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# Project Summary

# Use of Short-Term Bioassays to Evaluate Environmental Impact of Land Treatment of Hazardous Industrial Waste

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A four-phase study was conducted to evaluate the utility of short-term bioassays in monitoring the environmental impact of land treatment of hazardous industrial waste. During phase one, bioassays were conducted using *Bacillus subtilis, Salmonella typhimurium,* and haploid and diploid forms of *Aspergillus nidulans* to define the chronic toxic potential of each waste selected for study. The acid, base, and neutral fractions of each of the three wastes studied induced genetic damage in at least two of the three bioassays.

Phase two involved adding 2-nitrofluorene or benzo(a)pyrene to the soil. This phase was conducted to evaluate efficiencies of the blender and Soxhlet extraction procedures, as well as potential interactions between known mutagens and soil components. Results indicate that while greater quantities of hydrocarbons were extracted using the Soxhlet method, there was no appreciable difference in mutagenicity of the extract using either procedure. In addition, when pure compounds were added to the soil, the extraction efficiency using the blender procedure averaged greater than 85%, as measured by High Pressure Liquid Chromatography (HPLC). There was no statistical difference in mutagenicity of the pure compound or the extract of the soil plus the compound.

Phase three consisted of a greenhouse study in which each of three wastes was applied to two soils. Soil and runoff samples were collected at various times over a 360-day or a 540-day interval. Results from chemical analyses indicated that waste constituents were degraded in the soil. Bioassays of soil and water extracts indicated increased mutagenic activity, caused perhaps by the degradative process forming direct-acting mutagens and converting indirect-acting mutagens to direct-acting compounds. When compared on an equivalent volume basis, the mutagenic potential of waste-amended soils was reduced over time, and in some cases, it was reduced to a nonmutagenic level.

A wood-preserving bottom sediment was applied to barrel-sized lysimeters in the final phase of the project to compare results of soil-core and soil-pore liquid monitoring. Leachate and soil samples were collected prior to as well as 30 and 90 days after waste application. Different types of compounds were detected in soil-core and soil-pore liquid samples. These results indicate that if a land treatment facility is not properly managed, mutagenic constituents from land-applied waste may migrate through the soil.

This study was undertaken to demonstrate that short-term bioassays can be used to trace the environmental fate of mutagenic constituents in land applied hazardous industrial wastes.

*This Project Summary was developed by EPA's Robert S. Kerr Environmental Research Laboratory, Ada, OK, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).*

## Introduction

Over the past three decades, concurrent with the development of numerous new chemicals, the United States has experienced rapid industrial expansion, with its inevitable byproduct, generation of large volumes of hazardous wastes—150 million metric tons annually according to a 1983 estimate by the U.S. Environmental Protection Agency (EPA).

Recently, in many areas, the three most widely used disposal methods—deep sea dumping, incineration, and landfilling—have been replaced by land treatment, i.e., incorporation of waste into the surface layer of soil resulting in the degradation or attenuation of hazardous waste constituents.

Final interim regulations promulgated by the EPA (1982) state that a waste cannot be land applied unless the waste is rendered less or nonhazardous by chemical or biological reactions in the soil. Before land treatment can become a viable method of hazardous waste management, techniques are needed for the monitoring of hazardous constituents and their metabolites. Use of a combined biological and chemical testing protocol may provide the most practical means of efficiently monitoring a hazardous waste land treatment site. Use of chemical analyses alone fails to account for interactions of components of a complex mixture, production of mutagenic metabolites via degradative pathways, and chemical reactions between nontoxic precursors that may result in formation of mutagenic compounds. An appropriately selected bioassay should be capable of integrating these effects. Use of biological analyses alone, however, could fail to account for artifacts generated in the collection or extraction process.

The objectives of this current research were to characterize genotoxic constituents of three hazardous wastes, monitor waste degradation in soil, and determine the environmental fate of mutagenic waste constituents following land application. The project was divided into four main phases to meet these objectives, and to develop a set of test protocols to be used to monitor environmental contamination. The first phase, waste characterization, included an acute toxicity evaluation of ten wastes, a complete characterization of the mutagenic potential of seven subfractions of three selected wastes, and a chemical characterization of major organic constituents. In phase two, direct- and indirect-acting mutagens were added to the soil in order to quantify extraction procedures and determine the effect of soil components on the activity of mutagenic compounds. Phase three consisted of a greenhouse study in which three wastes were applied at one loading rate to two soil types packed in boxes. Simulated rainfall was applied, and runoff and soil samples were collected at various time intervals during a 360- or 540-day experimental period.

Results from phase three of the project were used to evaluate the effect of degradation on the mutagenic activity of waste-amended soil and the potential for removal of mutagens in runoff water. In the final study phase, one waste was applied at one loading rate to an undisturbed soil enclosed in lysimeters. Movement of mutagens through soil was monitored by collecting soil-core and soil-pore liquid samples.

## Materials and Methods

### Wastes

Initially, thirteen wastes were collected for use in the project. Included were two wood-preserving wastes, four refinery wastes, four petrochemical wastes, a pulp and paper waste, an alum sludge, and a paint sludge (Table 1). Selection of three wastes for use in the waste characterization and greenhouse studies was based on results from chemical characterization and acute toxicity testing.

### Extraction

Two methods were used for extraction of hydrocarbons from wastes and from waste-amended soils. The majority of samples were extracted using the blender technique. Comparisons were made with a limited number of samples using a Soxhlet extractor.

Dichloromethane was selected from a group of agents to extract organic fractions of the wastes and soil. Dichloromethane consistently provided the greatest extraction efficiency for the type of anticipated materials. Hydrocarbons were extracted from the waste or waste-amended soils using procedures described in the literature and partitioned into acid, base, and neutral fractions.

