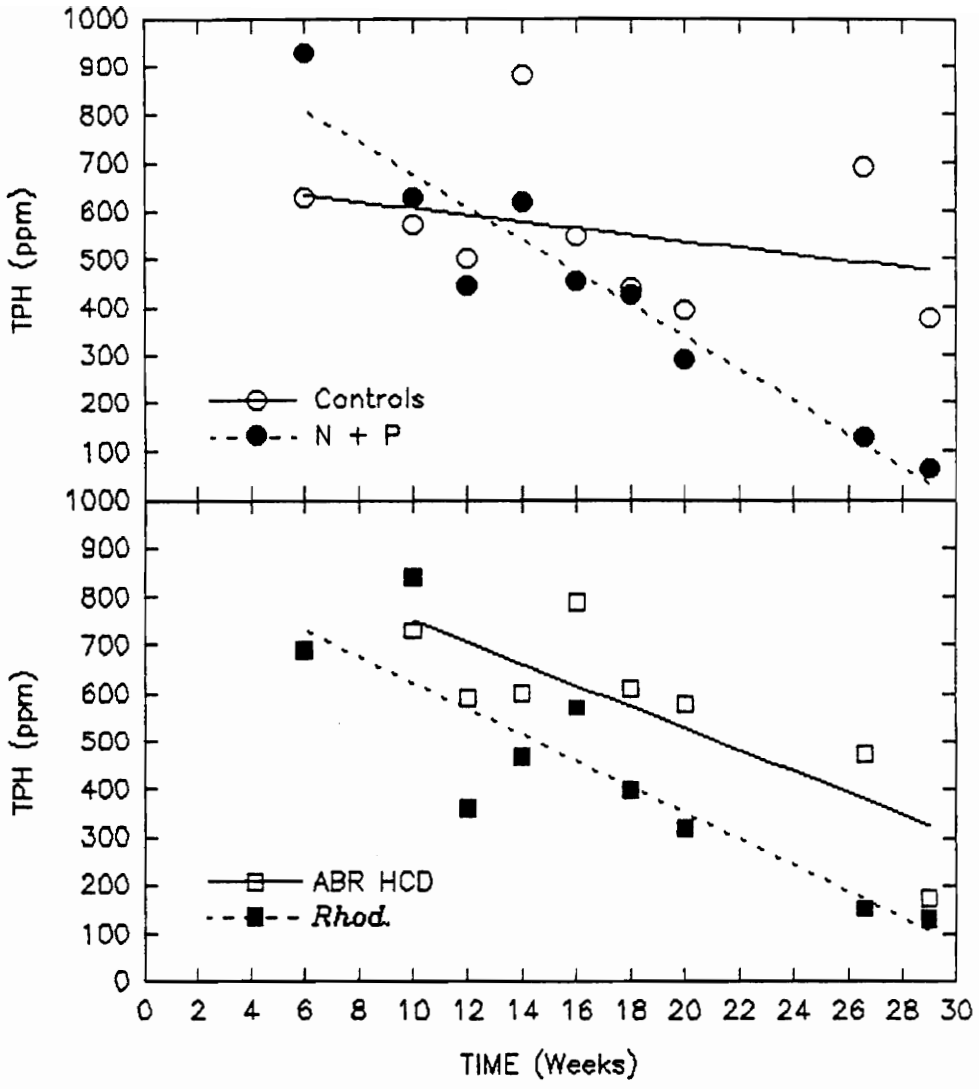

Figure 7: Sybron field experiment - TPH degradation, measured by the MeCl/GC method, in units without nutrient addition (controls) and nutrient enhanced treatment units without organism addition (N+P), with addition of ABR Hydrocarbon Degraders (ABR HCD), and *Rhodococcus sp.* inoculum (*Rhod.*). Samples were taken from the middle (M) of treatment units.

Similar to the freon/IR data significant deviation of the TPH was seen. However, considering each data point represents a single measurement, these deviations can be accounted for by random measurement errors, soil heterogeneity and an uneven distribution of the fuel oil.

No difference in TPH degradation was observed between indigenous and autochthonous microorganisms in the nutrient enhanced treatment units. Several reasons may be responsible:

The quantity and activity of hydrocarbon degrading microorganisms may not have been the primary limiting factor. Other limiting factors, such as oxygen and substrate availability or transport limits may have controlled the degradation reaction.

Introduced organisms may have been subject to predatism and competition by indigenous organisms and were therefore not effective.

The indigenous soil organisms were as effective as introduced organisms in degrading petroleum hydrocarbons. The soil had been subject to petroleum spills in the past and the soil microorganisms were thought to be well acclimated to a mixture of petroleum hydrocarbons. The added fuel oil did not contain specific toxic or undegradable compounds, that required a specially adapted microbial population.

No evidence was found for enhanced biodegradation effect due to the biosurfactant production of *Rhodococcus sp.*. Two explanations can be cited and appear likely.

First, biosurfactant production was not effective at solubilizing immobile hydrocarbons. The biosurfactant concentrations may have been too low, and the surfactants may have been cell-bound or adsorbed to the soil, and therefore not effective at solubilizing the free phase hydrocarbons.

An alternate explanation is that biosurfactant production occurred equally in all treatment units. *Rhodococcus sp.* has been found abundantly in petroleum contaminated marine and soil environments (Sorkoh *et al.*, 1990) and may be part of the indigenous soil population. In addition, there is no evidence that the surfactants produced by *Rhodococcus sp.* are especifically efficient at mobilizing insoluble hydrocarbons. Other microorganism have likewise been found to produce biosurfactants (Schulz *et al.*, 1990, Kosaric *et al.*,1990, Bosch *et al.*, 1988).

Degradation of petroleum hydrocarbons was found to be faster in units which received nutrients compared to the control units. Nutrient availability appears to be the limiting factor in the controls. The relatively slow decline of TPH to target treatment level even in the nutrient enhanced treatment units however points to the existence of other

limiting factors. The low permeability of the soil may have inhibited the transport of nutrients. Other potentially limiting factors are oxygen and substrate availability.

Dissolved oxygen (DO) was provided in the circulated water, which could equilibrate with the atmosphere before percolating through the soil. However, the high clay content and consequently low permeability of the soil may have limited the transport of DO and created partially anaerobic conditions. In addition, field conditions were heterogeneous with respect to the hydraulic properties of the soil. Water in some treatment units was observed to be pooled on top, while other units were relatively dry. Channeling of the water may have prevented an even circulation of the water. Oxygen limitation could account for the variation of TPH data and cannot be excluded per se.

The substrate consisted of petroleum hydrocarbons from an old contamination event and from recent contamination with No. 6 fuel oil. The original petroleum contamination had been subject to physical, chemical and biological action for a considerable time and the remaining hydrocarbon components were the less biodegradable and less mobile. No. 6 fuel oil contains relatively long chain hydrocarbons with more than 30 carbon atoms and is consequently very insoluble. In addition, because of the high clay content of the soil, the hydrocarbons are likely to be adsorbed to the soil surface. Bioremediation

of hydrocarbons adsorbed to the clay fraction of soil in slurry reactors has been shown to be very slow (Bhandhari, 1992). The rate of hydrocarbon solubilization and desorption may therefore have limited the biological degradation. Solubility limited degradation could be expected to follow a zero order rate as indicated by the TPH results.

A number of soil samples, collected by Sybron and stored frozen, were analyzed at VPI&SU using the MeCl/GC method. The TPH degradation in the soil samples, originating from the top (sample T) of a *Rhod.* treatment unit (unit d), is shown in Figure 8. In comparison TPH concentrations of samples from the middle (M) of a *Rhod.* treatment unit (unit 4) are presented. Hydrocarbon degradation, measured in samples T, appeared to be rapid within the first 10 weeks of the experiment with a decline from above 600 ppm to around 300 ppm. Subsequently concentrations declined more slowly to target treatment level of about 100 ppm in week 29.

The TPH data of the stored samples T (Fig. 8) indicate two distinct phases of zero order degradation reaction. If the degradation in the nutrient enhanced treatment units was limited by the substrate solubility, the initial rapid TPH decline would correspond to the degradation of the more soluble fraction of hydrocarbons, the subsequent slower decline to the degradation of less soluble hydrocarbons.

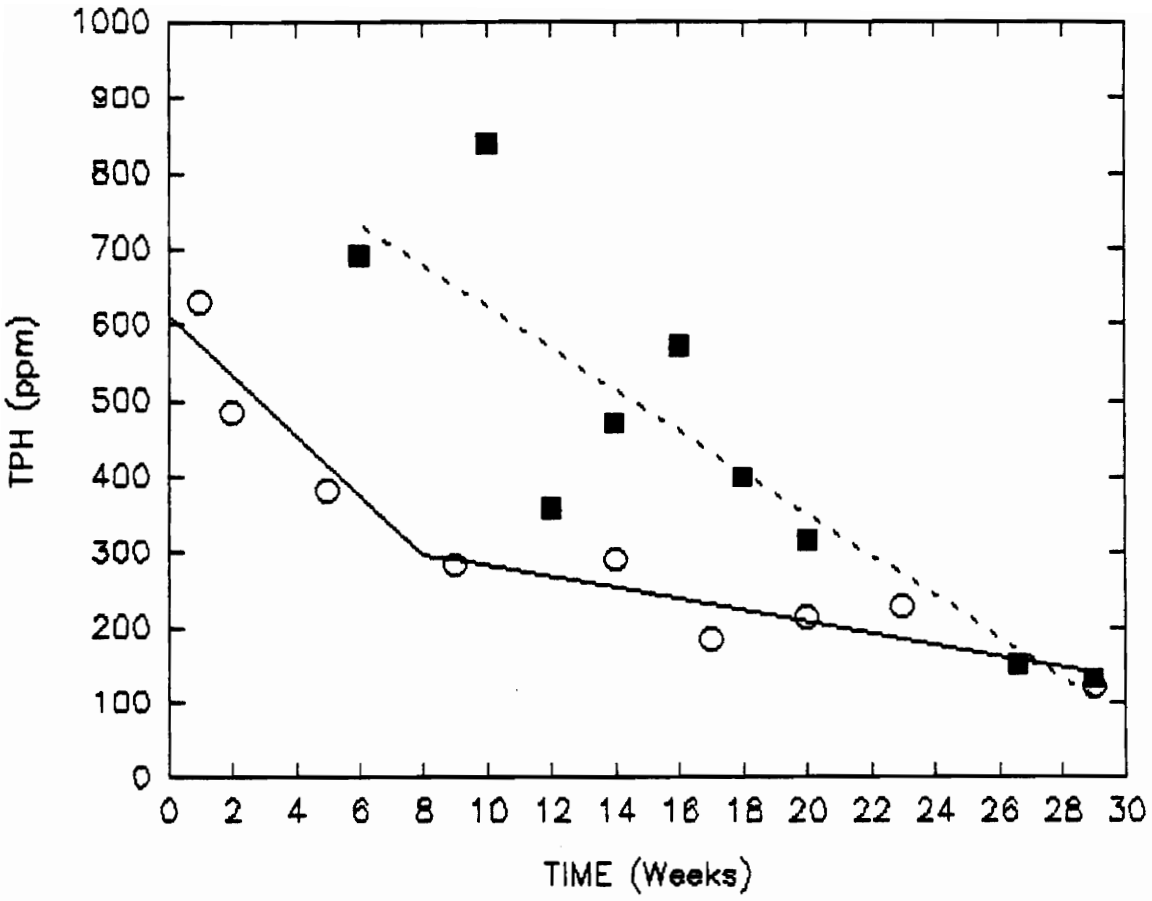

Figure 8: Sybron field experiment - TPH degradation in nutrient enhanced and *Rhodococcus sp.* inoculated treatment (*Rhod.*), measured by the MeCl/GC method. Soil samples compared were taken from the top (T) of treatment unit d and middle (M) of treatment unit 4.

The degradation rates, about 38 ppm/week initially, subsequently 7 ppm/week, were quantified by a linear regression of the data.

A more rapid and steady decline of hydrocarbon concentrations was observed in the soil samples from the top (T), compared to samples from the middle (M) of a treatment vessel. In addition to volatilization, a faster and more homogenous biodegradation may have occurred due to better oxygen and nutrient availability. Dissolved oxygen concentrations (DO) in the recirculated water can be assumed to be highest at the top of each unit. The impermeability of the soil, limiting the transport of oxygen and nutrients, was expected to be more significant in the compressed bottom region of the soil. Mathematical models have shown that the biodegradation of recirculated hydrocarbons takes place predominantly in the upper region of the soil bed (Wu *et al.*, 1990)

The TPH results strongly indicate that nutrient addition accelerated the biological degradation of petroleum hydrocarbons under the simulated field conditions, while introduced microorganisms such as ABR Hydrocarbon Degraders or *Rhodococcus sp.* did not increase degradation rates.

4.1.3 Biodegradation Potential - Correlation to TPH Degradation

The evolution of $^{14}CO_2$ due to the mineralization of radio-labelled hexadecane gave a direct measurement of the degradation potential of soil organisms in different treatment units. The method will be referred to as ^{14}C-tracer technique. Biodegradation activity of units without nutrient addition (controls) and nutrient enhanced treatment units without organism addition (N+P), with addition of ABR Hydrocarbon Degraders (ABR HCD) and *Rhodococcus sp.* (*Rhod.*) was measured until week 20 and is shown in Figure 9. Standard deviations are shown as vertical error bars.

