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Determination of Petroleum Hydrocarbon Toxicity with Microtox®

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Groundwater is becoming increasingly contaminated with petroleum hydrocarbons as a result of spills and leaking underground storage tanks. In many cases, in situ bioremediation is being considered or used for the spill remediation. Knowledge of the toxicity before and after such treatment would be of value to the treatment specialist by providing a means of evaluating the effectiveness of the remediation effort. Tests which are available for assessing the toxicity of contaminants usually use aquatic organisms. Such tests, however, are time consuming and require test organisms which are difficult to maintain in the laboratory. The Microtox system, which uses luminescent bacteria as bioassay organisms (Bulich 1979), was examined as a means for the rapid analysis of toxicity of petroleum fuels and components. Other investigators have used Microtox^R for environmental samples and hydrocarbon contaminants (Atkinson et al. 1985, Chang et al. 1981, Ribo et al. 1983, Kaiser 1984, Thomas et al. 1986).

Toxicity tests are usually conducted with single chemicals. However, environmental contamination more commonly involves mixtures of chemicals rather than single compounds. For instance, petroleum fuels often consist of more than 300 components (Smith 1981). Results from toxicity tests involving combinations of chemicals may be different from those of single chemicals if synergistic or antagonistic interactions occur. The Microtox^R system has been shown to be a system which can assay the toxicity of pure compounds and complex effluents (Bulich et al. 1981).

The goal of this toxicity investigation was to determine the toxicity of petroleum fuels and fuel components which are likely to contaminate the subsurface environment. Various fuels and individual components, water soluble fractions of fuels, and soil leachates were tested for toxicity using the Microtox^R. The fuels used included diesel, unleaded gas, and the aviation fuels JP4 and JP5. Individual components were chosen from the following chemical groups: alkanes, cycloalkanes, alkenes, alkylbenzenes, and polynuclear aromatic hydrocarbons.

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MATERIALS AND METHODS

The Microtox^R toxicity analyzer (Microbics Corporation, Carlsbad, California) uses lyophilized marine bacteria (Photobacterium phosphorium) which emit light upon reconstitution (rehydration). The Microtox^R analyzer is equipped with a precision photometer for light measurements. When a potential toxicant is added to the reconstituted bacteria, the level of luminescence, relative to a reagent blank, is diminished in direct proportion to the concentration of the toxicant. During the testing procedure, the bacteria are exposed to four dilutions of the toxicant that have been osmotically adjusted to 2% sodium chloride. After an exposure period, the light output of each dilution is measured and compared with initial light output and reagent blanks. This determines the percent sample concentration which will produce a specified percent light reduction. Such a concentration is called the effective concentration (EC). Each effective concentration is listed with the percent light reduction created, e.g. EC50, for an effective concentration which produces a 50% light decrease. The EC50 corresponds to the LC50 of other bioassay organisms concentration.

All tests on the Microtox^R instrument were done at 15°C. The standard assay protocol followed for toxicity determination is described in the Microtox System Operation Manual (Beckman 1982). However, to determine the required primary dilution for the toxicant for the standard assay, initial screening runs were conducted for each sample. This protocol is similar to the one described in the Microtox System Operation Manual with the following modifications. For each screening run, 10 uL of the toxicant was added to 10 uL reconstituted bacteria in 500 uL of diluent from Microbics Corporation. The percent light reduction after 5 min with the toxicant was used to calculate the appropriate dilution series as follows: if the screening run showed less than 20% light reduction, then the toxicity run was done with no sample dilution and only osmotic adjustment (1800 uL toxicant, 200 uL Microtox Osmotic Adjusting Solution, or MOAS, 22% NaCl). If the screening run showed 20 to 60% light reduction, then the toxicity run was done with a 5:1 dilution and osmotic adjustment (40 uL toxicant, 50 uL MOAS and 1560 uL diluent). And if the screening run showed greater than 60% light reduction, the toxicant run was done with a 10:1 dilution and osmotic adjustment (2000 uL toxicant, 20 uL MOAS, 1780 uL diluent).

