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Effect of River Humic Acid on 1-Aminopyrene Ecotoxicity in a Dynamic Solar Photolysis Process

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Polycyclic aromatic hydrocarbons (PAHs), a group of organic compounds consisting of three or more fused benzene rings, are listed as U.S. Environmental Protection Agency priority pollutants (Keith and Telliard 1979) because some of them are known mutagens or carcinogens (IARC 1983; ATSDR 1995). 1-Aminopyrene (1-AP), an amino-PAH, was reported as a major metabolite of 1-nitropyrene transformed by microflora in natural environments and in living tissues (Manning et al. 1986). Under UV-A irradiation, 1-AP has been shown to cause light-induced DNA single-strand cleavage (Dong et al. 2000).

In the presence of natural or simulated sunlight, photo-induced toxicity of PAHs has been reported to occur to aquatic organisms and may impact environmental health of aquatic habitats, especially those in highly contaminated areas with shallow or clear water (McConkey et al. 1997; Hatch and Burton 1998). Since the water solubility of PAHs usually increases after photolysis, aquatic organisms may be affected more by the photoproducts than the parent PAHs. Therefore, photo-induced toxicity may present a greater hazard to aquatic organisms and ultimately humans, because of direct exposure and/or bioaccumulation through food chains. Indeed, this phenomenon (i.e., photo-induced toxicity of PAHs) should be considered relative to the promulgation of adequately protective water quality criteria.

Humic substances (HS) are ubiquitous in the natural environment. They are formed during the microbiological and abiotic transformations of animal and plant materials. Among them, humic acid (HA) is the fraction of HS that is not soluble in water under acidic conditions (< pH 2), but is soluble at higher pHs. The typical average molecular weight is 2,000 to 3,000 (Matthews et al. 1995). HA in aquatic ecosystems has been shown to influence the bioavailability, toxicity and fate of organic xenobiotics including PAHs. For example, Matthews et al. (1995), Wu et al. (1999) and Weinstein and Oris (1999) reported that HS or HA could interact with test organic contaminants and cause a significant decrease in their toxicities. The extent of alteration, however, depends on the concentration and source of dissolved humic substances. Conversely, HS were also reported to increase the toxicity of several organic contaminants to the test organism (Stewart 1984).

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It is known the intensity of global UV-irradiation is increasing due to ozone depletion in the stratosphere. Therefore, it is necessary to understand the UV-mediated chemical processes occurring in natural humic waters and the influence of these processes on the fate and effects of PAHs in the natural aquatic environment. Since bacteria are usually the main degraders of organic contaminants, they are used as the surrogate for assessing the environmental impact of 1-AP in this study. The main objective of this study is to compare the effect of a HA on the toxicity of 1-AP to natural aquatic bacterial assemblages in the presence or absence of solar irradiation.

Two bacterial bioassays, plate count (spread plate method) and measurement of heterotrophic mineralization of ^{14}C-glucose, were used to determine the toxicity of 1-AP to bacterial assemblages in river water. Plate count measures viability of selective heterotrophic bacteria and heterotrophic mineralization reflects general metabolic activity of all bacterial assemblages, respectively (Hwang et al. 2000). Experimental parameters include photolysis process (PP), time course of exposure (TC), concentration of HA and presence of 1-AP. Experimental data were analyzed with SAS (Cary, NC, USA) to check the interactions between and among the various parameters involved in the experimentation. In our previous study (Balarezo et al. 2002), a river water sample containing 1-AP and/or HA was exposed to solar irradiation to generate photoproducts first before the natural bacterial assemblages were inoculated. To contrast with that static ecotoxicity research, in this study the bacterial assemblages were enclosed in the test river water at time zero. Therefore, the research results herein reflect the integration of the dynamic interactions between 1-AP, HA, bacterial assemblages and UV-irradiation.

