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EVALUATION OF THE POTENTIAL USE OF MICROORGANISMS IN THE CLEANUP OF PETROLEUM HYDROCARBON SPILLS IN SOILS

by

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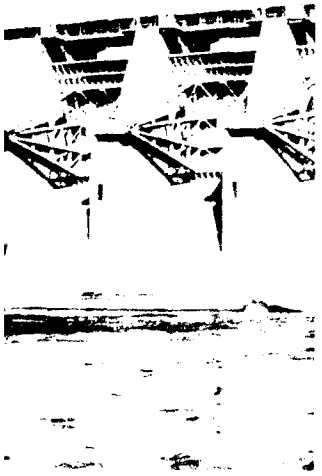

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A study was undertaken to examine the feasibility of using native soil microflora to degrade diesel fuel, fuel oil, and motor oils within the soil matrix; to isolate and identify those environmental factors controlling the rate and extent of degradation; and to develop procedures to optimize the rate and extent of biodegradation achieved.

The work demonstrated that native soil microorganisms were able to bring about moderate to substantial removal of petroleum hydrocarbons from soils contaminated with JP-5 and diesel fuel. Some removal was noted upon addition of water only, but addition of water to the point of flooding did not necessarily stimulate degradation. Fertilization was effective in stimulating degradation under dry (unflooded) conditions for both JP-5- and diesel-contaminated soils.

Fertilization was also sometimes important where biotreatment was effective under anaerobic conditions, but some of the biotreatment under anaerobic

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conditions may have resulted from initial oxidation of ammonium in the fertilizer to supply nitrate as an alternate electron acceptor.

Based on the results of this work, use of native soil microflora to degrade diesel fuel, fuel oil, and motor oils within the soil matrix is feasible. Those environmental factors most important in controlling the rate and extent of degradation are moisture, nutrients, and oxygen, and, under anaerobic conditions, nitrate. Procedures to optimize treatment should focus on methods to determine the exact amounts of fertilizer and nitrate to add, and the rate at which addition should occur.

PREFACE

The study reported herein was conducted by the Environmental Laboratory (EL) of the US Army Engineer Waterways Experiment Station (WES), Vicksburg, MS. The research was sponsored by the Department of the Army In-House Laboratory Independent Research (ILIR) Program for fiscal years 1990 and 1991, under ILIR Project No. A91D-LR-004.

The study was conducted by Dr. Douglas Gunnison of the Aquatic Processes and Effects Group (APEG), Ecosystem Research and Simulation Division (ERSD), EL. Technical assistance was provided by Ms. Cynthia B. Price of the APEG and contractors Ms. Patricia Reilly, Ms. Jane M. Brazil, and Mr. Glenn Myrick. Analytical chemistry laboratory assistance was provided by Ms. Ann Strong, Mr. Richard Karn, and Mr. Robert Jones of the Analytical Laboratory Group, Environmental Engineering Division (EED), EL. Drs. Judith C. Pennington and James M. Brannon of the APEG and Mr. Mark E. Zappi of the EED provided technical reviews. The report was edited by Ms. Jessica S. Ruff of the WES Information Technology Laboratory.

The study was conducted under the direct supervision of Dr. Thomas L. Hart and Dr. Richard E. Price, Chief and Acting Chief, respectively, of the APEG, and under the general supervision of Mr. Donald L. Robey, Chief, ERSD, and Dr. John Harrison, Chief, EL.

COL Larry B. Fulton, EN, was the Commander and Director of WES. Dr. Robert W. Whalin was Technical Director.

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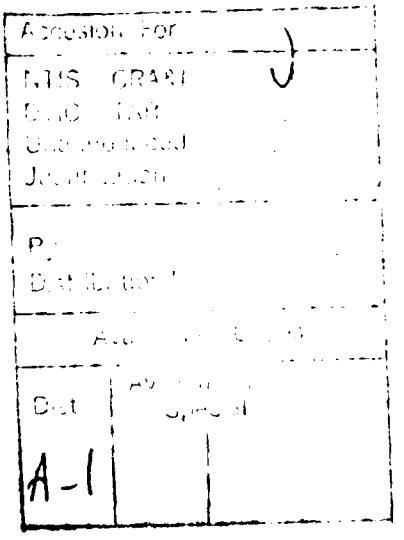

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EVALUATION OF THE POTENTIAL USE OF MICROORGANISMS IN THE CLEANUP OF PETROLEUM HYDROCARBON SPILLS IN SOILS

PART I: INTRODUCTION

Background and Relevance

1. Soils and sediments at many military facilities have been contaminated with petroleum hydrocarbons (gasoline, lubricating oil, diesel fuel, aviation fuel), often as a consequence of spills occurring during storage and/or active use. Various elements of the military are required to clean up contamination resulting from any activity on lands under their jurisdiction.

2. Numerous examples of such spills have occurred at Army, Air Force, and Navy storage facilities and at sites of active use, such as airfields and motorpools. Leaks occurring in underground storage tanks situated in or near groundwater aquifers can be a particularly serious problem, resulting in contamination of groundwater. The presence of petroleum hydrocarbon contaminants in flooded soils and sediments can pose unacceptable hazards to the environment, both by their availability to resident organisms and through their release to adjacent waters.

3. Current cleanup procedures are expensive and labor intensive because they require physical removal and incineration or confinement of contaminated soils and sediments. Recently, microbial degradation of certain contaminants in soils and sediments, including many petroleum hydrocarbons, has begun to be explored. Utilization of in situ biodegradation is currently an area of intense interest. Materials and/or organisms capable of stimulating degradation are added to the soil, either by injection through wells or to groundwater that is recirculated. For treatment of contaminated soils at or near the surface, these amendments may be mixed with the surface soil, although this type of treatment is more properly termed landfarming.

4. The same process may be extremely useful for biodegradation of more recalcitrant petroleum hydrocarbons, such as diesel fuel and motor oils, and the Dutch have applied a variation of the procedure to the cleanup of petroleum-contaminated soils from refinery areas. In this case, the soils were physically transported to a site specifically prepared for aerobic

degradation of the petroleum hydrocarbons, rather than using in situ biodegradation.

5. Universities and the oil industry have conducted investigations of petroleum biodegradation in the past, but technology is just now reaching the point at which it can be readily applied to ongoing cleanup activities. Furthermore, biodegradation of various gasoline components has been demonstrated; however, successful degradation of diesel fuel and motor oils at degradation rates acceptable for routine cleanup activities is uncertain. Stimulation of the activity of native microorganisms in soils, flooded soils, and sediments may enhance biodegradation rates to achieve rapid cleanup of spilled fuel oil and motor oils.

Objectives

6. The study had the following objectives: (a) to determine the feasibility of using native soil microflora to degrade diesel fuel, fuel oil, and motor oils within the soil matrix; (b) to isolate and identify those environmental factors controlling the rate and extent of in situ biodegradation; and (c) to develop procedures to optimize the rate and extent of biodegradation achieved.

PART II: LITERATURE REVIEW

Microbial Degradation of Petroleum Hydrocarbons

7. Petroleum is a complex, heterogenous mixture of organic compounds varying from easily degraded to recalcitrant. According to Atlas (1981), the mixture can be separated into several classes based on structure. These include a saturate or aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction. Saturated hydrocarbons include the n-alkanes (linear or straight-chain molecules), branched alkanes, and cycloalkanes (naphthenes). Aromatic compounds are ring-containing compounds based on benzene as the parent type and may be considered as derivatives of benzene. Polar compounds are highly complex structures that are difficult to analyze.

8. Most of the petroleum hydrocarbons of military importance are fuels. Fuels consist of groups of compounds within a specified range of molecular weights. Fuels most commonly used in the Army at present are gasoline and diesel oil, although heating oil is also important for military installations located in temperate regions. The following ranges of carbon are present in gasoline, heating oil, and diesel oils (Song, Wang, and Bartha 1990).

Fuel	Carbon Range
Gasoline	C_6-C_{11}
Heating oil	C_9-C_{22}
Diesel oil	C_9-C_{23}

9. The metabolic pathways for biodegradation of petroleum hydrocarbons have been recently reviewed (see Atlas 1981, 1984). Most studies have focused on the aquatic environment because of the severe problems encountered with spill containment in water (Jones 1977, Bossert and Bartha 1984). Spills on land can also be a problem because of lateral and vertical spreading and potential access to the water table (Atlas 1981), but petroleum hydrocarbon degradation in soils and soil enrichments is limited.

Degradation of Major Groups of Petroleum Hydrocarbon Components

10. A large body of information exists on the biodegradation of components of the various groups of compounds present in fuel. This section summarizes relevant portions of this information. Details on degradation of each of the classes are available in the references cited.

11. N-alkanes are thought to be the most readily degradable portion of a petroleum mixture (Atlas 1981). Biodegradation of the n-alkanes has been demonstrated for compounds having molecular weights ranging up to 44 carbon atoms (Haines and Alexander 1974). The following summary of the microbial metabolism of straight-chain and branched-chain alkanes is based on a review by Singer and Finnerty (1984). Short- and long-chain alkanes are oxidized through one of three mechanisms. Degradation of n-alkanes usually occurs by sequential monoterminal alkane oxidation (oxidation of one end of the chain) mediated by mono- or di-oxygenases to the corresponding alcohol, aldehyde, and monobasic fatty acid, and then to acetate by beta oxidation. Acetate degradation can produce shorter fatty acids and, eventually, CO_2. The environmental conditions required for this type of activity have not been established.

12. Alternatively, some organisms attack both ends of an alkane through a diterminal or omega oxidation process. Still other organisms perform subterminal oxidation (oxidation near the end of the molecule), forming a secondary alcohol and then a ketone. This apparently is a minor degradative pathway. Branched-chain alkane degradation also occurs; however, the metabolic process becomes more complicated because the presence of chains sometimes hinders microbial oxidation of the molecule, resulting in variable degradability. For example, 2-methyl branched compounds are normally capable of supporting good growth, while 3-methyl branched compounds are attacked by very few microorganisms.

13. Cycloalkanes are particularly resistant to microbial degradation; complex forms, such as the tripentacyclic compounds, are extremely persistent (Atlas 1981). The following summary of the metabolism of cycloalkanes, otherwise known as alicyclic or cycloparaffinic compounds, is derived from the reviews of Perry (1984) and Trudgill (1984). Representative cycloalkanes present in gasoline include cyclopentane, cyclohexane, methylcyclopentane, and trimethylcyclopentane (Perry 1984). These substances, actively produced by both plants and bacteria, are components of chemical substances in plants,

microbial lipids, and insect secretions (Trudgill 1984). In spite of the fact that these chemicals have existed for millions of years and are produced by a myriad of organisms, investigators have failed to demonstrate the presence of large numbers of cycloalkane-utilizing microorganisms. Apparently, alicyclic hydrocarbons resist microbial degradation more strongly than most other hydrocarbons (Trudgill 1984).

14. Trudgill (1984) and Perry (1984) speculate that this may be the result of several different factors. One possibility is that the broad specificity of enzymes that convert cycloalkanes to cycloalkanones causes cycloalkanes to be metabolized rapidly. Consequently, these compounds may not have ever accumulated sufficiently to select for microorganisms capable of using them. Alternatively, certain cycloalkanes may be highly complex, precluding utilization by pure cultures of organisms, and requiring instead the presence of two or more different organisms to bring about degradation.

15. Cerniglia (1984), Gibson and Subramanian (1984), and others (Edwards 1983, Bossert and Bartha 1986) have summarized the results of recent investigations of aromatic hydrocarbon degradation. Light aromatic hydrocarbons undergo both evaporation and microbial degradation while in the dissolved state (see Kappeler and Wuhrmann 1978a,b). The fate of polycyclic aromatic hydrocarbons (PAHs) is particularly important; many of them are toxic, carcinogenic, and occur naturally, perhaps playing a structural role in the humic substances (Martin, Haider, and Bondietti 1972).

16. While many bacteria, fungi, and algae are enzymatically capable of metabolizing aromatic compounds, the hydroxylation mechanisms among these groups of organisms are fundamentally different (Cerniglia 1984). Moreover, most of the studies of microbial metabolism of aromatic hydrocarbons have been conducted with pure cultures and/or purified enzymes. This work provides a good understanding of the ability of microorganisms to degrade these compounds and the mechanisms involved in the degradation, but does little to evaluate the environmental conditions required for degradation.

17. At the time Cerniglia's article was written, the predominantly aerobic degradation pathways had been established. According to Cerniglia (1984) and Gibson and Subramanian (1984), many of the pathways for the more complex compounds require participation by several organisms, although there are exceptions. For example, Heitkamp, Franklin, and Cerniglia (1988) recently isolated a *Mycobacterium* able to completely mineralize pyrene. Bacteria oxygenate compounds using mono- or di-oxygenases to form dihydrodiols

having a *cis*-configuration, while fungi use a mono-oxygenase to form *trans*-dihydrodiols. Later work by Mihelcic and Luthy (1988) demonstrated that several PAHs are readily degraded under anaerobic conditions in the presence of nitrate.

18. Recent research has shown that mononuclear and polynuclear aromatic hydrocarbons can also be transformed by anaerobic microbial communities under denitrifying, fermentative (including methanogenic), and in some cases, sulfate-reducing conditions (Grbic-Galic 1990).

19. The rate of microbial degradation of individual and complex mixtures of hydrocarbons is considerably reduced because of the low solubility of many of the individual components. Several microorganisms are known to use hydrocarbons as substrates to produce a number of different surfactants and emulsifiers, some of which are derived from the hydrocarbons being degraded (Rosenberg et al. 1979; Zajic and Mahomedy 1984; Desai, Patel, and Desai 1988). Surfactants and bioemulsifiers undoubtedly provide the formative microorganism with the means to better obtain these insoluble carbon sources (Desai, Patel, and Desai 1988).

Degradation of Fuels in Soils

20. A wealth of information exists on microbial degradation of individual petroleum hydrocarbon components and the pathways responsible for degradation of these compounds. Considerably less information on the rate and extent of fuel degradation in soil is available in the literature. Existing information includes the reviews of Atlas (1981, 1984) and the articles of Jones (1977), Westlake, Jobson, and Cook (1978), Dibble and Bartha (1979), Fedorak and Westlake (1981), Aamand et al. (1989), and Carberry and Lee (1990). Reports in the gray literature include many site-specific studies as, for example, the work of Ridgeway et al. (1988). Soils contaminated by petroleum fuel spills are considered hazardous wastes, and the disposal of large volumes of contaminated soil by burial in secure sites or incineration is very expensive. By contrast, land treatment of oily refinery sludges has been conducted for years with generally good results (Song, Wang, and Bartha 1990).

21. Bossert and Bartha (1984) summarized the structure, degradability, and toxicity of hydrocarbons in soil, based on the reviews of Bartha and Atlas (1977), Atlas (1981), and the National Academy of Sciences (1984). According to this information, petroleum components including the n-alkanes, the

n-alkylaromatics, and the aromatic compounds in the C_{10}-C_{22} range are the least toxic and the most biodegradable. The n-alkanes, alkylaromatics, and aromatic hydrocarbons in the C_5-C_9 range are degradable at low concentrations, but are highly toxic and are removed primarily by volatilization. The branched alkanes in the C_{10}-C_{22} range are less biodegradable because branching hinders microbial attack.

22. As indicated above, biodegradation of the cycloalkanes requires some sort of interaction by two or more microbial species. The complex aromatic and cycloparaffinic compounds having four or more condensed rings are extremely resistant to degradation, and degradation requires active participation by several species of microorganisms (Cerniglia 1984, Gibson and Subramanian 1984).

23. Initial steps of hydrocarbon degradation are either oxygen dependent or proceed much more rapidly under aerobic conditions (Bossert and Bartha 1984). Mihelcic and Luthy (1988) were able to demonstrate anaerobic degradation of some PAHs under denitrifying conditions in soil slurries; however, other authors have not been as successful. Ward and Brock (1978) and DeLaune, Hambrick, and Patrick (1980) have indicated that anaerobic degradation of petroleum hydrocarbons is either negligible or nonexistent.

24. DeLaune, Hambrick, and Patrick (1980) examined degradation of radiolabeled hydrocarbons in estuarine sediment and found up to 15 percent degradation at oxidation-reduction (redox) potentials of -220 mV and pH 8.0. However, biodegradation reached considerably higher levels at more positive redox potentials. Bossert and Bartha (1984) expressed reservations about the results of this work because measurements were made of volatile $^{14}CO_2$ trap results only; other volatile compounds were not assessed.

25. Dibble and Bartha (1979), Atlas (1981), and Bossert and Bartha (1984) examined the environmental factors influencing biodegradation of petroleum hydrocarbons in soils. Soils do not always provide the necessary combinations of nutrients, moisture, pH, and temperature for optimal microbial activity. Too much moisture interferes with oxygen movement into the soil, inhibiting aerobic degradation. However, the growth of fungi, some of the most effective agents of degradation, can be limited by too much water.

26. While sulfate can serve as an alternate electron acceptor for anaerobic degradation, many soils do not have high sulfate levels. Unlike many other situations on military installations where organic compounds are found at very low levels, petroleum hydrocarbon spills can inundate the soil

with high levels of carbon-rich compounds. Under these circumstances, nitrogen and phosphorus can easily become limiting, as has been evidenced by the vast improvement in degradation brought about by addition of fertilizers (Dibble and Bartha 1979).

27. Several reports in the literature deal with fuel spills and/or are focused on degradation of one or more components of more complex fuel mixtures (see Ridgeway et al. 1988, Wilson et al. 1988, Aamand et al. 1989, Ostendorf 1990). Song, Wang, and Bartha (1990) investigated a bioremediation approach for cost-effective land treatment for five fuels--gasoline, jet fuel, heating oil, diesel oil, and bunker C. They found that biodegradation makes only a modest contribution to the disappearance of gasoline from soil, while volatilization can play a major role. A similar role for volatilization was reported by Ostendorf (1990), who indicated that volatilization may account for 30 percent of the original gasoline spilled at the US Coast Guard Air Station, Traverse City, MI. Song, Wang, and Bartha (1990) also found that bunker C was structurally recalcitrant (resisted degradation), with nearly 80 percent persisting for a year after initiating treatment.

28. The three medium distillates (jet fuel, heating oil, and diesel oil) increased in persistence in that order, but responded well to bioremediation treatment. Song and Bartha (1990) examined the effects of jet fuel spills on soil microbial community structure and function. Disappearance of this fuel from surface soil was much more rapid than from poorly aerated subsurface soil. Jones (1977) studied the effects of kerosene production on soil and found very little change in the polluted sites. He speculated that soil permeability was reduced because of hydrocarbons and fatty end products decreasing the rate of decomposition by preventing gas exchange at the soil surface. The studies of Song, Wang, and Bartha (1990), Song and Bartha (1990), and Jones (1977) dealt with fuel spills restricted to the upper 0.5 m or less of the soil surface. Conditions here are much different from those in the deeper soils that will be the focus of most in situ work.

Role of Environmental Factors in Petroleum Hydrocarbon Biodegradation

29. Several authors have delineated the physical and chemical factors affecting the biodegradation of hydrocarbons. The following information is based on information presented by Bossert and Bartha (1984) and by Leahy and

Colwell (1990), who have each described major groups of environmental factors playing a dominant role in regulating degradation in soil. These factors include (a) chemical composition of the oil or hydrocarbon mixture, (b) physical state of the oil or hydrocarbon mixture, (c) concentration of the oil or hydrocarbon mixture, (d) temperature, (e) oxygen and moisture, (f) inorganic and organic nutrients, and (g) pH. Major facets of these factors are considered briefly in the following paragraphs.

