A STUDY ON MICROBIAL ECOTOXICITY AND MUTAGENICITY OF 1-AMINOPYRENE AND 1-HYDROXYPYRENE

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) enter natural environment from a variety of sources, such as forest fires, volcanic eruption, incomplete combustion of fuel and other materials, industrial effluents and seepage or spillage of petrochemical products (Manahan 2000; Johnson et al. 1985; Grimmer and Pott 1983; Hites, Laflamme, and Windsor Jr. 1980). PAHs are toxic and many of them are known carcinogens and mutagens (IARC 1983; UDHHS 1995). They are also listed as the U.S. Environmental Protection Agency priority pollutants (Keith and Telliard 1979). Photolysis transforms many organic pollutants to less toxic or harmless products. However, in the presence of natural or simulated sunlight, many PAHs become more toxic to aquatic organisms at concentrations that are well below the PAHs aqueous solubility limits (Landrum et al. 1987; McConkey et al. 1997; Hatch and Burton Jr. 1998). This may occur via photosensitization reactions (e.g., production of singlet oxygen) (Ankley et al. 1994) and photooxidation of the compounds to more toxic products such as diols and quinnones (David and Boule 1993). Since the water solubility of PAHs increases after photolysis, aquatic organisms may be exposed to higher concentrations of the photoproducts than the parent PAHs. Therefore, the photoinduced toxicity may present a greater risk to aquatic organisms and ultimately humans, because of direct exposure and/or bioaccumulation through food chains.

Biological monitoring of PAHs exposure has been mostly performed by measuring metabolites of PAHs in urine. 1-Hydroxypyrene (1-HP), a major metabolite of pyrene, has been proposed and used as a biological marker for monitoring occupational and environmental exposure to PAHs (Jongeneelen et al. 1986; Jongeneelen 1994; Angerer, Mannschreck, and Gündel 1997; Merlo et al. 1998). Recently, 1-HP was reported to be acutely toxic and genotoxic (Hauser, Schrader, and Bahadir 1997) and appeared to be more toxic to the test organisms than its parent compound-pyrene (Lambert, Kremer and Anke 1995). 1-nitropyrene (1-NP), a known mammalian and bacterial mutagen and a tumorigen in animals, is one of the most abundant nitro-polycyclic aromatic hydrocarbons in the environment (Herreno-Saenz 1995). 1-Aminopyrene (1-AP), a reduction product of (1-NP), has been reported to be a major metabolite during biotransformation of 1-NP by microflora in natural environment and in the guts of animals and humans (Tahara et al. 1995; Kinouchi et al. 1982; El-Bayoumy et al. 1983). Under UVA irradiation, both 1-AP and 1-HP have been shown to cause light-induced DNA single stranded cleavage (Dong et al. 2000).

Toxic metals are generated from both natural anthropogenic sources and become common contaminants in natural waters (Kuo and Genthner 1996; Moore and Ramamoorthy 1984). Their presence may significantly affect potentially important microbial transformation of organic chemicals in natural environment (Said and Lewis 1991; Kuo and Genthner 1996). The complexation of metal ions with organic ligands or species is a subject of great environmental relevance, because the availability and toxicity of both metals and organic chemicals to organisms strongly depend on their speciation (Sunda and Huntsman 1995; Xue and Sigg 1999). Copper and manganese were chosen in this study to assess their effect on PAHs mutagenicity, due to their importance in a variety of aquatic environments (Lasier, Winger, and Bogenrieder 2000; Markwiese and Colberg 2000).

Bacterial *in vitro* assays have emerged as important ecotoxicological screening tools to monitor the hazrads of chemical contaminants in natural environment. In this study, the possible photo-induced genotoxicity and ecotoxicity of 1-HP and 1-AP were measured with a variety of bacterial bioassays, including viable bacteria counting, bacterial direct counting, heterotrophic mineralization of glucose and Mutatox Test. Mutatox determines the genotoxicity of chemical

contaminants in liquid phase from environmental samples by measuring the changes of light emitted by a dark mutant strain of bioluminescent bacteria *Vibrio fischeri* strain –M169 (AXZUR). The test has been used as a rapid screening tool for detecting the mutagenicity of DNA-damaging substances (Sun and Stahr 1993; Johnson 1998). Glucose mineralization reflects bacterial heterotrophic activities, while spread plate counting and direct counting measure viability of heterotrophic bacterial populations and total number of bacterial populations respectively (Hwang, McCullum, and Slaughter 1998).