The degradation potential in the controls without nutrient or organism additions, remained consistently low. All soil samples from nutrient enhanced treatment units (N+P, ABR HCD, *Rhod.*), showed a significantly higher activity. N+P displayed a steadily increasing biodegradation potential, which was higher than ABR HCD and *Rhod.* treatment between week 6 and 10, and then declined. In both soils inoculated with organisms (ABR HCD and *Rhod.*) a relatively high initial activity was measured. *Rhod.* activity was seen to increase temporarily after reinoculation at weeks 3 and 10, but otherwise tended to decrease gradually. The biodegradation activity of ABR HCD remained comparatively high throughout the 20 weeks.

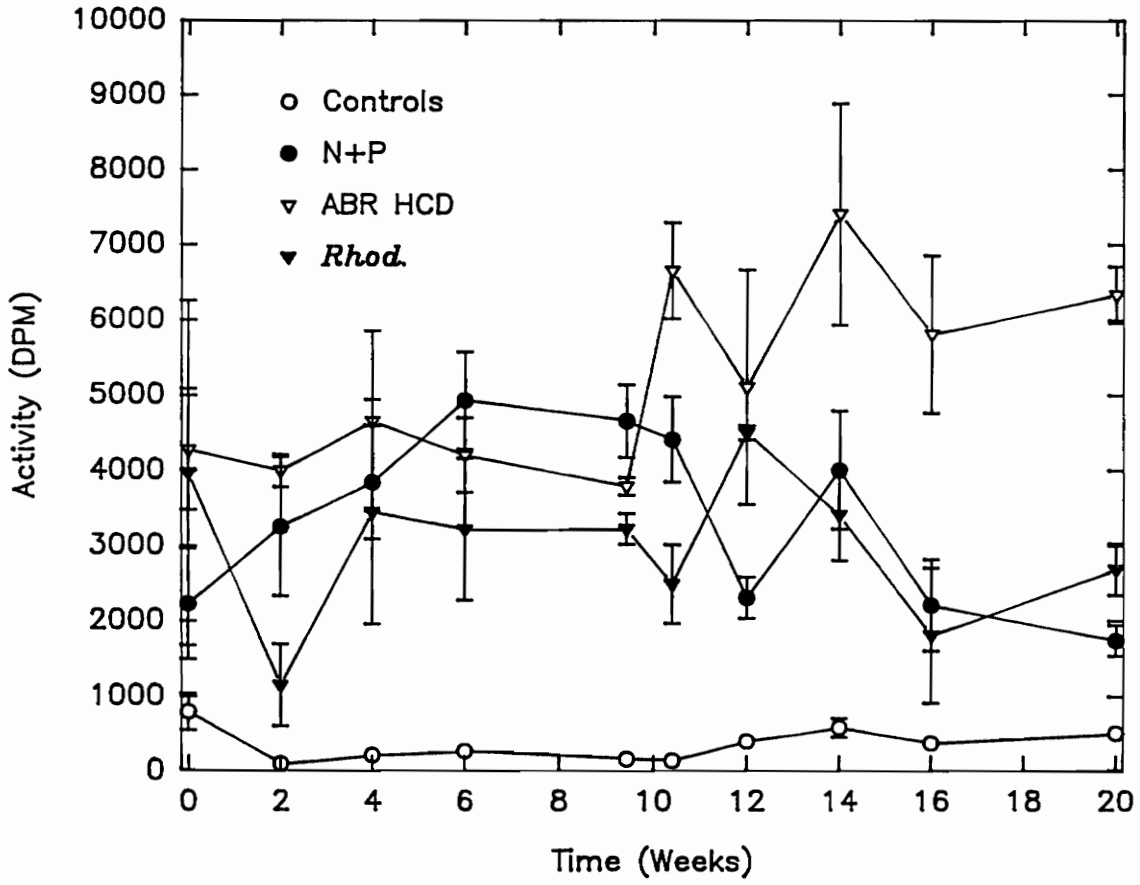

Figure 9: Sybron field experiment - Biodegradation potential vs time, measured by the ^{14}C-tracer technique. Treatments compared were: treatment without addition of nutrients (control), and nutrient enhanced treatments without introduced organisms (N+P), with addition of ABR Hydrocarbon Degraders (ABR HCD), and *Rhodococcus sp.* (*Rhod.*)

It increased significantly after the inoculation at week 10 and remained higher than the activity of other microbial populations until week 20.

Nonnutrient-limited degradation potential was measured in flasks with additional diammonium phosphate (DAP) supplement. Cumulative activity of soil organisms from different treatment units after four and seven days is shown in Figure 10. The N+P unit had the highest activity, however, the differences in degradation potential do not appear significant. The increase of measurements to day four and day seven was roughly linear, which suggested a zero order degradation reaction. It also indicated that the reaction in the flasks was not oxygen or substrate limited. The results prove that the biodegradation activity of soil microorganisms from all treatment units, indigenous and autochthonous, is potentially identical under nonnutrient-limited conditions. The rates of ^{14}C-hexadecane mineralization by indigenous soil organisms from control treatment units were consequently nutrient-limited in the flask assay without DAP supplement.

The average biodegradation potentials of different treatments compared to the average degradation rates determined by linear regression analysis of the MeCl/GC data are shown in Figure 11.

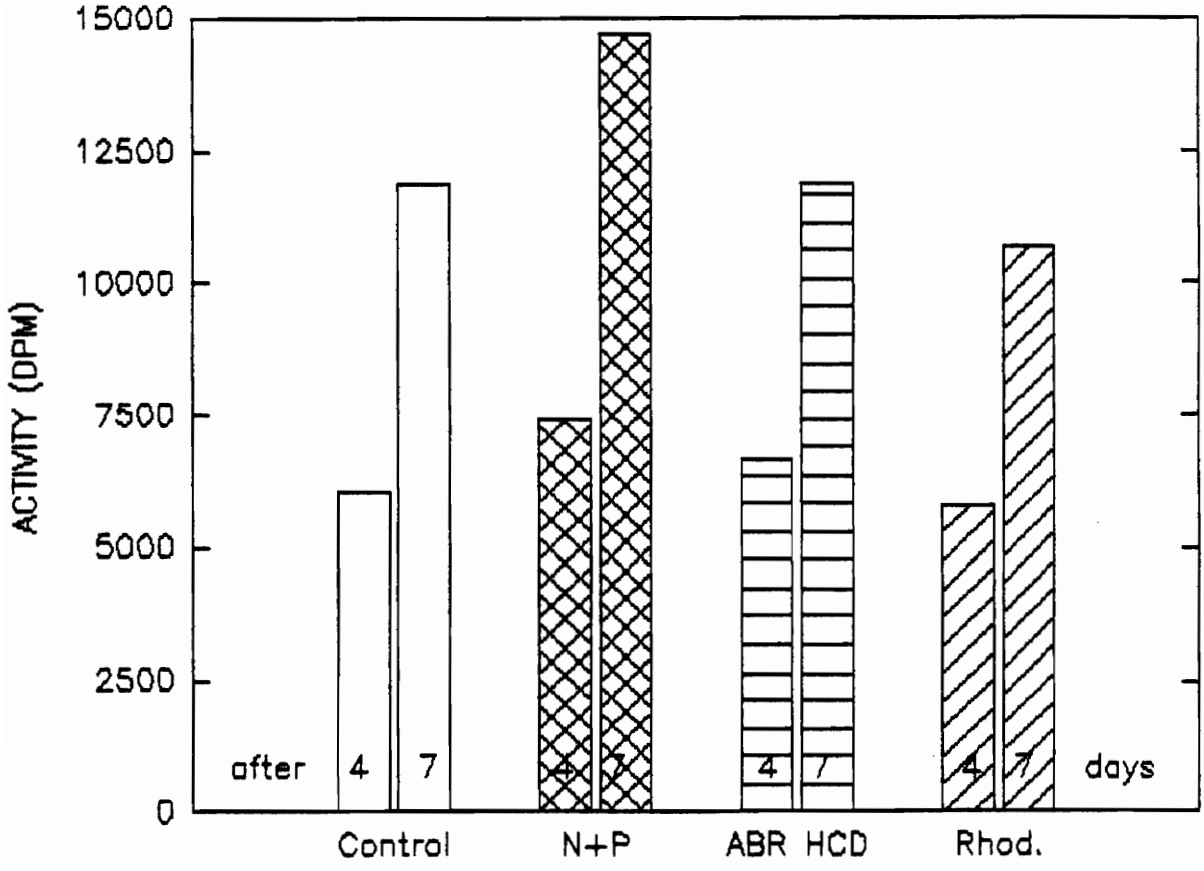

Figure 10: Sybron field experiment - Nonnutrient-limited biodegradation potential, measured by the ^{14}C-tracer technique. Treatments compared were: treatment without addition of nutrients (control), and nutrient enhanced treatments without introduced organisms (N+P), with addition of ABR Hydrocarbon Degraders (ABR HCD), and *Rhodococcus sp.* (*Rhod.*)

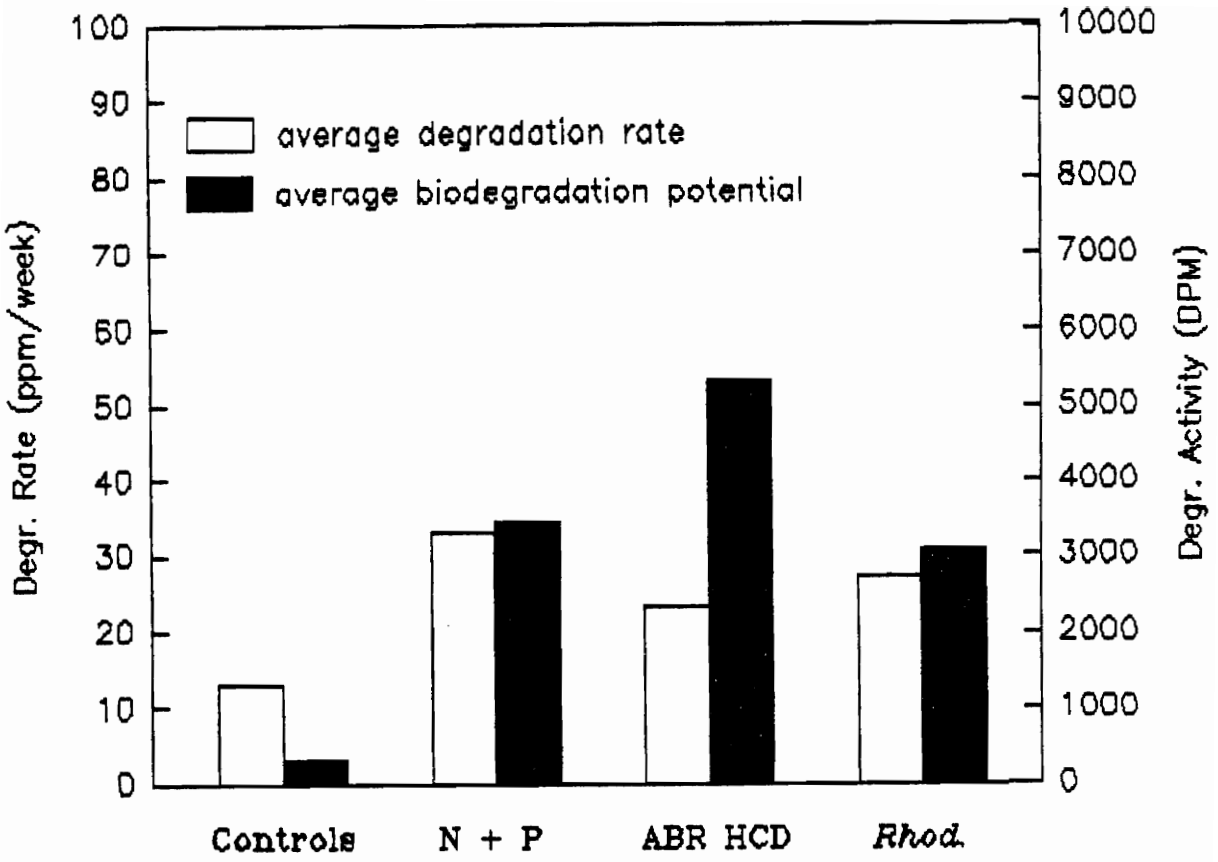

Figure 11: Comparison of average TPH degradation rates, measured by MeCl/GC, and biodegradation potential, measured by the ^{14}C-tracer technique. Treatments compared were: treatment without addition of nutrients (control), and nutrient enhanced treatments without introduced organisms (N+P), with addition of ABR Hydrocarbon Degraders (ABR HCD), and *Rhodococcus sp.* (*Rhod.*).

The increased biodegradation activity in nutrient enhanced N+P, ABR HCD, and *Rhod*. treatment units, qualitatively corresponds to the increased TPH degradation rates, seen in units with nutrient addition. The potential for biodegradation, however, does not correlate quantitatively with the TPH degradation rates. Actual biodegradation in the control units was not as slow as the degradation activity measurements suggest. In addition, ABR Hydrocarbon degraders (ABR HCD) have significantly higher degradation potential than indigenous organisms (N+P) and *Rhodococcus sp.* (*Rhod.*), although actual degradation rates of all these nutrient enhanced treatment units were roughly identical.

Laboratory flask experiments impose changes on the soil sample and create artificial conditions different from the natural environment of the microbial soil population. The ^{14}C-tracer technique may not have been adequately calibrated with two factors that limit in situ biodegradation:

Substrate availability, was thought to be limited by solubility and desorption rate of heavy petroleum compounds in the field experiment. Hexadecane, a more soluble and degradable substrate than fuel oil, was not strongly sorbed to the soil and provided at higher concentrations in the flask assay.

Oxygen transport in the field scale treatment units may have been limited due to the impermeability of the clay loam.

Oxygen limitation has not been reported for laboratory experiments such as the ^{14}C-tracer technique (Atlas, 1979, Langschwager, 1985) and appears particularly unlikely because of the relatively large flask volume used.

