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Effect of Methyl *tert*-Butyl Ether on the Bioconcentration and Photoinduced Toxicity of Fluoranthene in Fathead Minnow Larvae (*Pimephales promelas*)

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The use of methyl *tert*-butyl ether (MTBE) in reformulated gasoline in motorized watercraft can lead to the presence of both unburned MTBE and fossil fuel combustion products in aquatic environments. MTBE has been added to unleaded gasoline to decrease vehicular carbon monoxide emissions in the atmosphere since the 1980s. Fluoranthene is among the most prevalent polycyclic aromatic hydrocarbons (PAHs) identified in the exhaust of internal combustion engines, and it has toxic effects on organisms in the presence of near-ultraviolet radiation. Because the interactions between MTBE and PAHs were not known, the toxicities of MTBE and fluoranthene together in fish were studied. Four-day-old fathead minnow larvae were exposed to combinations of fluoranthene and MTBE in a laboratory system under simulated sunlight for 96 h. Fish exposed to both MTBE and fluoranthene experienced higher mortality as compared to fish exposed only to fluoranthene. Fish exposed to MTBE alone did not exhibit increased mortality as compared to no-exposure controls. Co-exposure to MTBE was associated with significantly increased uptake rates and significantly decreased elimination rates for fluoranthene. The bioconcentration factor in fish exposed to 20 μg of fluoranthene/L in the presence of 40 μg of MTBE/L was 1.7−2.2 times higher than that for fluoranthene alone. The observed pattern of mortality reflected the different body residues of fluoranthene in fish. We conclude that, because of enhanced bioconcentration factors, the photoinduced toxicity of fluoranthene and potentially other PAHs will be greater in aquatic environments where reformulated gasoline containing MTBE is utilized in motorized watercraft.

Introduction

The activity and emissions of motorized watercraft can result in exposure of aquatic organisms to complex mixtures of unburned and burned fuel components. The impacts of such exposures are not well-studied, and there is a need to examine the toxicity of mixtures of commonly co-occurring chemicals in the aquatic environment.

Since 1992, the Clean Air Act has required areas where carbon monoxide concentrations exceed national ambient air-quality standards to use reformulated, oxygenated gasoline. Oxygenates include substances such as ethanol, methanol, methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), *tert*-butyl alcohol (TBA), and *tert*-amyl methyl ether (TAME) (*1*). In the late 1990s, oxygenates were added to more than 30% of the gasoline used in the United States (*2*), and MTBE made up approximately 85% of all oxygenates used nationally (*3*). MTBE (CAS No. 1634-04-4), in a typical gasoline formulation, has an equilibrium water concentration of approximately 5000 mg/L at room temperature and readily partitions into aquatic systems (*4*). The toxicity of MTBE is low as compared to environmental exposures in both mammals and aquatic organisms (*5*−*8*). Thus, it has been suggested that the risk of adverse effects because of MTBE in aquatic systems is minimal (*8*).

There has been an increasing frequency of detection of MTBE in both groundwaters and surface waters, and the taste and odor of MTBE renders water supplies unpalatable at very low concentrations (20−40 μg/L). The U.S. Geological Survey (USGS), detected MTBE in 41 (6.9%) of 592 stormwater samples collected in 16 cities and metropolitan areas in the United States from 1991 through 1995 (*9*). Concentrations of MTBE ranged from 0.2 to 8.7 μg/L, with a median of 1.5 μg/L. A more recent study detected MTBE in 47% of samples, with a reported maximum concentration of 14 μg/L (*10*). The frequent presence and associated problems of MTBE in surface water and groundwater resulted in a request from the U.S. EPA to Congress to consider legislation that would significantly reduce or eliminate the use of MTBE (*11*).

In watercraft powered by carbureted two-cycle engines, between 20 and 30% of the fuel that enters the combustion chamber is released in the exhaust unburned (*12*), and between 3 and 10% of MTBE is released directly into water (*13*). It has been suggested that motorized watercraft exhaust is one of the most important sources of MTBE in California lakes and reservoirs (*14*−*16*) with detected MTBE concentrations ranging from <1 μg/L to as high as 88 μg/L. Prior to a ban on the use of MTBE in motorized watercraft fuel in the Lake Tahoe Basin, the USGS detected MTBE in 100% of the samples taken from Lake Tahoe, Calfornia/Nevada, in concentrations ranging from 0.18 to 4.2 μg/L at depths up to 30 m (*14*).