MATERIALS AND METHODS

Surface freshwater samples were collected from the Ross Barnett Reservoir, Ridgeland, Mississippi. The pH of the water samples ranged from 7.0 to 7.5, and the temperature ranged from 15°C to 28°C. All chemicals used were of analytical or HPLC grade. 1-AP and 1-HP (Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in dimethyl sulfoxide (DMSO; HPLC grade, Fisher Scientific) with sodium phosphate buffer, added to 50 mL of water sample in 150-mL quartz flasks (GM Associates, Inc., Oakland, California) and incubated in triplicate. Degradation of the PAHs was measured by quantifying disappearance of the parent compounds (transformation rate). At the termination of incubation, aliquots of water samples were filtered (0.45 µm; Schleicher and Schuell, Keene, New Hampshire) and the quantification was performed by using a Waters 996 HPLC system. The system is equipped with a photodiode array detector and a reverse-phase Supelcosil LC-8 column (Supelco Co., Bellefonte, Pennsylvania), under isocratic condition with acetonitrile-water (50:50, v/v) at a flow rate of 1 mL/min. Detection wavelength was set at 254 nm. The final concentration of PAHs ranged from 0.8-10 µM. The quartz flasks allowed 85% and 100% transmission of light at wavelength of 285 and $\geq$ 300 nm, respectively. The flasks were suspended in an outdoor tub which contained continuous running water for maintaining the water temperature at 27±3°C. The water level in the flask was about 3 cm below the surface of the cooling water. A research radiometer (model IL 1700, International Light Inc., Newburyport, Massachusetts) was placed beside the tub to measure UV irradiance. The dark exposure group consisted of flasks being wrapped with aluminum foil. Killed control was accomplished by autoclaving at 250°F at 15 psi for 20 minutes. All bottles were capped with silicon stoppers. Difference in experimental data between different treatment groups was determined with student-t-test ($p \leq 0.05$).

In a separate experiment, the eco-toxic effect of the PAHs and their photoproducts on bacterial assemblages in reservoir water was measured with spread plate counting (nutrient agar) and heterotrophic mineralization of ^{14}C-UL–D-glucose (S.A.: 265mCi/mmol; 99% purity; Moravek Biochemicals, Brea, California). PAHs were added to 25 mL of autoclaved water in quartz flasks to make the final concentration 1.6-16 µM. The water samples containing the PAHs were exposed to irradiation of a UVA lamp (Dong et al. 2000) indoors or midday sunlight outdoors for up to 1.5 hr. Equal amount of fresh unamended water was then added to the photo-exposed water to make the final concentration 0.8-8 µM. Water samples were incubated in darkness for 1 day at 25°C in the laboratory. Treatments included: darkness & light controls (no PAHs), darkness & light exposures (exposure to PAHs in darkness or light). Radiolabeled glucose (final concentration 1 µg/L) dissolved in ethanol was added to the water samples after the pre-exposure and incubated at 25°C in darkness for 1 hr. At the termination of the incubation, 0.5 mL of 2N H_2SO_4 was added to the sample, and the $^{14}CO_2$ produced was trapped with 2-phenylethylamine-soaked filter papers (Hwang and Maloney 1996). The radioactivity of the filter paper was measured with liquid scintillation spectrometry (Packard Instrument; model TR 1600).

Genotoxic effect of 1-AP and 1-HP (1 mM) was measured with the Mutatox test according to the Mutatox genotoxicity test (Microbics Mutatox Manual 1995). Cu^{2+} (100 µM) and Mn^{2+} (5 mM) were added in the form of cupric nitrate and manganese chloride to assess their effect on PAHs mutagenicity test. The mutagenic response of the reconstituted bacteria with serial 2 fold-dilutions was determined after 16, 20 and 24 hr of incubation at 27°C. Activation with S-9 enzymes received 1 hr incubation at 35°C. Light emitted from the media control, solvent (4% DMSO) control, positive controls (phenol for direct acting and bezo[a]pyrene for S-9 acting) and samples receiving different treatments were measured. Positive mutagenic agents are defined as those samples which induce, increase light levels to at least two times the average control light reading in at least two consecutive sample dilute (concentration) cuvettes from two test results.

