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# Bioluminescent Toxicity Assay of Synfuel By-Product Waters

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Large amounts of complex by-product waters, potentially toxic to biological systems, are generated during in situ recovery of fuels from oil shale, coal, and tar sand deposits. Toxicity assessment is an essential component to an effective management program for synfuel waters. Since these by-product waters may contain a broad spectrum of organic and inorganic pollutants (Jackson et al. 1975; Braunstein et al. 1977), analysis of individual compounds may not always be practical. More importantly, toxicity of a complex mixture cannot always be predicted from knowledge of its constituents, due to potential antagonistic and/or synergistic interactions. Separation of complex by-product waters into environmentally interpretable hydrophilic (HP) and organophilic (OP) fractions can, however, provide a useful approach for initial toxicity evaluation. Because most HP compounds have less affinity for soil than OP compounds (Leenheer and Stuber 1981), the former are expected to show greater mobility. However, OP compounds often have a greater bioaccumulation potential because of their higher lipid affinity.

The Microtox assay was developed by Beckman Microbics Operations (Bulich 1979; Bulich and Isenberg 1981). For this assay, reduction of bacterial luminescence upon exposure to potential inhibitors was used as an index of toxicity. Microtox is useful as a pollutant monitor and as a rapid screening device to precede more comprehensive bioassays.

A number of studies have compared the Microtox assay with microbial, invertebrate, and fish bioassays. Dutka and Kwan (1981) concluded that four microbial testing procedures, including Microtox, exhibited unique sensitivity patterns and could not be readily correlated. Results of Microtox and fish bioassays have generally shown good agreement (Bulich et al. 1981; Lebsack et al. 1981; Curtis et al. 1982; Qureshi et al. 1982). In a recent evaluation of complex wastes, Microtox indicated toxicity in 81% of samples toxic to fathead minnows and 62% of samples toxic to Daphnia pulex (US EPA 1981). These comparative studies suggest that the Microtox assay can be useful as an exploratory screening test.

Although considerable effort has been directed toward evaluation of synfuel whole waters using traditional bioassay designs (e.g., Anderson et al. 1980; DeGraeve et al. 1980), application of the Microtox system to synfuel by-product waters and defined chemical fractions has received only limited attention (Lebsack et al. 1981; Peake and MacLean 1982). This paper presents results of the Microtox assay on eight whole waters and their HP and OP fractions, derived from oil shale, coal, and tar sand recovery processes.

## MATERIALS AND METHODS

Brief descriptions of the eight waters tested are listed in Table 1. Information on collection, storage, and amounts of selected chemical constituents contained within whole waters has been compiled (LETC 1979-82). Gas and liquid chromatographic analyses for these whole waters and their HP and OP fractions have been presented elsewhere (Delistraty et al. 1983).

Waters were separated into HP and OP fractions, based on the solvent extraction method of Tobben et al. (1982). Raw waters were filtered through a 0.45 μ membrane, adjusted to pH 7.0, and extracted four times with methylene chloride ($CH_2Cl_2$). Resultant aqueous and organic phases were designated, HP and OP, respectively. In order to perform Microtox testing, the OP fraction was transferred into aqueous phase by evaporation of $CH_2Cl_2$ and exchange for an equal volume of water. Residual $CH_2Cl_2$ was then removed from both HP and OP fractions by vacuum and stirring. Although some volatile components may have also been removed during this step, it was necessary to reduce the amount of $CH_2Cl_2$ to a low background level. The pH of test waters was readjusted to 7.2 ± 0.5, and NaCl was added to a final concentration of 2% (w/v) to osmotically protect the bacterial reagent of marine origin.

The toxicity assay was performed and data were reduced, as detailed in the Microtox system operating manual (Beckman 1982). The Microtox Analyzer (Model 2055) was provided by Beckman Instruments (Carlsbad, CA). Absorbance correction cuvettes and Microtox Reagent, a lyophilized strain of marine luminescent bacteria most closely resembling Photobacterium phosphoreum, were purchased from Beckman. Median effective concentration (EC50), the toxicant concentration inhibiting luminescence by 50%, was determined for each sample with a 2% NaCl blank and four sample dilutions (each in duplicate) after a 5 minute exposure period at 15°C. Gamma, the ratio of light lost to light remaining, was used as the dependent variable, in lieu of simple light loss. Calculation of EC50 has been shown to be more precise with use of the gamma function (Johnson et al. 1974). Correlation coefficients, ranging from 0.97 to 0.99, were highly significant ($P < 0.002$) for logarithmic regressions of sample concentration vs. gamma.