The ^{14}C-tracer technique proves the potential for biological degradation of hydrocarbons in all soils. The results confirm that the control units without fertilizer addition in the Sybron field experiment were nutrient limited. The results also suggest a different biological degradation potential of indigenous microorganisms, ABR Hydrocarbon Degraders and *Rhodococcus* in nutrient enhanced treatment units. However, the differences and changes of degradation potential over time did not correspond to actual TPH degradation. The quantity and activity of the microorganisms may therefore not be the rate limiting factor for nutrient enhanced treatment in the field scale experiment.

4.1.4 Surface Tension - Correlation to TPH Degradation

Surface tension of the circulated water in different units, measured until week 20, is shown in Figure 12. The surface tension of distilled water is 72 dynes/cm. During the field experiment the surface tension of the treatment without nutrient addition (control), with nutrient addition (N+P) and

with ABR Hydrocarbon degraders (ABR HCD) fluctuated between about 65 and 70 dynes/cm. The trend of surface tension for all three treatments was uniform. The surface tension of the water circulated in the *Rhodococcus sp.* inoculated treatment unit (*Rhod.*) remained between 65 and 70 dynes/cm for most of the time it was monitored. However, between week 4 and 6, as well as week 9 and 10, it declined temporarily to around 58 dynes/cm.

The lowered surface tension indicated that microbial surfactant production occurred in all treatment units. No correlation was seen between surface tension and TPH data, measured by the MeCl/GC method (Fig. 7), for any of the treatment units. No correlation between surface tension and biodegradation potential (Fig. 10) was observed for control, N+P, and ABR HCD units. The decline of surface tension in *Rhod.* units, however, did coincide with an increase of biodegradation activity. It occurred shortly after inoculation at week 3 and 10. This may be explained by the fact that *Rhodococcus* produces certain biosurfactants only under growth conditions (Kim *et al.*, 1990). The subsequent rise of surface tension indicated that microbial production of biosurfactants stopped. *Rhodococcus* may have started to form agglomerates, which limited further growth (Kim *et al.*, 1990), or was outcompeted by indigenous organisms.

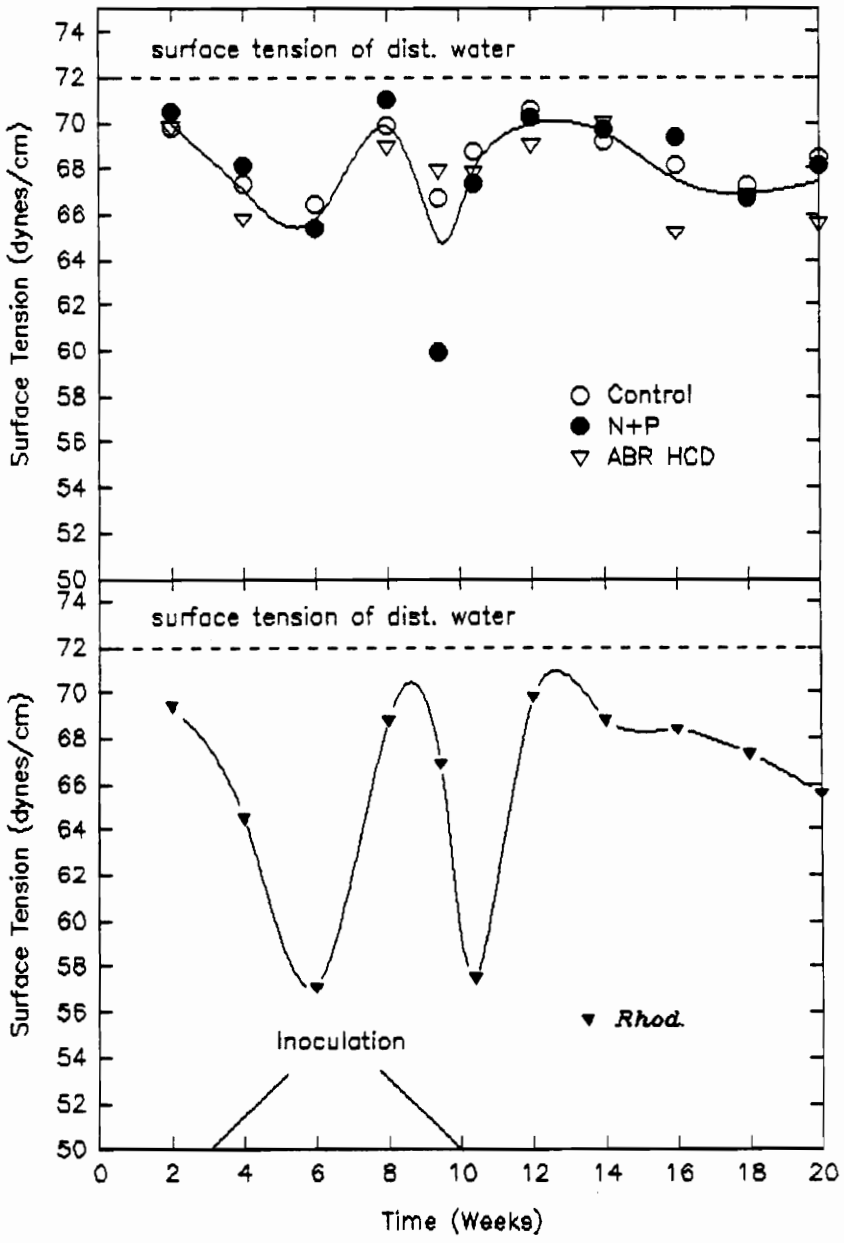

Figure 12: Sybron field experiment - Surface tension of circulated water vs time in units without nutrient addition (control) and nutrient enhanced treatment units, without organism addition (N+P), with addition of ABR Hydrocarbon Degrader Blend (ABR HCD), and *Rhodococcus sp.* inoculum.

4.2 Microcosm Study

Two microcosm studies with different soils were carried out, the initial with spiked Sybron soil from the field experiment, a second study with screened Sybron and Hillcrest soil. Microcosms with spiked Sybron soil were to simulate TPH degradation in the field experiment. The more homogenous, screened soils were used to obtain a more steady TPH trend and compare degradation in the acclimated Sybron and unacclimated Hillcrest soil.

Treatments compared were no addition (controls), nutrient addition without introduced organisms (N+P), with ABR Hydrocarbon Degraders (ABR HCD), and *Rhodococcus* (*Rhod.*). Hydrocarbon concentrations in the soil were monitored by the MeCl/GC method. During initial weeks biodegradation potential of the soil population was determined by the ^{14}C-tracer technique.

4.2.1 TPH Degradation

The TPH concentrations in spiked Sybron soil, shown in Figure 13, declined from about 1000 ppm to around 600 ppm during the initial 10 weeks for all treatments. Subsequently TPH fluctuated, but declined slowly to around 400 to 500 ppm

in controls, N+P and *Rhod.* microcosms. In contrast the TPH in ABR HCD microcosms appeared to increase slightly after week 10. TPH concentrations in the sterilized controls declined from 1000 ppm to 650 ppm at week 26.

Because of the poor detection limit of the original MeCl/GC method developed at VPI&SU, the procedure was modified after week 6. A larger soil sample, 5 g instead of 1 g, was analyzed. Data of week 4 to 6 with 1 g sample size was too close to the detection limit to be considered.

In the scale of the microcosms, the spiked Sybron soil, prepared for the field scale study, was considered to be heterogeneous. In addition, a fine dispersion of the No. 6 fuel oil was difficult because of its viscosity. Soil heterogeneity and uneven distribution of the contaminant therefore account for the significant variations in the TPH trend, particularly of N+P treatment. Although the samples analyzed were composite samples, a larger sample population and replication of the microcosms is recommended for further studies.

The decline of TPH in autoclaved controls may partly be due to abiotic decomposition of petroleum hydrocarbons or volatilization. Primarily, the sterilization may not have been effective in completely preventing biological degradation.

The low solubility of the fuel oil was expected to limit the degradation. A zero order rate, as indicated by the

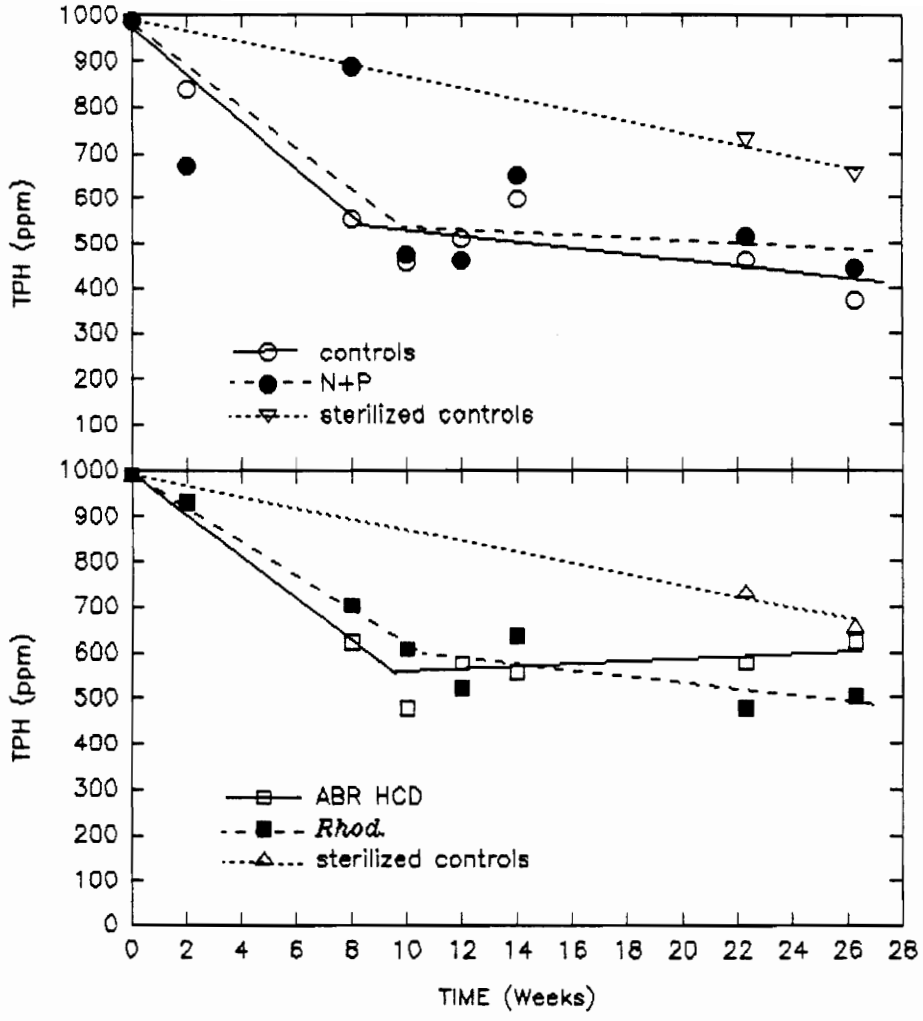

Figure 13: Microcosms with spiked Sybron soil - TPH degradation, measured by the MeCl/GC method. Treatments compared were: microcosms without nutrient addition (controls), and nutrient enhanced microcosms without introduced organisms (N+P), with ABR Hydrocarbon Degrader Blend (ABR HCD), and with *Rhodococcus sp.* inoculum (*Rhod.*).

^{14}C-tracer technique (Fig. 9), may be assumed for solubility limited degradation of hydrocarbons. The TPH data (Fig. 13) suggest that the more soluble fraction of the fuel oil, approximately 40% of the total initial TPH, was degraded rapidly over the first 8 to 10 weeks. The more insoluble fraction of the fuel oil and the old petroleum contamination in the soil were degraded with a much slower rate. Consequently the TPH decline can be approximated with two linear response curves. Initial and subsequent degradation rates were approximately 54 and 7 ppm/week (controls), 43 and 3 ppm/week (N+P), 46 and -3 ppm/week (ABR HCD), 38 and 8 ppm/week (*Rhod.*) respectively.

Considering the degree of variation and incompleteness of the data in initial weeks, the results are not conclusive with regard to the effect of nutrient addition and introduced microorganisms on the biodegradation.