Combustion of fuel in gasoline engines produces a complex mixture of polycyclic aromatic hydrocarbons (PAHs). PAHs are common environmental contaminants consisting of two or more fused benzene rings and have been detected in surface water, sediments, soils, plants, animals, and both rural and urban air. In addition to emissions from motorized watercraft (*17*−*19*), PAHs can reach the aquatic environment through industrial and domestic sewage effluent, surface runoff from land, deposition of airborne particulates, and spillage of petroleum and petroleum products into bodies of water (*20*).

Studies have shown that in the presence of natural or simulated sunlight, PAHs are acutely toxic to aquatic organisms at concentrations that are well below the PAH aqueous solubility limits (*21*). Phototoxic potency is directly proportional to both the concentration of PAH in an organism's body and the dose of actinic radiation (*22*−*24*). Fluoranthene (CAS No. 206-44-0) is often among the most prevalent compounds in environments contaminated by PAHs (*25*). In the presence of near-ultraviolet radiation, fluoranthene causes photoinduced toxicity to a wide variety of aquatic organisms (*23*, *24*, *26*−*29*), and death is due to photosensitized

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disruption of cellular (e.g., gills) membranes (*28*). Fluoranthene was the main phototoxic component of PAH mixtures attributed to motorized water craft emissions in Lake Tahoe in field experiments conducted in 1997 (*30*).

Because the exhaust of most marine engines is injected directly into the surrounding water and because this exhaust consists of both burned and unburned fuel components, it is likely that aquatic organisms will be exposed to unburned fuel oxygenates and PAHs. Considering the possibility of co-existence of fluoranthene and MTBE in water bodies as a result of boating activities (*15, 17*), the objective of this study was to determine the toxic effect of co-exposure to these chemicals in an aquatic organism. To fulfill this objective, the effects of MTBE on the uptake, elimination, and bioconcentration of fluoranthene and on the photoinduced toxicity of fluoranthene to larval fish were examined.

Experimental Methods

Experimental Organisms. Fish larvae were obtained from the fathead minnow (*Pimephales promelas*) culture in the Department of Zoology, Miami University. Dechlorinated and carbon-filtered tap water (temperature, 22.1 ± 2.4 °C; dissolved oxygen, 7.6 ± 1.1 mg/L; pH, 7.6 ± 0.2; alkalinity, 252 ± 32 mg/L $CaCO_3$; conductivity, 0.70 ± 0.04 μS/cm; ammonia, 0.04 ± 0.05 mg/L NH_3; hardness, 248 ± 78 mg/L $CaCO_3$; mean ± SD, $n = 32$) was used to hold the fish and for the toxicity tests. Fathead minnows were fed twice a day with ground Purina Trout Chow and brine shrimp (O.S.I. Marine Lab Inc.) (*23, 26*). Eggs were collected from the breeding tanks that held 2 male and 6 female adult fish and incubated until hatching (*31*). Toxicokinetic and toxicity tests were initiated 4 days post-hatching.

Lighting System. To simulate natural sunlight, visible light and UV radiation were produced in the laboratory by a bank of fluorescent bulbs that produce a spectral distribution similar to natural sunlight (Vita-Lite, Durotest Inc., Fairfield, NJ) (*23, 26*). An Optronics model 754 scanning spectroradiometer equipped with a 15.2 cm diameter integrating sphere (Optronics Laboratory, Orlando, FL) was used for the quantification of UV radiation of natural sunlight and laboratory lightings. For the measurement and recording of the spectral irradiance in the air (natural sunlight and laboratory lighting), the interval of bandwidth was 2 nm. The spectroradiometer was calibrated prior to the measurements with a NIST-traceable tungsten lamp (OL-752-10 Irradiance Standard) (*23*).

