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# Mutagenic Potential of Water Concentrates from the Effluent of A Waste Oil Storage Pond

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A consequence of the rapid expansion of the petrochemical industry over the past thirty years has been the generation of large quantities of hazardous waste. The EPA (1979) estimates that approximately 40 million tons of hazardous waste will be generated in 1980.

Many hazardous wastes are stored on site prior to disposal in a pit, pond, or lagoon. JOSEPHSON (1980) states that many of the more than 132,000 surface impoundments in the United States are unlined, and underlain by permeable soils, resulting in a potential for groundwater contamination due to seepage of contaminants. Another source of environmental contamination from surface impoundments occurs when excessive rainfall results in the overflow of a storage basin. Overflow and seepage water may contain a wide variety of hazardous constituents which may be toxic, carcinogenic, teratogenic, or mutagenic.

At present, there are few standardized testing protocols which can be used to define the total genotoxic potential of a complex mixture. Chemical analysis of a complex mixture fails to account for the antagonistic, synergistic, or additive effects of the components of a complex mixture (GEHRS *et al.* 1978). The analysis of a complex mixture utilizing both chemical and biological methods may provide identification of both the types of compounds present, and the specific hazard associated with the mixture. The combined use of chemical and biological analyses was employed COLEMAN *et al.* (1980) to identify the mutagenic compounds in concentrated drinking water samples. Complex mixtures identified as mutagenic by microbial assays include shale oil (PELROY & PETERSON 1979), crude oil (EPLER *et al.* 1978), and aqueous industrial effluents (COMMONER 1976).

This investigation was begun as a feasibility study to compare the mutagenic effects of water samples collected before and after a contaminated pond was dredged and to evaluate the utility of bioassays for the determination of the mutagenic potential of a complex mixture. The contaminated pond contained runoff water and waste oil of an unknown origin which was being used to ignite training fires at a fireman's training school. The pond was in a location which would result in overflow into

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a nearby stream when excessive rainfall occurred. In order to prevent environmental contamination, the training school desired to remove for disposal the contaminated sludge from the pond and use only diesel fuel in future operations.

## MATERIALS AND METHODS

Water samples collected from the pond were analyzed in two biological systems capable of detecting mutagens and potential carcinogens. The *Salmonella*/microsome assay as developed by AMES *et al.* (1975) has been found to be from 80 to 95% efficient for detecting carcinogenic organic chemicals as bacterial mutagens (McCANN & AMES 1975; PURCHASE *et al.* 1976). In addition, the *Bacillus subtilis* DNA repair assay (FELKNER *et al.* 1979) was used to measure the ability of various extracts to produce increased lethal damage in DNA repair deficient strains. This test has been found to be more sensitive than the *Salmonella* assay to certain types of pesticides (SHIAU *et al.* 1980). The *Bacillus* assay was used to improve the efficiency of the biological analysis by allowing the evaluation of the effects of a sample on several different DNA repair mechanisms. These tests were supplemented with a chemical analysis to detect hexachlorobenzene (HCB), and polychlorinated biphenyls (PCB).

### Water Samples

Samples used in this study were subsamples of two water samples collected from a retention pond. Two 8-L samples were stored in amber glass bottles at 4°C for a maximum of two weeks until processed. These were labeled contaminated samples 1 and 2. Also included in this report are data obtained from a runoff sample collected from an uncontaminated area at the Texas A&M University Research Farm. This sample served as a negative control. The retention pond was subsequently dredged and the pond allowed to refill with a less contaminated diesel-water mixture. Once the pond had refilled, a third sample was collected for analysis.