Effect of chemical composition

30. Major classes of petroleum hydrocarbons include the saturates, the aromatics, the asphaltenes (esters, fatty acids, ketones, phenols, and porphyrins), and the resins (amides, carbozoles, pyridines, quinolines, and sulfoxides). These substances can be ranked as to availability for microbial degradation, but the pattern varies based on the system being considered, and the reports cited by Leahy and Colwell (1990) were generally for freshwater and marine systems. Rates of biodegradation of these classes of compounds occur in the following order of decreasing susceptibility: saturates > light aromatics > high molecular weight aromatics and polar compounds. When placed in terms of decreasing susceptibility of groups of compounds to degradation, this becomes: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes.

Physical state of oil
or hydrocarbon mixture

31. According to Bossert and Bartha (1984), major influences on petroleum hydrocarbon degradation in soil and aquatic systems are related to movement and distribution of the oil and the presence or absence of particulates. These each affect the physical and chemical nature of the mixture and its susceptibility to microbial degradation.

32. In aquatic systems the mixtures tend to spread horizontally and are subject to high levels of evaporative losses of volatile hydrocarbons. In soils, the mixtures tend to move vertically, cutting down on evaporative losses of compounds that are toxic to microorganisms. Sorption to particle surfaces can decrease the toxicity of the components of the mixture. However, the sorption process may also contribute to persistence through formation of complexes that are inaccessible to microbial attack. Microorganisms are capable of producing biosurfactants that form emulsions which enhance the susceptibility of hydrocarbons to microbial attack. Microorganisms effective

in biodegradation of petroleum hydrocarbon mixtures are often found to possess strong emulsifying activity.

Concentration of oil
or hydrocarbon mixture

33. As discussed by Leahy and Colwell (1990), the rates of uptake of organic compounds by microorganisms are driven by the concentration of the compound in solution. Low molecular weight hydrocarbons have comparatively high solubility, and this regulates the access of microorganisms to these materials. The degradation of higher molecular weight compounds is related to aqueous solubilities, rather than to concentration of substrate in solution. The degradation of hydrocarbons is made more difficult by the fact that, above certain critical levels, the concentration of hydrocarbons may reach toxic levels.

Temperature

34. Temperature can have unforeseen effects on degradation. Temperature is generally not of concern within the soil matrix because soil temperatures, while generally low, are often quite stable. However, it is important to understand the consequences of changes in temperature on degradation, particularly when surface-level and aboveground treatment processes are to be used.

35. Increases in temperature are usually expected to double the microbial degradation rate for every 10 °C increase in temperature (termed "the Q_{10} effect"). However, there are exceptions, particularly at temperatures below the 20 °C. Thus, Best et al. (1990) found that biodegradation rates for a plant material underwent substantially larger increases over the temperature range from 5 to 10 °C (20 percent) than in the range of 10 to 18 °C (2 percent).

36. Others have found temperature to have variable effects on degradation according to the particular aspect being examined. For example, warmer temperatures might be expected to increase the volatilization of lower molecular weight compounds, resulting in a decrease in biodegradation due to decreased substrate concentration. However, Walker and Colwell (1974) reported that the decreased toxicity present at lower temperatures may actually enhance biodegradation of petroleum at these temperatures. By contrast, Atlas (1981) suggested that lower temperatures may retard volatilization of low molecular weight compounds that inhibit degradation.

Oxygen and moisture

37. As indicated above, oxygen is a requirement for effective degradation of aliphatic, cyclic, and aromatic hydrocarbons by oxygenases. Anaerobic degradation of specific hydrocarbons has been shown to occur in the presence of suitable alternate electron acceptors. Anaerobic transformation of certain individual petroleum hydrocarbons has been demonstrated to occur through the processes of methanogenesis (Grbic-Galic 1990) and nitrate-supported oxidation (Mihelcic and Luthy 1988). However, the importance of anaerobic biodegradation of aromatic hydrocarbons in natural systems is not understood. Moreover, the ability of anaerobic metabolism to remove other individual hydrocarbons or mixtures of hydrocarbons under methanogenic, sulfate-reducing, or denitrifying conditions is not presently known.

38. Microorganisms all require some amount of moisture to conduct their activities, and lack of sufficient moisture will inhibit or prevent degradation. Too much moisture, however, affects oxygen transport to the site of the activity. This is particularly important in soils where waterlogging reduces the volume of air-filled pore spaces, reducing the oxygen supplies to that level available in the soil solution.

Inorganic and organic nutrients

39. The addition of petroleum hydrocarbons to natural systems tends to overwhelm the microflora with available carbon without a concomitant increase in nitrogen and phosphorus. While soils are better able to provide these nutrients than aquatic systems, this is not always the case. The presence in the soils of other sources of nutrients, including nitrogen-fixing microorganisms and organic reserves of nitrogen and phosphorus, may provide the necessary reservoir of materials. Alternatively, highly sandy soils low in organic matter may lack the required nutrients. Addition of nutrients to aquatic systems almost always stimulates degradation. Because of the varied composition of soil, the addition of nutrients to soil systems may or may not have a stimulatory effect.

pH

40. With the exception of acid mine-influenced streams, pH in aquatic systems is fairly stable. In soil systems, however, pH can vary to a great extent, ranging from highly alkaline (11 or greater in alkaline deserts) to acidic (2.5 or less in acid mine tailings). Most bacteria and fungi prefer a pH nearer to neutrality or slightly alkaline (>5 to 8), and biodegradation of petroleum hydrocarbons proceeds much more rapidly in this range.

Microbial Limitations in Soil

41. In addition to the actual sorption of a contaminant to soil, diffusion within the soil matrix is an important factor. While soil can be a highly porous medium, the microorganism must be able to get to the substrate. Moreover, tortuosity increases the difficulty of the organism and target compound coming together, by increasing the complexity of the path as well as the physical distance between microbe and substrate. Also, many microorganisms are physically unable to get through micropores in the soil, and therefore cannot get to the substrate. Moreover, certain microorganisms themselves are known to adversely affect movement of fluids through rocks (Updegraff 1983) by physically blocking pores. The same is probably also true for soils.

42. This means that other methods will have to be used to achieve penetration; examples include the use of electrolytic treatment to enhance mobility in soils and application of chemical measures that will shrink the soil fabric, effectively increasing the pore size. Another example is the potential use of smaller microorganisms that are better able to more through soil pores. Recent research has shown that many bacteria occur in the natural environment as small filterable particles that are induced by starvation. These organisms, the so-called ultrabacteria (Roszak and Colwell 1987) are sometimes less than 0.08 μm in diameter and are recovered under conditions of minimal nutrition and prolonged incubation.

43. Lappin-Scott, Cusack, and Costerton (1988) found that these organisms could be resuscitated and then grown in sandstone cores, demonstrating the ability of the ultrabacterial forms to move into small pores. MacLeod, Lappin-Scott, and Costerton (1988) further demonstrated that the starved bacteria penetrated much deeper into sandstone core sections than their vegetative counterparts, but that the recovered cells could produce reductions in sandstone core permeability due to cell growth and polymer production.

44. Another mechanism whereby a microbial cell that is too large to move through soil pores may obtain its target compound is through the release of surfactants. These compounds may solubilize the compound, making it available for attack. Also, certain microorganisms may have the ability to use the compound within a solvent phase that permits direct physical contact between the organism and the compound.

45. Superimposed on the problem of sorption of target contaminants to surfaces is the fact that microorganisms themselves prefer to live attached to

surfaces (Marshall 1980). Soil surfaces tend to attract microorganisms from groundwater because of net charge differences (Marshall 1980). Particle surfaces are ideal microbial habitats because of their ability to scavenge nutrients from the water, enhancing microbial nutrition with little or no energy requirements from the organism. Life on surfaces also provides access to the nutritional environment occurring on the particle surface and to oxygen and other gases dissolved in the ambient liquid.

46. Life on surfaces, however, also complicates the problem of access to target contaminants. A cell attached to a surface is not able to move around and find its substrate. The substrate itself may be sorbed, as discussed above. The enzymes required for degradation, if extracellular in nature, may themselves be immediately immobilized on the particle surface via sorption (see Alexander 1971).

47. On the other hand, Arvin et al. (1988) have shown that while the attached biomass level in an aquifer may be much higher than the free-living biomass, the latter is very important for degradation, if the attached bacteria are fixed to fine soil particles. This occurs because the flow of water and the resulting target contaminant movement to the attached bacteria are relatively small because of the low hydraulic conductivity in the fine soil fractions. However, others have shown that bacteria sorbed to soil surfaces and free in the soil solution-phase degrade soluble contaminants with about equal efficiency (Orgam et al. 1985).

Conclusions Based on the Literature

48. A large body of information exists on the microbial degradation of individual compounds in petroleum hydrocarbon mixtures, including crude oil and fuels. The mechanisms and pathways for many of these compounds have been established, and several of the responsible microorganisms have been identified. By contrast, only a limited amount of information is available on degradation of these mixtures in soil. A substantial amount of information exists on fuel and petroleum spills in water.

49. The information available on individual petroleum components indicates that, under the proper circumstances, the linear alkanes and aromatic hydrocarbons degrade fairly readily, with the cyclic alkanes being considerably more resistant to degradation. Most of the known pathways of degradation require the presence of oxygen. However, recent research indicates that, for

the aromatic hydrocarbons at least, oxygen may be supplied by nitrate, sulfate, or even iron oxyhydroxides. Whether anaerobic degradation supported by these alternate electron acceptors is capable of removing enough hydrocarbons to achieve acceptable levels of bioremediation in situ remains to be seen.

50. While a potential exists for petroleum hydrocarbon removal under anaerobic conditions, the most complete degradation of the constituent hydrocarbons can be obtained when sources of oxygen, necessary nutrients, and the required microorganisms are all present in the contaminated soil (Wilson et al. 1985, Aamand et al. 1989, Grbic-Galic 1990). This may not always be enough, however. In a recent study assessing the potential for in situ biotreatment of hydrocarbon-contaminated soils, Morgan and Watkinson (1990) found that while available data indicated that biotreatment of a gasoline and lubricating oil-contaminated site was viable, the addition of nutrients inhibited mineralization in the soils.

51. For these reasons, research on biotreatment of flooded soils contaminated with petroleum hydrocarbons should focus on the development of procedures to determine the best means to provide oxygen to the degrading microflora and on the assessment of limiting factors present at a given site.

52. Several environmental factors will be examined in the project. A mesotrophic temperature range (20 to 25 °C) will be used, based on the fact that the mesophilic (20 to 40 °C) levels produce the fastest degradation rates. The effect of nutrient addition and the ability of native soil microflora to degrade petroleum hydrocarbons will be determined under flooded as well as aerobic conditions at field moisture capacity. Degradation will be examined in the presence and absence of added nitrogen and phosphorus sources and with the use of nitrate as an alternate electron acceptor under flooded conditions.

53. To determine the relative importance of volatilization to the removal of total petroleum hydrocarbons in soil, mercuric chloride ($HgCl_2$) will be added to aerobic fertilized controls to inhibit the growth and activity of microorganisms.

PART III: METHODS AND MATERIALS

Soil Collection and Analysis

Collection and initial preparation

54. Soil samples were obtained from the locations described below. Upon receipt at the US Army Engineer Waterways Experiment Station (WES), all soils were stored at 4 °C until used in the studies.

55. Fort Devens soil contaminated with No. 4 fuel oil was collected by Mr. Tim Pryor of the Emergency Management Office, Fort Devens Military Reservation, Fort Devens, MA, from an area in the vicinity of surface storage tanks. The samples were mixed in 55-gal* drums and redispensed into 5-gal buckets for shipment to WES at ambient temperature. The soil was sandy, wet, and had a fuel oil odor. Upon receipt at WES, the soil was stored at 4 °C. A sample of the No. 4 fuel oil was also sent to WES. This heavy fuel oil-contaminated soil was substituted for the motor oil-contaminated soil originally proposed.

56. Soil samples contaminated with JP-5 jet fuel oil from Fallon Naval Air Station (NAS), Fallon, NV, were collected by Mr. Ron Hoeppel of the Naval Civil Engineering Laboratory, Port Hueneme, CA. These samples were taken from several locations, including leach fields and areas in the vicinity of wells. Samples were placed into individual 1-qt (0.95-ℓ) jars and shipped to WES on ice. Upon receipt, these samples were combined, mixed well, and placed into a 5-gal plastic bucket for storage.

57. A sample of WES soil obtained near the Ecosystem Research and Simulation Division Laboratory facility was amended with 700 ml of commercial auto diesel fuel to 20 ℓ of the soil. After addition of 4 ℓ of minimal salts medium (MSM), the soil was mixed several times and aerated. (MSM contains, per liter of glass-distilled water: NH_4NO_3, 0.4 g; K_2HPO_4, 0.5 g; $MgSO_4*7H_2O$, 0.2 g; and $FeCl_3*6H_2O$, 0.05 g. MSM is adjusted to pH 7.0 $\pm$ 0.2 with 10 percent HCl.) To permit a natural diesel fuel-degrading microflora to develop, the contaminated soil was incubated for 1 month out of doors at ambient temperature (approximately 15 °C). Following this, the soil was held at 4 °C until used.

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* To convert gallons (US liquid) to liters, multiply by 3.785412.

Initial soil analysis

58. Soils were analyzed for particle size composition using the method of Patrick (1958). Total organic carbon content in soil samples was determined by dry combustion (Allison 1965). Soil moisture content was determined gravimetrically following oven drying at 105 °C.

Preparation of Flooded Soil Pans and Soil Metabolism Chambers

59. Except for the anaerobic biological controls set up in soil metabolism chambers to assess volatile hydrocarbon losses from flooded soils, all flooded soil studies were carried out in 16.5-cm-wide by 25.8-cm-long by 3.5-cm-high baking pans. A total of 1,376 g of the Fort Devens soil was placed into each pan, and the pans were set up in triplicate. For Fallon NAS and WES diesel-treated soils, 700 g of each soil was used in each pan. Pans were set up according to the following treatment scheme:

- Flooded soil only (untreated flooded soil).
- Flooded soil treated with 0.07 percent $(NH_4)_2SO_4$ (flooded soil plus fertilizer).
- Flooded soil treated with 0.2 percent KNO_3 (flooded soil plus nitrate).
- Flooded soil treated with 0.2 percent KNO_3 and 0.07 percent $(NH_4)_2HPO_4$ (flooded soil plus fertilizer plus nitrate).

All soils were flooded with 1.9 cm of reverse osmosis water and sealed with Saran Wrap to minimize evaporation.

60. Soil metabolism chambers were designed to permit a slow, even flow of air across the soil surface and to collect volatiles in the air leaving the chamber (Figure 1). Soil metabolism chambers were set up according to the following treatment scheme:

- Soil only (untreated dry soil held at field water-holding capacity).
- Soil flooded with 3/4 in. (1.9 cm) of distilled water (flooded control).
- Soil treated with 0.07 percent $(NH_4)_2HPO_4$ (fertilized soil).
- Soil treated with 0.07 percent $(NH_4)_2HPO_4$ and 1.0 percent $HgCl_2$ (inhibited soil).

61. Inhibited soils were treated with fertilizer to accelerate the growth of microorganisms and their subsequent uptake of the mercuric chloride inhibitor. All soils were adjusted to field capacity with distilled water and

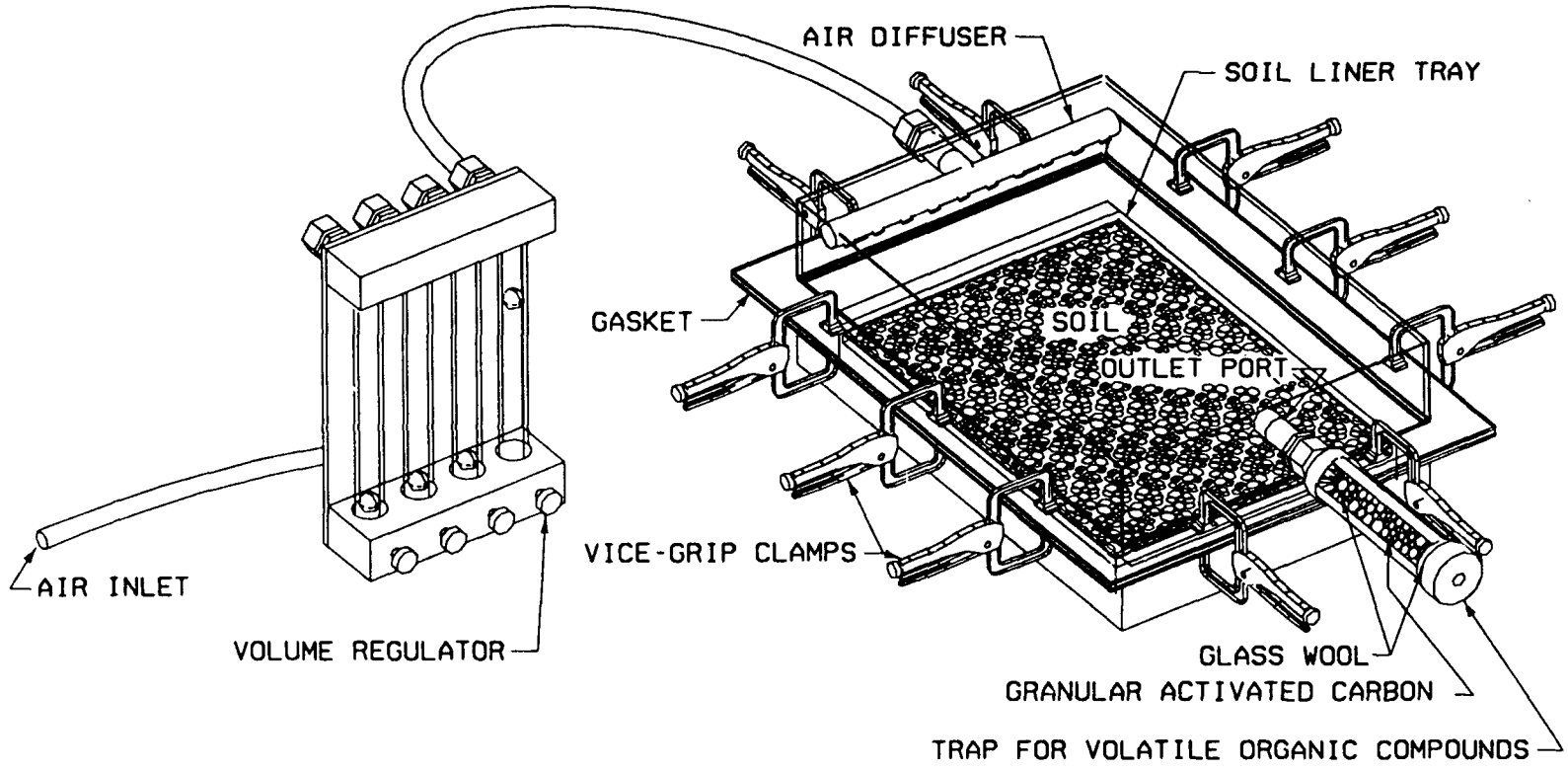

Figure 1. Soil metabolism chamber

maintained at this moisture level throughout the incubation period. Soils maintained at field water capacity are hereafter referred to as "dry" soils.