TPH concentrations over time in the microcosms with screened Sybron soil are presented in Figure 14. The TPH decreased rapidly to around 1000 ppm in all microcosms with nutrient addition within the first 6 weeks and in microcosms without nutrient addition within the first 8 weeks. From week 8 to 18 TPH concentrations remained stable for N+P and *Rhod.*, but increased slightly for treatment nutrient controls and ABR HCD. At week 18 TPH in N+P microcosms is lowest, followed by

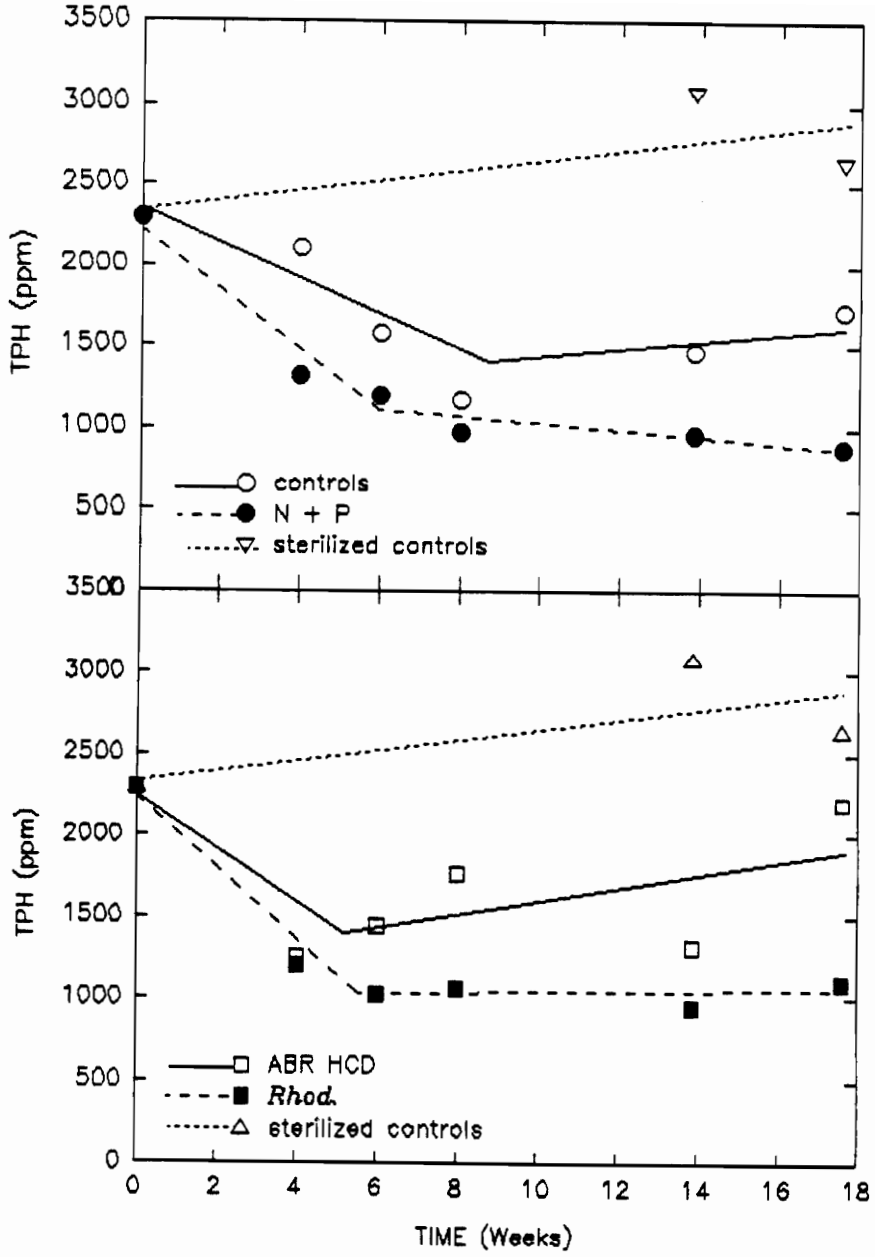

Figure 14: Microcosms - TPH degradation in screened Sybron Soil, measured by the MeCl/GC method. Treatments compared were: microcosms without nutrient addition (controls), and nutrient enhanced microcosms without introduced organisms (N+P), with ABR Hydrocarbon Degrader Blend (ABR HCD), and *with Rhodococcus sp.* inoculum (*Rhod.*)

Rhod., controls and ABR HCD.

The rate of TPH decline in each degradation phase was quantified with a linear regression curve. Initial and subsequent degradation rates for each treatment were approximately 110 and -20 ppm/week (controls), 190 and 10 ppm/week (N+P), 170 and -40 ppm/week (ABR HCD), 220 and 0 ppm/week (*Rhod.*) respectively.

The results indicate that in the previously contaminated soil nutrient addition enhanced the biological degradation of petroleum hydrocarbons. The addition of organisms was not found to accelerate the degradation.

TPH degradation in the Hillcrest soil is presented in Figure 15. The TPH concentrations declined rapidly to approximately 1000 ppm within the first 8 weeks in controls and N+P microcosms, within the first 6 weeks in ABR HCD and *Rhod.* microcosms. Subsequently TPH of treatment N+P and *Rhod.* remained stable, while TPH of treatment controls and ABR HCD appeared to increase. At week 18 the TPH concentration of *Rhod.* is lowest, followed by ABR HCD and controls.

The TPH trend was approximated with two linear regression curves. Initial and final degradation rates were about 39 and -25 ppm/week (controls), 68 and -4 ppm/week (N+P), 87 and -44 ppm/week (ABR HCD), and 90 and 0 ppm/week (*Rhod.*) respectively.

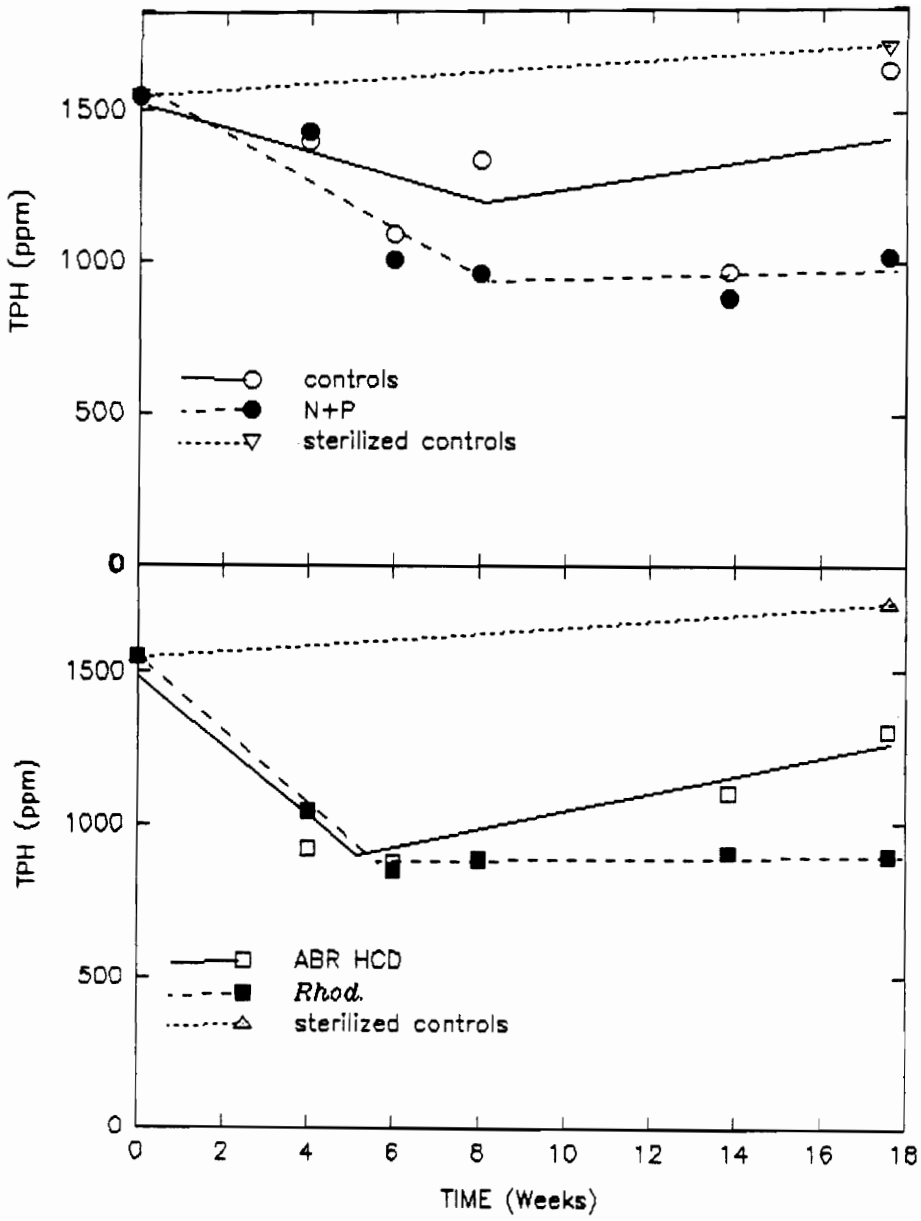

Figure 15: Microcosms - TPH degradation in screened Hillcrest soil, measured by the MeCl/GC method. Treatments compared were: microcosms without nutrient addition (controls), and nutrient enhanced microcosms without introduced organisms (N+P), with ABR Hydrocarbon Degrader Blend (ABR HCD), and *with Rhodococcus sp.* inoculum (*Rhod.*)

Initial TPH degradation in soil microcosms with addition of autochthonous microorganism - ABR Hydrocarbon Degraders and *Rhodococcus sp.*- was significantly higher than degradation by indigenous soil organisms. However TPH in microcosms with nutrient addition (N+P) ultimately declined to about the same level as microcosms with introduced microorganisms. Microcosms without nutrient addition had the lowest degradation rates.

The results suggest an initial lag phase of TPH decline in the previously uncontaminated soil compared to hydrocarbon degradation by introduced organisms, which showed microbial activity more rapidly.

In both screened soils nutrient limitation is thought to be responsible for the slow and overall low TPH decline in the controls. The screened Sybron and Hillcrest soils were relatively fine soils with a high clay content and consequently impermeable. The high remaining TPH in the nutrient enhanced, static microcosms may therefore be due to limited oxygen transport.

The more soluble and degradable fraction of the petroleum hydrocarbons, approximately 40 % to 60 % of the initial TPH, was found to degrade readily. The remaining hydrocarbon fraction may be very insoluble, undegradable or sorbed to the soil. The increases of TPH in some microcosms, seen in both soils after the initial decline, may be due to systematic

measurement errors originating from the standard curve. In addition, desorption may have resulted in a higher extraction efficiency.

4.2.2 Biodegradation Potential

Average biodegradation activity in the contaminated Sybron soil, measured by the ^{14}C-tracer technique during the first 6 weeks of the study, is shown in Figure 16. The activity of the controls was very low. Nutrient enhanced microcosms, N+P, ABR HCD and *Rhod.*, had a high, nearly identical biodegradation activity.

The ^{14}C-tracer technique suggested a nutrient limitation in control microcosms. The differences in degradation activity measurements are not correlated with the nearly identical TPH degradation rates in the contaminated Sybron soil microcosms, but correspond to the TPH and activity results from the field experiment.

Average biodegradation potential in screened Sybron soil, measured initially and at week 2 by the ^{14}C-tracer technique, is shown in Figure 17. Biodegradation activity was very low in controls without nutrient addition. The significantly higher

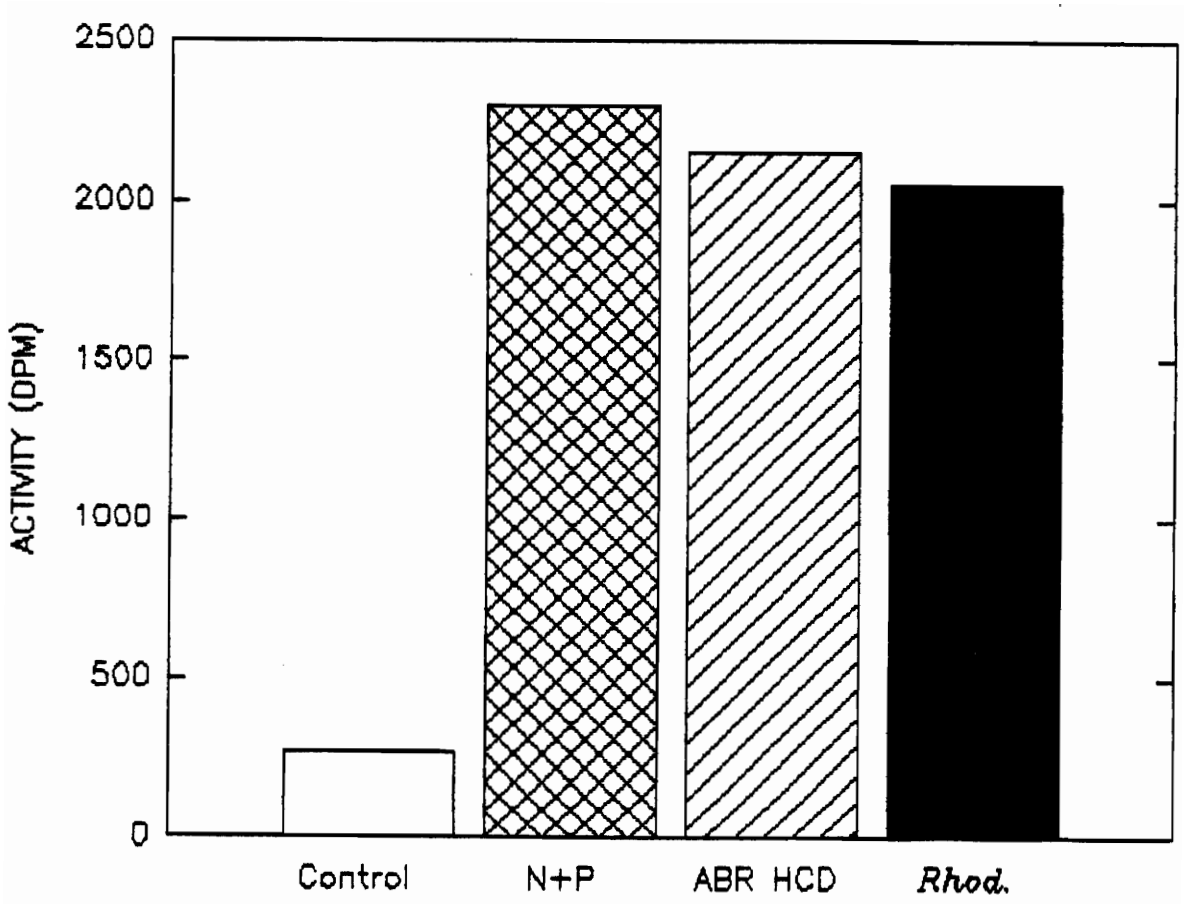

Figure 16: Microcosms - Average biodegradation activity in spiked Sybron soil, measured by the ^{14}C-tracer technique. Treatments compared were: microcosms without nutrient addition (controls), and nutrient enhanced microcosms without introduced organisms (N+P), with ABR Hydrocarbon Degrader Blend (ABR HCD), and *with Rhodococcus sp.* inoculum (*Rhod.*)