MOAS, diluent, lyophilized bacteria and reconstitution solution were purchased from Microbics Corporation and are described in the Microtox System operation Manual (Beckman 1982).

EC50 values are calculated using the data reduction program from Microbics Corporation (Bulich et al. 1981).

The fuels tested were diesel, unleaded gasoline, aviation gasoline 1130 (Av-gas 1130), JP4 and JP5. All fuels were obtained from the U.S. Navy. The fuel components tested were n-octane, cyclohexane, cyclohexene, benzene, toluene, o-xylene , and naphthalene. All fuel components were purchased from Sigma. The dilutions were vigorously mixed with a vortex mixer immediately prior to adding aliquots to the reaction cuvette.

The water soluble fraction (WSF) of the fuels and fuel components were prepared by modifying procedures of Klein and Jenkins (1983). One part of the fuel or component was diluted with nine parts of deionized water in a separatory funnel. The dilution was shaken vigorously for 5 min twice a day for three consecutive days, then left quiescent for at least 30 days at room temperature. Since the water

had a greater density than the hydrocarbons, 1-mL aliquots of the water containing WSF were easily collected from the exit port of the separatory funnel. WSFs were prepared for diesel, Av-gas 1130, unleaded gas, JP4, JP5, n-octane, cyclohexane, cyclohexene, benzene, toluene, o-xylene and naphthalene.

Glass columns (Cole-Parmer) were used to provide leachate for toxicity testing. Each column was filled with pure and clean sand (#16 borosilicate sand, washed three times with deionized water and autoclaved), or pure and clean sand with organic matter in the form of sawdust, or soil from a spill site were used to provide leachate for toxicity testing. Columns were 2 ft x 2 in. (ID) with Teflon plugs on each end. Peristaltic pumps (Masterflex 7553-3 0) with Tygon tubing were used to pump deionized water from 5-gal carboys through the columns at a rate of 1.5 mL/min from top to bottom of the column. Each column contained approximately 1.5 kg sand or soil. The different column treatments are given in Table 1. Once a column was filled with the substrate of choice, the fuel was added at the top and pumped through the system. Four controls were used, specifically, one with sand only, one with soil from a contaminated site, and two with two different percentages of added sawdust.

Table 1. Column Treatment

Column	Substrate	Fuel	Amount Fuel, mL
1	sand (wet)*	JP5	250
2	sand (dry)**	JP5	250
3	sand (dry)	control	—
4	sand (wet)	JP5	100
5	sand (dry)	JP5	100
6	sand (wet)	JP4	100
7	sand (wet)	unleaded gas	100
8	soil	contaminated	—
9	soil	control	—
10	soil	contaminated	—
11	sand, 3.3% org+ (wet)	JP5	100
12	sand, 0.3% org (wet)	JP5	100
13	sand, 3.3% org (wet)	control	—
14	sand, 0.3% org (wet)	control	—

*sand was wet with water when fuel was added
**sand was dry when fuel was added
+sawdust was the organic matter (org), which was rinsed and then autoclaved with deionized H_2O. After autoclaving, the water was decanted. This procedure was done twice.

Effluent samples were collected at pre-determined intervals in 4-mL vials which were sealed with Teflon-lined septa caps. Vials were filled leaving no headspace, then stored upside down until tested to prevent any offgassing of volatiles. Samples were refrigerated until tested, which was within seven days of collection.

The effect of volatilization of the toxicant on the calculated EC50 was examined. Toxicity analysis was conducted on samples at 2.5, 5, 7. 5, 10 and 15 min while sitting in the Microtox^R instrument at 15°C. J P5, benzene and n-octane were used as the toxicants.