The organic compounds in the water samples were concentrated on a non-polar XAD-2 resin (Applied Science Lab., State College, PA*) using the methods of HOOPER *et al.* (1978). The XAD-2 resin was washed prior to use three times with ten volumes each of acetone, methanol, and distilled water. Glass econo-columns (15 x 20 mm) from Bio-Rad (Richmond, CA) were packed with 10 $cm^3$ (3.0 g) of XAD resin and rinsed with 30 bed volumes of distilled water prior to loading the water sample. Water samples were allowed to pass through the column by gravity flow at about 50 mL/min. Residual water was removed from the column with a stream of nitrogen, and the column was washed with three bed volumes of distilled water. Adsorbed compounds were

*Mention of brand names does not constitute endorsement.

removed with 20 mL of acetone which has been previously reported to be the best single eluant (HOOPER *et al.* 1978).

The eluate was collected in a 25 x 250 mm culture tube and was taken to dryness under a stream of nitrogen. The concentrated extract was redissolved in dimethylsulfoxide (Grade I, Sigma) at a rate of 0.5 mL DMSO/L of unconcentrated water. The resultant solution was then filtered through 0.2 µm pore diameter, 13 mm Teflon filter (Millipore-Fluoropore, Bedford, MA). Water extracts were tested at dose levels equivalent to 10 to 400 mL of the unconcentrated water per plate.

Analysis of Genetic Toxicity

The mutagenic potential of water extracts was measured with two microbial systems capable of detecting compounds which produce point mutations and lethal damage to cellular DNA. The *Salmonella*/microsome assay of AMES *et al.* (1975) was used to monitor the mutagenic activity of concentrated water samples. The *Salmonella* strains were kindly supplied by Dr. Bruce N. Ames. The methods used were the same as AMES *et al.* (1975), except that overnight cultures were prepared by inoculation into 5 mL of Oxoid broth (KC Biological, Inc. Lenexa, KS) and incubated in a 125-mL Erlenmeyer flask for 16 h at 37°C. Extracts were tested in the standard plate incorporation assay at a minimum of 4 dose levels of the sample with and without enzyme activation (0.3 mL rat liver/mL S-9 mix). Aroclor 1254 induced rat liver was obtained from Litton Bionetics, (Kensington, MD). Positive controls included 2 µg/plate N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Co., St. Louis, MO), for TA 1535 and TA 100, 25 µg 2-nitrofluorene (Aldrich Chemical Co., Milwaukee, WI) for TA 1538 and TA 98, and 10 µg/plate 2-acetylaminofluorene (Sigma), which was used to verify the functioning of the metabolic activation system. All reagents and extracts were tested for sterility; DMSO was used as a negative control.

Additional analyses were conducted with the DNA repair assay described by FELKNER *et al.* (1979). Toxicity of water extracts was compared in six strains of *Bacillus subtilis* supplied by Dr. I. C. Felkner. These strains were deficient in different recombination (Rec$^-$) and/or excision (Exc$^-$) repair. These included the Rec$^-$ strains recA8, recE4, and mc-1; Exc$^-$ strain hcr$^{-9}$, and Rec$^-$ Exc$^-$ strain fh 2006-7. These strains are all isogenic with *B. subtilis* strain 168 which has all repair intact. Cultures were inoculated into Difco Brain-heart infusion broth and incubated for 16 h at 37°C. Inocula from the overnight cultures were streaked radially on a nutrient agar plate to a centered sensitivity disc containing 50 µL of the water extract. The plates were then incubated overnight at 37°C, and the zone of growth inhibition from the disk measured. Positive controls for all strains were 10 µg mitomycin C (Sigma), 2 µL methylmethanesulfonate (Eastman). The excision repair deficiency was verified by sensitivity to ultraviolet light. The solvent DMSO served as negative controls in addition to a

runoff sample from an uncontaminated area.

Chemical Analysis

A chemical analysis of these samples was performed by the Center for Trace Characterization at Texas A&M University using a computerized gas chromatography/mass spectrometry system. The analysis indicated that samples 1 and 2 contained both hexachlorobenzene and trace quantities of polychlorinated biphenyls, while sample 3 contained neither chemical.

## RESULTS AND DISCUSSION

Mutagenicity Assay

The results of the *Salmonella* plate incorporation assay were inconclusive. The two effluent samples collected before dredging were applied to the plates as concentrates at dose levels equivalent to 20, 100, 140, and 200 mL of unconcentrated effluent per plate. Dose levels greater than 140 mL eq were bactericidal in all strains (data not shown).