### Waste Extraction

Runoff samples were extracted and passed through a mixed bed of 4.0 g of XAD-2 and 6.3 g of XAD-7, or approximately 20 $cm^3$ of each resin. After loading the water sample, dry nitrogen was introduced into the column to remove the residual aqueous phase. The column was washed with 120 ml of distilled water to remove residual histidine. The adsorbed organic compounds were eluted with 160 ml of acetone.

### Chemical Analysis

Chemical analyses of waste and soil-waste extracts were conducted by the

*Table 1. Gross Characteristics of Hazardous Wastes Collected for Study*

| Waste | EPA No. | Extractable[1] Hydrocarbons (%) | Physical[2] Form | Use in Study[3] |
|---|---|---|---|---|
| Wood-Preserving Bottom Sediment (PENT S) | K001 | 27 | Sludge | A,W,G,L |
| Wood-Preserving Wastewater | – | NT | Liquid | A |
| Slop-Oil Emulsion Solids | K049 | 86 | Liquid | A,W |
| Combined API Separator, Waste Treatment Sludge (COMBO) | K051 | 41 | Sludge | A,W,G |
| Storm-Water Runoff Impoundment (SWRI) | – | 21 | Sludge | A,W,G |
| Dissolved Air Flotation | K048 | 5 | Sludge | A |
| Acetonitrile | K013 | 2 | Liquid | A,W |
| Methyl Ethyl Ketone | – | 97 | Liquid | A,W |
| Phenol Production | – | 0.2 | Liquid | A |
| Agricultural Chemicals—Biosolids Waste | – | 0.2 | Liquid | A |
| Primary Clarifier Pump and Papermill | | NT | Solid | N |
| Alum Sludge | | NT | Liquid | N |
| Paint Sludge | | NT | Sludge | N |

[1]Percent by weight, extracted with dichloromethane; NT = not tested.
[2]Physical form estimated from visual observation.
[3]A = acute toxicity; W = waste characterization; G = greenhouse; L = lysimeter; N = not used.

EPA's Robert S. Kerr Environmental Research Laboratory. Compounds were identified using a Finnigan OWA Automated GC/MS. The GC capillary column used was a J & W Scientific DB-5-30W.

## Biological Analysis

The ability of samples to induce genetic damage was measured in three microbial test systems. A eukaryotic bioassay employing *Aspergillus nidulans* (a fungus) was used to detect point mutations and small deletions induced in a haploid genome. The diploid organism was used to detect chromosome aberrations, mitotic recombination, gene mutation, nondisjunction recombinogenic events, recessive lethals, and spindle poisons. A sample was considered mutagenic if there was a positive slope on the mutation induction curve, or if the induced mutation frequency for at least two exposure times was more than twice the spontaneous mutation frequency.

A microbial DNA repair assay was used to measure the capacity of a sample to produce increased lethal damage in DNA repair deficient strains. Six strains of *B subtilis* deficient in different recombination ($Rec^-$) and/or excision ($Exc^-$) repair were used to test for lethal DNA damage. These included $Rec^-$ strains *recA8, recE4; mc-1*, $Exc^-$ strain *hcr-9;* and $Rec^-/Exc^-$ *fh2006.7*. All of these strains are isogenic with *B subtilis* strain *168* which has all repair intact A response was considered positive if the distance of growth inhibition was more than 2 5 mm greater in one of the repair deficient strains than in the repair proficient strain *168*. Fractional survival ($N/N_0$) was determined for those strains showing the greatest sensitivity (inhibition) to the test chemical

A *Salmonella*/microsome assay was used to evaluate mutagenic activity of waste fraction samples

## Results and Discussion

Biological and chemical analyses were employed in the study to evaluate mutagenic potential of the acid, base, and neutral fractions of three hazardous industrial wastes A summary of the results obtained in the different biological test systems is provided in Table 2

For the wood-preserving bottom sediment, the maximum level of genotoxic activity was detected in the base fraction With metabolic activation, the base fraction induced the maximum response in the *B subtilis* DNA repair assay, the *Salmonella*/microsome assay (strains TA98, TA100, TA1538), and the *Aspergillus* methionine assay. In the absence of metabolic activation, the acid fraction induced the maximum response in the *Aspergillus* diploid assay and the *Bacillus* DNA repair spot test. Chemical analyses identified a variety of potentially genotoxic waste constituents, including pentachlorophenol. Thus, biological analyses detected genotoxic compounds in the wood-preserving waste, and chemical analyses detected compounds that could be mobile and recalcitrant. These results indicate that land application of a wood-preserving waste should proceed with caution, preferably at a low application rate.

***Table 2.*** *Summary of Results Obtained from Testing Waste Fractions in Biological Test Systems*

| Sample | | Bioassay[1,2] DNA | | SALM | | BAC PM | | ASPMT | | ASPDP | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | S9[3] | – | + | – | + | – | + | – | + | – | + |
| PENT S | Acid | + | + | – | + | – | + | + | ++ | + | 0 |
| | Base | – | ++ | – | ++ | – | + | + | ++ | ± | 0 |
| | Neutral | – | – | – | + | – | – | + | + | + | 0 |
| SWRI | Acid | – | – | – | ++ | 0 | 0 | + | ++ | + | 0 |
| | Base | – | – | – | + | 0 | 0 | + | ++ | + | 0 |
| | Neutral | – | – | – | ++ | 0 | 0 | + | + | + | 0 |
| COMBO | Acid | – | + | – | ++ | 0 | 0 | + | + | + | 0 |
| | Base | – | – | – | ++ | 0 | 0 | + | + | ± | 0 |
| | Neutral | – | – | + | ++ | 0 | 0 | + | ++ | + | 0 |