62. For Fort Devens soil, 1,897 g of soil was placed into each of three chambers. Difficulty was experienced in removing oily residues from the chamber walls upon completion of the Fort Devens study. For this reason, soils from the Fallon NAS and WES diesel-treated sites were placed into 16.5-cm-wide by 25.8-cm-long by 3.5-cm-high baking pans modified to fit inside each chamber, and 1,400-g amounts of soil were added to each pan. Chambers for these studies were set up in duplicate.

63. All chambers were equipped with granular activated carbon (GAC) traps to retain any hydrocarbons volatilized from the soil surface. Traps consisted of 50-ml all-glass syringes loaded with glass wool and GAC and held in place with a glass wool plug, as shown in Figure 1. Each syringe was loaded with 20 g GAC (12 × 30 mesh). Inflowing air was metered to each chamber through a flowmeter at a flow rate of approximately 1.0 ml/min.

64. Incubation of the chambers and pans was carried out in a controlled-temperature environment at approximately 25 °C. Flooded pans were checked weekly to determine water levels, and additional water was added, if required.

Sampling Procedure

65. Soil total petroleum hydrocarbon (soil TPH) concentration and microbial enumeration were conducted initially (time zero sampling) and then at 7, 21, and 42 days of incubation. All GAC was removed from each soil metabolism chamber trap and replaced with an equal amount of fresh GAC at 7 and 21 days; final GAC samples were taken at 6 weeks.

66. Fallon NAS and WES diesel-treated soils were incubated for 84 days. Samples for soil TPH, volatile TPH, and volatile organic hydrocarbon (VOC) were taken initially and at 12 weeks. Soil TPH samples and volatile TPH samples were also taken at 21 and 42 days.

Soil and GAC extraction and handling procedures

67. Soil TPH content was assessed using EPA Method 413.2 for Total Recoverable Oil and Grease (USEPA 1982). Twenty grams of each soil was extracted with 20 ml of freon. Freon extracts of Fort Devens soil contaminated with No. 4 fuel oil had a dark brown color, precluding soil TPH

determination with infrared analysis. To obtain an acceptable sample for analysis, these extracts were diluted serially with freon to the point of minimal coloration (1:1,000). Extracts of Fallon NAS and WES diesel-treated soils had minimal coloration upon extraction and were analyzed at full strength. Extracts were placed into sealed I-Chem vials and held at 4 °C until analyzed.

68. GAC was extracted in freon at the rate of 10 ml freon/g GAC. Extraction was accomplished by placing the GAC and freon in sealed glass centrifuge tubes and agitating the tubes on a reciprocating shaker for 30 min at 150 strokes per minute. The volatile TPH extract was placed into a sealed I-Chem vial and held at 4 °C until analyzed.

69. Soil samples for VOC analysis were placed into sealed I-Chem jars and held at 4 °C until analyzed.

Microbial enumeration

70. Microbiological enumeration was conducted by determining the number of colony-forming units developed from each dilution in the following series: (a) 10 g of soil sample in 90 ml of sterile tap water (10^{-1} dilution); (b) 10 ml of dilution (a) added to 90 of sterile tap water (10^{-2} dilution); and (c) 10 ml of dilution (b) added to 90 ml of sterile tap water (10^{-3} dilution). The process was continued until a dilution of 10^{-9} of the original soil sample was attained.

71. One milliliter of each dilution was spread onto agar plates containing one of the following media using standard microbiological techniques: peptone-tryptose-yeast extract-glucose agar (PTYG), potato dextrose agar (PDA), or MSM solidified with 1.5 percent agar (referred to as MSA) and containing 0.1 percent benzo[a]pyrene. Growth on naphthalene, a highly volatile PAH, was determined by spreading the dilution onto plates of MSA that were incubated in a sealed plastic bucket containing an open beaker of naphthalene crystals.

72. Microorganisms isolated from Fort Devens soil were also isolated on MSA overlaid with pure No. 4 fuel oil. In addition to growth on PTYG, PDA, and MSA in naphthalene fumes, microorganisms isolated from Fallon NAS soil were grown on MSA overlaid with JP-5 fuel oil, cyclohexane, or decane, instead of No. 4 fuel oil. In addition to growth on PTYG, PDA, and MSA in naphthalene fumes, microorganisms isolated from WES soil contaminated with diesel oil were grown on MSA overlaid with diesel fuel oil, cyclohexane, and decane, instead of No. 4 fuel oil.

73. All plates were incubated at room temperature for 5 days to 3 weeks, depending on the rate of growth. All plates were examined, and dilutions having an acceptable range of colonies were counted manually. Automatic enumeration of the colonies was precluded because of the variety of characteristics and sizes of colonies occurring on each medium and because several of the media had irregular and multicolored surfaces resulting from the hydrocarbon overlays.

74. PTYG agar is a rich medium supporting growth of a wide range of heterotrophic bacteria and, for this reason, was used to enumerate total heterotrophic microorganisms. While best suited for isolation of fungi, many bacteria taken from the hydrocarbon-contaminated soils also grew very well on PDA. For this reason, microorganisms enumerated on PDA were considered another form of heterotrophs, although the isolation occurred at a more acidic pH than with PTYG.

Analytical Procedures

75. Volatile organic hydrocarbons from benzene through undecane were determined following elution from the Tenax columns into a Hewlett Packard Model 5993 Gas Chromatograph-Mass Spectrometer. Alternatively, soil samples were extracted in an acetone-methyl alcohol mixture and then subjected to gas stripping, using the Hewlett Packard Model 5993 Gas Chromatograph-Mass Spectrometer. Both procedures permitted separation and identification of the lower molecular weight gasoline hydrocarbons, including ethane through dodecane.

PART IV: RESULTS AND DISCUSSION

General Physical and Chemical Properties of the Soils

76. Table 1 summarizes the physical properties and organic matter levels of each of the soils. The Fort Devens material was predominantly sand, the Fallon NAS soil a silty sand, and the WES diesel-contaminated soil a clayey silt. Organic matter content also varied from 2.54 percent in the Fallon NAS soil to 5.75 percent in the WES diesel-contaminated soil.

Degradation of Petroleum Hydrocarbons

Numbers of microorganisms

77. Examination of the changes in levels of microorganisms over the course of incubation indicated that microbial populations generally remained within one or two orders of magnitude when compared between treatments for the same soil. Complete data for all of the microorganisms examined are presented as line graphs showing all sampling points in Figures A1-A15 (see Appendix A). Growth on PTYG and on MSA containing the contaminating fuels specific for the soils examined are summarized as bar graphs in Figures 2 and 3, respectively.

78. As indicated in the methods section, PTYG is a general medium for isolation of heterotrophic microorganisms. For this reason, it is ideal for assessing the general growth pattern for microorganisms present in a soil. In Fort Devens soil, the average number of microorganisms recovered on PTYG was not different in any of the treatments, except for the inhibited treatment (Figure 2). The latter showed a slightly lower level of microorganisms, consistent with the expectation that mercuric chloride kills or inhibits the growth of microorganisms. However, a substantial amount of growth occurred in the inhibited treatment, suggesting that the level of mercuric chloride used was not great enough to suppress all microbial activity.

79. Growth of microorganisms isolated from Fallon NAS soil on PTYG agar also showed little or no difference in the average number of microorganisms recovered among the various treatments, except for the inhibited treatment. The latter showed a nearly two order of magnitude decrease. The number of organisms present in the dry fertilized treatment increased slightly over the number present in the initial soil and remaining treatments. The amount of variation was nearly the same in each of the treatments, except for that in

Table 1

Summary of Physical and Chemical Properties of Contaminated Soils

Soil	Textural Composition			Organic Matter Content, %
	% Sand	% Silt	% Clay	
Fort Devens	95.0	2.5	2.5	4.41
Fallon NAS	75.0	17.5	7.5	2.54
WES diesel-contaminated	0	80.0	20.0	5.75

the flooded fertilized soil with nitrate. The reason for the large variation in this treatment was not apparent from the data.

80. Recovery of microorganisms from the various treatments of WES diesel-contaminated soil showed a different pattern from the other two soils. The level of microorganisms present in the soil prior to incubation was the same as the levels found in the dry untreated and dry fertilized treatments; however, the means for the flooded treatments were approximately an order of magnitude less than the initial soil level. The flooded fertilized soil with nitrate again had the largest variation, and the inhibited soil had the lowest level of microorganisms, although not significantly less than any of the flooded soil treatments.

81. As evident from Figure 3, recovery of microorganisms on MSA containing the fuel substance specific for the contaminated soil of origin showed patterns very similar to those obtained on PTYG. This verified what was expected--that the majority of microorganisms able to survive and grow on MSA containing specific hydrocarbons should be able to grow on PTYG also.

82. Microorganisms that could utilize all of the tested hydrocarbons were found in each of the soils. Based on this, suitable microorganisms were present for biotreatment. However, the lack of stimulation of microbial growth in the treated soils was surprising, since the numbers of microorganisms recovered from the treated soils on PTYG and on MSA plus fuel were expected to increase. This suggests that either the levels of the fertilizer and other amendments were too low to provide stimulation or some other factor limited growth. These possibilities are explored in detail in the section on factors affecting degradation.

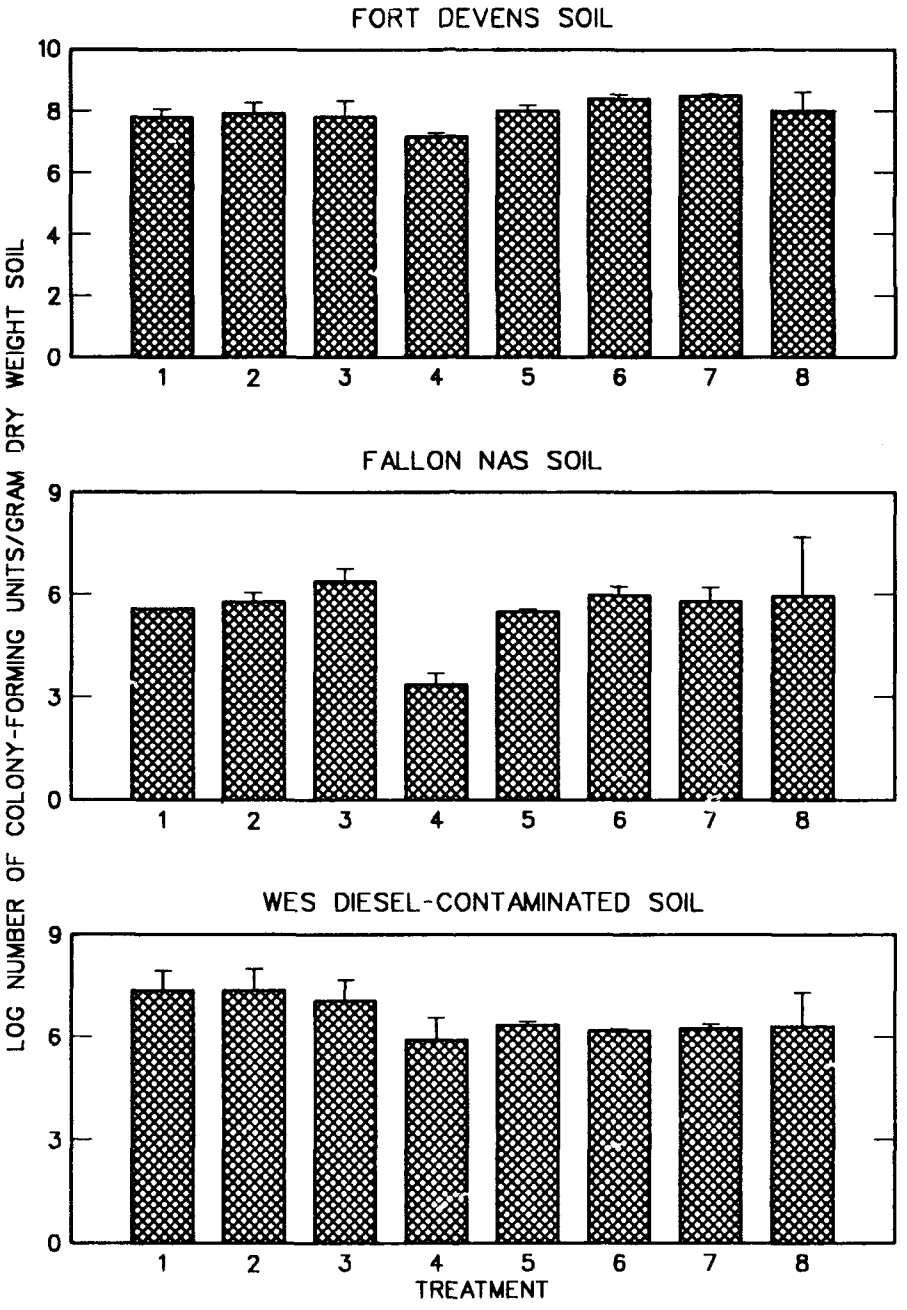

Figure 2. Mean numbers of microorganisms recovered on PTYG agar during incubation

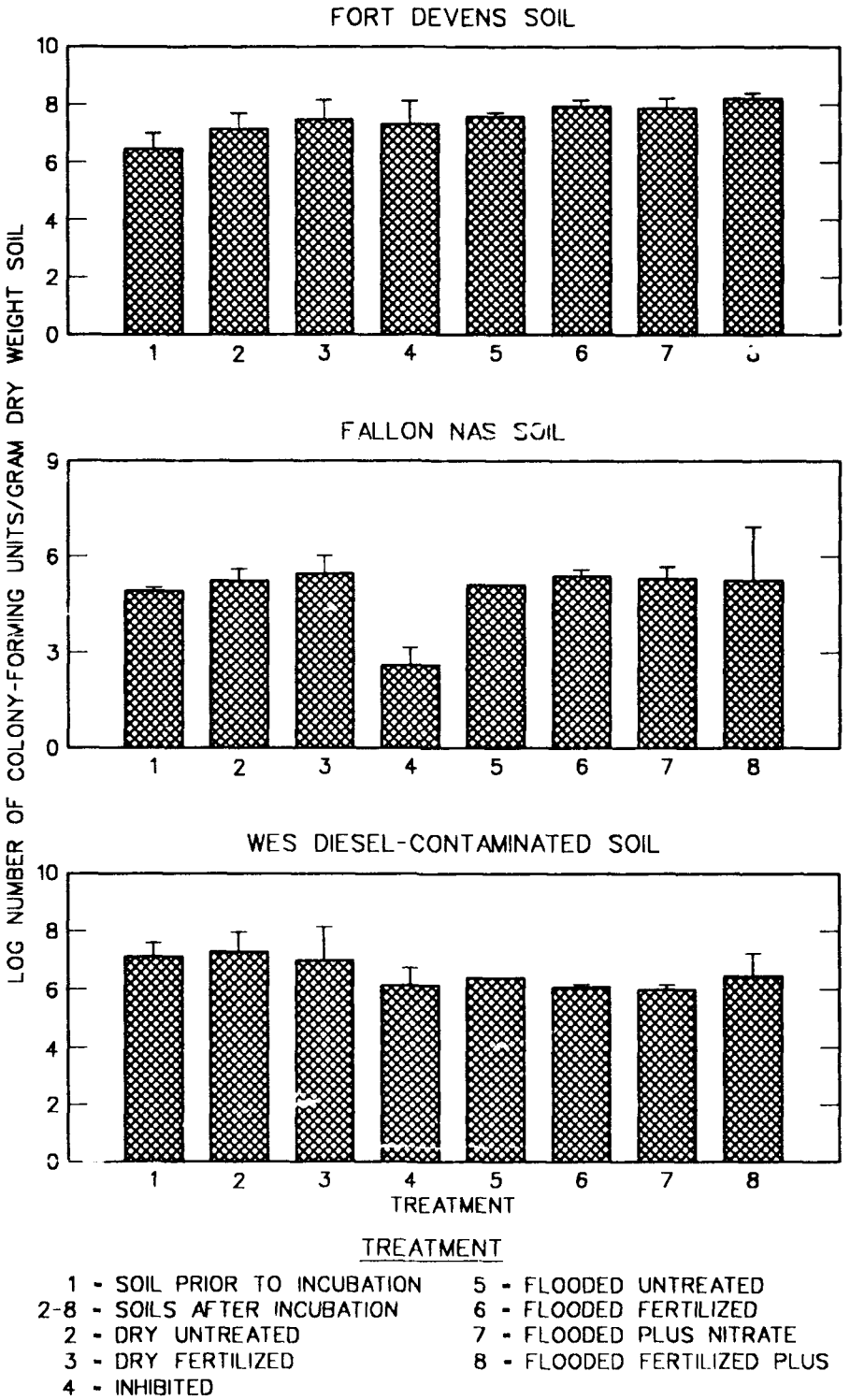

Figure 3. Mean numbers of microorganisms recovered on MSA containing petroleum hydrocarbons specific for source of contamination

Soil TPH concentration

83. Figure 4 presents a summary comparison of initial TPH levels prior to and after incubation of each of the treatments for the three soils. Figures A16-A18 (Appendix A) present graphs of the data for each soil over the incubation period.

84. No removal of TPH in any of the Fort Devens soil treatments was observed. By contrast, each of the treatments demonstrated an increase in TPH levels over the original sample. All treatments exhibited the same effectiveness in Fort Devens soil. These findings may be a result of the character of No. 4 fuel oil and the site history. No. 4 fuel oil is a very heavy oil, made up predominantly of high molecular weight aromatic compounds that are typically hard to degrade. This situation is complicated by the fact that the spill has been in place for several years, and many of the lighter molecular weight components have had the opportunity to be lost through natural degradation, leaching, or volatilization. In addition, the level of fuel oil contamination in Fort Devens soil is very high, an order of magnitude greater than the freshly diesel-contaminated WES soil and three orders of magnitude greater than the Fallon NAS soil. Great removal would have to occur for treatment effectiveness to be observed in this soil.

85. Finally, the remaining fuel oil constituents have had a long period to sorb to the soil, making microbial access to them difficult. The soil sample that was analyzed before incubation was taken as the test chambers and pans were being loaded, without any further agitation or mixing. All of the postincubation treatments, including the untreated soils, had been subjected to mixing and an increased moisture content. There is a possibility that these factors, perhaps in combination with surfactant production by the soil microflora stimulated by improved moisture and mixing, may have increased the extractability of TPHs from Fort Devens soil during the incubation period. This may explain why higher TPH levels were obtained at the end of incubation.

86. The three dry treatments yielded removals of TPHs from the Fallon NAS soil. The dry treatments showed depression of TPH levels to one sixth (dry fertilized) and one third (dry untreated and inhibited) the original value. By contrast, all of the flooded treatments had TPH levels that did not significantly differ from the initial TPH concentration. This indicated that, for the Fallon NAS soil, the flooded treatment did not result in removal of TPHs.