RESULTS AND DISCUSSION

Rates of photochemical and microbial degradation of 1-AP and 1-HP (8 μM) were monitored with HPLC analysis. Photolysis half-lives of the compounds were computed to compare the relative toxicity and used to determine the photolysis treatment periods for conducting ecotoxicity and mutagenicity measurements. After 90 minutes of solar photolysis, 1-AP was transformed by 69%, 92% and 98% in darkness (microbial degradation only), photolysis only, and combined photolysis and microbial degradation, respectively. 1-HP was transformed by 0%, 80% and 100%, respectively after the corresponding treatments. Therefore, 1-HP was more resistant to microbial degradation and synergistic interaction between photolysis and microbial degradation for the PAHs was observed. After 1 day of exposure to the 90- minute photoproducts (i.e., photoproducts generated after 90 minutes of photolysis in outdoor irradiation) of 1-AP and 1-HP, viability counts of heterotrophic bacterial assemblages were significantly enhanced by comparison to the light control group (Figure 1). The enhancement was highest (4.75 folds) with exposure to the 1-HP outdoor photoproducts. However, no effect was seen for the exposure to the parent compounds. Therefore, the net response of the bacterial assemblages is utilizing the mixture of the photoproducts as the growth substrates. This was also reported for photolysis and bacterial transformation of other organic contaminants (Hwang, McCullum, and Slaughter 1998). Concurrent measurement of the total bacterial numbers indicated the similar response, with the exception that bacterial number with exposure to 1-HP parent compound was only 15.6% of that exposing to 1-AP parent compound (results not shown). Apparently, 1-HP is more toxic to the overall microbial community than 1-AP. The effect of the test PAHs and their photoproducts on bacterial heterotrophic activity of glucose mineralization is shown in Figure 2. With comparison to the control groups, microbial mineralization activity of D-glucose was inhibited by up to 64% and 75% after exposing to PAHs photoproducts and the parent compounds, respectively. Based on the results in Figures 1 and 2, we speculate that the inhibition on heterotrophic activity by the photoproducts was due to substrate competition between glucose and mixture of photoproducts (Hwang, McCullum, and Slaughter 1998), while the parent compounds of the test PAHs imposed inhibition on bacterial metabolic activity by virtually toxicity.

Mutatox test of 1-AP and 1-HP was conducted for both direct acting (in direct growth medium) and pro-mutagens (growth medium containing S-9 enzymes). Both test compounds exhibited positive responses in direct medium. However, only 1-AP also scored positive response to the test in S-9 medium. Therefore, all other Mutatox tests for various treatment structures were conducted with direct medium. 1-HP was reported by Hauser, Schrader, and Bahadir (1997) to respond positively in both direct medium and S-9 medium. The discrepancy could be due to the difference in the substrate concentration (i.e., 1.4- 11.5 μM in their system vs. 0.0625-0.8 μM in our system) and solvent concentration (2% DMSO in their system vs. 4% DMSO in our system) used in the tests. Results of the Mutatox test on 1-AP and 1-AP (10 μM), photoproducts of 1-AP and 1-HP, mixtures of Cu^{2+}, Mn^{2+} and the parent compounds or photoproducts of 1-AP and 1-HP are shown in Table 1. As indicated in the Table, 1-AP and 1-HP are both mutagenic, with the lowest observed effective concentration (LOEC) of 1-AP and 1-HP being 1.25 μM and 0.0625 μM, respectively. Photo-transformation by solar irradiation (for 90 minuites) changed the light reading of both 1-AP and 1-HP significantly. Specifically, photo-transformation lowered the LOEC of 1-AP and increased the light reading at lower concentration ranges (i.e., 1.25-2.5 μM), while the effect on 1-HP was the opposite. 1-Nitropyrene, a potent bacterial and mammalian mutagen (Manning, Cerniglia, and Fedferle 1986), was reported to derive from 1-AP after photo-transformation by near ultraviolet light (Okinaka et al. 1986) and appeared to be more mutagenic than 1-AP (Kinouchi et al. 1982). Identification of the photoproducts of 1-AP and 1-HP is underway.