[1] *DNA* = B. subtilis *DNA repair assay; SALM* = S. typhimurium *reverse mutation assay; BAC PM* = B. subtillis *reverse mutation assay; ASPMT* = A nidulans *methionine assay; ASPDP* = A. nidulans *diploid assay*

[2] *Response 0 = not tested; – = <2 times background, ± =>2 <2 5 times background; + = >2.5 <5 times background, ++ =≥5 times background*

[3] *S9 = 9000 x g supernatant from Aroclor 1254 induced rats*

Although chemical analyses were unable to identify any of the major organic constituents of the storm-water impoundment (SWRI) waste, biological analyses detected mutagenic activity in all three waste fractions. The maximum response observed in the *Salmonella* and *Aspergillis* mutagenicity assays was induced by the acid fraction, while no response was observed in the *Bacillus* DNA repair assay with any of the fractions All three fractions induced a positive response in the diploid assay, with the maximum response induced by the base fraction. Thus, biological analyses detected genotoxic compounds in all three fractions of the SWRI waste; however, these compounds were present in quantities that were below the detection limits of chemical analyses.

In the combined API separator/slop-oil emulsion (COMBO) waste, results of chemical and biological analyses indicate that the neutral fraction had the greatest mutagenic potential. Both mutagenesis assays detected the maximum response in the neutral fraction. The maximum response in the DNA repair assay was induced by the acid fraction, and the base fraction induced the maximum response in the diploid assay. These results indicate that the COMBO waste contained compounds capable of inducing a range of genetic damage. Although chemical analyses were unable to identify all of the genotoxic waste constituents, both biological and chemical analyses indicated that the neutral fraction has the greatest mutagenic potential.

Biological analyses of the organic extract of three soils which have been used solely for agricultural purposes demonstrated the presence of low concentrations of mutagens and potential carcinogens. Chemical analyses of two of the three soils used in this research were unable to conclusively identify the mutagenic contaminants. However, the past history of these soils indicates that the most probable source of mutagenic activity is trace quantities of partially oxidized residues from previous biocide applications. Mutagenic activity of these trace contaminants also may have been enhanced by the presence of promoters and cocarcinogens.

Biological and chemical analyses of two soils amended with either 2-nitrofluorene or benzo(a)pyrene indicated that the blender extraction procedure provides adequate recovery of mutagenic compounds. Results from the *Salmonella*/microsome assay indicate that there was

no appreciable difference in mutagenic activity of the pure compound or extract of the soil amended with the pure compound at equivalent dose levels. In addition, extraction efficiency as measured using HPLC analyses averaged greater than 85% for both chemicals at all treatment levels on both soils. This combined chemical/biological analytical approach has demonstrated that for the compounds, levels and soils evaluated, there are no interactions with soil compounds, and that the blender procedure provides efficient extraction of mutagenic compounds from soil.

Degradation, infiltration, and removal will influence the quality and quantity of hazardous organic compounds in runoff water from a land-treatment facility. In order to evaluate influence of these factors on mutagenic potential of runoff water, results from waste-amended and control soils were compared on the basis of equivalent volumes. A comparison of results from testing the equivalent of 10 ml of runoff water from the wood-preserving waste-amended Norwood soil in the *Salmonella* assay indicates that mutagenic potential increases consistently from immediately after waste application through 540 days after waste application (Figure 1). Contrasting results were observed in the *Aspergillus* assay (Figure 2). In the wood-preserving waste-amended Norwood soil, mutagenic activity of runoff water collected 360 days after application decreased to a level approximately 16% of that detected immediately following waste application (Figure 2). The surviving fraction in *Aspergillus* increased significantly over the 360-day period. If this effect also occurred in the *Salmonella* assay, the induced mutants per survivor measured immediately after waste application would be three to four times greater than the net reversion frequency, while the induced mutants per survivor and the net mutation frequency measured 540 days after application would be approximately the same. Thus, the results from the two bioassays may be comparable.

In the Bastrop soil, the mutagenic response in both bioassays decreased from immediately after waste application through 540 days after waste application (Figures 2 and 3). In both bioassays, the response induced by the sample collected 360 days after application was at a level that was only slightly greater than twice background. However, 540 days after application, there was an appreciable increase in both the amount of extractable hydrocarbon and mutagenic potential of