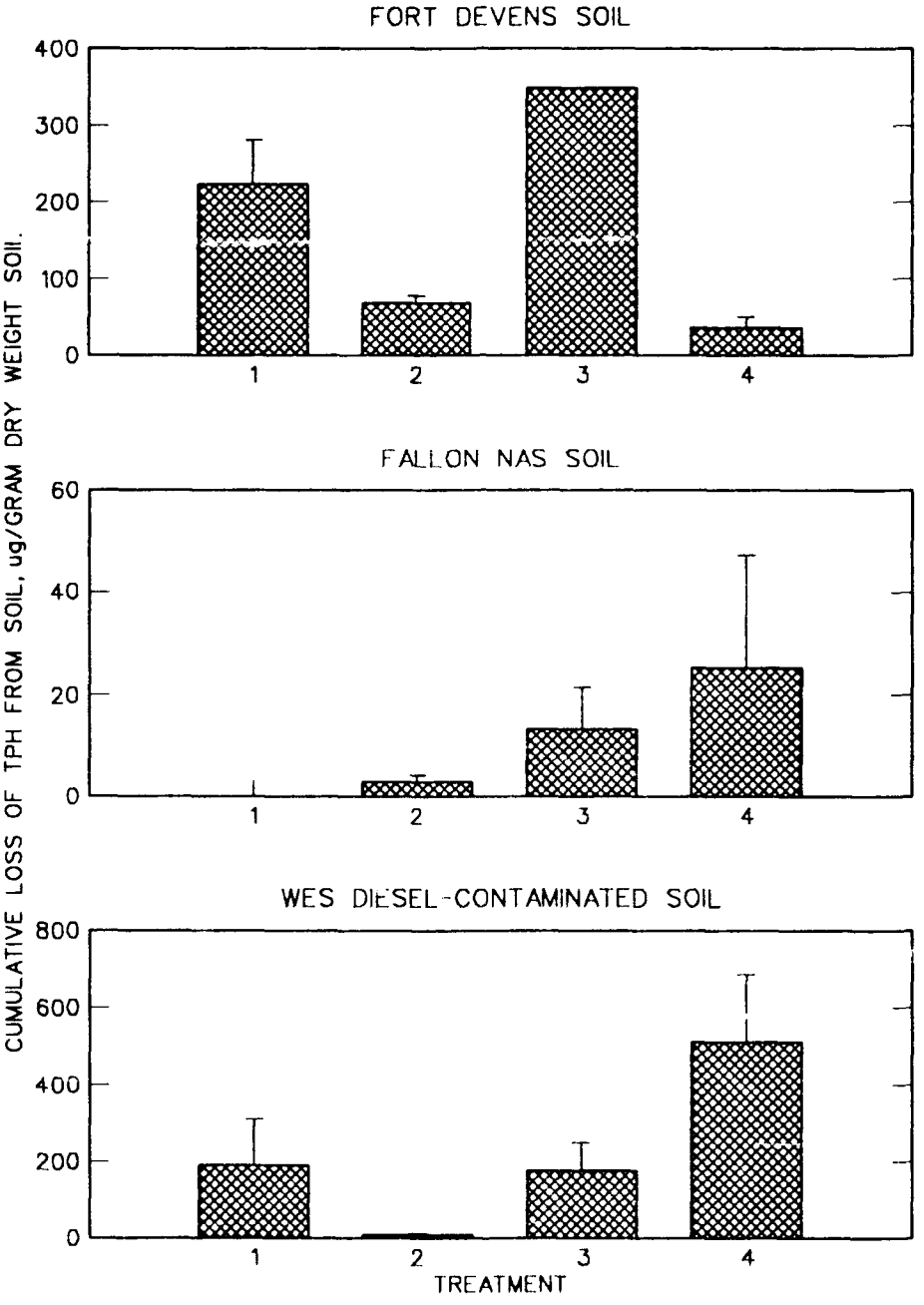

Figure 4. Comparison of total petroleum hydrocarbon levels in soils before and after incubation

87. The WES diesel-contaminated soil also had a varied pattern in TPH levels remaining after treatment. The dry untreated soil (treatment 2) and flooded untreated soil (treatment 5) showed approximately the same or slightly greater TPH levels as before incubation. The standard error associated with each of these two untreated soils was large enough to hide any differences between these and the two remaining dry soil treatments (3 and 4). The reasons for this variation are not clear, but may indicate that manipulation influenced TPH extractability or did not consistently enhance removal. The dry fertilized (3), inhibited (4), flooded fertilized (6), flooded plus nitrate (7), and flooded fertilized plus nitrate (8) treatments all reduced TPH levels below that present in the soil before incubation (1). Both the dry fertilized (3) and inhibited dry (4) treatments reduced TPH levels approximately one third. The flooded treatments (6-8) appeared to be the most effective, each reducing TPH levels to less than half of that originally present.

88. The reason for the apparent effectiveness of the three flooded treatments is not apparent. The flooded treatment with fertilizer only did not have an added alternate electron acceptor (AEA). However, the levels of AEAs such as nitrate, sulfate, and oxidized iron and manganese were not determined in this study, and one or more of these may have been high enough to support anaerobic degradation.

89. Results for these three soils indicate that TPH removal is treatment specific. Removal is also hydrocarbon specific and may be soil specific, but it is not possible to determine which of these two factors is predominant.

Volatile petroleum hydrocarbon losses

90. Cumulative TPH losses. Cumulative volatile TPH losses from the soils could be assessed only for those treatments incubated in the soil metabolism chambers. For this reason, data are available only for the dry untreated, dry fertilized, inhibited, and untreated flooded treatments (Figure 5).

91. Volatile TPH losses from Fort Devens soil were greatest in the inhibited soil. The dry untreated (1), dry fertilized (2), and untreated flooded (4) soils lost approximately two thirds, one fifth, and one eighth of the TPHs of the inhibited soil, respectively. The inhibited soil (3) was expected to lose the most volatiles, since the utilization of volatile TPHs by microorganisms was minimized, while volatilization into the overlying water layer was not a factor. The flooded soil (4) was expected to lose the least volatiles, if the majority of the hydrocarbons present are of high molecular

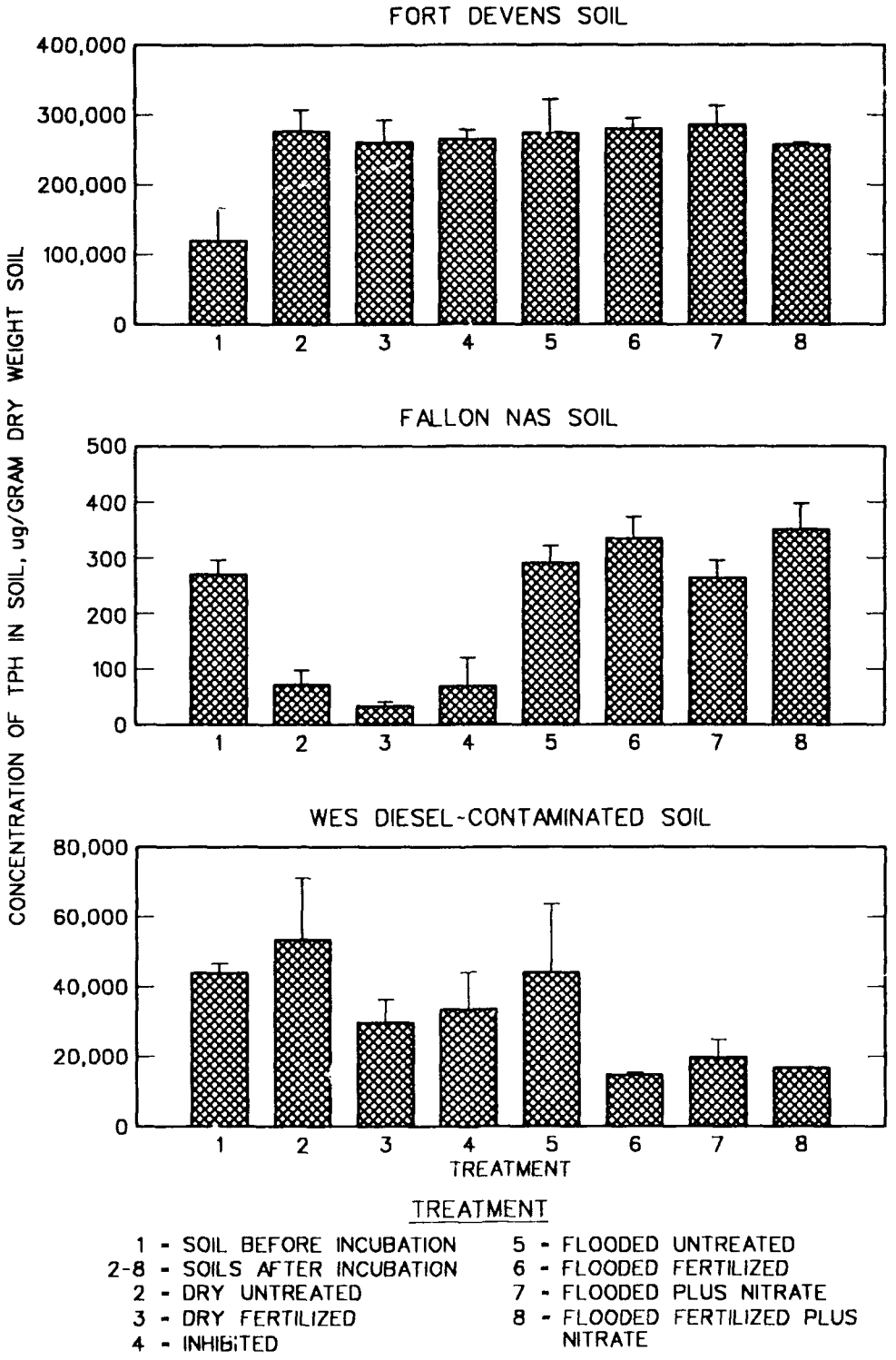

Figure 5. Comparison of cumulative volatile total petroleum hydrocarbon losses during incubation

weight and very poorly soluble. The dry untreated soil (1) should lose less than the inhibited soil (3), because some of the volatiles are being utilized, but not as much as in the fertilized soil (2) where the active consumption of volatiles is stimulated. While this order of volatile loss follows a pattern predicted by treatment, the magnitude of loss is, at most, three orders of magnitude below the TPH level present in the soil.

92. Losses of volatile TPHs from Fallon NAS soil did not follow the order observed for Fort Devens soil. The Fallon soil lost a significantly higher percentage of the amount present. For the untreated flooded soil, the loss was roughly 10 percent of the original TPH level. The Fallon NAS soil contained JP-5, a jet fuel that has a substantial complement of lower molecular weight aromatic hydrocarbons; these are somewhat more soluble in water than the hydrocarbons in No. 4 fuel oil. The flooded untreated soil (4) has a high degree of variability, i.e., the standard error was nearly the same as the mean value. As in the Fort Devens soil, the Fallon NAS soil inhibited treatment (3) again lost more TPH than did the dry untreated and dry fertilized treatments.

93. The flooded untreated soil released the highest level of TPHs among treatments for the WES diesel-contaminated soil. This soil, which was the most recently contaminated of all three soils, undoubtedly contained a large amount of volatiles. However, the maximum level of cumulative volatiles trapped (treatment 4) was only approximately 1 percent of the volatile TPH levels present in the soil before incubation (based on the preincubation levels present in this soil) (Treatment 1 of WES diesel-contaminated soil in Figure 4). No difference was observed between the dry untreated (1) and the inhibited (3) treatments, while the dry fertilized treatment lost no volatile TPHs, which is a pattern expected if the microflora in the fertilized soil were consuming most of the volatile TPHs.

94. Changes in volatile organic hydrocarbon composition. Volatile organic hydrocarbon losses were assessed only for the Fallon NAS and the WES diesel-contaminated soils. Because of the generally lower molecular weight range of readily volatile alkanes and aromatic hydrocarbons in JP-5 and auto diesel fuel, higher losses of VOCs were anticipated for these soils than for the Fort Devens soil. Results of the VOC analyses of Fallon NAS soil prior to and following incubation are shown in Table 2. With the exception of undecane, the levels of all VOCs in the dry soils decreased. Undecane accumulated in all soils relative to the initial levels. Except for hexane, the

Table 2

Volatile Organic Hydrocarbons (µg/g Dry Weight of Soil)in Fallon Naval Air Station Soil Initially and After 84 Days of Incubation

Compound	Initial Value	Dry Soil			Saturated Soil			
		Untreated Soil	Soil + Fertilizer	Inhibited Soil	Untreated Soil	Soil + Fertilizer	Soil + Nitrate	Soil + Fertilizer + Nitrate
Hexane	0.300 ±0.110	0.023 ±0.017	0.034 ±0.002	0.028 ±0.008	0.180 ±0.000	0.490 ±0.040	0.325 ±0.045	0.300 ±0.080
Octane	0	0	0	0	0.445 ±0.265	0.650 ±0.120	0.400 ±0.090	0.390 ±0.010
Nonane	0.023 ±0.011	0	0	0	27.9 ±27.7	69.1 ±3.70	40.2 ±13.2	22.6 ±4.66
Decane	0.101 ±0.019	0.018 ±0.010	0.013 ±0.001	0.023 ±0.019	0.650 ±0.060	0.650 ±0.120	0.400 ±0.090	0.390 ±0.010
Undecane	1.22 ±0.280	3.50 ±1.62	1,014 ±1,013	3.86 ±3.68	39.4 ±16.2	69.1 ±3.70	40.2 ±13.2	22.6 ±4.55

levels of each VOC in each of the flooded soils increased with respect to the initial value. This was particularly evident in the case of nonane, where a 1,000-fold increase in the unamended saturated soil occurred.

95. The reasons for these increases were not apparent from the data, but accumulation of lower molecular weight hydrocarbons may represent products released from degradation of higher molecular weight compounds. Ridgeway et al. (1988) have observed that addition of salts (i.e., as fertilizers, nitrate, mercuric chloride) can also cause release of these compounds from the soil, enhancing their recovery during analysis.

96. Removal of all compounds in the dry untreated and fertilized treatments of the WES diesel-contaminated soil was substantial (Table 3). All compounds but undecane were completely removed from the untreated soil, and only 23.9 percent of the latter substance remained at 84 days. All compounds but decane and undecane were lost from the fertilized soil, and only 4.6 percent of the decane and 16.4 percent of the undecane remained at 84 days. Several compounds were left after 84 days in the inhibited soil, but levels of each of these were diminished from those originally present. Analyses of the inhibited soil for microbial growth, as discussed above, indicated that microorganisms were present in the inhibited soils during the incubation period. Therefore, removal of VOCs from the inhibited soil is not surprising.

97. Substantial amounts of all compounds but undecane were removed from most of the treatments of the flooded WES diesel-contaminated soil. Exceptions included hexane in the fertilized treatment and nonane in the nitrate and fertilizer plus nitrate treatments. The effectiveness of removal varied extensively between treatments and compounds. T-xylene decreased substantially in the unamended soil, and was completely removed from each of the other treatments. The levels of hexane present in the initial and untreated soils were not different. Hexane in the fertilized soil was greater than in any of the remaining treatments and the initial sample. By contrast, hexane in the remaining flooded soil treatments was roughly one half the initial value. Heptane remaining after 84 days of incubation varied from 19.6 to 32 percent, with the fertilized soil losing the greatest amount of this compound.

98. Soil treated with fertilizer only lost the most heptane. Nonane levels decreased by 26.3 and 33.6 percent in the untreated flooded soil and flooded soil amended with fertilizer, respectively, but increases of 307 and 487 percent were observed in the soils amended with nitrate only and with

Table 3

Volatile Organic Hydrocarbons (µg/g Dry Weight of Soil) in WES Diesel-Contaminated Soil Initially and After 84 Days of Incubation

		Dry Soil			Saturated Soil			
Compound	Initial Value	Untreated Soil	Soil + Fertilizer	Inhibited Soil	Untreated Soil	Soil + Fertilizer	Soil + Nitrate	Soil + Fertilizer + Nitrate
T-xylene	85.9 ±7.90	0	0	0	3.30 ±0.617	0	0	0
Hexane	16.8 ±2.81	0	0	15.9 ±8.10	12.7 ±7.00	27.6 ±0.500	8.40 ±0.900	9.2 ±0.100
Heptane	134 ±5.60	0	0	0	26.2 ±14.2	29.0 ±6.15	40.2 ±3.75	43.0 ±5.55
Octane	252 ±28.0	0	0	4.80 ±1.8	80.4 ±43.8	59.2 ±8.45	51.0 ±1.75	52.2 ±1.65
Nonane	121 ±0.700	0	0	4.45 ±1.45	31.8 ±14.7	40.7 ±1.50	372 ±10.0	589 ±35.0
Decane	118 ±2.84	0	5.40 ±2.65	11.8 ±6.75	48.4 ±19.8	96.1 ±20.9	51.0 ±1.75	52.2 ±1.65
Undecane	173 ±16.8	41.4 ±1.41	28.4 ±1.41	142 ±6.00	862 ±267	1,820 ±660	373 ±9.0	589 ±35.0

nitrate plus fertilizer. Decane fell to between 41.0 and 44.2 percent of the original level in all but the fertilizer-amended soil, where 81.4 percent remained at 84 days. The concentration of undecane increased in each of the treatments, with the amounts accumulated ranging from 216 percent in the nitrate amendment to well over 1,000 percent in the fertilizer-amended soil. Possible factors responsible for these increases are the same as those discussed for similar results in the Fallon NAS soil.

Losses due to
degradation and volatilization

99. Losses due to degradation and volatilization were computed for those soils used in the soil metabolism chambers, where volatiles were trapped (Table 4). Based on the results of these computations, degradation was obtained in dry untreated, dry fertilized, and inhibited Fallon NAS soils and in the dry fertilized and inhibited WES diesel-contaminated soils. Expressed in terms of percent losses due to degradation, the Fallon NAS fertilized dry soil treatment was most effective, followed by the untreated dry soil and inhibited dry soil treatments for this soil. Degradation effectiveness for the WES diesel-contaminated soils was much lower, but the fertilized dry soil treatment was again the most effective, followed by the inhibited soil treatment.

100. Based on the literature and the work presented here, diesel fuel and JP-5 fuel oil were removed to some extent through microbial activity, while No. 4 fuel oil degraded little, if at all, within the time frame used in this study.

Environmental Factors Controlling Degradation

101. Based on the results of this study and the literature review, it appears that the most important factors regulating the rate and extent of degradation are the nature, concentration, and physical state of the hydrocarbons present; temperature; oxygen and moisture status, nutrients; and pH. Under the conditions examined here, the predominant factors were the nature of the contaminated soil and the fuel associated with it, the presence or absence of nutrients, moisture status (i.e., saturated or held at field moisture holding capacity), and oxygen. All native soil pH values were within the desirable range of 5 to 8.

Table 4

Computation of Losses Due to Degradation for Fallon NAS and WES Diesel-Contaminated Soils Based on Changes in TPH Levels*

Condition	Concentration of Total Petroleum Hydrocarbon, µg/g Soil			
	Initial Soil Concentration	Final Soil Concentration	Total Loss to Volatilization	Loss Due to Degradation
Fallon NAS Soil				
Untreated dry soil	270	78.1	0	191.9 (71.1%)
Fertilized dry soil	270	33.4	13.3	223.3 (82.7%)
Inhibited soil	270	78.2	25.2	166.6 (61.7%)
Untreated saturated soil	270	284	2.71	No loss
WES Diesel-Contaminated Soil				
Untreated dry soil	43,781	53,413	191	No loss
Fertilized dry soil	43,781	30,092	177	13,512 (30.9%)
Inhibited soil	43,781	33,412	509	9,860 (22.5%)
Untreated saturated Soil	43,781	44,444	5.57	No loss

* Based on the equation: Loss due to degradation = Original concentration - Final concentration - Loss due to volatilization. Losses due to degradation are assumed, since other mechanisms for loss cannot be accounted for. Values in parentheses are losses due to degradation expressed as percent of original. "No loss" indicates total recoveries from soil at end of treatment and/or volatile traps exceeded original levels in soil. Computations were made on mean values only.