Fluoranthene Column. Fluoranthene (Aldrich Chemical Co., Milwaukee, WI; purity >99%) dissolved in water was delivered via a once-through elution column filled with fluoranthene-coated sand (*23, 26*). Fluoranthene-coated sand was made by mixing 1 kg of dry acetone-washed silica sand and 10 g of fluoranthene dissolved in 300 mL of acetone to a 4-L uncapped chemical bottle, which was placed on a Rollacell (New Brunswick Scientific, New Brunswick, NJ) rolling mixer and rolled at a low speed until the sand was completely dry. The fluoranthene-coated sand was placed into a 7.5 × 30 cm glass column connected into a flow-through diluter system. Flow of water was initiated, and the column was rinsed for 24 h to remove loose fluoranthene crystals prior to use in toxicokinetic or toxicity tests. Fluoranthene eluted from the column at its water solubility (approximately 120 μg/L at 24° C) and was diluted with clean water to the target concentrations used in all tests.

Toxicokinetic Test. Toxicokinetic measurements were made using a flow-through exposure system, consisting of two fiberglass bins, each containing three specially modified, flow-through 250-mL crystallizing dishes (*32*). Flow of water into each exposure system was approximately 170 mL/min. Fluoranthene was delivered to the dishes from dilution of water from the sand column, and MTBE was delivered to the dishes via peristaltic pump from a concentrated stock solution of MTBE dissolved in water. For the toxicokinetic measurements, the target fluoranthene concentration was 20 μg/L, and target MTBE concentrations were 0 and 40 μg/L. Twenty fish were placed in each of the crystallizing dishes under cool white fluorescent lighting, which contained little to no UV radiation. Two fish from each dish (total of six fish from each fiberglass bin) were removed at 0, 2, 4, 8, 16, and 24 h. Fish were pooled in groups of three, and the fluoranthene concentration in fish was measured as described below ($n = 2$ per time period). After 24 h, the remaining fish were moved to clean water, and the concentrations of fluoranthene in fish were determined at 0, 2, 4, 8, 16, and 24 h to examine the elimination phase. Fish were provided with a small amount of food (brine shrimp) for 30 min each day. At the end of the feeding period, the remaining brine shrimp were siphoned from the chambers.

Toxicity Tests. Toxicity tests were conducted in the same exposure system as for the toxicokinetic tests. Three concentrations of fluoranthene (0, 5, and 15 μg/L) and two MTBE concentrations (0 and 40 μg/L) were used. Six treatment combinations were thus tested: (i) no fluoranthene + no MTBE, (ii) no fluoranthene + 40 μg of MTBE/L, (iii) 5 μg of fluoranthene/L + no MTBE, (iv) 5 μg of fluoranthene/L + 40 μg of MTBE/L, (v) 15 μg of fluoranthene/L + no MTBE, and (vi) 15 μg of fluoranthene/L + 40 μg of MTBE/L. Twenty larval fish were placed in 250-mL crystallizing dishes. There were two replicate dishes in each treatment. For the first 24 h, sunlight-simulating lights were kept off to allow for uptake of fluoranthene. After this uptake period, fish were given constant sunlight-simulating laboratory lighting and the appropriate fluoranthene and/or MTBE combination in 96-h flow-through toxicity tests (*32*). Mortality was checked every 2−4 h for 96 h (*23*). Fish were considered dead if no opercular movement was observed.

Fluoranthene Analysis. Fish collected for fluoranthene analysis were extracted into a cyclohexane:acetone (2:1) solution according to the protocol described in Weinstein and Oris (*23*). Water collected for fluoranthene analysis was pipetted directly into HPLC sample vials and was analyzed without further extraction or concentration. Concentrations of fluoranthene in fish and in water were measured using reverse-phase HPLC (*23*). Fifty microliters of water sample, tissue extract, or standard solution was injected onto a Waters 3.9 mm × 15 cm μBondpak C_{18} column at 30 °C. An isocratic elution was performed with 8:2 acetonitrile:water at 1.0 mL/min. Fluorescence emission was detected at 460 nm using a Waters 474 scanning fluorescence detector with an excitation wavelength of 360 nm. Peaks were recorded and quantified using a microcomputer-based data station. The detection limit for fluoranthene using this method was 0.2 pg/μL of injected sample or standard, and the presence of MTBE at the concentration levels tested did not interfere with fluoranthene analysis.