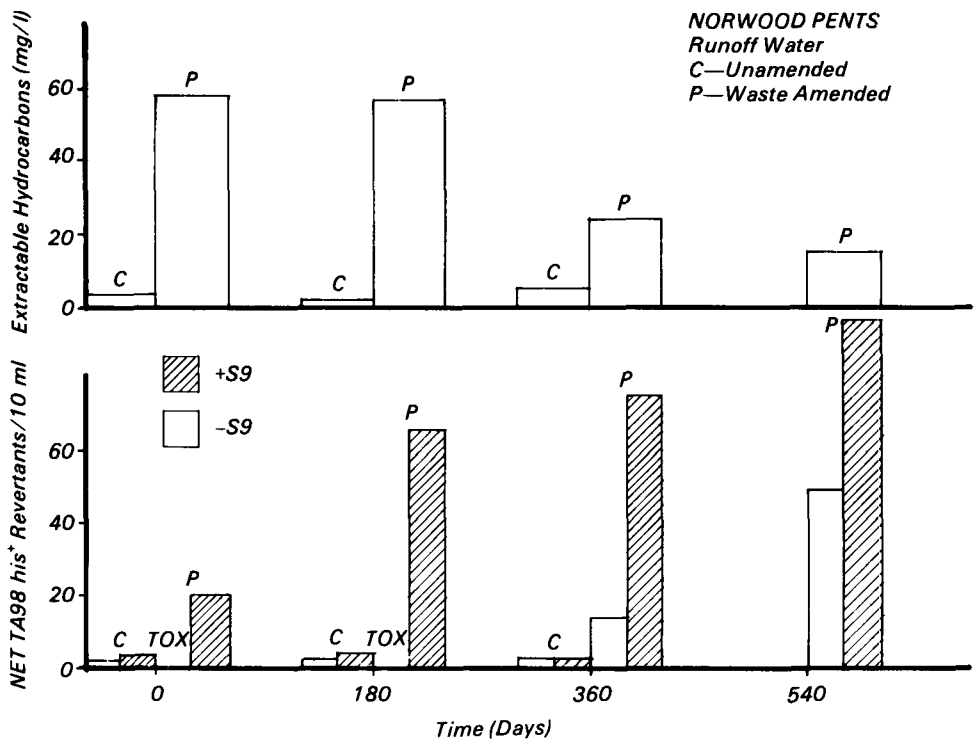


***Figure 1.*** *Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/10 ml) in runoff water from PENT S waste-amended soil.*

runoff water from the wood-preserving waste-amended Bastrop soil. The increased mutagenicity observed in the sample collected 540 days after application may have been a result of degradation increasing the reactivity of residual compounds. Degradation may have reduced the concentration of nonmutagenic compounds, while increasing the relative concentration of mutagenic compounds. The results obtained from the runoff water from either soil amended with the wood-preserving waste as measured with the *Salmonella* and *Aspergillus* assays indicate that significant levels of mutagenic activity are detectable 360 days after waste application. However, the overall results indicate that the mutagenic potential of the runoff water was appreciably reduced 360 days after waste application.

In order to determine if a waste is rendered less or nonhazardous by soil incorporation, a comparison of mutagenic potential of equal volumes of waste-amended soil is necessary. In the *Salmonella* assay, the mutagenic potential (Figures 4 and 5) was determined by calculating the mutagenic activity ratio of two nontoxic dose levels from a five member dose-response curve and adjusting according to the rate of degradation. Utility of these data lies in their ability to define hazardous characteristics of a waste-amended soil. By comparing mutagenic potential of equivalent volumes of waste-amended soil over time, determination of whether a waste is rendered less or nonhazardous by soil incorporation is possible.

Results from evaluating the effect of soil degradation on the mutagenic potential of wood-preserving waste-amended soils are presented in Figures 4 and 5. In the Norwood soil, mutagenic potential of the base and neutral fractions decreased to below the significant level in both bioassays. In the acid fraction, mutagenic potential with metabolic activation was reduced to below a level at which the response would be considered mutagenic in both bioassays. In contrast, the response without activation in *Aspergillus* was increased by more than 50% from immediately after waste application to 360 days after application. Mutagenic potential of the neutral fraction from wood-preserving waste-amended Bastrop soil was also decreased to below significant levels in both bioassays. In the acid and base fractions from wood-preserving waste-amended Bastrop soil, mutagenic potential increased during the 180 days following waste application. The response in *Aspergillus* from both fractions was

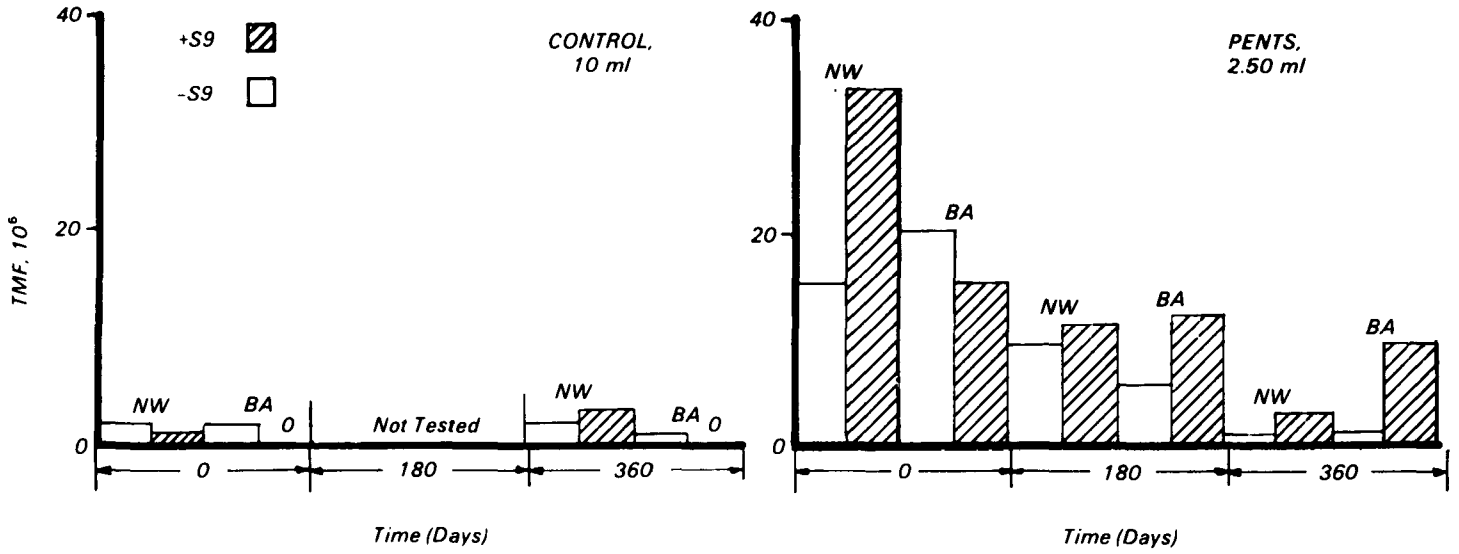


*Figure 2.* *Total mutation frequency per $10^6$ survivors in A nidulans induced by the extractable hydrocarbons in runoff water from PENTA S waste-amended soils*