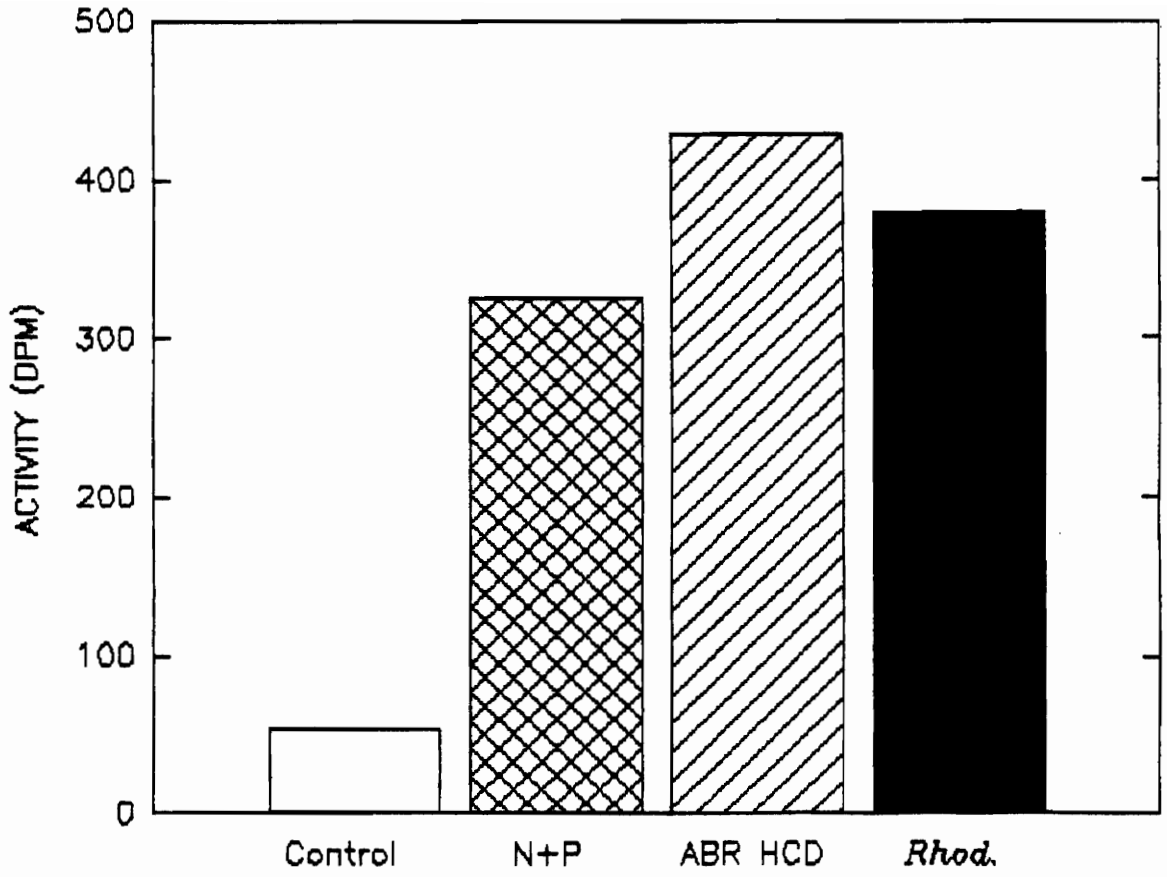

Figure 17: Microcosms - Average biodegradation activity in screened Sybron soil, measured by the ^{14}C-tracer technique. Treatments compared were: microcosms without nutrient addition (controls), and nutrient enhanced microcosms without introduced organisms (N+P), with ABR Hydrocarbon Degrader Blend (ABR HCD), and with *Rhodococcus sp.* inoculum (*Rhod.*).

activities in nutrient enhanced treatments, N+P, ABR HCD, and *Rhod*. were approximately identical.

The results correspond qualitatively to the TPH degradation rates in the microcosms (Fig. 14) and confirm that in the previously contaminated soil nutrient addition accelerated biodegradation, while introduced organisms did not. In proportion the degradation potential of different treatments is in accordance with data from contaminated Sybron soil microcosms and the field experiment. The absolute $^{14}CO_2$ evolution in screened Sybron soil was however substantially lower, which confirms that disturbance of soil samples due to the soil preparation may significantly alter the quantitative results of laboratory flask assays.

Average biodegradation potential of week 0 and 2 in screened Hillcrest soil, measured by the ^{14}C-tracer technique, is shown in Figure 18. While biodegradation activity in controls was very low, it was significantly higher in N+P, ABR HCD, and *Rhod*. A lower considerably lower activity was observed in nutrient enhanced microcosms without organism addition (N+P), compared to the nearly identical activities in microcosms with ABR HCD and *Rhod*.

The results correspond to the TPH degradation rates in the microcosms (Fig. 15) and confirm that nutrient addition accelerated biodegradation. The addition of organisms was

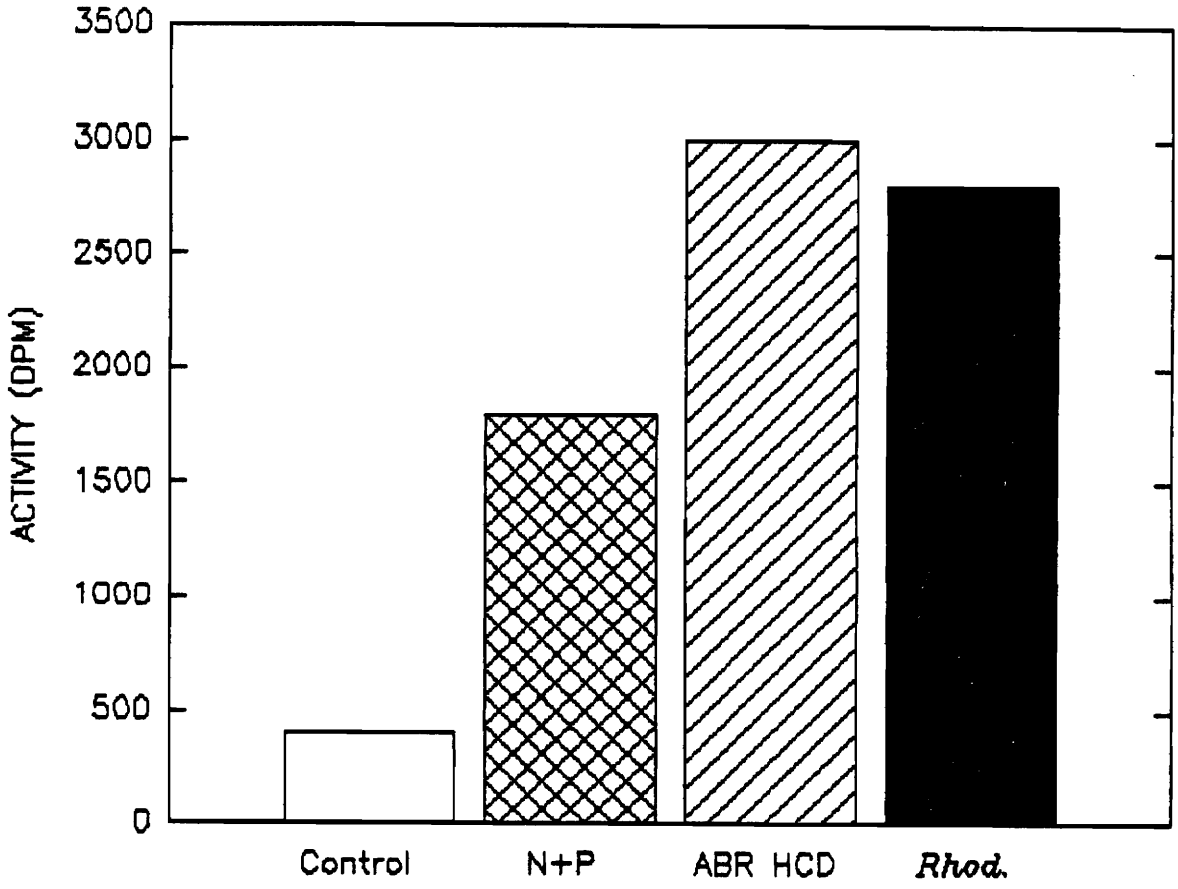

Figure 18: Microcosms - Average biodegradation activity in screened Hillcrest soil, measured by the ^{14}C-tracer technique. Treatments compared were: microcosms without nutrient addition (controls), and nutrient enhanced microcosms without introduced organisms (N+P), with ABR Hydrocarbon Degrader Blend (ABR HCD), and with *Rhodococcus sp.* inoculum (*Rhod.*)

found to increase degradation potential in previously uncontaminated soil during the initial weeks.

4.3 Soil Column Study

In order to investigate the nature of TPH variations seen in the field experiment, the large scale treatment units were simulated in the laboratory with soil columns, through which water and contaminants were recirculated.

The soil from the Sybron site, which had been subject to earlier, uncharacterized hydrocarbon spills, will be referred to as unspiked soil. Unspiked Sybron soil was treated to investigate, whether the weathered hydrocarbon contamination accounted for desorption effects leading to an increase of TPH seen in the Sybron field experiment. Spiked Sybron soil had been screened and contaminated in the laboratory to create quasi homogenous conditions, and redundant units were provided to allow the collection of multiple samples. The experiment was designed to minimize random variation of the data and measure actual TPH degradation in the spiked soil as accurately as possible.

Nutrient enhanced treatment (N+P) without addition of autochthonous organisms was applied to both unspiked and spiked Sybron soil.

4.3.1 TPH Degradation

TPH concentrations in spiked and unspiked Sybron soil were monitored over a period of 13 weeks, using the MeCl/GC method, and are shown in Figure 19. Standard deviations were calculated and are represented as vertical error bars. The average TPH concentrations in the unspiked soil decreased steadily but slowly over time and reached the target treatment level of 100 ppm after 13 weeks. Average TPH of the spiked soil decreased rapidly from about 700 to 300 ppm until week 6, then more slowly to approximately 200 ppm in the following seven weeks. A linear regression was fitted to the TPH data of the uncontaminated soil. The degradation rate in the uncontaminated soil was approximately 12 ppm/week. The two phases of TPH decline in the spiked soil were quantified with two linear regressions. The rates of initial rapid and subsequent slower decline were approximately 60 ppm/week and 12 ppm/week respectively.

The results verify that the degradation of No. 6 fuel oil hydrocarbon in the soil takes place in two phases. First the soluble and degradable compounds are rapidly metabolized before more insoluble or undegradable undergo a much slower decomposition. The degradation rate and level of TPH, at which the slow decline in spiked soil set in, corresponded to the decline in the unspiked soil.

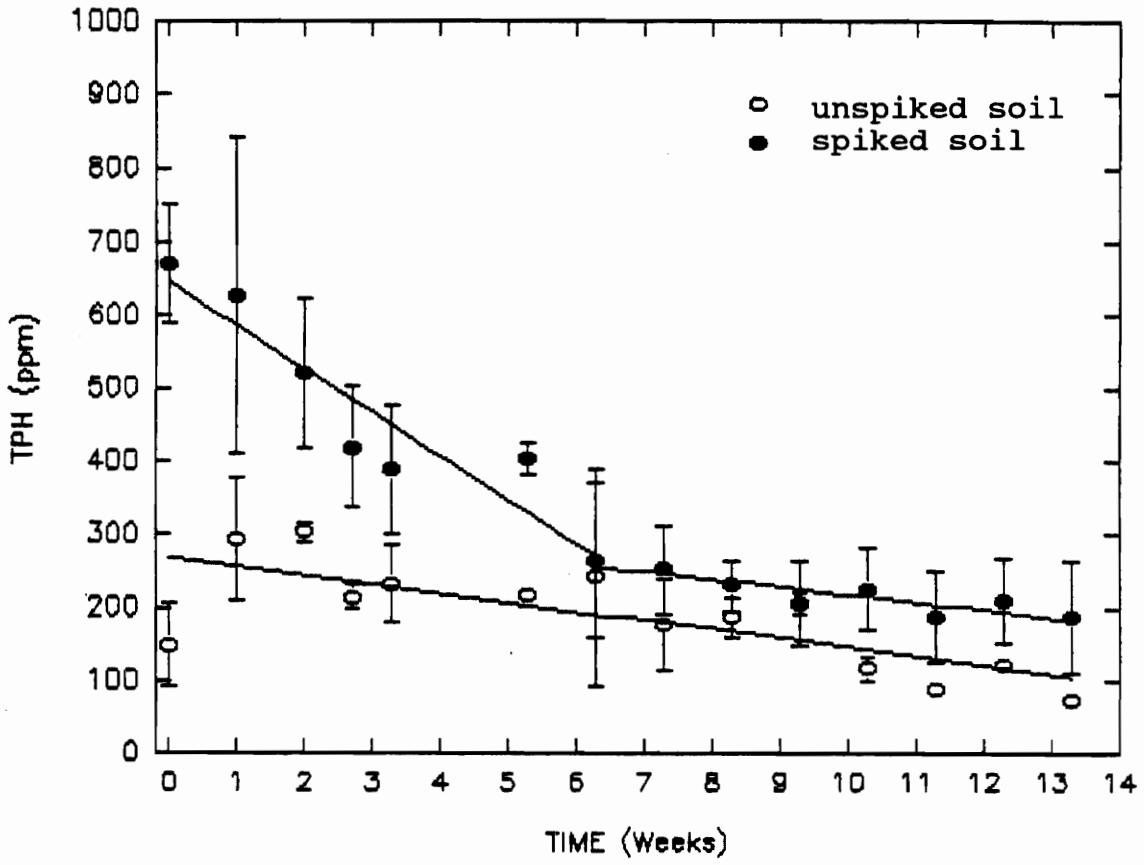

Figure 19: Soil columns - TPH degradation in unspiked and spiked Sybron soil, measured by the MeCl/GC method.