Table 1. Synfuel source and recovery process, by-product water, dissolved organic carbon (DOC), and 5-minute EC50 with 95% confidence limits (CL).

| Synfuel | | By-Product Water | | | |
|---|---|---|---|---|---|
| Source | Recovery Process | Designation | Fraction | DOC (mg/l) | EC50(CL) (%) |
| Oil Shale | Simulated In Situ Retort | 150 Ton-R17 | Whole | 3014 | 1.3(1.2-1.5) |
| | | | HP | 1990 | 16(14-17) |
| | | | OP | 980 | 1.9(1.7-2.1) |
| Oil Shale | In Situ Retort | Geokinetics-17 | Whole | 1833 | 1.4(1.2-1.6) |
| | | | HP | 1147 | 11(6.7-19) |
| | | | OP | 839 | 5.3(4.4-6.4) |
| Oil Shale | Modified In Situ Retort | Oxy-6-Process | Whole | 2742 | 1.3(1.1-1.4) |
| | | | HP | 2051 | 23(22-24) |
| | | | OP | 725 | 13(8.9-19) |
| Oil Shale | Modified In Situ Retort | Oxy-6-Condensate | Whole | 453 | 1.0(0.78-1.4) |
| | | | HP | 132 | 18(16-19) |
| | | | OP | 264 | 15(12-18) |
| Oil Shale | Surface Retort | Paraho-77/78 | Whole | 40734 | 0.30(0.24-0.37) |
| | | | HP | 36452 | 1.5(1.0-2.3) |
| | | | OP | 2928 | 1.8(1.3-2.4) |
| Coal | In Situ Gasification | Hanna-IVB-Condensate | Whole | 4843 | 0.074(0.069-0.079) |
| | | | HP | 1079 | 11(9.5-13) |
| | | | OP | 3282 | 0.17(0.11-0.25) |
| Tar Sands | In Situ Combustion | Tar Sand-2C | Whole | 670 | 2.8(2.4-3.2) |
| | | | HP | 541 | 23(22-25) |
| | | | OP | 159 | 12(12-13) |
| Tar Sands | In Situ Steam Injection | Tar Sand-1S | Whole | 76 | 70[a](61-80) |
| | | | HP | 55 | >100[a] |
| | | | OP | 42 | >100[a] |

[a]Extrapolated values (see text for explanation)

It was shown that trace amounts of $CH_2Cl_2$ remaining from the extraction procedure exerted an inhibitory effect on bacterial luminescence in OP fractions but not in HP fractions. Accordingly, a 2% NaCl solution which had contacted $CH_2Cl_2$ was incorporated as the blank in the assay of OP fractions. A correction procedure (Beckman 1982) was applied to visually colored samples in order to eliminate the effect of color quenching on bioluminescence.

Dissolved organic carbon (DOC) analysis was performed on all test waters. Since DOC analysis required aqueous samples, $CH_2Cl_2$ was exchanged for an equal volume of water in OP fractions. Samples were then acidified (pH < 2) and sparged to remove inorganic carbon. DOC was measured by injection of a 200 µl sample (in duplicate), combustion of organic carbon, and coulometric titration of resultant $CO_2$. A Coulometrics Inc. (Wheat Ridge, CO) Total Carbon Apparatus (Model 5020) and $CO_2$ Coulometer (Model 5010) were used for DOC analyses.

## RESULTS AND DISCUSSION

Five-minute EC50 values and associated 95% confidence limits are listed in Table 1. A lower EC50 indicates greater toxicity. Microtox 5-minute EC50 values for synfuel whole waters ranged from 0.074 to 70% in this study (Table 1). Other studies have reported Microtox 5-minute EC50 values for synfuel whole waters, ranging from 0.11 to 5.3% (Lebsack et al. 1981) and 0.30 to 11% (Peake and MacLean 1982). Differences in synfuel source and recovery processes, along with compositional changes of the waters occurring during storage, preclude strict comparisons between studies.

Comparing whole waters from three synfuel sources examined in this study (Table 1), the coal water exerted greatest toxicity, presumably due to the presence of large amounts of phenolic compounds and ammonia (Humenick and Mattox 1982). Tar sand waters exhibited the least toxicity, and oil shale waters were intermediate. Low levels of DOC (Table 1) and ammonia (LETC 1979-82) may have accounted for the relatively low toxicity of Tar Sand-1S. With the exception of Paraho-77/78, a similar range of toxicity was apparent among oil shale waters. Paraho-77/78 contained over ten times the DOC content as other oil shale waters (Table 1), due to production by a surface retorting process (Jackson et al. 1975). The other oil shale waters were generated in in situ retorting processes.