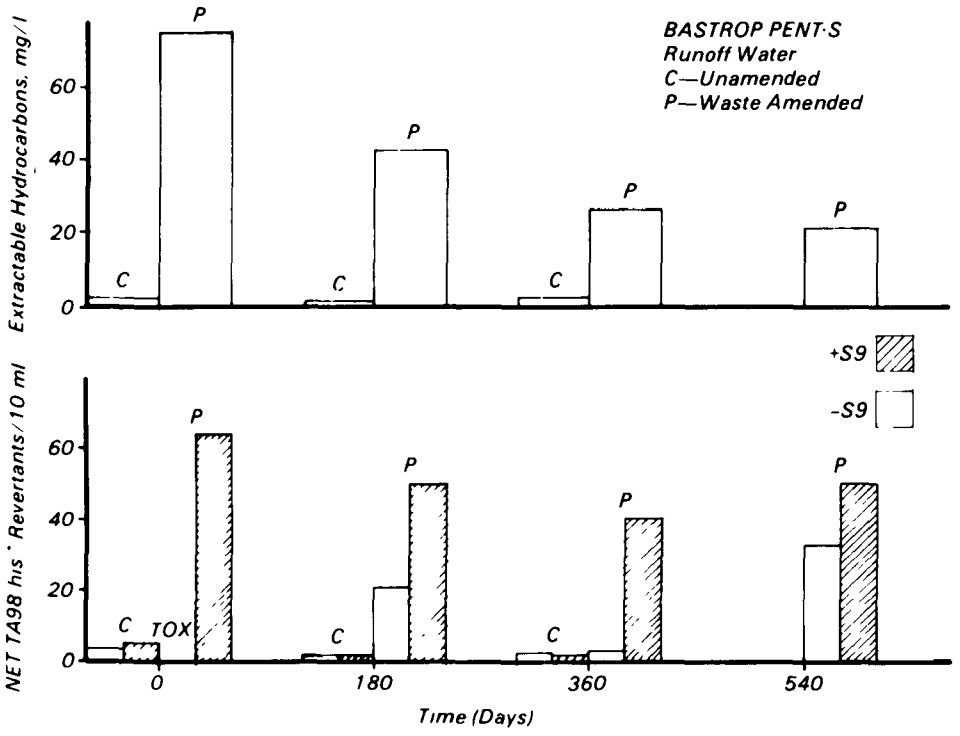


*Figure 3.* *Extractable hydrocarbons (mg/l) and mutagenic activity (revertants/10 ml) in runoff water from PENT S waste-amended soil*

reduced the 360th day following application to less than that observed immediately after application. In *Salmonella*, a mutagenic potential of the base fraction was reduced the 360th day following application, while the response from the acid fraction did not decrease until 540 days after application. Thus, both bioassays detected constituent(s) of the acid fraction of wood-preserving waste-amended soil which were resistant to degradation and highly mutagenic. The bioassays also indicated that the bulk of total extractable hydrocarbons were rendered nonhazardous by land treatment during the time of the study.

Chemical and biological analyses of soil and leachate water from control and waste-amended lysimeters indicate that certain constituents of the wood-preserving bottom sediment are capable of migrating through the soil. Analysis of soil-core samples from control and waste-amended lysimeters over various depths indicated that greater quantities of residual hydrocarbons and mutagenic activity were present in waste-amended samples up to a depth of 45 cm (Figure 6). There was no appreciable difference in mutagenic potential of soil from control and waste-amended lysimeters at the 45 to 90 cm depth, although greater quantities of residual hydrocarbons were recovered from the waste-amended soil at this depth. Mutagenic potential of the soil-core sample from a depth of 0 to 15 cm was greatest in the presence of metabolic activation, whereas the soil at 15 to 45 cm gave approximately the same response with or without metabolic activation (Figure 6).

Mutagenic compounds identified in the surface soil sample included methylnaphthalene, dihydro acenaphthylene, fluoranthene, pyrene, and benzanthracene. Other compounds with a potential influence on genetic activity of the soil extract included pentachlorophenol, several alkane promoters and cocarcinogens, and hexadecane, which is an inhibitor. Only one compound, dihydro-acenaphthylene, was