MATERIALS AND METHODS

Standard River HA (Suwannee River; 1996 batch) was obtained from the International Humic Substances Society (IHSS; St. Paul, MN). Elemental analysis data indicate that % of C:H:O:N:S:P = 52.55:4.40:42:53:1.19:0.58:<0.01. Concentrations of total carbohydrates and amino acids are 16 μg/mg and 89 nmol/mg, respectively. HA stock aqueous solution was prepared at 2.5 g/L. Considering the environmental concentration of lightweight PAH and its aqueous solubility, 1-AP (Aldrich Chemical Co., Milwaukee, WI) was prepared at 10 μM in dimethylsulfoxide (DMSO, 4%; HPLC grade, Fisher Scientific). ^{14}C-D-glucose was dissolved in 95% ethanol (S.A: 265 mCi/mmol; Moravek Biochemicals, Brea, CA) and about 10^4 disintegration per minute (dpm) was added to each sample. Fresh river water was collected from the Ross Barnett Reservoir, Ridgeland, Mississippi. Water samples were processed within one hour of collection. The pH of the water samples ranged between 7.0 and 7.5. The following physical and chemical properties were determined with a PASTEL UV Spectrophotometer (ESCOMAN, France): total organic carbon content (TOC): 30 to 60 ppm; total suspended solids: 152-162 mg/L ; nitrates: < 1.0 mg/L; biochemical oxygen demand (BOD): 34.5-36.0 mg/L; chemical oxygen demand (COD): 16.0-16.8 mg/L; and surfactants: 3.2-3.8 mg/L. The ambient concentration of PAHs was not measured in this study.

In March 2002, 49 mL of fresh river water was added to 150 mL Erlenmeyer quartz flasks (Quartz Scientific, Fairport Harbor, Ohio) and incubated without shaking. Four groups of flasks (triplicate samples for each group) were prepared by adding HA solutions to reach three different nominal concentrations: 0, 20, and 60 ppm. 1-AP was added to two of the four groups to reach a final nominal concentration of 10 μM. Afterwards they were incubated outdoors in a tub under natural sunlight at noontime during sunny days at Jackson, Mississippi (32^0 19' N; 90^0 5' W). The incubation temperature for all flasks in the tub was maintained at 27±3°C with tap water flowing from a faucet in the lab. The surface water level in the flask was about 3 cm below the surface of the cooling tap water. The irradiation was not significantly attenuated by the cooling tap water and the incubating flasks were assumed to receive the same amount of irradiation and maintained at the same temperature. A research radiometer (model IL 1700, International Light Inc., Newburyport, MA) was placed beside the tub to measure UV-A irradiation. The average UV-A light intensity of the solar irradiation was 2.5 ± 0.3 mJ/cm^2.

Two groups containing 0 μM or 10 μM 1-AP were incubated for a period of 18 minutes or 90 minutes, respectively. Therefore, the experimental parameters included HA, 1-AP, time course (TC) of light exposure, and photolysis process (PP). Darkness incubation was conducted by wrapping the flasks with a sheet of aluminum foil. The influence of HA on 1-AP toxicity during the solar photolysis process was measured with two methods: (a) bacteria plate count and (b) heterotrophic mineralization of ^{14}C-D-glucose. A killed control was accomplished by autoclaving at 121.1^0C for 15 minutes. Aliquots of 1 mL of all replicates were used to determine the heterotrophic bacteria counting with the spread plate method using nutrient agar (Balarezo et al. 2002). Plates were incubated in a growth chamber for 24 hours at 25°C before counting the viable colony forming units (cfu). After sampling for spread plate counting, radiolabeled ^{14}C-D-glucose solution (~1 μg/L) was added into each sample. Heterotrophic mineralization of ^{14}C-D-glucose was measured with the same procedure described in Balarezo et al. (2002).

To detect the interactions between and among different parameters in the experiment, statistical analysis of results was conducted by factorial arrangement of treatments in a complete randomized design using General Linear Model by SAS. LSMEANS were used to separate means or combinations of means. Significance of the treatment effect or the interactions between experimental parameters was determined at $p \leq 0.05$.

RESULTS AND DISCUSSION

Time-course exposure to sunlight irradiation was conducted to determine whether there was any time-dependent toxicity effect. The exposure intervals (18 minutes and 90 minutes) were determined according to chemical analysis with HPLC (Balarezo et al. 2002). Half or all of the 1-AP parent compound was photochemically transformed after 18 or 90 minutes, respectively. The data for the colony forming units (cfu/mL) of viable heterotrophic bacteria under different

treatment structures are listed in Table 1. SAS was used to analyze the treatment effect of individual parameter of photolysis process (PP), time course of exposure (TC), concentration of HA (HA) and presence of 1-AP (1-AP), plus two-way, three-way and four-way interactions among the four parameters. The p values of all treatment sources/interactions are listed as following: HA (0.015), AP (< 0.0001), TC (0.306). PP (0.0003), HA*AP (0.118), HA*TC (0.288), HA*PP (0.771), AP*TC (0.051), AP*PP (< 0.0001), TC*PP (0.262), HA*AP*TC (0.107), HA*AP*PP (0.092), HA*TC*PP (0.197), AP*TC*PP (0.746) and HA*AP*TC*PP (0.732). In short, viability count was significantly affected by (1) the individual treatment with HA, 1-AP or PP, and (2) 1-AP*PP: a combination of treatments with 1-AP and photolysis process (PP). Therefore, exposure to solar irradiation (PP) and presence of 1-aminopyrene interacted with each other and significantly affected viability count data (1-AP*PP; $p < 0.05$). Significant interactions in light exposure groups may be different from those in darkness exposure groups. However, SAS analysis results indicate the overall trends with respect to specific parameters.