Nature of the contaminating fuel

102. Use of naturally contaminated soils such as the Fort Devens and Fallon NAS soils carries with it two disadvantages. First, environmental samples inherently have a high degree of variability, creating difficulties in demonstrating effective treatment. Second, obtaining samples from the field having different types of petroleum hydrocarbon contamination on soils of identical composition is impossible. As was the case in the present study, the soil is a source of variation along with the different types of fuel examined. On the other hand, when a soil is freshly contaminated with different kinds of fuel, as was done here with the WES diesel-contaminated soil, the investigator does not normally have the luxury of waiting several months to a few years for the contaminating fuel to weather and for long-term incorporation of the fuel into the soil. However, this type of incorporation is believed to make microbial access to the bound hydrocarbon much more difficult, resulting in decreased degradation compared to that occurring with hydrocarbons that have not been incorporated into soil. Thus, contamination of soils in the laboratory does not necessarily reflect characteristics of the field.

103. In spite of this, the differences between the degradation obtained in the present study were similar to those obtained by Song, Wang, and Bartha (1990), who used freshly contaminated soil. The results obtained by these investigators show most effective degradation for jet fuel, less effective for diesel oil, and least effective for bunker C. The bunker C that was used is considered closer to No. 4 fuel oil, which contaminated Fort Devens soil, than to No. 2 fuel oil, which Song, Wang, and Bartha (1990) also used. The jet fuel and the diesel oil responded well to bioremediation treatment. By contrast, 80 percent of the bunker C persisted a year after initiating treatment.

Effect of nutrient addition

104. Much information is available demonstrating the positive effects of nutrient addition on biodegradation. Petroleum hydrocarbons, while rich in carbon, are extremely poor in nitrogen and phosphorus. In the studies conducted here, addition of a fertilizer containing sources of nitrogen and phosphorus enhanced the treatment losses of TPHs. In addition, substantial losses were also obtained in the inhibited soils where fertilizer was added to stimulate microbial growth, so that uptake of mercuric chloride by the soil microflora would be enhanced, thereby increasing its toxicity to the soil microflora. Evidently, the increased numbers of microorganisms resulting from

fertilization outstripped the losses due to poisoning, resulting in some positive treatment effects. The present investigation did not include studies to assess the optimum level of fertilization; whether the amount of degradation observed here was low or high is not known.

Moisture status

105. The removal of TPHs from the Fallon NAS soil appeared to be affected more by the amount of water present than by any of the remaining factors. No significant removal of soil TPHs was observed from any of the amendments used in the flooded Fallon NAS soil. By contrast, except for hexane, major accumulations of all VOCs were measured in flooded Fallon NAS soils. The causes of this are not apparent from the data, but may be related to the observation by Ridgeway et al. (1988) about the effects of salts on leaching of fuel components, as discussed above.

106. The effect of water on petroleum hydrocarbon removal from Fallon NAS soil was also apparent in the results of volatile TPH analysis, where the untreated flooded soil released less material than the inhibited dry soil. By contrast, the unamended and the fertilized dry soils released at most only very low levels of volatile TPHs.

107. In agreement with the results for the Fallon NAS soil, significant losses of soil TPHs from the WES diesel-contaminated soil occurred in the fertilized dry soil and in the inhibited soil. Based on the observation of the TPH levels in the flooded soils, substantial TPH losses also occurred from the saturated soil treated with fertilizer, with nitrate, and with fertilizer plus nitrate. Monitoring volatile TPH losses from all of the flooded soils was not possible, so the overall losses due to treatment could not be assessed.

108. Despite the lack of significant decreases in soil TPHs in the untreated dry and the inhibited dry WES soil, substantial to moderate removals of soil VOCs from these soils were observed. Also, in spite of significant losses of soil TPHs from the flooded soils amended with fertilizer, nitrate, and fertilizer plus nitrate, only low removals of soil VOCs were obtained from these soils. This evidence further suggests that, while active degradation occurred in this soil, accurate quantification of losses would be very difficult.

Effect of oxygen

109. The most effective removal of petroleum hydrocarbons occurs under aerobic conditions, as attested to by the fact that these substances have

accumulated under anaerobic conditions in nature. Arthur et al. (1988) have observed that oxygen is the limiting factor establishing the active removal of jet fuel during in situ treatment. Others have observed that oxygen transport through aquifers is difficult to achieve (Raymond, Jamison, and Hudson 1976; Thompson and Ward 1989). Oxygen supply may have been limiting for the aerobic treatments. Soils in these treatments were mixed initially, and a continuous stream of air was moved over the soil surface. However, the soils were not mixed during the treatment period and, for all treatments, oxygen could have been readily consumed below the first few millimeters of soil surface. A lack of oxygen could explain the lack of substantial increases in microbial growth observed in these treatments, which could act to decrease degradation.

110. Many investigators have found nitrate-supported anaerobic degradation of petroleum hydrocarbons. To make up for the probable lack of oxygen in the flooded soils, nitrate was added as an alternate electron acceptor to two of the four treatment combinations. Addition of nitrate did not stimulate degradation in the Fort Devens and Fallon NAS flooded soils and did not appear more effective than addition of fertilizer alone in the WES diesel-contaminated soil. Nonetheless, as indicated above, the levels of AEAs in these soils were not determined, and substantial amounts may have been present. Moreover, aerobic conditions present during initial flooding in conjunction with ammonium present in the fertilizer would have allowed for nitrification of the ammonium to supply nitrate in the WES soil containing fertilizer, but lacking nitrate. This may explain the removal of TPHs and VOCs observed in the flooded WES diesel-contaminated soils receiving fertilizer and/or nitrate and the lack of treatment in the untreated flooded soil.

PART V: SUMMARY AND CONCLUSIONS

111. The present work demonstrated that native soil microorganisms were able to bring about moderate to substantial removal of petroleum hydrocarbons from soils contaminated with JP-5 and auto diesel fuel. Some removal was noted upon addition of water only, but addition of water to the point of flooding did not necessarily stimulate degradation. Fertilization was noticeably effective in stimulating degradation under dry (unflooded) conditions for both Fallon NAS and WES diesel-contaminated soils. Fertilization was also sometimes important where biotreatment was effective under anaerobic conditions, but some of the biotreatment under anaerobic conditions may have resulted from initial oxidation of ammonium in the fertilizer to supply nitrate as an alternate electron acceptor. Biotreatment did not remove detectable levels of petroleum hydrocarbons from a No. 4 fuel oil-contaminated soil, but the literature suggests that incubation of this type of material well beyond the time frame used in the present investigation may be required.

112. Based on the results of this work, use of native soil microflora to degrade diesel fuel, fuel oil, and motor oils within the soil matrix is feasible. Those environmental factors most important in controlling the rate and extent of degradation are moisture, nutrients, and oxygen, and, under anaerobic conditions, nitrate. Procedures to optimize treatment should focus on methods to determine the exact amounts of fertilizer and nitrate to add, and the rate at which addition should occur. Also, mechanical stirring of soil during aerobic treatment may be necessary to overcome the formation of oxygen gradients.

113. Finally, because growth of microorganisms is only slightly inhibited by 1 percent $HgCl_2$, the concentration of $HgCl_2$ used to inhibit microbial activity should be raised to a minimum of 5 percent in subsequent studies.

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APPENDIX A: CHANGES IN LEVELS OF MICROORGANISMS AND TOTAL PETROLEUM HYDROCARBONS

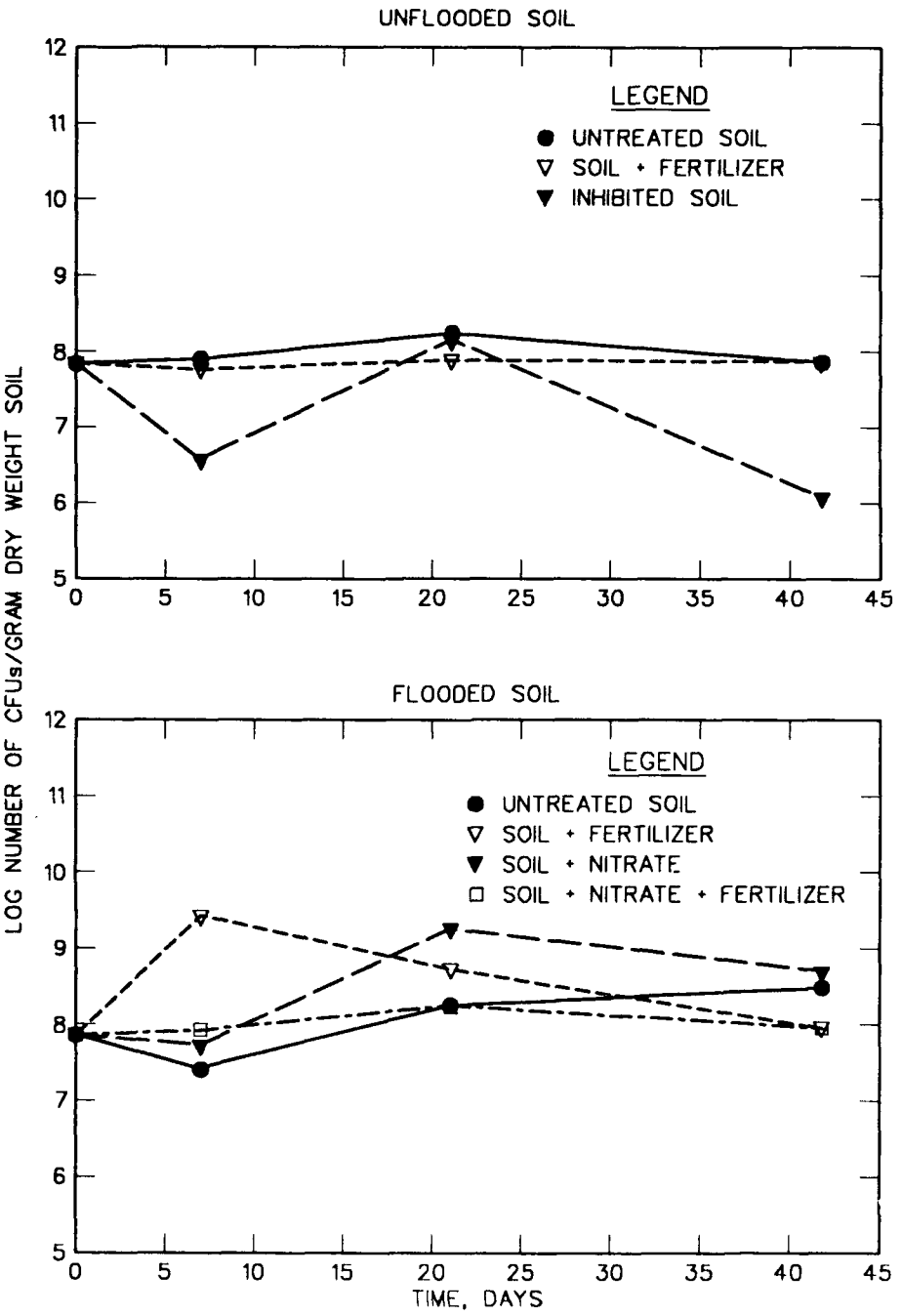

Figure A1. Levels of total heterotrophic microorganisms recovered on PTYG from Fort Devens soil

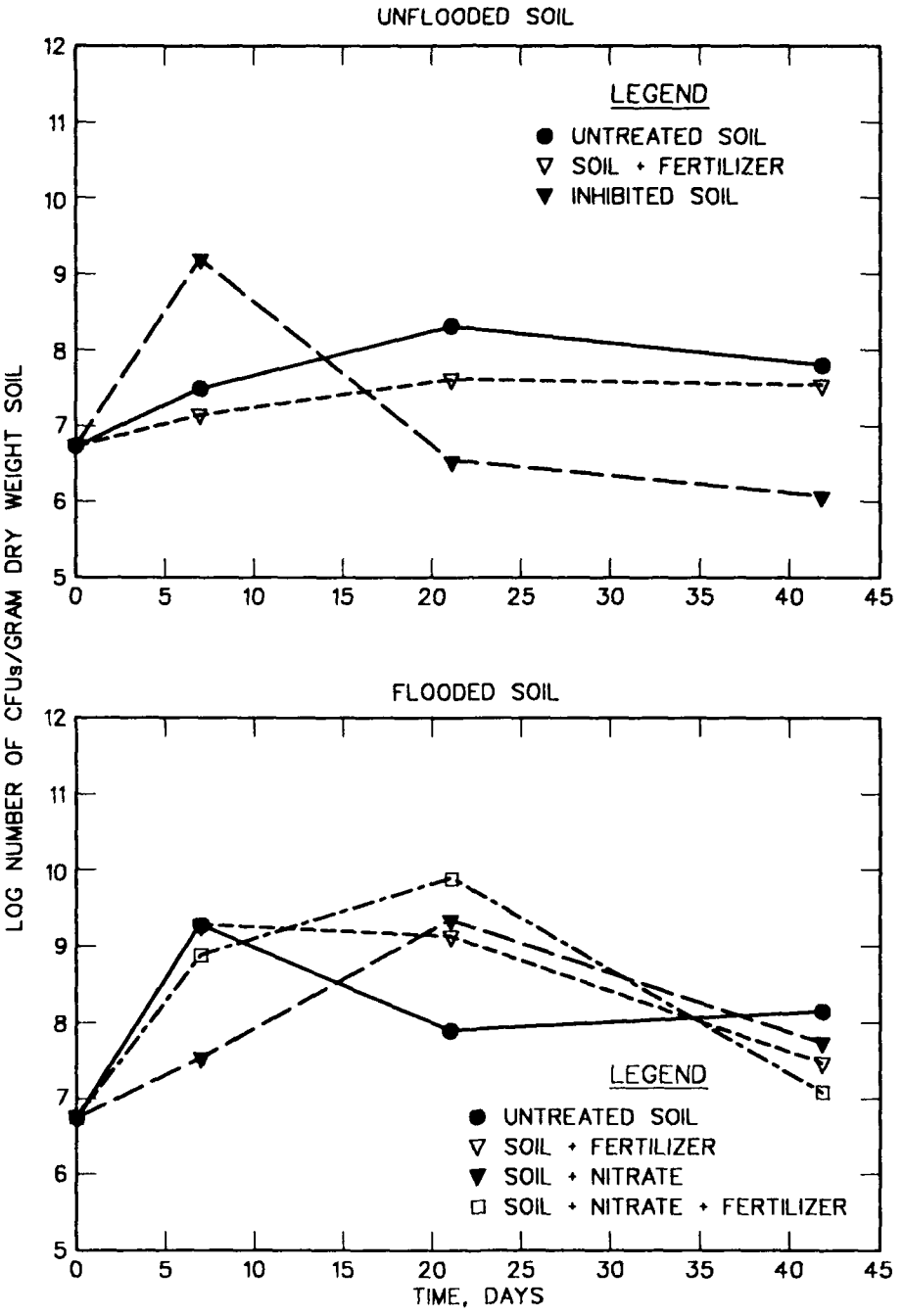

Figure A2. Levels of total heterotrophic microorganisms recovered on PDA from Fort Devens soil

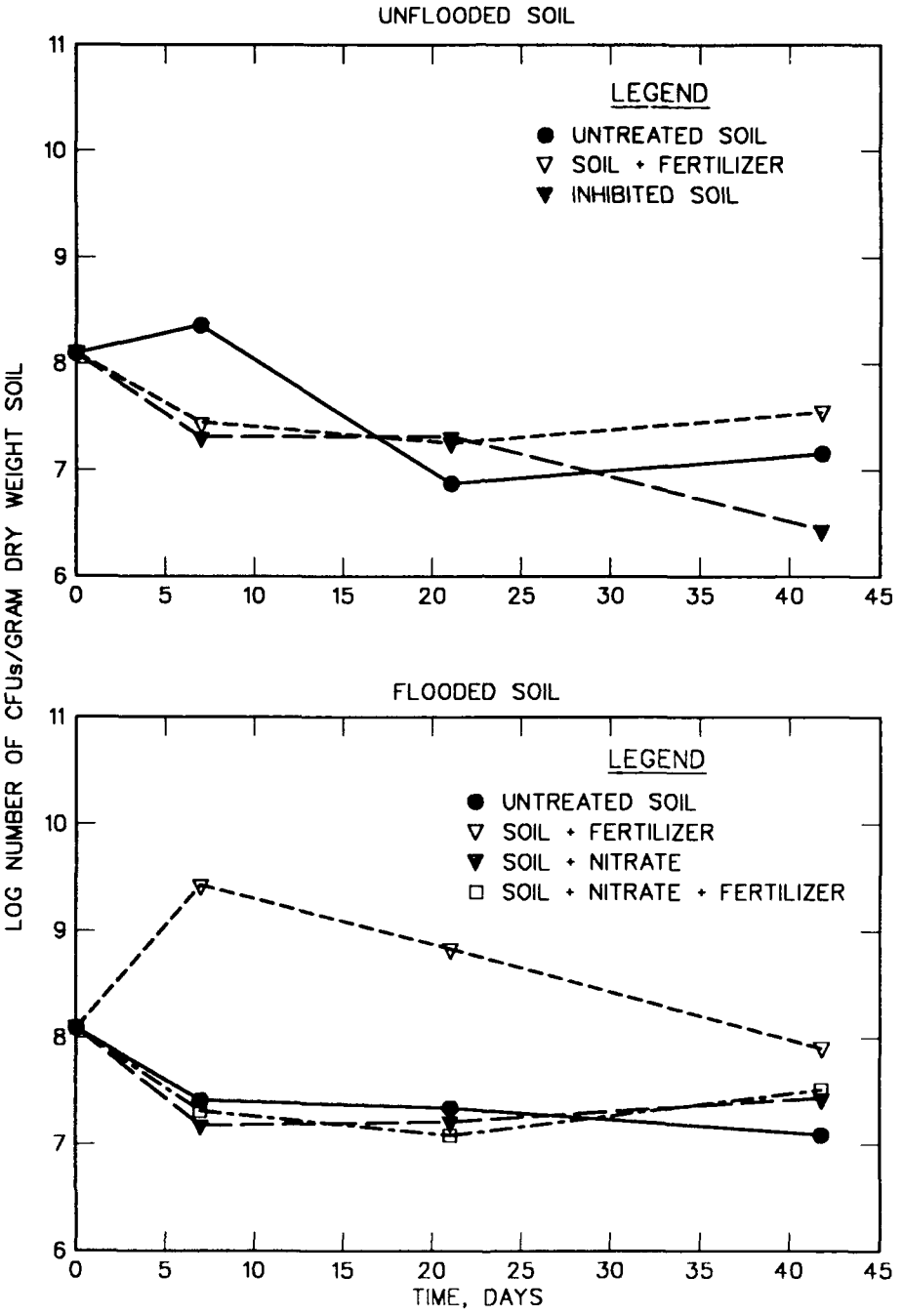

Figure A3. Levels of benzo[a]pyrene-degrading microorganisms recovered from Fort Devens soil