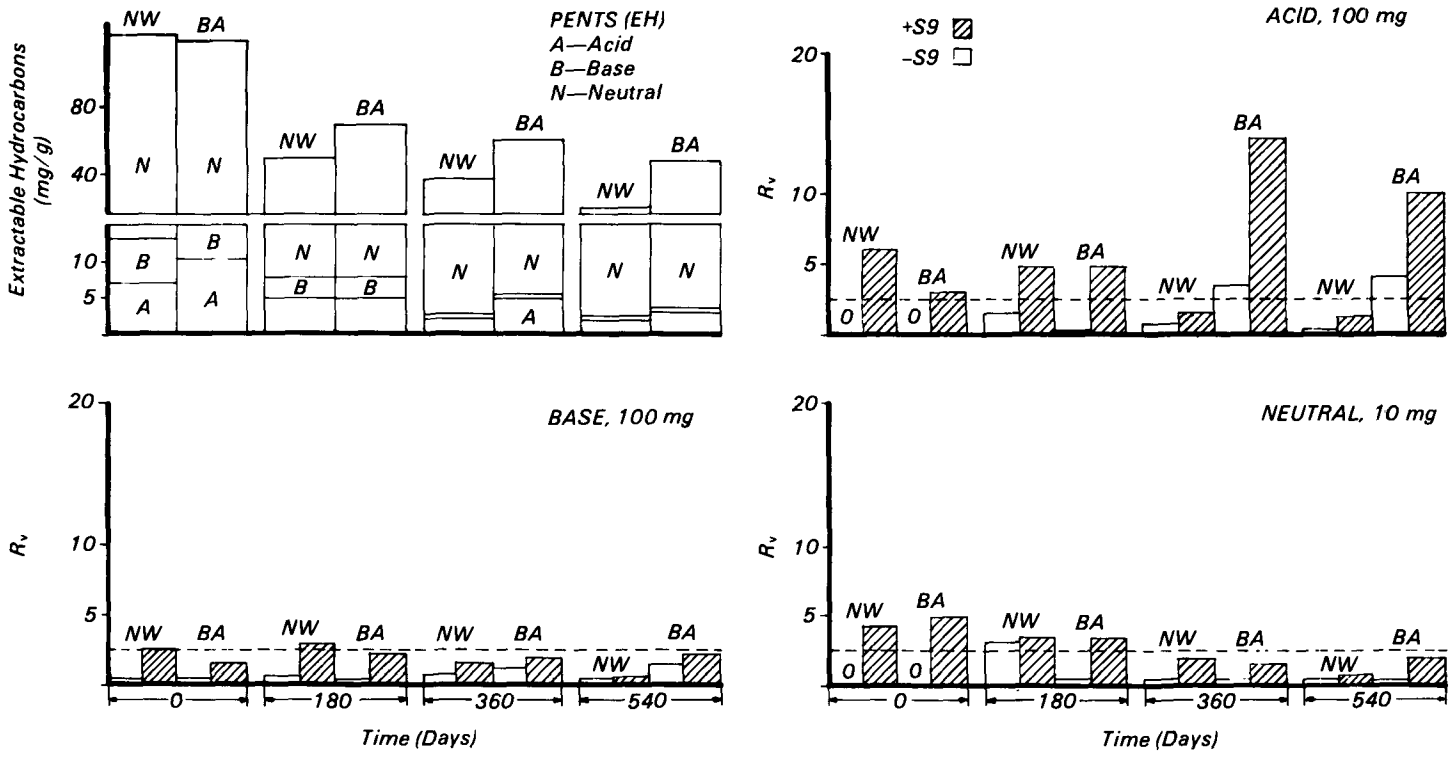


*Figure 4. Total extractable hydrocarbons and mutagenic potential of equivalent volumes of PENT S waste-amended Norwood (NW) and Bastrop (BA) soils as measured with* S. typhimurium *strain TA98 with and without metabolic activation. Dashed line (---) is equal to 2.5 times solvent control.*

consistently identified in the waste and soil-core samples at all depths.

When compared to soil-core samples, analyses of soil-pore liquid samples provides a slightly different perspective on the capacity of wood-preserving waste samples to migrate through soil. Biological analysis with *S. typhimurium* strain TA98 indicated no detectable mutagenic activity in the leachate water collected from control and waste-amended lysimeters prior to waste application. Mutagenic potential of the soil-pore sample collected at a depth of 90 cm 30 days after application was approximately seven times greater than the control. The sample collected 90 days after application had a mutagenic potential approximately ten times the control (Figure 7). Thus, the bioassays detected significantly greater quantities of mutagenic activity in soil-pore samples from wood-preserving waste-amended lysimeters than in samples from control lysimeters, both on 30 and 90 days after waste application.

There were ten compounds in soil-pore samples from wood-preserving waste-amended lysimeters, including anthracene and pentachlorophenol which were also detected in the 0 to 15 cm soil-core sample collected 90 days after waste application. Tetrachlorophenol was also detected in the leachate sample collected 90 days after application. This compound was not present in waste- or soil-pore samples. As a result, tetrachlorophenol may have been transformed from pentachlorophenol at the soil-water interface and subsequently leached into the soil-pore water 90 cm below the soil surface. Analysis of samples from lysimeters amended with wood-preserving bottom sediment indicated that mutagens and potential carcinogens are capable of migrating to a depth of 75 cm below the zone of incorporation within 90 days following waste application. Mobility of waste constituents may have been influenced by the high waste application rate and high amount of rainfall (12.66 cm) that occurred during the 90-day study.

## Conclusions

This research has demonstrated the utility of a combined testing protocol using biological analyses to measure genotoxic potential of waste fractions and chemical analyses to identify major organic constituents. These results have also demonstrated the inability of chemical analyses to provide a comprehensive evaluation of the genotoxic potential of a hazardous industrial waste. While it is possible that more intensive chemical analyses could have identified genotoxic compounds present in trace concentrations, information would still be lacking as to interactions of waste constituents. However, the results also indicate that chemical analyses are a necessary component of a hazardous-waste analytical protocol. Chemical analyses are necessary to identify waste constituents and to verify the absence of artifacts generated in the collection or extraction process.

The efficiency of the blender technique for extracting the diagnostic mutagens, 2-nitrofluorene or benzo(a)pyrene, averaged greater than 85% as measured by HPLC. In addition, there was no appreciable difference in the mutagenic activity of the pure compound and the extract of soil amended with the pure compound.