Toxicokinetic Model. A first-order, diffusion-based pharmacokinetic model (*33*) was used to characterize the bioconcentration of fluoranthene in fish. Uptake and elimination of fluoranthene was modeled on the basis of first-order diffusion kinetics using the following relationship:

$$dC_{fish}/dt = k_u C_{water} - k_e C_{fish} \quad (1)$$

where C_{fish} was the concentration of fluoranthene in fish tissue (μg/g), t was time (h), dC_{fish}/dt was the rate of change of fluoranthene concentration in fish over time ($\mu g\ g^{-1}\ h^{-1}$), k_u was an uptake rate constant (mL g^{-1} h^{-1}), C_{water} was the fluoranthene concentration in the exposure water (μg/mL), and k_e was an elimination rate constant (h^{-1}). An inherent assumption in first-order, diffusion-based models is that the rate constants are independent of concentration of chemical in water or fish (*34*).

Statistical Analysis. (a) Toxicokinetic Data. Recall that in the experimental protocol, fish were exposed to the fluoranthene for a set amount of time, 24 h. After this time period, the fish were removed from the fluoranthene exposure tank and placed in a clean tank. For sampling times up to 24 h, both uptake and elimination processes were operating, while after 24 h, only elimination processes were operating. Typically, C_{water} is held constant during the exposure period. This allows a closed-form integration of eq 1 and simplifies estimation of rate constants. However, because of a drop in fluoranthene concentrations in water between 16 and 24 h during this experiment, we implemented a model with the possibility of a discontinuity in fluoranthene concentration at time 24 h. This two-component uptake and elimination model of fluoranthene concentrations in fish was expressed as

$$C_{fish} = \frac{k_u}{ke} C_{water}(1 - e^{-k_e t}) I_1 + A e^{-k_e(t-24)} I_2 \qquad (2)$$

where C_{fish} was the average concentration of fluoranthene in fish at any time (t). As before, fluoranthene concentration in the water was denoted C_{water}, and the uptake and elimination rate constants were denoted k_u and k_e, respectively. The value 24 represented the predetermined stop-exposure time in the experiment (h). The indicator variable $I_1 = 1$ for all times up to the stop-exposure time (24 h) and $I_1 = 0$ for all times after the stop-exposure time ($\geq$24 h). Similarly, $I_2 = 0$ for all times <24 h and $I_2 = 1$ for all times $\geq$24 h. The fitted constant A (μg/g) represented the estimated fluoranthene concentration in fish at the initiation of the elimination period. The model specified by eq 2 was separately fit to the MTBE and no MTBE experimental data using the nonlinear regression SAS procedure NLIN (*35*).

Statistical differences between the MTBE- and no MTBE-exposed fish were established on the basis of comparisons of uptake rate constants, elimination rate constants, and bioconcentration factors (BCFs). The k_u and k_e values from the MTBE and no MTBE conditions were compared using an asymptotic Z-test based on point estimates and standard errors of these quantities (*36*). Bioconcentration factors, defined as the steady-state concentration of a chemical taken up by an organism relative to the concentration of chemical dissolved in water, equates to BCF $= k_u/k_e$ in the model above. A Z-test for equality of the BCF between MTBE and no MTBE conditions was based on methods described by Bailer et al. (*37*) and evaluated in detail by Wheeler and Bailer (*38*).

(b) Toxicity Data. The toxicity of the combination of fluoranthene and MTBE was assessed by comparing survival probabilities (*39*). Using the observed data, Kaplan-Meier estimates of the survival curve were generated and plotted versus time. Median lethal times and associated confidence intervals were estimated from survival curves. Log-rank tests were used to conduct pairwise comparisons between groups. The equality of survival between replicate dishes was tested within each fluoranthene-MTBE combination. If no difference between dishes was detected, data were pooled across dishes for comparing survival across fluoranthene-MTBE combinations. The S-Plus functions *kaplanMeier* and *survdiff* (*40*) and the SAS procedures LIFEREG and LIFETEST (*34*) were used to generate these estimates and tests.

Results and Discussion

For the uptake portion of the toxicokinetic test, fluoranthene concentrations in water were maintained close to the nominal fluoranthene concentration of 20 μg/L and were reasonably constant through 16 h of uptake (no MTBE = 16.8 ± 1.3 μg of fluoranthene/L, 40 μg of MTBE/L = 24.7 ± 0.6 μg of fluoranthene/L; mean ± SD). Between 16 and 24 h of uptake, fluoranthene concentrations in water declined (no MTBE = 15.2 ± 0.3 μg of fluoranthene/L, 40 μg of MTBE/L = 15.3 ± 2.5 μg of fluoranthene/L), and this decline was reflected in the measured body residues in fish (Figure 1). The change in fluoranthene concentration during the uptake portion of the toxicokinetic test necessitated the two-component toxicokinetic model used in this study.