This suggests that the slowly degrading fraction of TPH consisted mainly of the weathered contaminants, originating from the earlier hydrocarbon spills.

The steady TPH degradation in the relatively homogenous soil system confirmed that TPH fluctuations, measured in the field-scale treatment units, were due to soil heterogeneity, uneven distribution of the contaminants or random measurement errors. The results point to the necessity of analyzing large numbers of soil samples to obtain reliable TPH data, particularly in heterogeneous field situations.

When running a petroleum sample through a gas chromatograph, the lighter hydrocarbons come off quicker than the heavier compounds. The fraction of hydrocarbons eluting until 25 min, represents hydrocarbons with less than 20 carbon atoms (Bhandari, 1992). This light fraction of TPH was determined from the chromatograms, and the trend over time is shown in Figure 20. The light fraction accounted for approximately 85 % of the initial TPH, but decreased to below 60 % within the first 5 weeks of the experiment.

The decrease of light hydrocarbon compounds within the initial phase of the experiment fell together with the rapid overall decline of TPH. The results confirm that the lighter and more soluble hydrocarbons compounds were degraded rapidly.

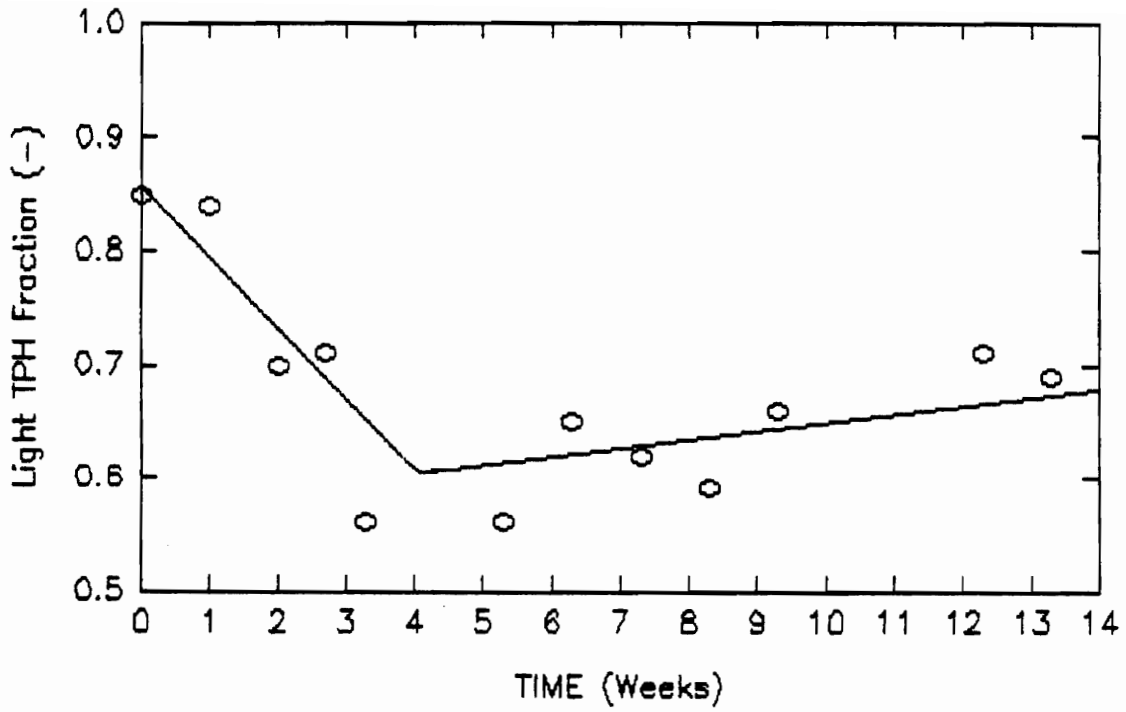

Figure 20: Soil columns study - Fraction of light hydrocarbons vs time.

4.3.2 Surface Tension - Correlation with TPH Degradation

Water collected from the soil columns with uncontaminated and uncontaminated soil was analyzed for surface tension approximately each week.

Surface tension of the circulated water, shown in Figure 21, fluctuated between 67 dynes/cm and 71 dynes/cm for the uncontaminated soil. Surface tension for the contaminated soil fluctuated between 66 and 69 dynes/cm. Except from week 2, 4 and 11 surface tension for the contaminated soil columns were lower than for the uncontaminated. A linear regression to the fluctuating surface tension measurements demonstrates a decreasing trend over time.

The surface tension measurements indicate that biological degradation of petroleum hydrocarbons is coupled with microbial production of biosurfactants. Biosurfactants are produced from the insoluble substrate, and the decrease of surface tension consequently corresponds to a decrease of TPH over time. Contaminated soil displayed lower average surface tension, compared to uncontaminated columns, because of the additional provision of No. 6 fuel oil as insoluble substrate.

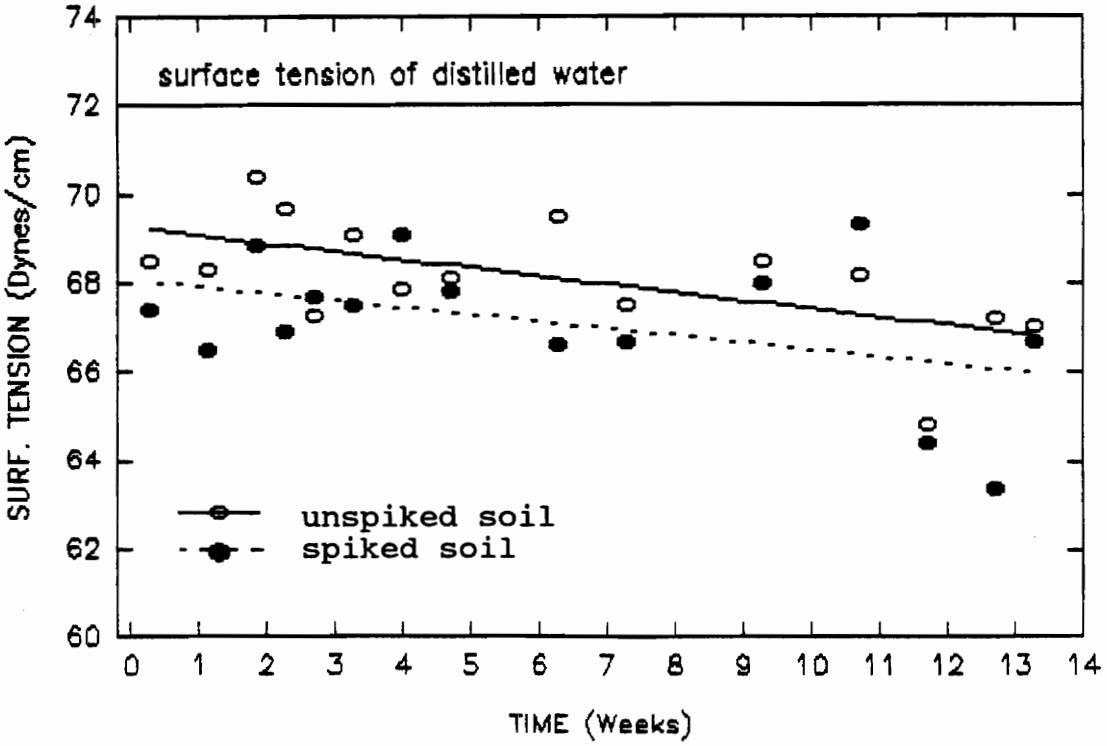

Figure 21: Soil column study - surface tension of circulated water vs time. Soils compared were: Unspiked and spiked Sybron soil.

4.4 Comparison of TPH Analysis by MeCl/GC and Freon/IR

The TPH measurements of the field scale study represented the primary evidence for the biological degradation of petroleum hydrocarbons.

Sybron's TPH analysis used multiple extractions of hydrocarbons with freon from a soil sample of 2 g. The soil and solvent were agitated in a separatory funnel for several minutes. Concentrations in the extracts were analyzed by infrared spectrophotometry. The method will be referred to as freon/IR.

The TPH analysis by VPI&SU involved solvent extraction with MeCl. A vial containing the solvent and a soil sample of 5 g was agitated for 24 hours. The hydrocarbon concentrations in the extracts were analyzed by gas chromatography. The method will be referred to as MeCl/GC.

The two analytical methods provided contradictory results with regard to the performance of the field-scale biotreatment. In comparison with the MeCl/GC data, the freon/IR was believed to be invalid for several reasons, which were confirmed by the laboratory experiments:

TPH data determined by the MeCl/GC method showed a gradual decline over time and suggested the occurrence of biodegradation. The potential of biological degradation was proven by the ^{14}C-tracer technique. In addition microcosm and

soil column data corresponded qualitatively with the field data.

Fluctuations of MeCl/GC measurements appeared to be random. Considering that a single sample was analyzed for each treatment, the variations of the data can be accounted for by random measurement errors. The results of the soil column study, where a larger sample population was analyzed, demonstrated that even under very homogenous conditions considerable deviations occur.

In contrast freon/IR measurements did not show any trend of TPH decline, rather a continuous fluctuation until the end of the study. The fluctuations of the data in duplicate as well as different treatment units were strongly correlated. As a large sample population was analyzed, the variation of the freon/IR data cannot be due to random measurement errors, but to a real phenomenon or a systematic measurement error. The fact that the TPH variations could not be verified with any of the laboratory experiments points to a significant systematic measurement error in the freon/IR method.

The analytical methods for the determination of TPH include the extraction using freon or MeCl and the analysis by IR or GC. Both steps of the procedure have to be examined to determine or rule out sources of systematic errors.

4.4.1 MeCl vs Freon Extraction

Hydrocarbon extractions with two different solvents, MeCl and freon, were carried out with a number of soil samples from the field experiment. These *Rhod.* samples from the top (T) of barrel d, had been collected by Sybron, originally analyzed using the freon/IR method and kept frozen.

TPH degradation using MeCl and freon extraction together with GC analysis is shown in Figure 22. The TPH determined by freon/IR is shown in comparison. Hydrocarbon concentration of MeCl extracts were consistently higher than of freon. Both extracts analyzed by GC show a steadily declining concentration trend, which appears to be parallel. This suggests correlation between TPH data of freon and MeCl extracts.

The TPH concentrations of freon extracts versus MeCl extracts are presented in Figure 23. A linear regression of the data had a slope of approximately 1.5 and an x-intercept at 100 ppm. Total petroleum hydrocarbon concentrations (TPH), determined by the MeCl/GC method, can be expressed as a function of TPH, measured by the freon/IR method in the form of the following equation:

$$\text{TPH (MeCl/GC)} = 100 \text{ ppm} + 1.5 \times \text{TPH (freon/GC)}$$

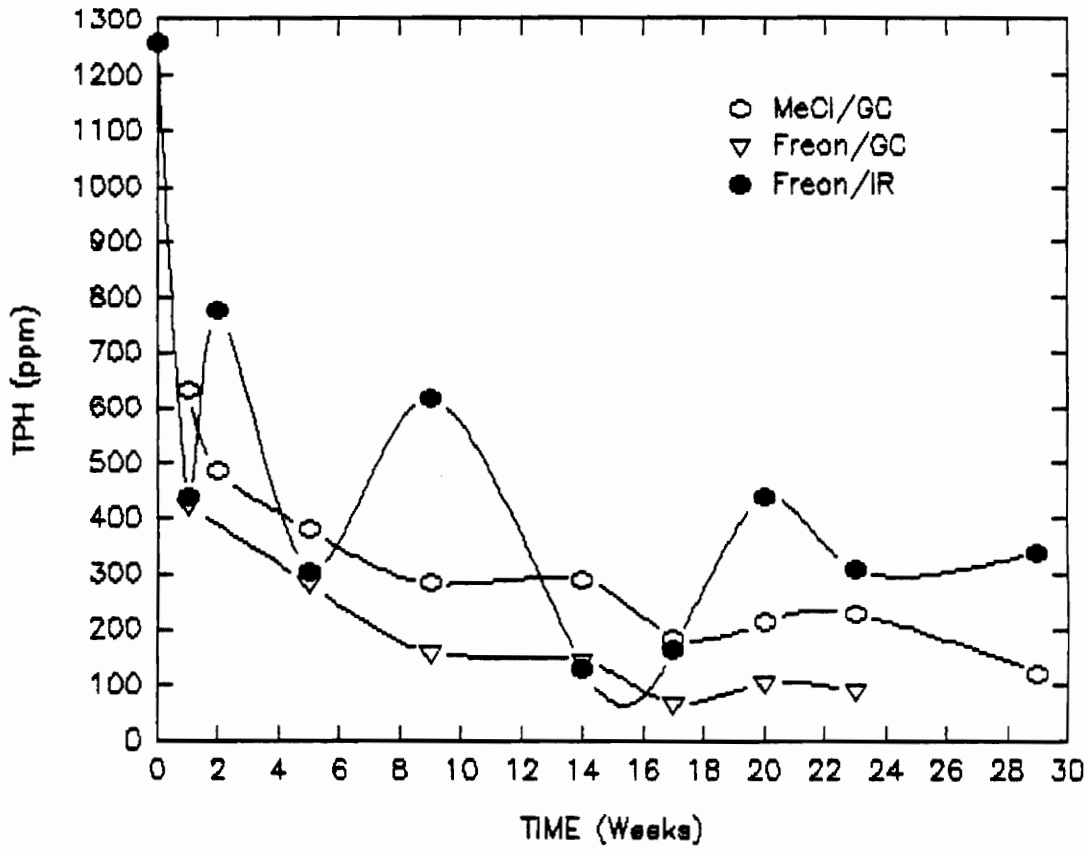

Figure 22: Comparison of TPH, measured by the MeCl/GC, freon/GC, and freon/IR method. Soil samples were taken from the top (sample T) of *Rhod*. treatment (unit d).