Table 1. Colony forming units per milliliter (cfu/mL)x10^3 (±STD) of viable heterotrophic bacteria after exposure to 1-AP in sunlight and darkness for 18 or 90 minutes in the presence or absence of HA.

Treatments:	cfu/mL x 10^3 (± STD)			
HA/1-AP/TC[a]	Light		Darkness	
0-0-18	9.3	± 2.2	5.5	± 0.4
20-0-18	10.2	± 0.2	5.6	± 1.8
60-0-18	10.4	± 0.3	7.6	± 0.7
0-10-18	1.3	± 0.3	7.5	± 0.9
20-10-18	1.0	± 0.7	7.3	± 0.5
60-10-18	2.8	± 0.2	8.3	± 0.9
0-0-90	8.8	± 1.4	5.7	± 0.7
20-0-90	13.6	± 4.0	8.6	± 1.1
60-0-90	10.1	± 0.6	8.9	± 2.1
0-10-90	0.53	± 0.05	7.5	± 0.9
20-10-90	0.9	± 0.5	7.3	± 0.5
60-10-90	0.8	± 0.4	8.3	± 0.9

[a]HA: ppm, 1-AP: μM, TC: time course (minutes)

Concentration of total organic carbon (TOC) can serve as an indicator of potential energy for bacteria; however, this information was used as the reference for adding HA at the nominal concentration 0-60 ppm. HA only (in the absence of 1-AP) under both light and dark incubations generally caused enhancement of viable bacteria count. The degree of enhancement generally increases with the HA

concentration. Solubility of organic matter by HA was reported to enhance substrate bioavailability to microorganisms. Increases in viable bacteria count in the groups containing only HA may occur via this mechanism (Holman et al. 2002). In addition, bacterial viability of light exposure control groups (e.g., 0-0-18 and 0-0-90) was higher than the darkness exposure control counterparts. The difference between the two control groups, however, decreases when TC increases from 18 to 90 minutes. This is speculated to result from depletion of dissolved nutrients in the incubation solutions.

In the presence of 1-AP (e.g., 0-10-18 and 0-10-90), the viability counts in the light exposure groups were reduced by 73% to 94% (Table 1) in comparison to the relevant control groups (e.g., 60-0-18 vs. 60-10-18 and 0-0-90 vs. 0-10-90). Concurrent measurement of heterotrophic activity also indicates a similar trend (Table 2). The decrease in bacterial viability and heterotrophic activity obviously reflects photo-induced toxicity of 1-AP, possibly through the mechanism of DNA damages caused by sunlight irradiation (Balarezo et al. 2002; Dong et al. 2000; Okinaka et al. 1986).

In comparison to the relevant 1-AP control groups, viable bacteria count generally decreased when both HA and 1-AP were present. For example, viable count decreased from $(10.4 \pm 0.3) \times 10^3$ to $(2.8 \pm 0.2) \times 10^3$ cfu/mL between the 60-0-18 group vs. 60-10-18 group in light (Table 1). A larger decrease was observed under two conditions: longer exposure time (i.e., 90 minutes) and light exposure (e.g., 60-10-18 vs. 60-0-18 and 60-10-90 vs. 60-0-90). This is in agreement with our previous report (Balarezo et al. 2002) in which the toxicity of 1-AP photoproducts and increased bioavailability of the photoproducts by HA were speculated to contribute to the increased inhibition of viable count. In a relevant study in our laboratory, the identified 1-AP photoproducts include 1-hydroxyaminopyrene, 1-nitrosopyrene, 1-NO_2P, 1-amino-x-hydroxypyrene, and three covalent dimmers (unpublished data). Among them, 1-NO_2P and 1-nitrosopyrene are genotoxic and 1-hydroxyaminopyrene can react with DNA to form covalent adducts (Fu et al. 1997; Fu and Herreno-Saenz 1999; Gu et al. 1999).