The environmental importance of complexation derives from the fact that the complexed chemical species will behave differently from the uncomplexed forms, as well as differently from other complexes.. Chemical properties, such as solubility, attenuation behavior on soils, bioconcentration factors, and toxicity are modified through complexation (Lyman 1995). Based on the reported average concentrations in aquatic environment (Stumm and Morgan 1981), manganese and copper ions were added during the Mutatox test at starting concentration of 5 mM and 100 μM, respectively, to study their effect on 1-AP and 1-HP mutagenicity. Mn^{2+} (5 mM to 312.5 μM) and Cu^{2+} (100 to 6.25 μM) are not mutagenic themselves (Table 1). However, Mn^{2+} (5 mM)

increases the light emitting of 1-HP and decreases the light emitting of 1-HP if irradiated with 1-HP or when added to 1-HP photoproducts. Mn^{2+} eliminates 1-AP & 1-HP photoproducts mutagenicity. Cu^{2+} (100 μM) decreases the light emitting of 1-AP and 1-HP when irradiated with either of them together. Metal ions were reported to cause oxidative DNA damage. For example, copper can induce single-base substitutions. Cu (II) binds preferentially to guanine residues and induces in combination with H_2O_2 oxidative damage at sites of two or more adjacent guanine residues (Sagripanti and Kramer 1989). In the presence of physiological concentrations of bicarbonate, manganese (II) forms a coordinate complex with amino acids and catalyzes the direct transfer of electrons from H_2O_2 to one of the bound amino acids. This will result in destruction of a molecule of amino acid with concomitant sparing of more important cellular targets. This novel pathway of H_2O_2 detoxification is used to explain that addition of Mn^{2+} to culture cells or experimental animals in the presence of amino acids and bicarbonate buffer affords strong protection against H_2O_2–induced injury (Varani et al. 1991). The changes in PAHs mutagenicity by metal addition are speculated to be due to the interactions (e.g., complexation) between the metal ions and the organic species of the 1-AP, 1-HP and/or their photoproducts.

In summary, photo-transformation changes the ecotoxicity and mutagenicity of 1-AP and 1-HP to aquatic bacterial assembalges and *Vibrio fischeri* strain –M169 strain of Mutatox test. Our finding of bacterial capability of utilizing the test PAHs photoproducts confirms the potential of using photolysis and biodegradation for remediating PAHs contamination. The modified genotoxic potential of the test PAHs by photolysis and metal ions indicates that the disappearance of PAHs in the environment does not always render them less ecologically hazardous. Close monitoring on the toxic or mutagenic properties of the complexed compounds and intermediates should be a necessary task for health regulatory professions.

ACKNOWLEDGMENTS

This research was supported by: (1) Department of Energy, under contract #DE-AC03-76SF00098 (to LBL); subcontract #6482515 to JSU; (2) NIH-RCMI 1G12RR12459-01 and NIH-MBRS S06GM08047 (to JSU); (3) Department of Energy DE-FG02-97ER62451 to UGA with subcontract #RR100-239/4891914 to JSU; and (4) The Army HPC Research Center under the auspices of the Department of the Army. Army Research Laboratory. The content does not necessarily reflect the position or policy of the government, and no official endorsement should be inferred.

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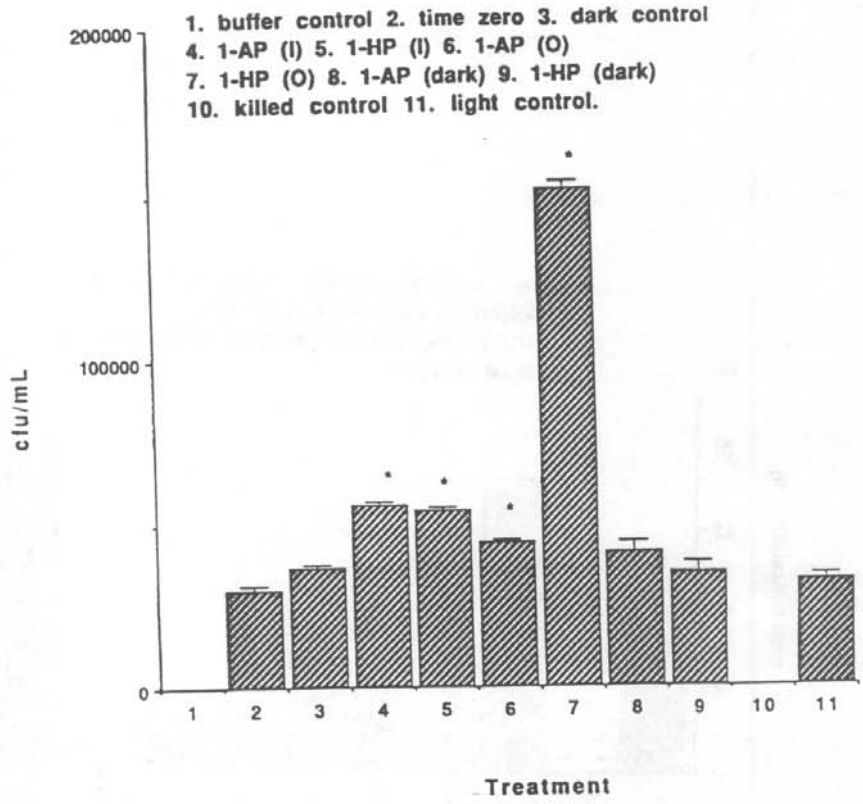