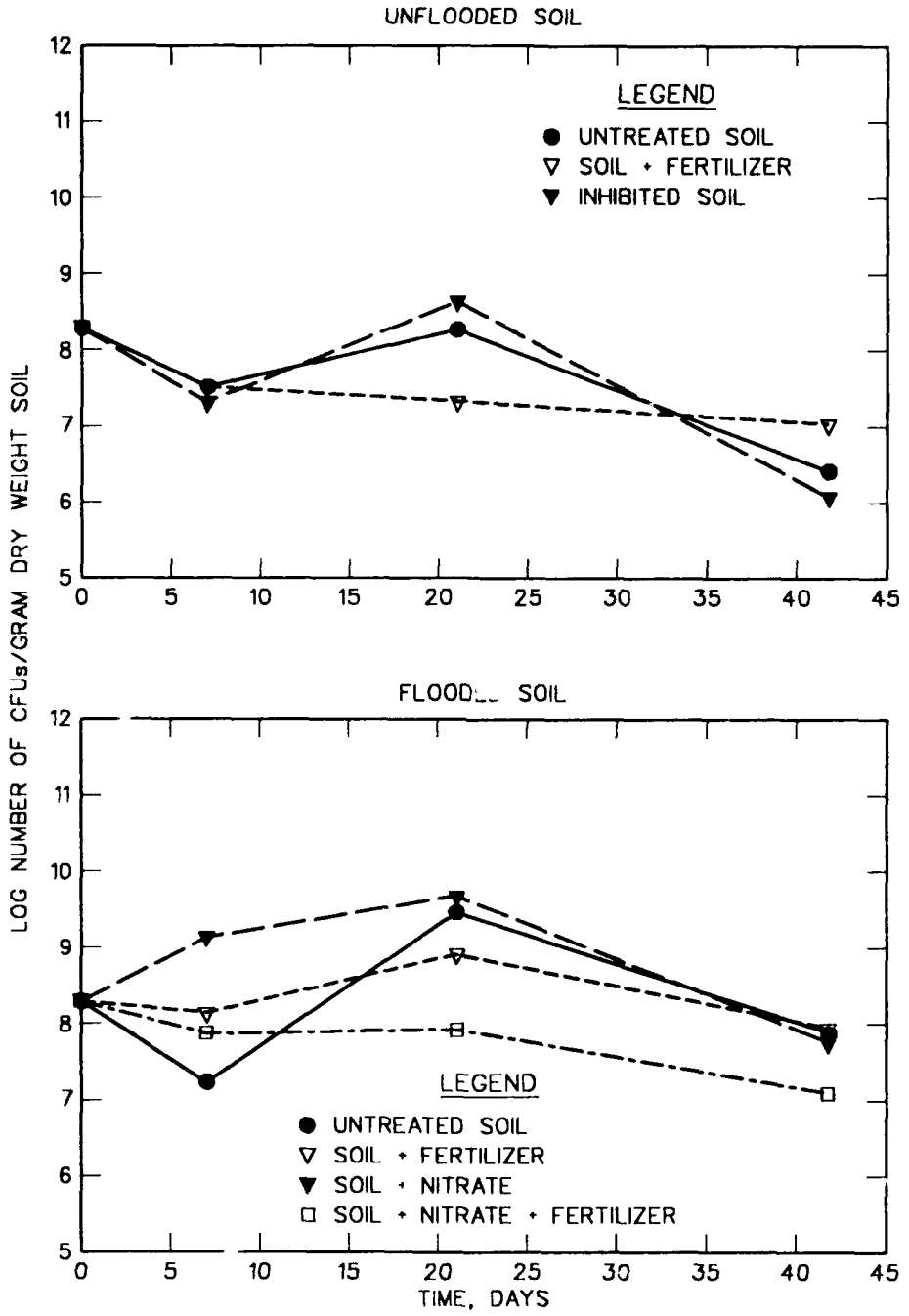

Figure A4. Levels of naphthalene-degrading microorganisms recovered from Fort Devens soil

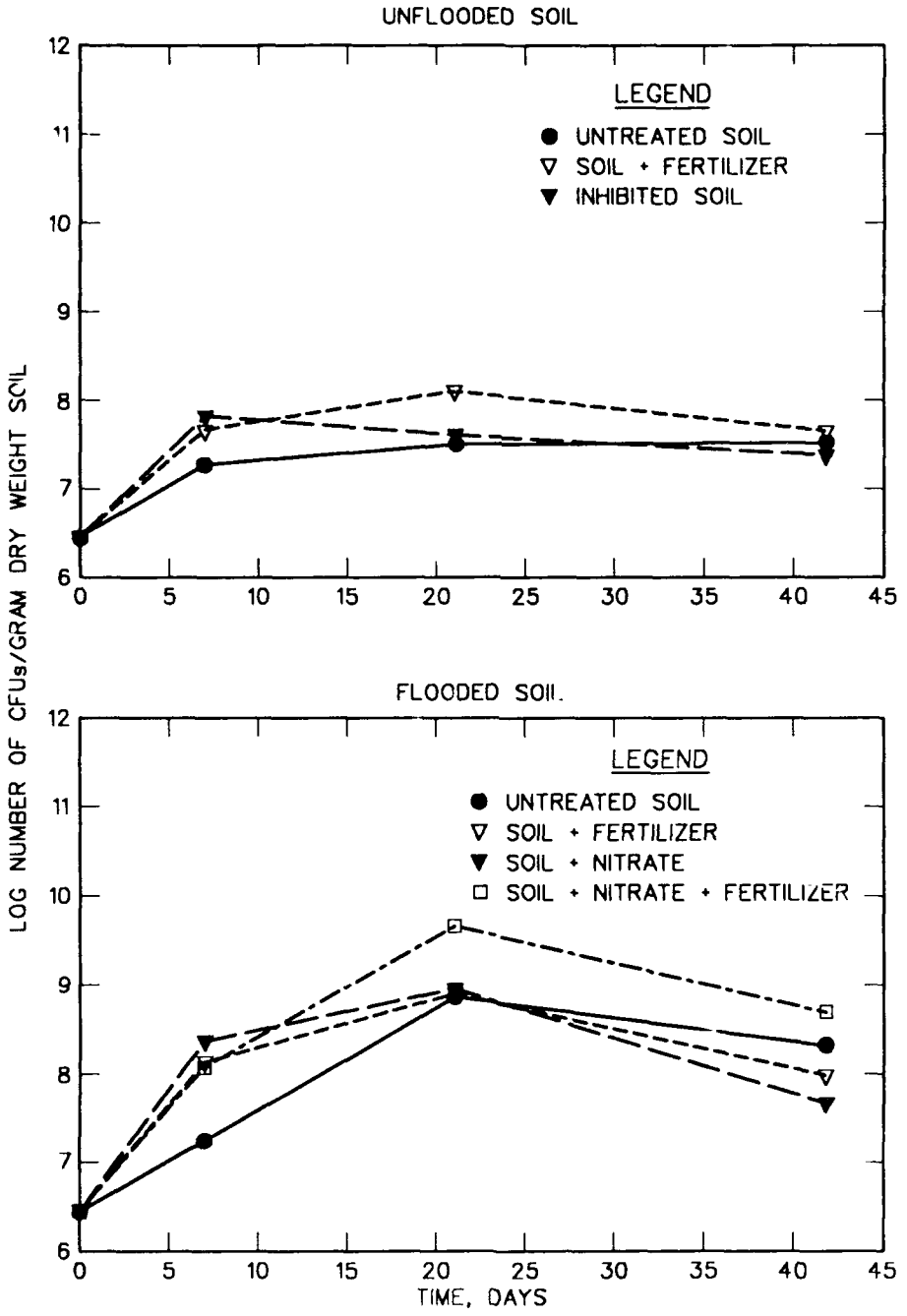

Figure A5. Levels of No. 4 fuel oil-degrading microorganisms recovered from Fort Devens soil

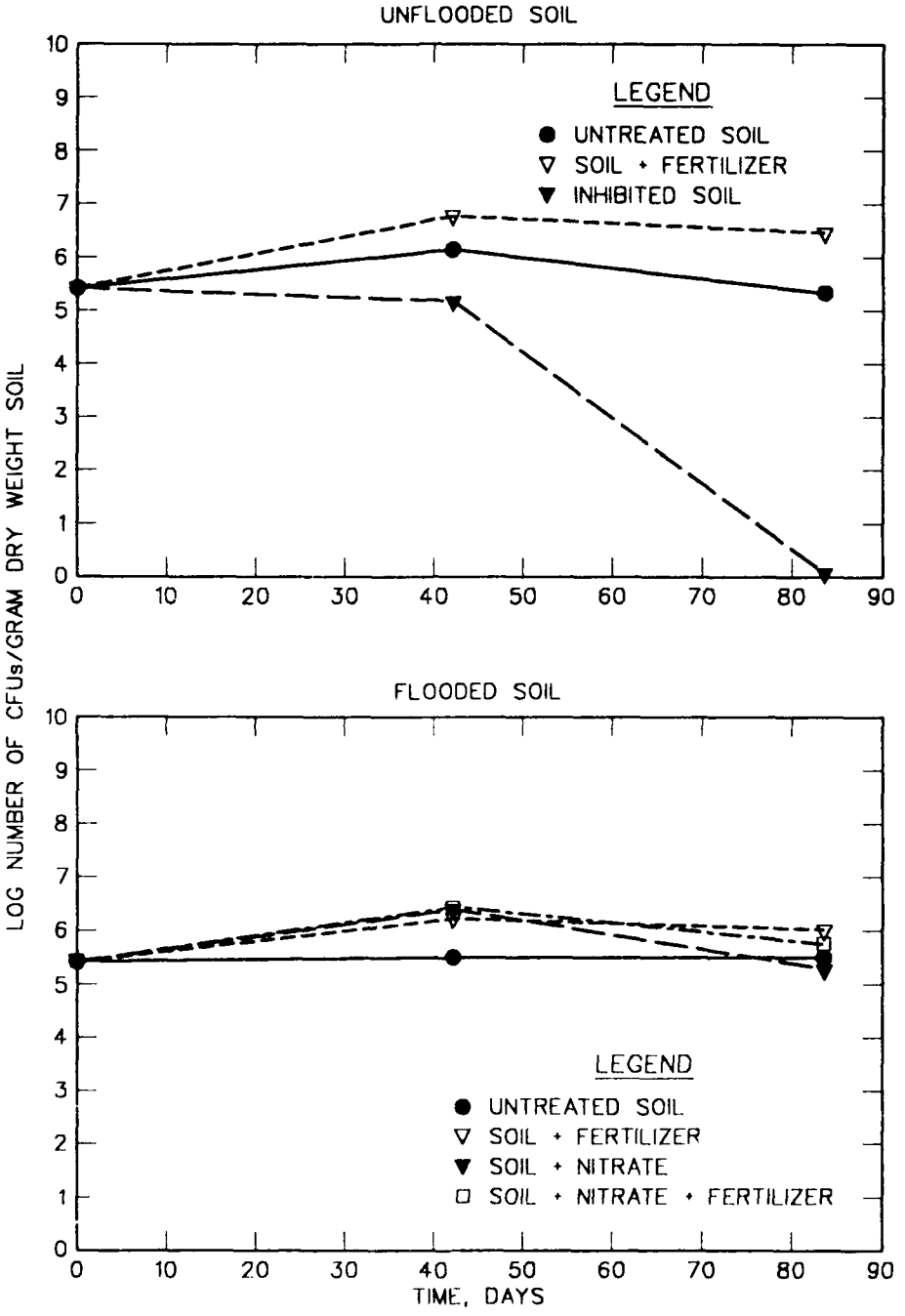

Figure A6. Levels of total heterotrophic microorganisms recovered on PTYG from Fallon NAS soil

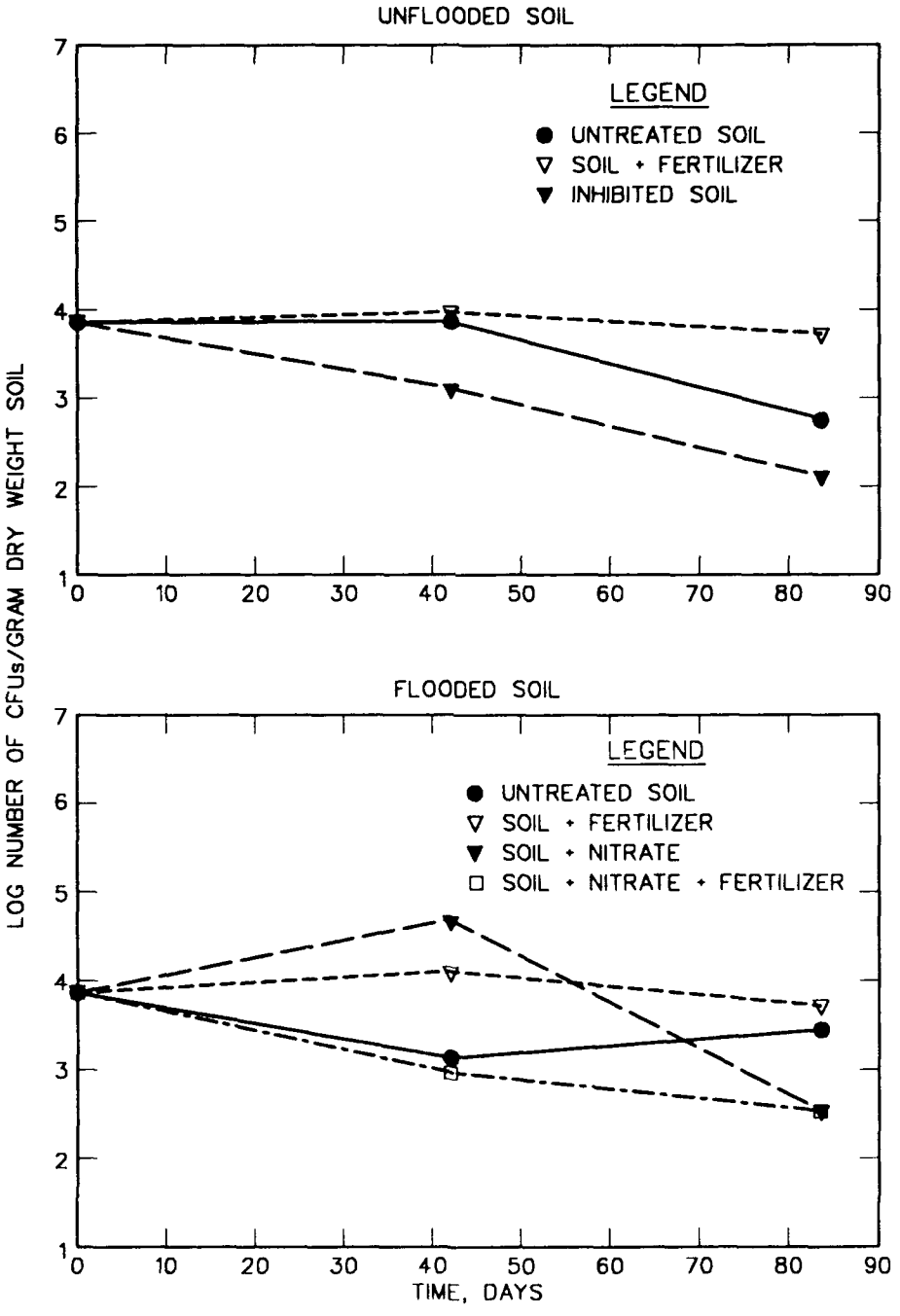

Figure A7. Levels of total heterotrophic microorganisms recovered on PDA from Fallon NAS soil

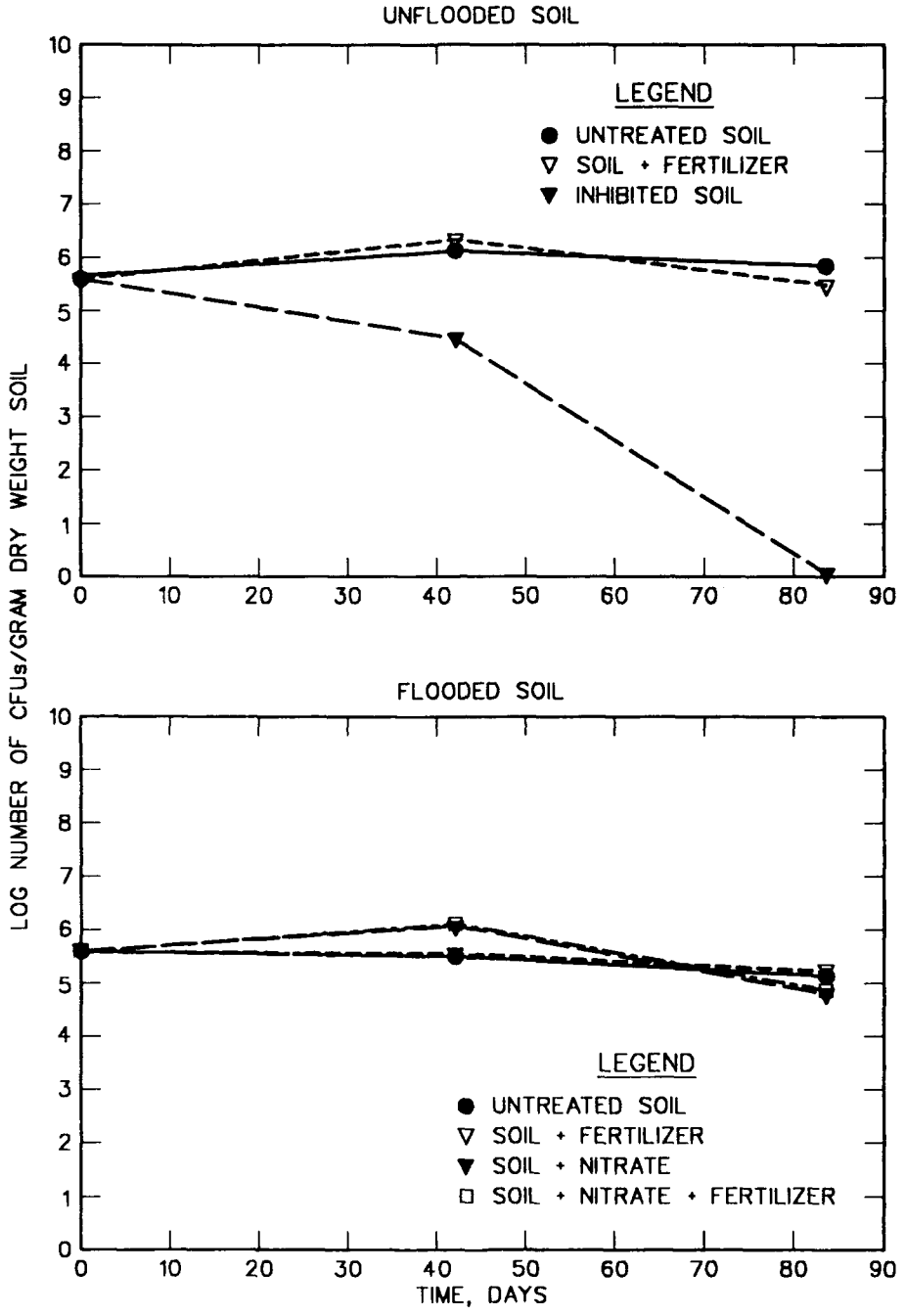

Figure A8. Levels of cyclohexane-degrading microorganisms recovered from Fallon NAS soil