RESULTS AND DISCUSSION

A major difficulty in working with petroleum fuels is that they are complex mixtures of hydrocarbons and contain both water soluble and non-soluble components. Tests with methanol dilutions of water soluble fractions yielded ambiguous results. Instead, consistent technique in the vortexing of samples was chosen as a means to evenly distribute the hydrocarbon in aqueous media. Immediately prior to any dilution, samples were vigorously vortexed for 5 sec. This procedure emulsified components sufficiently to ensure accurate serial dilutions both prior to testing and in the Microtox test procedure itself. Without vortexing, the hydrocarbons were clearly visible as layers on the water. With vortexing, the mixture was emulsified sufficiently to allow the pipette removal of a representative sample. For this reason, the units of parts per million in this paper are viewed as relative nominal values, not as absolute measurement of milligrams per liter. Just as "percent" is a ratio of relative value in units of hundred, in Tables 2, 3 and 4, the units are of relative value in units of million.

Table 2 lists the results of soil-column-effluent toxicity, which diminished over the four-week period examined. The greatest change in toxicity was within the first week; toxicity remained essentially unchanged after one week. Effluent toxicity was comparable to water soluble fraction toxicity.

Table 2. Soil Column Effluents

Toxicity in parts per thousand. All R-values were greater than or equal to 0.97. See note at bottom of table 3 regarding R-values

Col	Day 1	2	6	10	14	21	28
1	0.33	32	23	50	54	85	112
2	414	886	—	—	737	—	—
3	0.03	17	33	59	29	84	78
4	35	36	56	69	94	90	105
5	15	26	56	75	62	135	469
6	52	70	122	101	265	78	86
7	37	60	55	54	59	64	201
8	data inconclusive or inadequate sampling						
9	data inconclusive or inadequate sampling						
10	96	—	—	285	249	185	—
11	data inconclusive or inadequate sampling						
12	28	101	124	275	—	—	—
13	data inconclusive or inadequate sampling						
14	data inconclusive or inadequate sampling						

Many components of petroleum fuels are volatile, which could present a problem for toxicity testing. However, at the 15°C used in this investigation, volatilization of the fuel components did not significantly alter the results, as shown in Table 3. EC50 values were consistent over the test periods. Similar results were reported by Vasseur et al. (1986) with benzene.

Table 3. Volatility

Component	2.5 min EC50	5 min EC50	7.5 min EC50	10 min EC50
benzene	765	835	838	820
	1264	1443	1274	1009
	1788	1983	1774	1465
JP5	5	4	4	—
	9	7	5	4
	26	23	19	16
n-octane	1663	1196	1017	

For Table 2 and Table 3, the minimum R-value is >0.96
Below the value of 0.95, the data may become questionable

Five refined petroleum fuels and six hydrocarbon components of petroleum fuels were examined for toxicity, and the results are shown in Table 4. Stable EC50 values were obtained with 5-min runs for all toxicity tests. Extending the test time to 15-min did not significantly alter the EC 50 values; therefore, all results are reported for the 5 min test.

Table 4. EC50 Values* in ppt of Several Fuels and Components with Corresponding Water Soluble Fractions

	EC50 Value in ppt Fuel/Component	EC50 Value in ppt Water Soluble Fraction
JP5	0.015	269
Unleaded Gas	0.061	26
Diesel	0.074	120
JP4	0.087	41
o-xylene	0.200	21
Av-gas	0.250	57
Cyclohexene	0.323	32
Toluene	0.456	66
Cyclohexane	1.724	28
Benzene	1.999	33
n-octane	3.127	683

*EC50 is the effective concentration at which the light output is decreased by 50%

The difference between the toxicity of the water soluble fractions of each intact component and the toxicity of the intact components themselves indicates that either those components which are water soluble are not very toxic, or that very little of the fuel components partitioned into the water.

The Microtox system proved to be an effective and rapid means of assessing toxicity of the fuels, fuel components, water soluble fractions of these components, and of soil column effluents in these experiments. As many as five samples per hour were done, or as many as 25-40 per day, depending on operator fatigue. Vertebrate or invertebrate toxicity tests times are considerably longer, a factor which increases the attractiveness of the Microtox^R system as an efficient bioassay screening system.

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