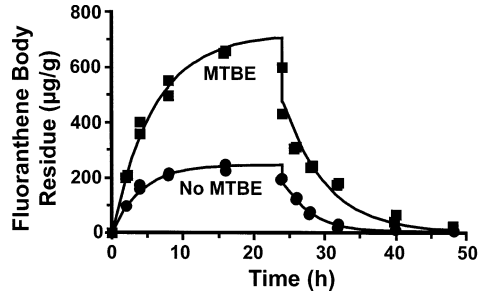

FIGURE 1. Plot of fluoranthene body residue as a function of time during the toxicokinetic test. The plotting symbols are squares for the 40 μg MTBE/L and dots for the no MTBE treatments (nominal fluoranthene concentration = 20 μg/L; measured values presented in Results and Discussion). Lines through the data represent the model specified by eq 2 fit to each of the MTBE and no MTBE experiments.

Throughout the uptake and elimination portion of the toxicokinetic test, body residues of fluoranthene in the 40 μg MTBE/L treatment were greater than those with no MTBE. A summary of the fit of the nonlinear regression model described by eq 2 is given in Table 1 and is also displayed in Figure 1. Since uptake and elimination rate constants from experiment to experiment are independent of fluoranthene concentration in water, the difference in fluoranthene concentrations between the two MTBE treatments did not affect the estimation of these parameters. An evaluation of Figure 1 suggests that the model provided a good summary of the time-related change in fluoranthene body residues. The observed test statistic for the k_u comparison between MTBE and no MTBE was $Z = 3.17$ (P value = 0.00151), while the observed test statistic for the k_e comparison between MTBE and no MTBE was $Z = -3.16$ (P value = 0.00155). This suggests that a significant difference existed between both uptake and elimination rate constants for the MTBE and no MTBE treatments. Thus, MTBE appeared to enhance the bioaccumulation of fluoranthene by both an increased uptake rate (greater k_u) and a decreased elimination rate (smaller k_e). These differences were reflected in a comparison of BCFs between the MTBE and no MTBE treatments. The BCFs were statistically different ($Z = 10.88$, P value < 0.0001). The BCF for fluoranthene was estimated to increase by 74−122% in the presence of MTBE.

Fluoranthene concentrations in water during toxicity tests were maintained close to nominal levels for both the 5 μg/L (5.49 ± 0.90, mean ± SD) and the 15 μg/L (13.7 ± 1.5) treatments. Concentration ranges for both fluoranthene and MTBE were at levels that have environmental relevance (*15*, *17*, *30*, *41*). During the 96 h of simulated sunlight exposure, the mean intensity ± SD for UV-A (320−400 nm) was 134.6 ± 5.2 μW/cm^2 and that for UV-B (285−319 nm) was 2.56 ± 0.07 μW/cm^2. Laboratory irradiance values were approximately 10-fold lower than maximum ambient terrestrial values. However, the spectral distribution of laboratory lighting was similar to natural sunlight, and laboratory intensities represented realistic values for a wide range of natural waters (*23*, *30*).

Under simulated sunlight, greater than 90% survival was observed in all 0 μg of fluoranthene/L treatments. Survival did not differ significantly between replicate dishes within a particular fluoranthene−MTBE combination, and thus data for replicate dishes were combined. The survivor functions

TABLE 1. Parameter Estimates, Standard Errors, and Confidence Intervals (CIs) from Fitting the Nonlinear Regression Model (Eq 2) for Fluoranthene Toxicokinetics in Fathead Minnow Larvae Exposed to Combinations of Fluoranthene and MTBE

experiment/ regression parameter	estimate	SE	95% CI[a] (lower, upper)	covariance[b] (k_u,k_e)
		No MTBE		
k_u (mL g^{-1} h^{-1})	3532	149.20	3218, 3847	1.74
k_e (h^{-1})	0.2381	0.0128	0.2111, 0.2651	
BCF	14836		14129, 15580	
A (μg/g)	189	5.59	180, 200	
		40 μg of MTBE/L		
k_u (mL g^{-1} h^{-1})	4837	383.10	4029, 5645	6.45
k_e (h^{-1})	0.1656	0.0190	0.1256, 0.2055	
BCF	29208		25854, 32998	
A (μg/g)	488	29.0	430, 550	