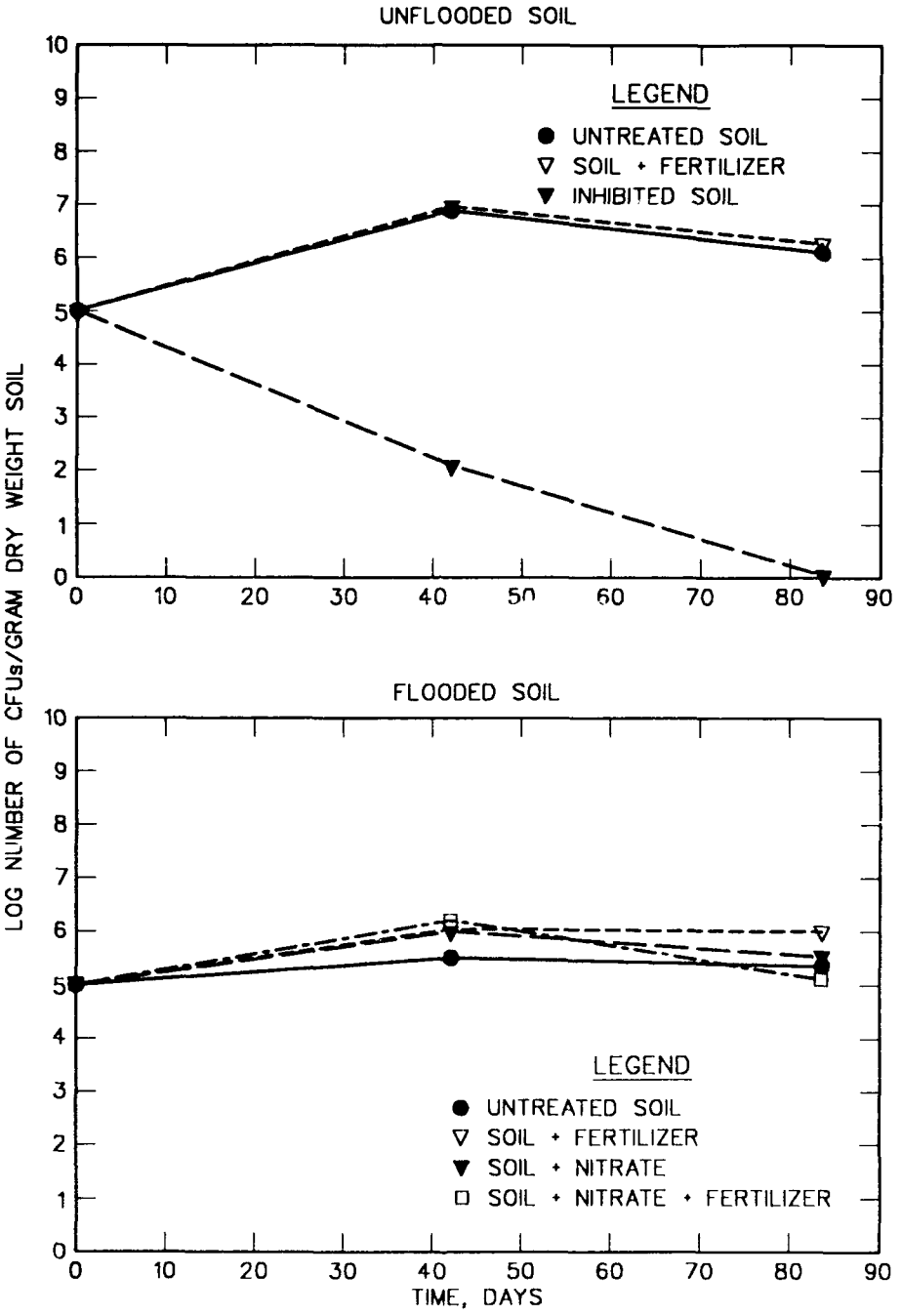

Figure A9. Levels of napthalene-degrading microorganisms recovered from Fallon NAS soil

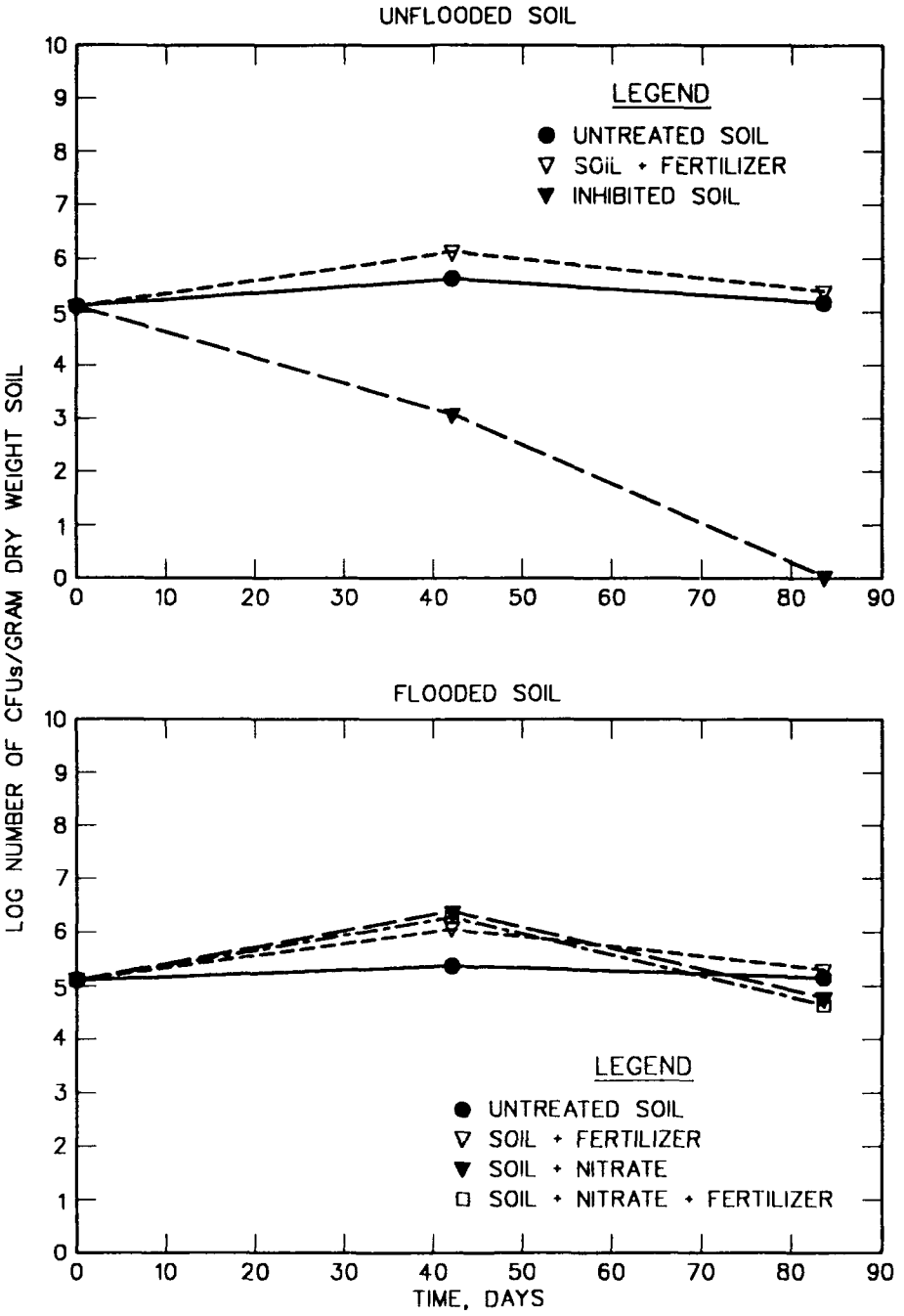

Figure A10. Levels of JP-5-degrading microorganisms recovered from Fallon NAS soil

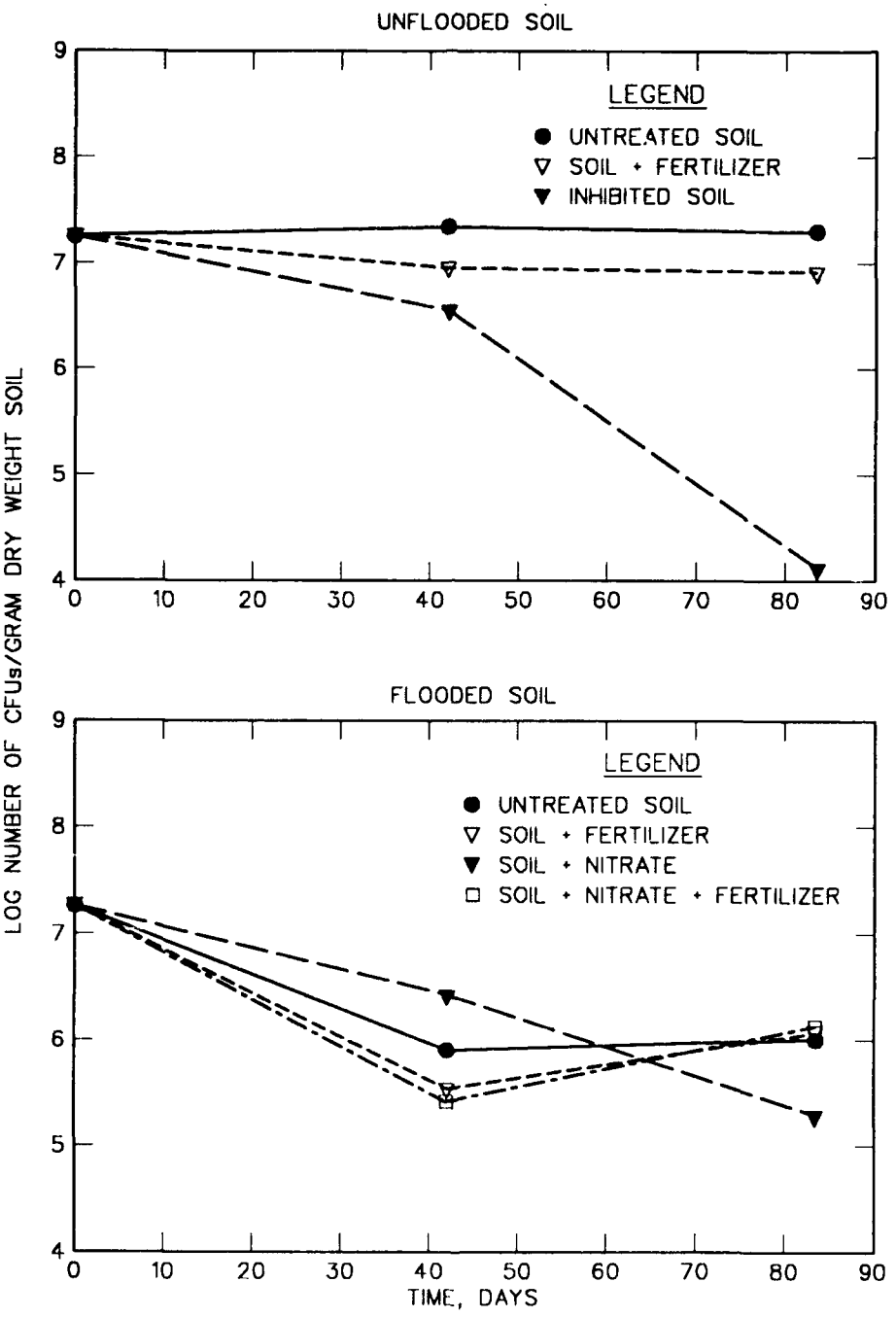

Figure A11. Levels of total heterotrophic microorganisms recovered on PTYG from WES diesel-contaminated soil

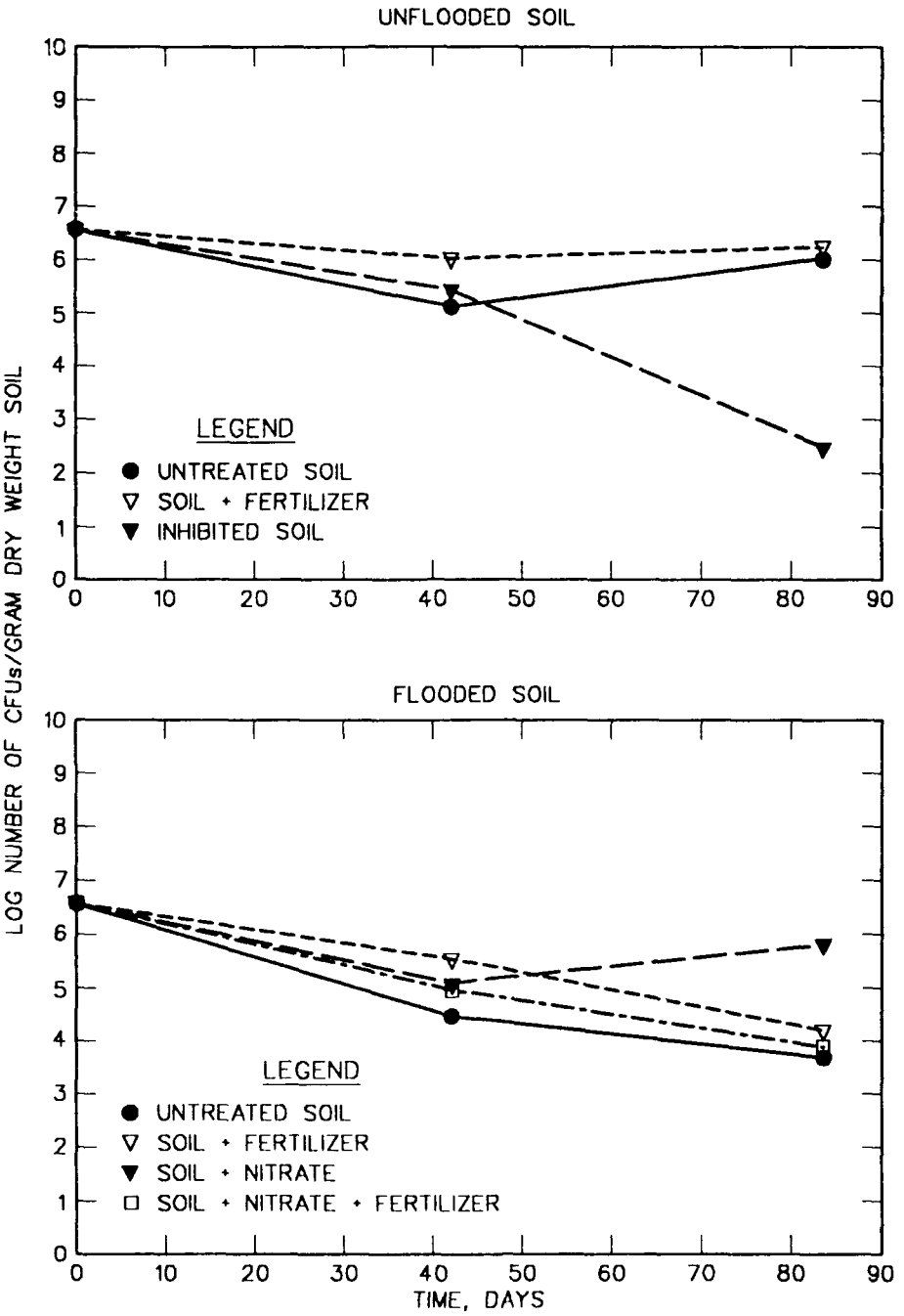

Figure A12. Levels of total heterotrophic microorganisms recovered on PDA from WES diesel-contaminated soil

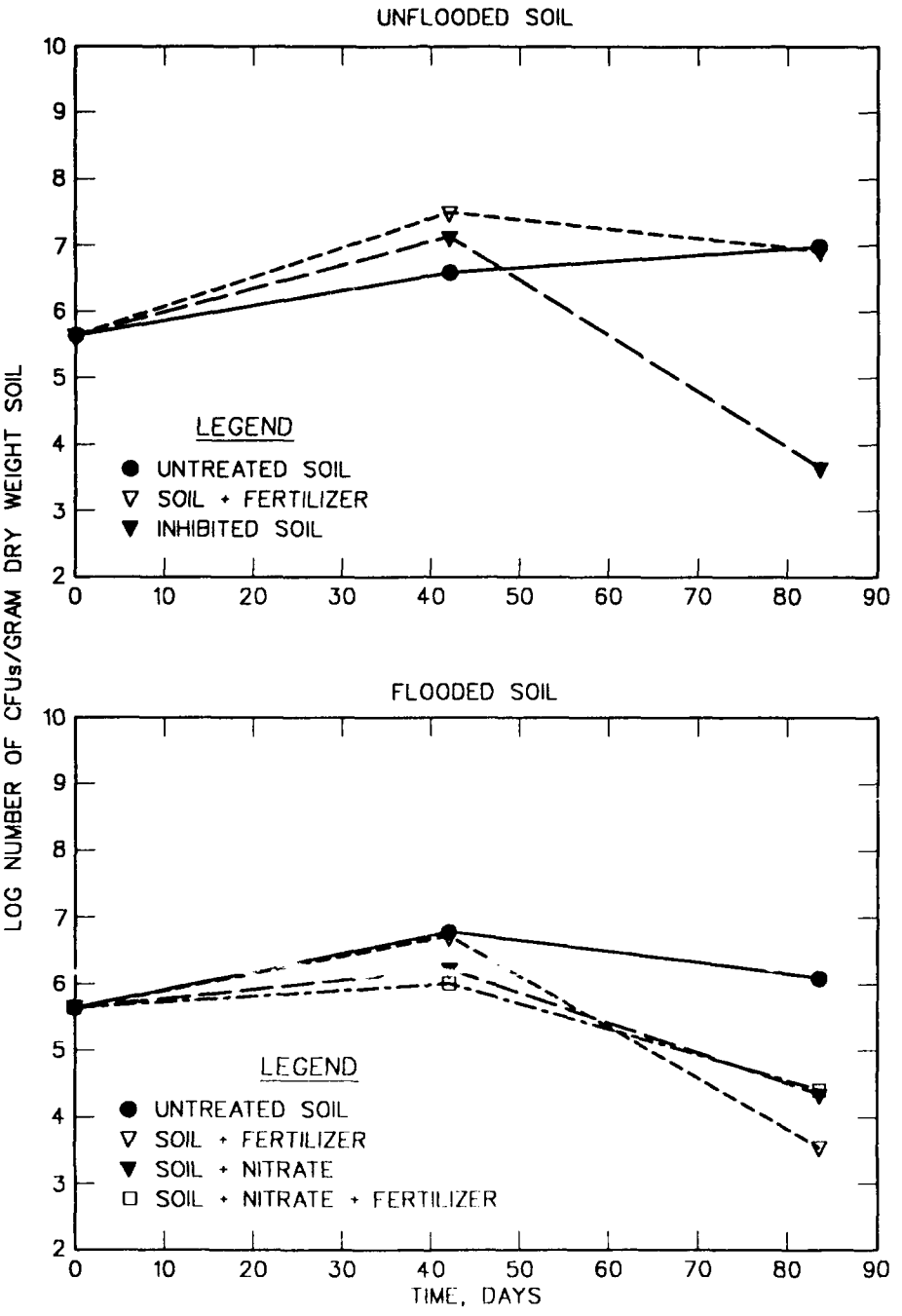

Figure A13. Levels of cyclohexane-degrading microorganisms recovered from WES diesel-contaminated soil