Results from the greenhouse study indicate that mutagenic potential of runoff water from hazardous waste-amended soils should eventually return to background levels. Major factors influencing mutagenic potential of residual hydrocarbons at a land treatment facility include the number of different compounds present, concentrations of these compounds,

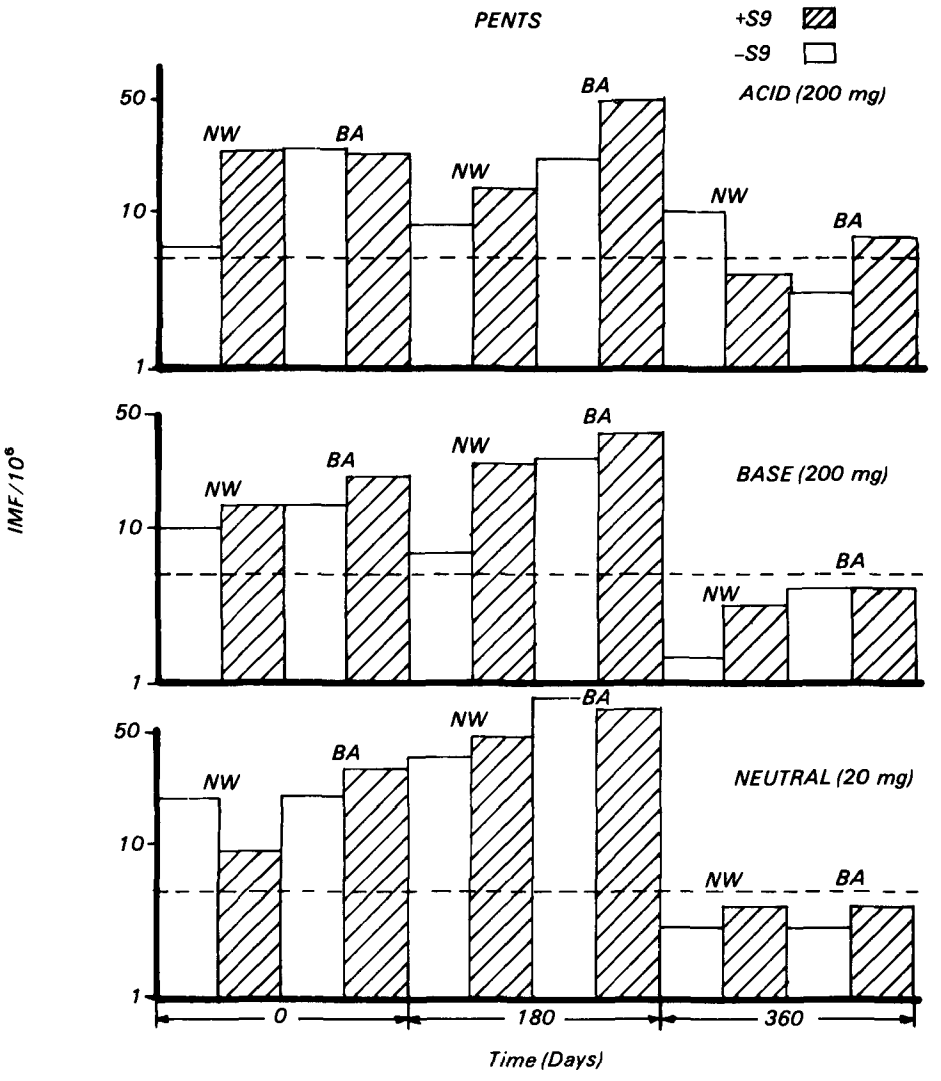


*Figure 5. Total induced mutation frequency of equivalent volumes of PENT S amended Norwood (NW) and Bastrop (BA) soils as measured in* A. nidulans *methionine system with and without metabolic activation. Dashed line (---) is equal to total induced mutation frequency of* $5.0/10^6$ *survivors.*

and toxic effects and interactions of these compounds. Results indicate that different soils will have substantially different capacities to retain and degrade organic compounds. Therefore, bioassays provide an effective analytical tool for evaluating when mutagenic potential of runoff water or soil from a hazardous waste land treatment facility has returned to background levels. While these results can only be applied to the wastes, soils, and loading rates employed, they do indicate that land treatment can render a waste less hazardous or nonhazardous through degradation or transformation in the soil.

## Recommendations

In order to obtain the most accurate evaluation of hazardous characteristics of a waste, both chemical and biological analyses should be used. The best results would be obtained if a battery of bioassays were used to define the genetic toxicity of a hazardous industrial waste. Bioassays used should include some that are capable of detecting point mutations, DNA repair damage, and chromosome damage. In addition, the testing protocol used to monitor hazardous-waste land treatment should include both chronic and acute toxicity bioassays.

This research has demonstrated that land treatment can be a viable disposal option for a variety of hazardous wastes. However, there is also an indication that mutagen and potential carcinogens can be released in runoff or leachate water. This information makes it imperative that a treatment demonstration, as suggested by the EPA in 1982, precede the application of hazardous waste to soil. This treatment demonstration should include degradation and leaching studies, as well as a demonstration of volatilization rates and the effects of management practices and repeat applications. Bioassays may be used a an integral part of the treatment demonstration and, more importantly, in operational monitoring of an active site.