A water sample which was collected after the dredging operation was tested at dose levels ranging from 10 to 400 mL eq. This sample did induce a slight increase in the mutation frequency in strain TA 98 at lower dose levels. However, there was no dose response effect, and at higher dose levels a toxic response was observed. A toxic response was observed in all strains at dose levels greater than 140 mL eq and in TA 100 at a dose level greater than 100 mL eq (data not shown).

The positive controls 2-nitrofluorene, N-methyl-N'-nitro-N-nitrosoguanidine, and 2-acetylaminofluorene produced the anticipated response in all strains.

DNA Repair Assay

In order to determine the potential of the samples to induce repairable DNA damage, the activity of each sample was compared in DNA repair deficient and proficient strains of *Bacillus subtilis*. The concentrates were added to the plates at a rate of 400 mL equivalent of the effluent concentrate per plate. The results of this analysis are presented in Table 1. These results clearly demonstrate the presence of a DNA damaging agent in the water extracts. The zone of inhibition was consistently higher in repair deficient strains than in the wild type. The greatest response was in the recombinant repair deficient strain recA8, in which the zone of inhibition was more than twice that produced in the repair proficient strain. The water sampled after the dredging operation failed to produce a large increase in the zone of inhibition in repair deficient strains. A comparison of the effect of the samples collected before and

Table 1. Comparison of Lethal Effect of Water Extracts on DNA Repair Deficient and Proficient Strains of *B. subtilis*.

| Sample | | Inhibition Radius (mm) 168 wt RP[1] | recA8 | mc-1 | recE4 | hcr-9 | fh 2006-7 | Response[2] |
|---|---|---|---|---|---|---|---|---|
| | | | repair deficient | | | | | |
| DMSO (Solvent) | | 0 | 0 | 0 | 0 | 0 | 0 | - |
| MMS[3] | 2 µL | 8 | 33 | 26 | 21 | 17 | 20 | +++ |
| Mit-C[4] | 20 µg | 5 | 14 | 13 | 10 | 8 | 9 | ++ |
| Sample #1 | 400 mL eq | 13 | 19 | 19 | 16 | 18 | 20 | ++ |
| Sample #2 | 400 mL eq | 15 | 36 | 21 | 21 | 17 | 16 | +++ |
| Sample #3 | 400 mL eq | 7 | 6 | 6 | 10 | 9 | 6 | ± |
| Runoff (Negative control) | 400 mL eq | 4 | 4 | 4 | 3 | 4 | 5 | - |

1 - Repair proficient.
2 - <3; ± = 3, 4; + = 5, 6, + + 6-10, + + + >10 (Difference in inhibition between wild type and repair deficient strains).
3 - Methyl methanesulfonate - 2 µL/plate.
4 - Mitomycin C - 10 µg/plate.

after the dredging operation does, however, indicate a substantial reduction in the capacity of the post-dredging sample to produce repairable DNA damage. These results indicate that the organic extract of the initial water samples was toxic to microorganisms at very low doses, and that one of the mechanisms for this toxicity is damage to DNA. Agents which damage DNA may be mutagenic, carcinogenic, or teratogenic (KADA *et al.* 1978).

In conclusion, although both groups of water samples contained compounds which were toxic to bacteria, the sample collected after the dredging operation exhibited a substantial reduction in its capacity to produce repairable DNA damage. Chemical analysis indicates that the reduction in repairable DNA damage may be due to the removal of chlorinated hydrocarbons. These results indicate the potential utility of bioassays for the detection of mutagens in environmental samples. The results were found to correspond with a chemical analysis which indicated the removal of HCB and PCB by the dredging of the storage pond. While chemical analysis cannot conclusively define the toxic potential of a complex mixture, biological systems can provide indicator organisms which respond to compounds which react with DNA. Biological analysis offers the advantage of a system which can detect genotoxic constituents in a complex mixture.

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