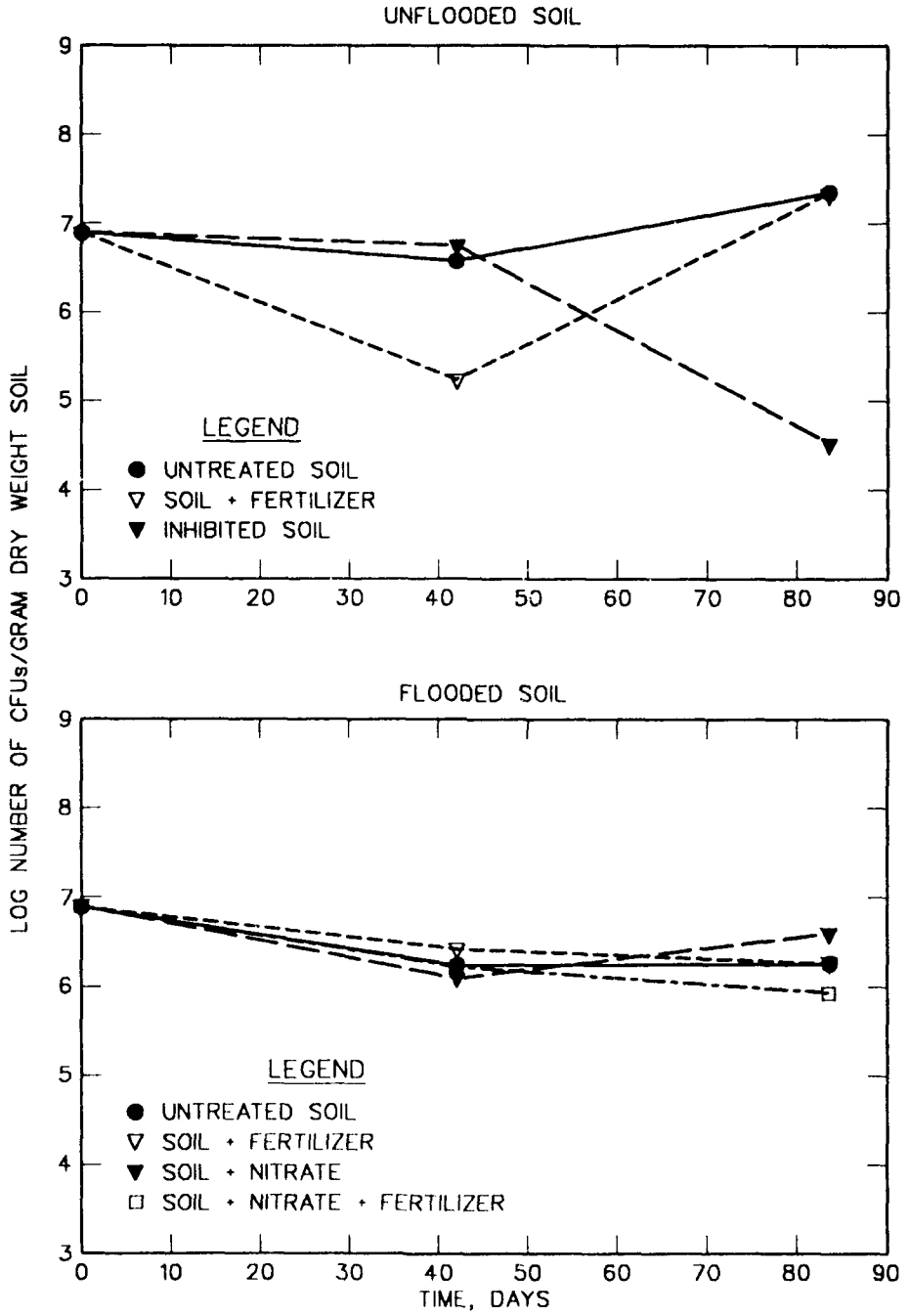

Figure A14. Levels of naphthalene-degrading microorganisms recovered from WES diesel-contaminated soil

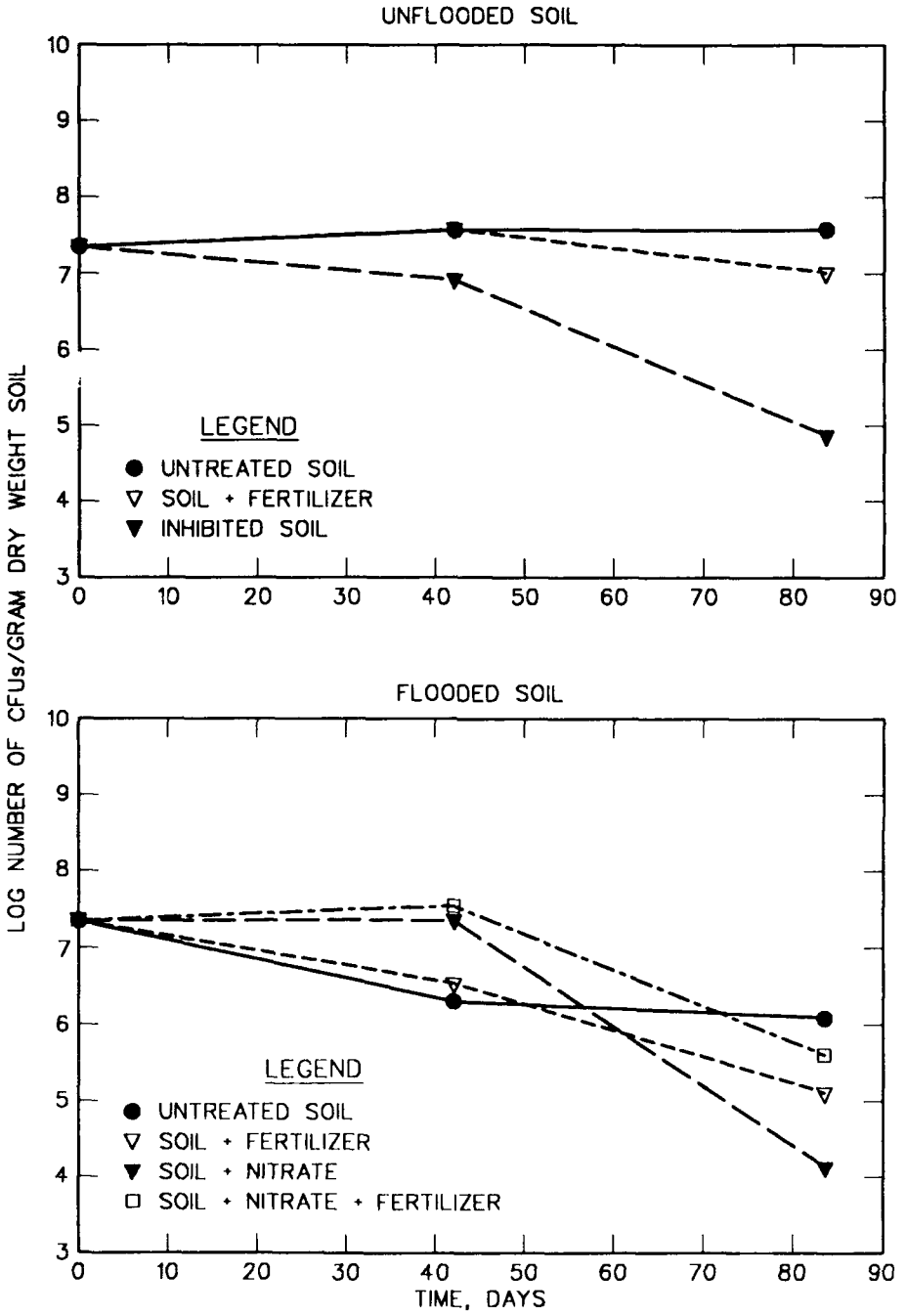

Figure A15. Levels of diesel fuel-degrading microorganisms recovered from WES diesel-contaminated soil

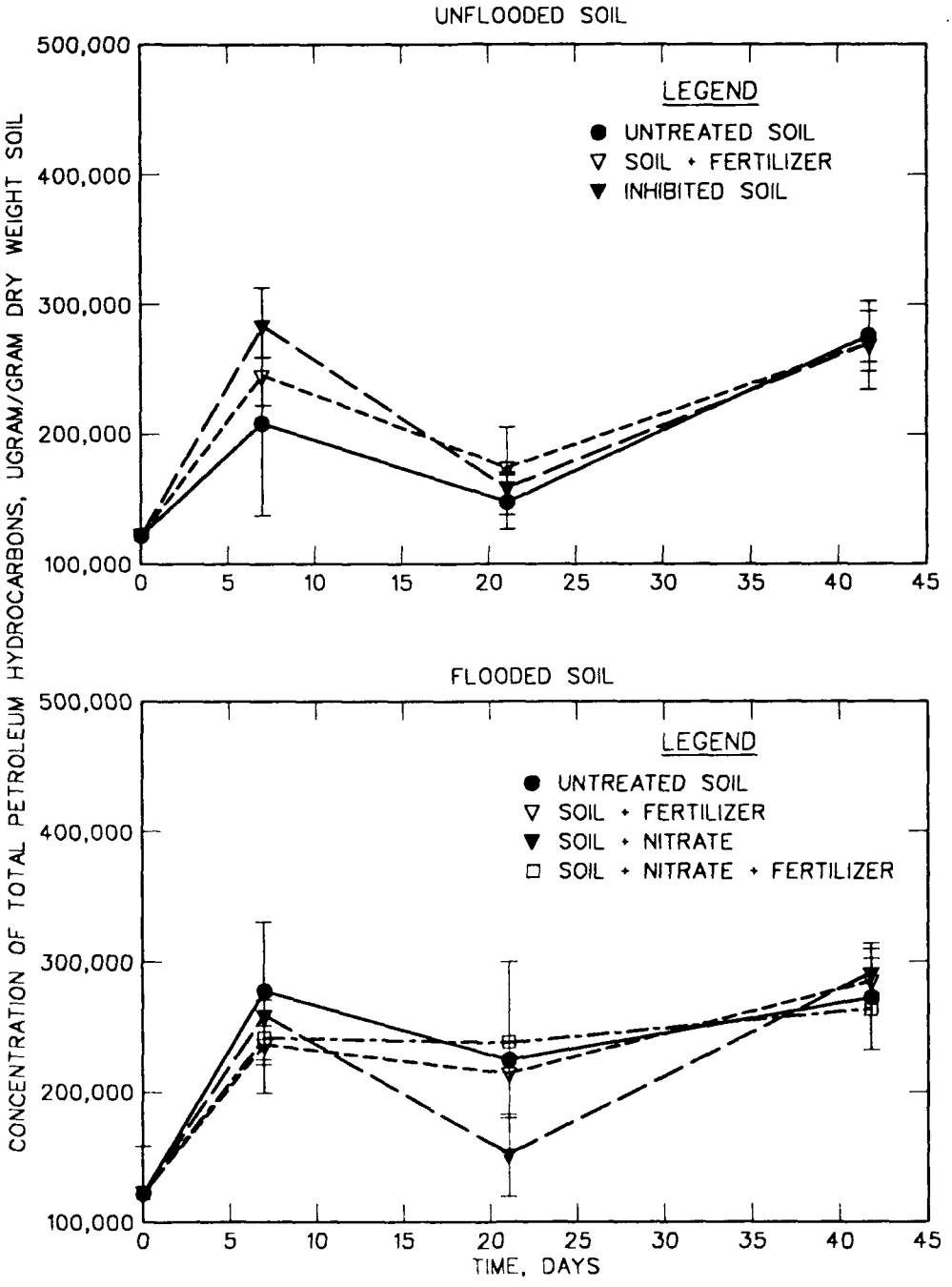

Figure A16. Total petroleum hydrocarbon levels in Fort Devens soil

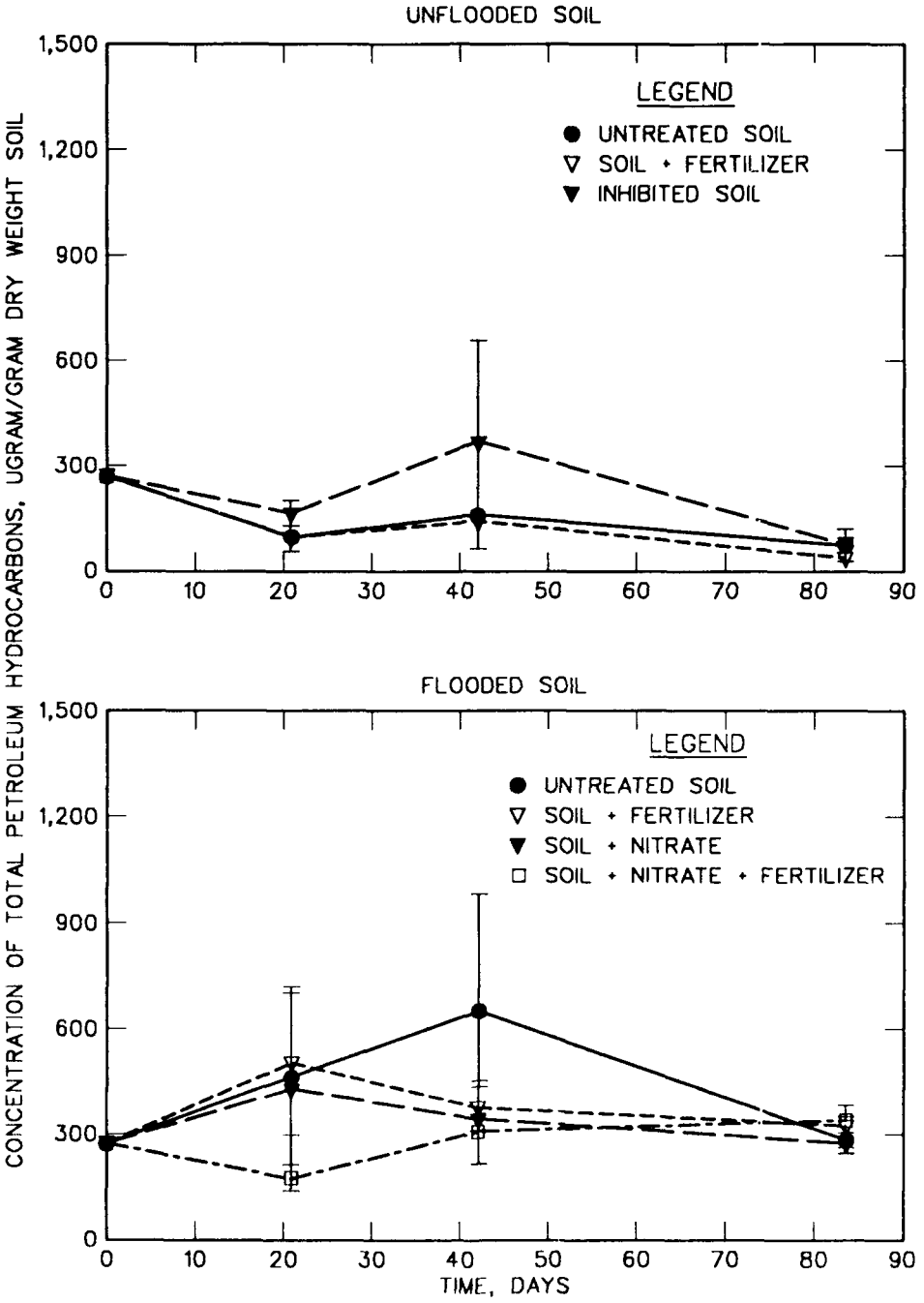

Figure A17. Total petroleum hydrocarbon levels in Fallon NAS soil

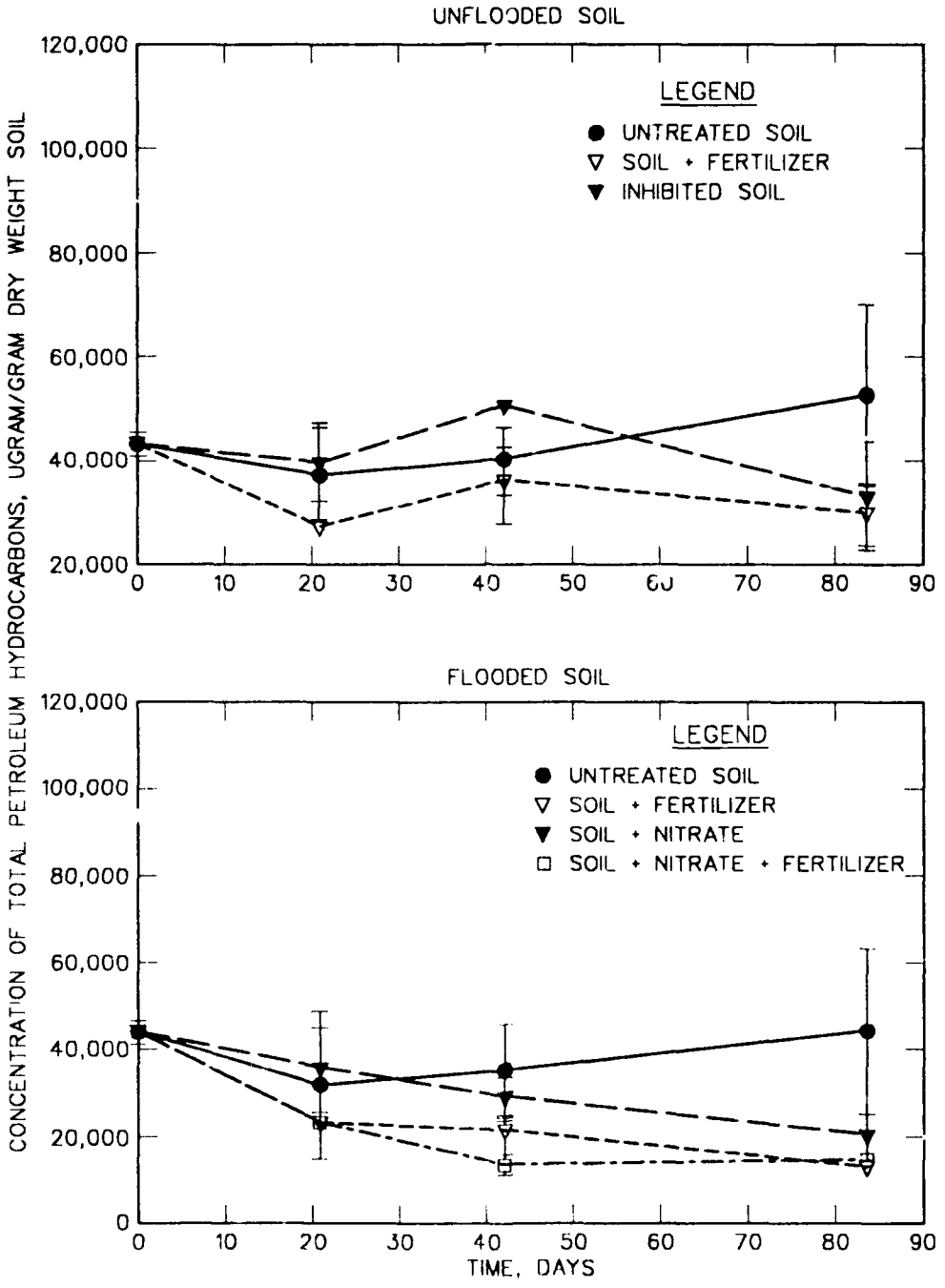

Figure A18. Total petroleum hydrocarbon levels in WES diesel-contaminated soil