Figure 1. The effect of the test PAHs on bacterial viability (1 day exposure, Nov. 1999). I: irradiated by an indoor UVA lamp; O: irradiated by midday sunlight. *: significantly different from the control group ($p \leq 0.05$; t-test).

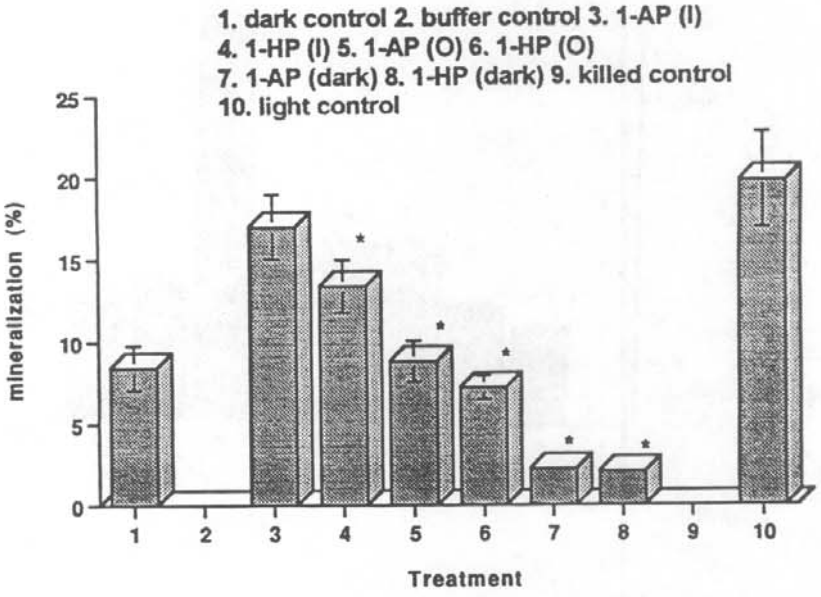

Figure 2. The effect of the test PAHs on bacterial mineralization of D-glucose (1 day exposure, Nov. 1999). I: irradiated by an indoor UVA lamp; O: irradiated by midday sunlight. *: significantly different from the control group ($p \leq 0.05$; t-test).

Table 1. Mutatox Test Reading Results*.

Med. Contr.	Positive Contr	1-AP	1-HP	Mn^{2+}	Cu^{2+}	1-AP + Mn^{2+}	1-HP + Mn^{2+}	1-AP irrad.	1-HP irrad.
11	1215	14	1	11	10	1	1	4	1483
10	11938	17646	62	8	8	1	1	46	1652
16	381	793	12310	6	7	386	1	1756	376
16	100	89	719	7	8	40	736	628	58
15	28	24	171	9	10	4	233	68	8

1-AP irrad. +Mn^{2+}	1-HP irrad. +Mn^{2+}	1-AP irrad. + Cu^{2+}	1-HP irrad. + Cu^{2+}	(1-AP + Cu^{2+}) irrad.	(1-HP + Cu^{2+}) irrad.	(1-AP + Mn^{2+}) irrad.	(1-HP + Mn^{2+}) irrad.	PP of 1-AP	PP of 1-HP
1	1	1	1	1	1	1	1	1	15
1	1	1	1	1	1	1	1	1	166
1	653	1	46	105	631	1	1	1	249
45	333	720	11	44	189	20	88	2520	102
13	14	1174	5	7	17	4	39	267	22

*PP: Photoproducts; Med.: medium; Contr: control; irrad.: irradiated.

The concentrations of the chemicals added (decreasing with 2-fold dilutions from the top to the bottom of each column): 1-AP and 1-HP: 10 µM; Mn^{2+}: 5 mM; Cu^{2+}: 100 µM.

All irradiated samples were exposed to midday sunlight for 1.5 hr before conducting the Mutatox test.