[a] Confidence intervals (CIs) for k_u and k_e are constructed as point estimate $\pm$ $t(0.975, 17) \times$ SE, where $t(0.975, 17) = 2.11$ is the critical value from a Student's t-distribution with 17 degrees of freedom that has 0.025 in the upper tail (*37*, *38*). [b] Estimated covariance between k_u and k_e, the two rate estimators.

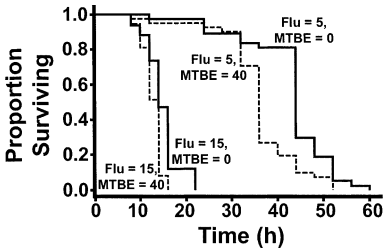

FIGURE 2. Estimated Kaplan–Meier survival curves from the photoinduced toxicity tests comparing fish exposed to nominal concentrations of 5 μg of fluoranthene/L or 15 μg of fluoranthene/L with 40 μg of MTBE/L or 0 μg of MTBE/L (Flu = fluoranthene). Measured fluoranthene concentration values are presented in Results and Discussion.

TABLE 2. Median Lethal Times (LT_{50}) and Associated 95% Confidence Intervals (CI) in Fathead Minnow Larvae Exposed for 96 h to Combinations of Fluoranthene and MTBE under Simulated Sunlight Conditions

fluoranthene[a] (μg/L)	MTBE (μg/L)	LT50 (h)	95% CI (upper, lower)
5	0	40.8	38.7, 43.1
5	40	35.0	33.2, 36.9
15	0	14.1	13.6, 14.7
15	40	12.1	11.6, 12.6

[a] Less than 10% mortality occurred in all 0 μg of fluoranthene/L treatments.

(Figure 2) and median lethal times (Table 2) for the four fluoranthene–MTBE treatments exhibited differences that were related both to fluoranthene and MTBE concentration. When comparing within each of the fluoranthene conditions, survival was reduced in the MTBE = 40 μg/L condition versus the MTBE = 0 μg/L condition. The log-rank test statistics (P values) for comparing survival in MTBE-exposed fish versus unexposed fish within the fluoranthene = 5 μg/L condition and the fluoranthene = 15 μg/L condition were $X^2 = 15.9$ (P value < 0.0001) and $X^2 = 11.7$ (P value = 0.0006), respectively. Thus, increasing fluoranthene and MTBE concentrations both appeared to cause increased toxicity in exposed organisms.

The photoinduced mortality of fish exposed to both MTBE and fluoranthene was higher than those exposed only to fluoranthene (Figure 2; Table 2). This result was explained in part by the bioconcentration of fluoranthene in fish. Reported values of log(BCF) of fluoranthene were 4.9, 3.24, 3.58, and 3.96 in amphipod *Pontoporeia hoyi*, *Daphnia magna*, juvenile *P. promelas*, and larval *P. promelas*, respectively (*23*, *26*, *42*, *43*). In the current study, the log(BCF) in the larvae exposed only to fluoranthene was 4.17 and that exposed both to fluoranthene and MTBE was 4.47. From the comparison of BCFs (Table 1), the BCF of fluoranthene co-exposed to MTBE in fish (29 208) was significantly greater as compared to that of fluoranthene exposure alone (14 836) and resulted in a nearly 2-fold increase in bioconcentration of fluoranthene.

Enhanced bioconcentration of fluoranthene due to co-exposure to MTBE increased the photoinduced toxicity of fluoranthene. Increased toxicity was observed because photoinduced toxicity is related to the body residue of fluoranthene or other PAHs (*22–24*). The current experiments have indicated that exposure to mixtures of burned and unburned fuel components can result in significantly greater larval fish mortality as compared to what would be predicted from toxicity tests conducted using single chemical exposures. We conclude that, because of enhanced bioconcentration factors, the photoinduced toxicity of fluoranthene and potentially other PAHs will be greater in aquatic environments where reformulated gasoline containing MTBE is utilized in motorized watercraft.

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