SAS analysis indicates the following p values for the following treatment sources/interactions: HA (< 0.0001), AP (< 0.0001), TC (< 0.0001). PP (< 0.0001), HA*AP (< 0.0001), HA*TC (0.0009), HA*PP (< 0.0001), AP*TC (0.525), AP*PP (0.730), TC*PP (0.0005), HA*AP*TC (0.800), HA*AP*PP (0.746), HA*TC*PP (< 0.0001), AP*TC*PP (0.348) and HA*AP*TC*PP (0.145). Therefore, bacterial heterotrophic mineralization activity of D-glucose was significantly affected by the following individual or combination of treatments:

HA	1-AP	TC	PP
HA*1-AP	HA*TC	HA*PP	TC*PP
HA*TC*PP			

Table 2 shows the % of ^{14}C-D-glucose mineralized by heterotrophic bacteria under the same treatment conditions as those in Table 1. Although significant two-way and three-way interactions were seen in this analysis, cautious

interpretation of the data is needed as bacterial heterotrophic activity can be affected by a variety of factors such as toxicity, interaction between the dissolved substrates in river water, substrate competition, and bioavailability (Hwang et al. 2000). For example, inhibition on bacterial heterotrophic activity was observed in the groups exposed to HA (e.g., 0-0-18 vs. 0-10-18) in light and in darkness (Table 2). Concurrent measurement of viability count, however, indicates the opposite trend (Table 1). Therefore, the inhibition of bacterial heterotrophic activity is most likely due to decreased glucose bioavailability by HA binding instead of toxicity factor.

Table 2. Percent of ^{14}C-D-glucose mineralized (± STD) by heterotrophic bacteria after exposure to 1-AP in sunlight and darkness for 18 or 90 minutes in the presence or absence of HA.

Treatments:	% DPM (± STD)			
HA/1-AP/TC[a]	Light		Darkness	
0-0-18	15.7	± 1.1	15.5	± 3.1
20-0-18	1.2	± 0.1	1.3	± 0.2
60-0-18	0.42	± 0.06	0.48	± 0.04
0-10-18	4.7	± 1.3	6.9	± 2.1
20-10-18	0.23	± 0.03	0.61	± 0.04
60-10-18	0.04	± 0.03	0.18	± 0.06
0-0-90	14.6	± 1.0	21.0	± 1.7
20-0-90	2.1	± 0.1	2.0	± 0.3
60-0-90	1.1	± 0.1	0.8	± 0.2
0-10-90	5.4	± 0.2	9.9	± 1.5
20-10-90	0.29	± 0.03	0.93	± 0.09
60-10-90	0.11	± 0.01	0.3	± 0.2

[a]HA: ppm, 1-AP: μM, TC: time course (minutes); Killed control: 0.21 ± 0.06

In comparison to the corresponding control group (e.g., 60-0-18 vs. 60-10-18), microbial heterotrophic activity was significantly inhibited in the groups receiving 10 μM of 1-AP in the absence or presence of HA (20-60 ppm) in both light and darkness. The inhibition for the groups in darkness is speculated to occur as the result of HA binding or substrate competition, as the data for viability count reflect the opposite trend (Table 1). Inhibition for the groups in light, however, must occur as the result of photo-induced toxicity of 1-AP. 1-AP induces its direct-acting mutagenicity in the Ames *Salmonella* strain TA98 plating assay after exposure to an artificial source of near UV light (Okinaka et al. 1986). In our laboratory with the Mutatox® Test, the mutagenic response of 1-AP increased after exposure to solar irradiation. 1-nitropyrene, a known potent mammalian mutagenic compound (Herreno-Saenz et al. 1995) was detected in the mixture of

1-AP photoproducts (unpublished data). Therefore, our finding was consistent with the report by Okinaka et al. (1986).

In summary, treatment with HA only under both light and dark incubations generally caused enhancement of viable bacteria count. Inhibition of bacterial heterotrophic activity was observed in the groups exposed to HA in both light and dark conditions. However, the inhibition was possibly caused by reduced glucose availability instead of a toxicity factor. Bacterial heterotrophic activity was significantly inhibited in the groups receiving 1-AP in the presence or absence of HA in light and in darkness. The inhibition could have been caused by HA binding or uptake competition from other organic substrates, as the data of viability count reflect the opposite trend. Photo-induced toxicity of 1-AP was observed in the groups receiving 1-AP in light, as indicated by both viability count and heterotrophic activity measurements. Photo-induced toxicity is caused and affected by the dynamic interactions between light irradiation, PAH, HA and target cellular materials. The endpoint response of this study reflected the net results of the dynamic interactions between and among the physical, chemical and biological parameters aforementioned. Statistical analysis of the experimental data can detect significant interactions between various parameters. Ecological interpretation of the interactions, however, has to depend upon information from multiple entities within the environment. This study serves as an attempt to better understand the complicated interactions among multiple environmental parameters in order to define the realistic hazards of PAHs released into natural aquatic ecosystems.

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