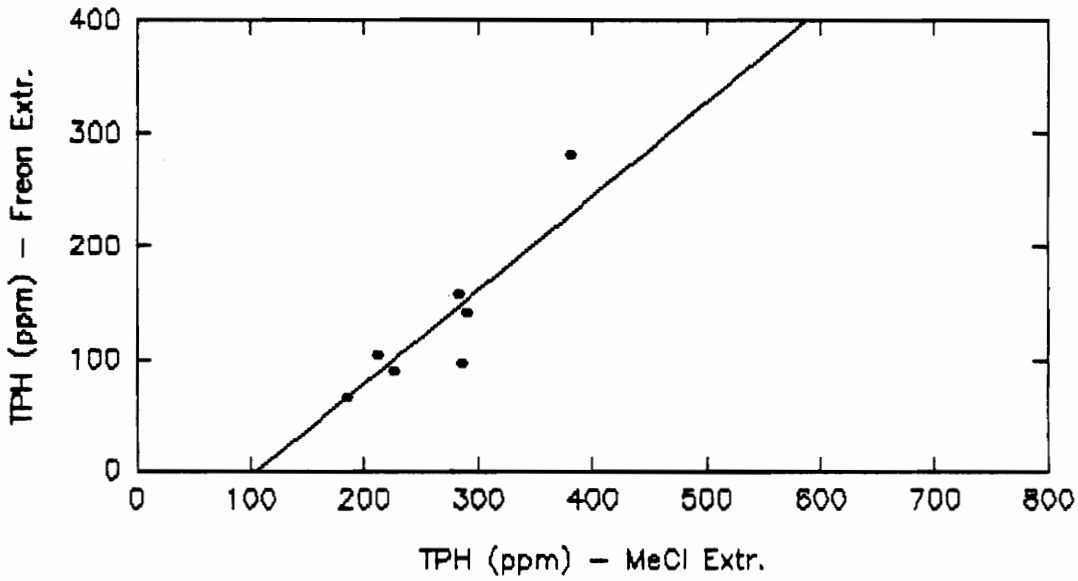

Figure 23: Comparison of MeCl and freon extraction. Soil samples originated from *Rhod*. treatment (Unit d, sample T).

The results show that MeCl extracts a larger amount of hydrocarbons from soil than freon. This observation is consistent with extraction efficiencies for the freon/GC and MeCl/GC method of 35% and 55 to 65% respectively, determined by P. Novak (1992). MeCl is known to be a better but also less selective solvent for hydrocarbons (Miller and Schmidt, 1991). In addition, the longer contact of solvent and soil sample in the case of the MeCl/GC method may have resulted in a higher extraction efficiency.

The fact that the TPH results of both methods were qualitatively similar indicates that the extraction step is not responsible for the variation of freon/IR data. Both solvents, freon and MeCl, are suitable for the extraction of petroleum hydrocarbons.

The results however suggest that the choice of extraction method and the selectivity of the solvent determine the efficiency and the overall detection limit of the TPH analysis. Clean-up target levels such as 100 ppm, mandated by regulatory agencies, are in a concentration range, where TPH measurements were found to be most sensitive to the extraction method used. Without specifying the method of analysis, these regulatory clean-up requirements appear very questionable.

4.4.2 GC vs IR Spectrophotometry

The freon extracts from the stored soil samples (unit d, sample T), originating from the Sybron field experiment, were analyzed at VPI&SU using the GC method described for MeCl extraction. The same freon extracts were analyzed by Sybron personnel using IR spectrophotometry.

The results of the GC and IR analysis are compared in Figure 24. IR measurements are consistently higher than GC measurements. The GC and IR data is not necessarily correlated, but a linear regression was used to quantify the ratio between freon/IR and freon/GC values as roughly 20.

In comparison to GC results from identical extracts, which are consistent with the approximate level of initial contamination, the incongruously high TPH measurements obtained by IR spectrophotometer appear to be incorrect. The interference of some of the extracted substances with the RI spectrophotometer is the suspected cause for the results.

The analytical step of the freon/IR method used by Sybron was identified as the cause for the erroneous TPH results.

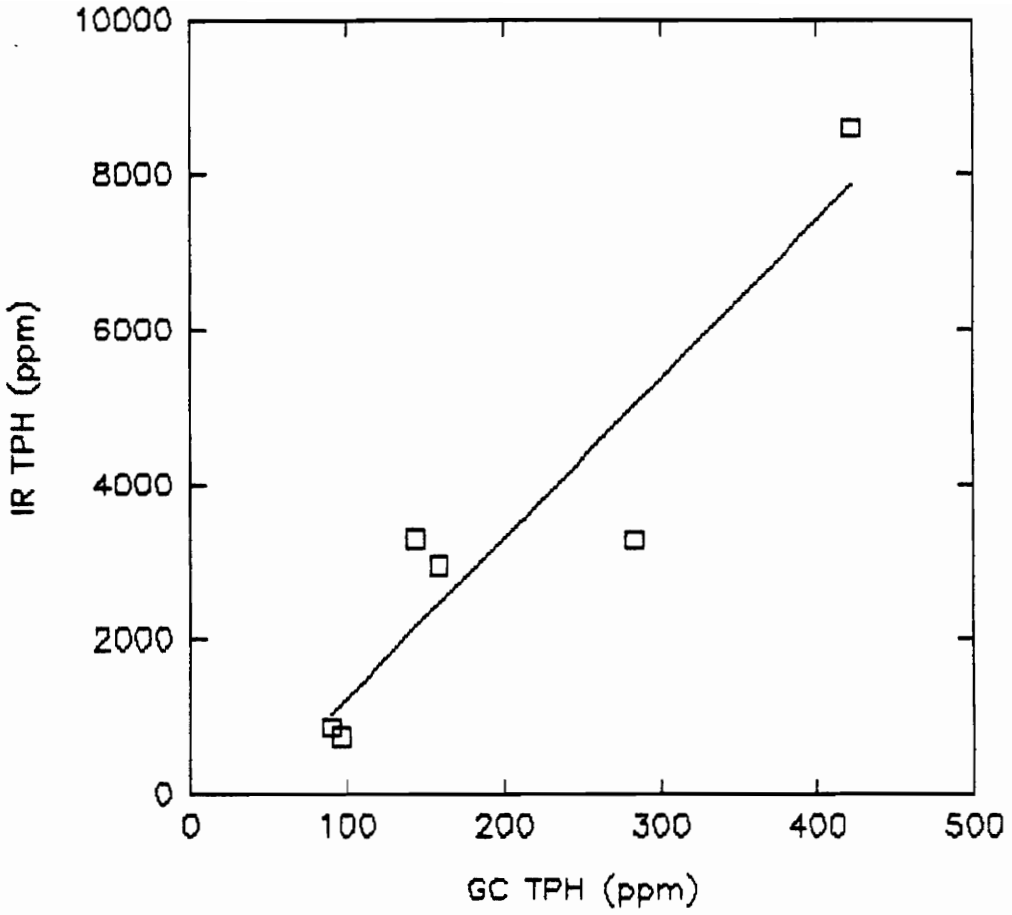

Figure 24: Comparison of TPH analysis by gas chromatography (GC) and infrared spectrophotometry (IR).

4.5 Comparison of Microcosms, Soil Columns and ^{14}C-tracer Technique as Tools for Predicting In Situ Biodegradation

Several laboratory experiments were used in this study, the ^{14}C-tracer technique, the microcosms and the soil column study. To evaluate the usefulness of these techniques for predicting in situ biodegradation, qualitative and the quantitative results of the Sybron field experiment will be summed up in comparison with the laboratory experiments .

The qualitative results observed in the field experiment regarded three factors limiting degradation reaction - the activity of microorganisms, nutrient and substrate availability.

Introduced organisms did not accelerate biodegradation in nutrient enhanced treatment units, which was shown also by microcosms with acclimated Sybron soil. In contrast the ^{14}C-tracer technique suggested a significantly higher degradation activity of ABR Hydrocarbon degraders.

Nutrient limitation in control treatment units was confirmed by the ^{14}C-tracer technique and microcosms with screened Sybron soil.

Substrate availability was suspected to be a limiting factor, and reaction rates close to zero order observed in all laboratory studies.

In order to evaluate the quantitative accuracy of different laboratory techniques, the degradation rates are compared in Figure 25. Nutrient enhanced treatment without introduced organisms (N+P) and with *Rhodococcus sp.* inoculum (*Rhod.*) are shown. The soil column study was only performed for (N+P) treatment, detailed field data from the stored samples obtained only for treatment *Rhod.* Both treatments, however, displayed nearly identical average degradation rates in the field experiment. The combined data for both treatments from microcosms, soil columns can therefore serve for comparison.

Microcosms and soil columns displayed two distinct phases of degradation with a rapid and subsequent slower reaction rate. The same trend was observed in TPH data from stored samples (unit d, sample T) of the *Rhod.* treatment unit. The initial and final degradation rates determined by linear regressions are shown.

Initial degradation rates are in the range of 40 to 60 ppm/week, final rates around 10 ppm/week. Soil columns - (N+P) treatment - have the highest degradation rates. Initial and final degradation rates in microcosms and field experiment are relatively similar. The same is true for MeCl or freon extraction method.

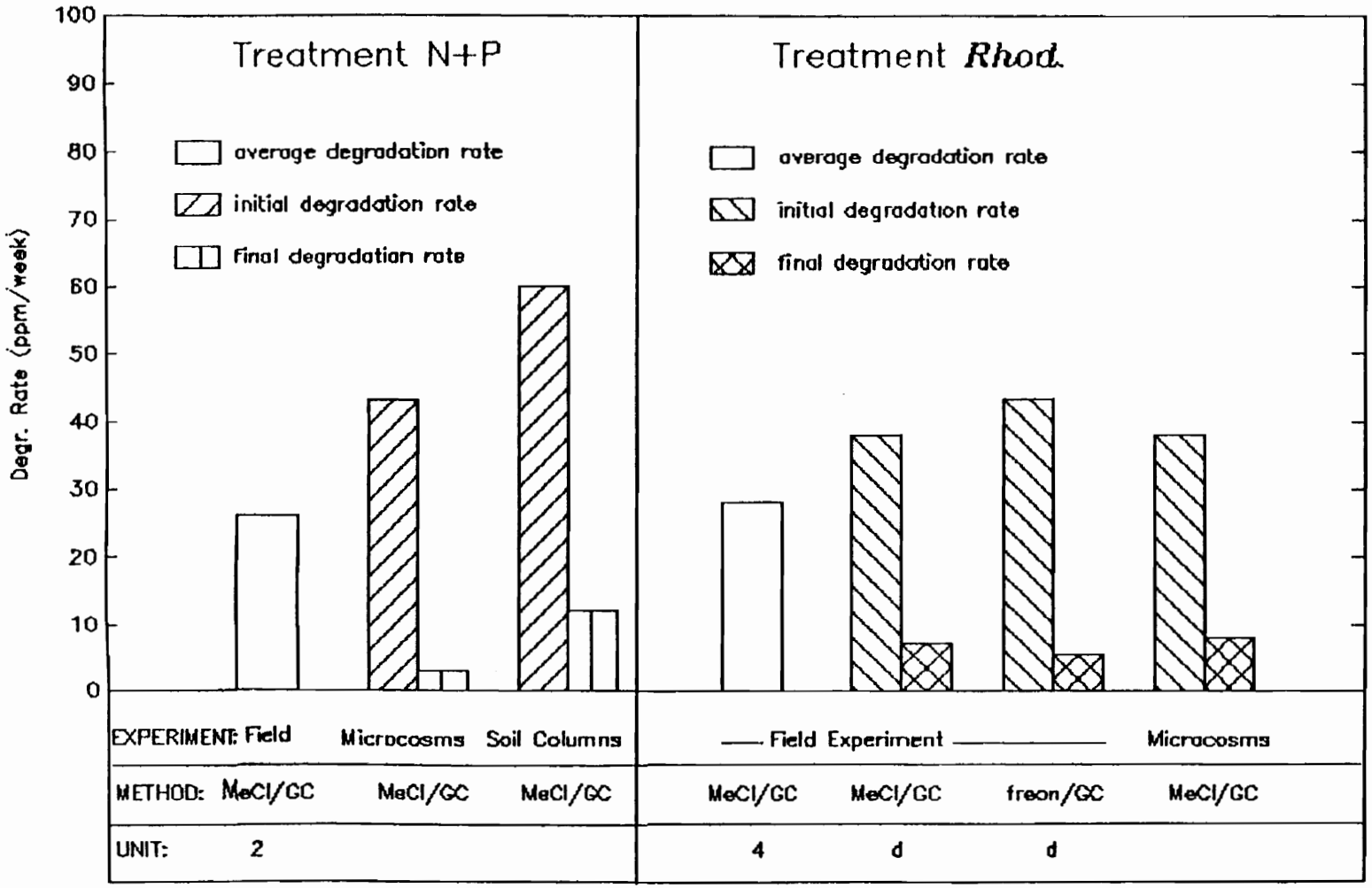

Figure 25: Comparison of degradation rates in field experiment, microcosms and soil column study.