With the exception of Paraho-77/78, toxicity of water fractions was as follows for each by-product water tested: Whole > OP > HP (Table 1). Paraho-77/78 HP and OP fractions exerted a similar degree of toxicity, as indicated by overlapping EC50 confidence limits. Whole waters showed greatest toxicity, since they contained all constituents in HP and OP fractions. Perhaps the higher lipophilicity of OP components in cytoplasmic membranes of tested bacteria accounted for greater OP toxicity, relative to HP fractions. For example, phenol (primarily an OP constituent) has been reported to interfere with enzyme systems and denature bacterial cytoplasmic membranes (Reynolds et al. 1973). Since the bacterial cytoplasmic membrane is the site of the electron transport chain (Atlas and Bartha 1981), which includes the bioluminescent pathway (Hastings 1978; Chang et al. 1981), it is clear that impairment of membrane function would adversely affect bioluminescence, the Microtox assay end point.

In contrast to results from this study, Geiger (1981) has reported greater toxicity associated with HP fractions in several oil shale waters. Ammonia was the major toxicant in HP fractions. Chemical fractions were generated with a column chromatography separation technique, test organisms included fish and invertebrates, and acute toxicity was assessed. These experimental differences may have accounted for the difference in observed results between the two studies.

Inorganic constituents were assumed to remain in the HP fraction, along with the more HP organic compounds. Since HP fractions generally showed less toxicity than OP fractions with the Microtox assay, it appears that inorganic constituents were relatively less toxic to bacteria than OP organic compounds. However, the presence of heavy metals, ammonia, and inorganic salts in the tested by-product waters (LETC 1979-82) undoubtedly contributed to the observed toxicity in whole waters and HP fractions.

Since a 50% solution was the highest concentration tested, EC50 values for Tar Sand-1S were calculated by extrapolation. As assessed with a 50% solution, Tar Sand-1S toxicity also followed the pattern of Whole > OP > HP. Since Tar Sand-1S HP and OP fractions failed to elicit a 50% reduction in bioluminescence even when extrapolated to full-strength (100% solution), their EC50 values were reported as >100% (Table 1).

Assuming an additive relationship, toxicity of HP and OP fractions should sum to their whole water toxicity, according to the following formula (Parkhurst in press):

$$(EC50_{Whole})^{-1} = (EC50_{HP})^{-1} + (EC50_{OP})^{-1}$$

With the possible exception of 150 Ton-R17, HP and OP toxicities did not entirely account for whole water toxicity (Table 1). This non-additivity may have been attributed to either synergistic interactions between HP and OP fractions or loss of toxic components during the fractionation procedure. The sum of HP and OP DOC concentrations was less than the whole water value for both Oxy-6-Condensate and Hanna-IVB-Condensate (Table 1). Negative DOC balance in these two condensates may have resulted from emulsion formation during extraction or partial loss of volatile organics during $CH_2Cl_2$ removal.

In a Microtox study which separated Canadian oil sand waters into acid, base, neutral, and phenolic fractions, neutral compounds (e.g., polycyclic aromatic hydrocarbons) were most frequently the major source of toxicity (Peake and MacLean 1982). However, in both the present study and that of Peake and MacLean (1982), no single class of organic compounds was solely responsible for the observed toxicity in whole by-product waters. In line with this observation, many organic compounds, belonging to a variety of chemical classes and known to be toxic, have been identified in synfuel waters from oil shale (Leenheer et al. 1982), coal

(Humenick and Mattox 1982), and oil sands (Peake and MacLean 1982). Furthermore, toxicity of synfuel by-product waters was most likely a function of inorganic, as well as organic constituents.

Combining chemical fractionation with short-term toxicity testing has proven useful in the evaluation of complex environmental mixtures, as a means of identifying toxic classes of compounds with similar physical and chemical properties (Parkhurst et al. 1979; Waters et al. 1982; Samoiloff et al. 1983). The simplicity, rapidity, small volume of toxicant required, statistical advantage (over $10^5$ bacteria per test), and cost-effectiveness of the Microtox assay make it a useful screening tool for toxicity evaluation. The EC50 data in Table 1 may be used to compare results of future studies, guide the course of further chemical fractionation (e.g., HP inorganics vs. HP organics), and determine the need for longer-term, more comprehensive bioassay of synfuel by-product waters.

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