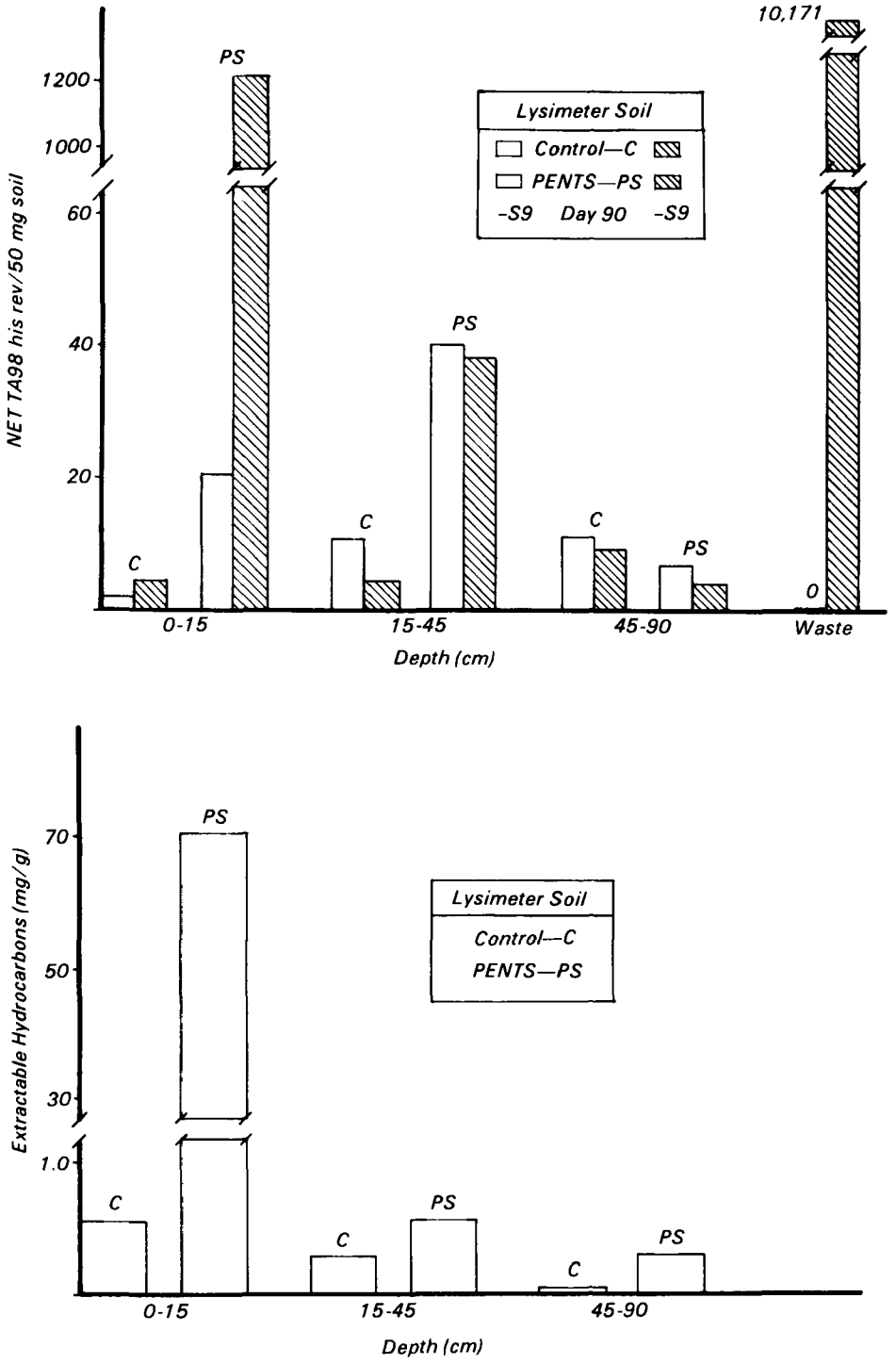


***Figure 6.*** *Extractable hydrocarbons and mutagenic activity from soil-core samples collected at various depths on day 90.*

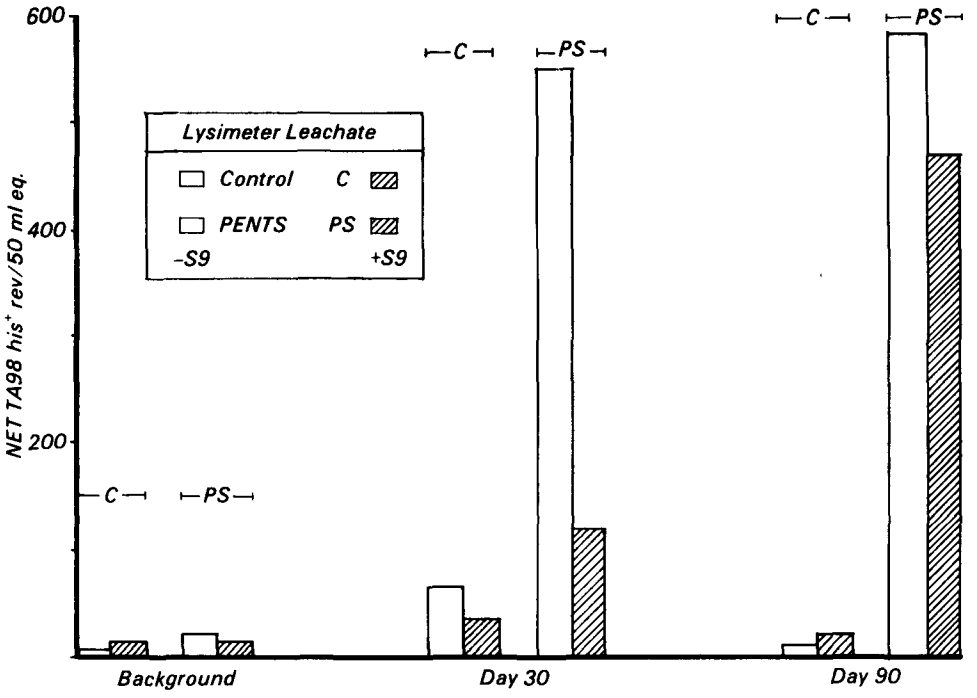


*Figure 7. Mutagenic activity of leachate water from control and PENT S waste-amended lysimeters.*

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*The complete report, entitled "Use of Short-Term Bioassays to Evaluate Environmental Impact of Land Treatment of Hazardous Industrial Waste," (Order No. PB 84-232 560; Cost: $29.50, subject to change) will be available only from:*
*National Technical Information Service*
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*Telephone: 703-487-4650*
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