None of the laboratory experiments simulated every aspect of the field experiment accurately. The ^{14}C-tracer technique and microcosms with a homogenous but differently prepared soil were found to qualitatively predict certain limiting factors, such as nutrient availability and the degradation activity of soil microorganisms. Soil microcosms with contaminated Sybron soil from the field experiment gave quantitatively accurate prediction of degradation rates. While soil columns simulate the recirculation of water, nutrients and contaminants most accurately, they appear to overestimate degradation rates. Nonoxygen-limited degradation, apparently occurring in the top of the treatment units, may be closely modeled by this system.

5 SUMMARY AND CONCLUSIONS

In situ bioremediation of petroleum contaminated soil using nutrient addition and introduced microorganism was simulated in a pilot scale study by Sybron Inc. and VPI&SU. TPH, biodegradation potential and surface tension of circulated water were monitored over time.

Soil microcosms were prepared with identical and a different soil to monitor TPH degradation and compare similar treatment options in a static system.

A soil column study was conducted to verify the occurrence of biodegradation and investigate the variability of TPH data. The following conclusions were drawn:

1. Measurements of mineralized C14-hexadecane proved the biological degradation potential of indigenous and added soil organisms.

2. Addition of inorganic nutrients, phosphorous and nitrogen, was found to enhance biodegradation of petroleum hydrocarbons.

3. The addition of ABR Hydrocarbon Degraders and *Rhodococcus* inoculum was not found to enhance biodegradation in previously contaminated soil both in the field experiment and the microcosm studies.

4. The microcosm study and ^{14}C-tracer technique suggested that in comparison to indigenous organisms the microbial activity in previously uncontaminated soil was seen sooner when inoculated with ABR Hydrocarbon degraders and *Rhodococcus sp*.

5. Microcosms and soil columns study suggest that biodegradation of No. 6 fuel oil was solubility limited and occurred in two phases with different rates. Initial degradation of soluble hydrocarbon compounds was rapid, subsequent degradation of less soluble compounds slower.

6. Lowered surface tension indicated production of biosurfactants, particularly by *Rhodococcus sp*. No evidence for increased degradation as a consequence of biosurfactant production was found.

7. The freon/IR method were found to be not applicable for the TPH analysis of Sybron soil. The MeCl/GC method gave satisfactory results, but the collection of a large number of samples is recommended under heterogeneous conditions.

8. The ^{14}C-tracer technique was found to be useful for predicting nutrient limitation of biodegradation. Microcosms predicted the degradation rates of soluble and degradable hydrocarbons accurately. Soil columns simulated the

biotreatment in a system with water circulation and the degradation of less soluble and degradable hydrocarbons best.

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APPENDIX A

TABLE A-1: Particle size analysis of Sybron soil.

Specification	Soil Analysis
% Sand	32.8
% Silt	40.0
% Clay	27.2
Textural Class	Clay Loam

TABLE A-2: Particle size analysis of screened Sybron soil.

Specification	Soil Analysis
% Sand	35.4
% Silt	42.0
% Clay	22.6
Textural Class	Loam

TABLE A-3: Particle size analysis of screened Hillcrest soil.

Specification	Soil Analysis
% Sand	25.7
% Silt	44.0
% Clay	30.3
Textural Class	Clay Loam

TABLE A-4: pH of Sybron and Hillcrest soil.

	pH
Sybron soil	6.4
Hillcrest soil	8.0

TABLE A-5: Bacterial enumeration of Sybron soil with Standard Methods Agar (SMA), Tryptic Soy Agar (TSA), and Soil Extract Agar (SEA).

Agar	SMA	TSA	SEA
Bacterial counts (10^6 cfu/g soil)	3	10	10

TABLE A-6: Bacterial enumeration of Hillcrest soil

Agar	SMA	TSA	SEA
Bacterial counts (10^6 cfu/g soil)	3.5	14	8

APPENDIX B

TABLE B-1: Preliminary study on biodegradation potential. Cumulative Activity of trapped $^{14}CO_2$ in flasks with 10 mL and 50 mL of BOD dilution water and 1 g, 2 g, and 3 g of unspiked Sybron soil.

Vol. of BOD water	50 mL		10 mL	
Soil sample size	1 g	1 g	2 g	3 g
Day	Activity (DPM)			
1			34	30
2	158	236		
3			352	257
4	172	249		
5			236	359
6	162	220		
7	155	150	554	406
Total Activity	648	854	1,175	1,053

APPENDIX C

TABLE C-1: Sybron field experiment - TPH concentrations, measured by MeCl/GC method.

Treatment	Controls	N+P	ABR HCD	*Rhod.*
Week	TPH (ppm)			
6	630	930	-	690
10	570	630	730	840
12	500	445	590	360
14	880	620	600	470
16	550	455	790	570
18	440	425	610	400
20	395	290	577	318
26.3	687	130	475	152
29	376	64	174	132

TABLE C-2: Sybron field experiment - Biodegradation activity, measured by the ^{14}C-tracer technique.

Treatment		Control		N+P		ABR HCD		*Rhod.*	
Week	Repl.	Activity (DPM)	Ave.		Ave.		Ave.		Ave.
0	A	590	750	1,900	2,200	5,100	4,200	1,500	3,900
	B	610		3,000		3,500		4,100	
	C	1,040		1,600		4,100		6,100	
2	A	110	90	3,800	3,500	3,400	3,500	500	1,100
	B	85		2,500		-		1,100	
	C	80		4,300		3,700		1,600	
4	A	210	210	3,100	3,800	5,300	4,600	4,800	3,400
	B	210		4,600		5,400		3,700	
	C	200		3,600		3,200		1,800	
6	A	200	250	5,600	4,900	4,500	4,200	3,500	3,200
	B	300		4,800		3,800		4,000	
	C	-		4,300		-		2,100	
9.4	A	200	160	5,000	4,700	3,700	3,800	3,400	3,200
	B	150		4,900		3,900		3,300	
	C	120		4,100		3,700		3,000	
10.4	A	220	130	4,500	4,400	6,900	6,600	3,000	2,500
	B	50		4,000		7,100		2,100	
	C	140		4,700		5,900		2,400	
12	A	380	440	2,100	2,300	6,600	5,100	3,500	3,500
	B	500		2,500		3,700		3,400	
14	A	550	580	3,400	4,000	5,800	7,400	2,700	1,800
	B	730		4,900		7,800		1,800	
	C	480		3,700		8,700		900	

16	A	400	370	2,900	2,200	7,000	5,800	2,300	2,700
	B	330		1,700		5,400		3,000	
	C	380		2,100		5,000		2,700	
20	A	490	510	1,800	1,700	5,900	6,300	2,700	3,400
	B	470		1,500		6,500		3,500	
	C	570		1,900		6,600		3,900	

TABLE C-3: Nonnutrient-limited biodegradation potential at week 8.

Treatment		Control		N+P		ABR HCD		*Rhod.*	
Day	Repl.	Activity (DPM)							
			Ave.		Ave.		Ave.		Ave.
4	A	5,600	6,100	8,700	7,400	7,000	6,700	6,400	5,800
	B	6,500		6,200		6,300		5,200	
7	A	6,600	6,600	8,100	8,500	5,800	5,800	6,300	3,300
	B	6,600		8,900		5,800		4,300	
	Total		12,700		15,900		12,500		9,100

TABLE C-4: Sybron field experiment - Surface tension of circulated water.

Treatment	Control	N+P	ABR HCD	*Rhod.*
week	Surface Tension (Dynes/cm)			
2	69.8	70.5	69.8	69.4
4	67.3	68.2	65.8	64.5
6	67.1	65.5	65.3	57.1
8	69.9	71.0	68.9	68.8
9.4	66.7	59.9	67.9	66.9
10.4	68.7	67.3	67.8	57.5
12	70.6	70.2	69.0	69.8
14	69.2	69.7	70.0	68.8
16	68.1	69.3	65.2	68.4
20	67.2	66.7	66.8	67.3

TABLE C-5: TPH concentrations in spiked Sybron soil microcosms.

Treatment	Controls	N+P	ABR HCD	*Rhod.*	sterilized controls
Week	TPH (ppm)				
0	990	990	990	990	990
2	840	670	-	930	
8	555	886	624	704	
10	457	473	479	607	
12	510	463	576	522	
14	596	651	558	637	
22.3	462	515	577	478	731
26.3	375	442	622	505	655

TABLE C-6: TPH concentrations in screened Sybron soil microcosms.

Treatment	Controls	N+P	ABR HCD	*Rhod.*	sterilized controls
Week	TPH (ppm)				
0	2,290	2,290	2,290	2,290	2,290
4	2,100	1,310	1,270	1,210	
6	1,580	1,190	1,440	1,030	
8	1,170	980	1,760	1,060	
14	1,460	960	1,320	950	3,080
18	1,720	880	2,200	1,100	2,640

TABLE C-7: TPH concentrations in screened Hillcrest soil microcosms.

Treatment	Controls	N+P	ABR HCD	*Rhod.*	sterilized controls
Week	TPH (ppm)				
0	1,550	1,550	1,550	1,550	1,550
4	1,400	1,430	930	1,050	
6	1,090	1,010	880	860	
8	1,340	960	880	890	
14	970	880	1,100	910	
18	1,640	1,020	1,310	900	1,720

TABLE C-8: Spiked Sybron soil microcosms - Biodegradation potential, measured by the ^{14}C-tracer technique.

Treatment		Control		N+P		ABR HCD		*Rhod.*	
Week	Repl.	Activity (DPM)	Ave.		Ave.		Ave.		Ave.
0	A	80	70	1,900	1,500	2,000	1,800	900	1,300
	B	50		1,300		2,000		2,200	
	C	80		1,200		1,400		800	
2	A	100	130	4,300	3,800	2,900	2,900	2,600	2,600
	B	140		2,500		3,000		2,200	
	C	140		4,600				3,100	
4	A	440	440	3,600	2,800	2,600	2,300	2,900	2,400
	B	400		2,600		2,100		1,900	
	C	470		2,300					
6	A	530	390	2,200	2,200	900	1,600	1,700 1,800	
	B	400		2,000		1,600		1,200	
	C	320		2,400		2,300		2,600	
Average			260		2,600		2,200		2,000

TABLE C-9: Screened Sybron soil microcosms - Biodegradation potential, measured by the ^{14}C-tracer technique.

Treatment		Control		N+P		ABR HCD		*Rhod.*	
Week	Repl.	Activity (DPM) Ave.		Ave.		Ave.		Ave.	
0	A	100	80	400	290	620	590	330	370
	B	70		330		500		420	
	C	90		130		650		360	
2	A	20	30	250	360	310	280	310	400
	B	30		470		240		480	
Total Ave.			60		330		430		390

TABLE C-10: Screened Hillcrest soil microcosms - Biodegradation potential, measured by the ^{14}C-tracer technique.

Treatment		Control		N+P		ABR HCD		*Rhod.*	
Week	Repl.	Activity (DPM) Ave.		Ave.		Ave.		Ave.	
0	A	660	550	2,200	2,100	3,900	3,700	2,900	3,000
	B	440		2,000		4,400		3,400	
	C					2,900		2,800	
2	A	330	260	1,300	1,600	1,700	2,300	2,500	2,600
	B	190		1,900		3,000		2,600	
Total Ave.			400		1,800		3,000		2,800

TABLE C-11: Soil columns - TPH concentrations in spiked and unspiked Sybron soil.

TPH (ppm)										
Soil	unspiked				spiked					
Week	A	B	C	Ave	A	B	C	D	E	Ave
0	190	110		150	730	610				670
1	230	350		290	640	610				630
2	310	310	290	300	510	390	640	550		520
2.7	200	230		220	490	440	330			420
3.3	190	270		230	370	310	480			390
5.3	220			220	450	380	410	420		400
6.3	140	350		250	200	420	230	210		330
7.3	140	220		180	170	240	260	360	230	250
8.3	210	170		190	230	180	250	260		230
9.3	140	180		160	220	190	250	250		230
10.3	100	130		120	180	200	310	220		230
11,3	180			180	130	130	280			150
12.3	130	110	130	120	150	210	280	230	140	200
13.3	70	80		70	220	280	150	100		190

TABLE C-12: Soil columns - Surface tension of circulated water.

Soil	unspiked	spiked
Week	Surface Tension (Dynes/cm)	
0.2	68.5	67.4
1.1	68.3	66.5
1.9	70.4	68.8
2.3	69.7	66.9
2.7	67.3	67.7
3.3	69.1	67.5
4	67.9	69.1
4.7	68.1	67.8
6.3	69.5	66.6
7.3	67.5	66.7
9.3	68.5	68.0
10.7	68.2	69.3
11.7	64.8	64.4
12.7	67.2	63.4
13.3	67.0	66.7

VITA

Joachim Ernst Dietrich Vogdt was born February 14th, 1967 in Hamburg, Germany to Ursula and Kurt Dietrich Vogdt. After graduating from the Gymnasium Isernhagen in 1987 with a Abitur (certificate of general qualification), he completed his compulsory service as a lance corporal and military driver in the 1st Armoured Division, Hannover. From October 1988 to October 1990 he studied at the Technische Universität Hamburg-Harburg, where he finished his Vordiplom in Maschinenbau (mechanical engineering). He continued his studies at the Universität Hannover until August 1991, when he was awarded a Fulbright scholarship and entered the environmental engineering program at Virginia Polytechnic Institute and State University, Blacksburg, VA, USA. Upon receiving the M.S. in environmental engineering, Joachim intends to return and work in Germany.