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THE PHOTOENHANCED TOXICITY OF ANTHRACENE TO JUVENILE SUNFISH (*LEPOMIS* SPP.)

JAMES T. ORIS and JOHN P. GIESY, JR.

Pesticide Research Center, Department of Fisheries and Wildlife, and Center for Environmental Toxicology, Michigan State University, East Lansing, MI 48824, U.S.A

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The acute toxicity of anthracene to juvenile sunfish (*Lepomis* spp.) in the presence of simulated sunlight has been assessed. Previously, anthracene was considered not to be acutely toxic to fish. However, in this study 96 h LC_{50} values were found to be 190–1800-times less, with concurrent exposure of anthracene and simulated sunlight, than a reported 24-h no-effect anthracene concentration for *L. macrochirus* derived under standard laboratory conditions. Opercular ventilation rate was significantly increased in UV light-anthracene treated fish, and histological evidence indicated that major structural changes occurred in the gills of these fish as compared to UV light-only controls. The site of toxic action does not appear to be limited to respiratory surfaces, however, since dorsal epidermal structure was also disrupted in UV light-anthracene treated fish as compared to UV light-only controls. A mathematical relationship to estimate the potential toxicity of anthracene to fish is presented which closely predicts the results of toxicity studies conducted under natural sunlight. It is concluded that solar radiation is an important accessory parameter that deserves consideration in the toxicity assessment of polycyclic aromatic hydrocarbons in the aquatic environment.

Key words: anthracene; PAH; sunlight; phototoxicity; ultraviolet radiation; *Lepomis macrochirus*

INTRODUCTION

Increasing non-point source inputs of polycyclic aromatic hydrocarbons (PAH) into aquatic systems from greater utilization of coal combustion and petroleum products (Gehrs, 1976) has created the need to develop an environmentally realistic assessment of the impact these compounds may have on fish and other aquatic organisms. Data from laboratory toxicity tests have indicated that most PAH are not acutely toxic to fish, and therefore emphasis has been placed on the chronic effects and potential carcinogenicity of these compounds to humans (Neff, 1979). Most studies of PAH in aquatic organisms have dealt with the fate and biotransformation in organisms as vectors of exposure to humans. However, by accounting for an important environmental parameter, solar ultraviolet radiation (SUV), it has been shown that anthracene, a linear, 3-ring PAH, is acutely toxic to juvenile sunfish (*Lepomis* spp.), at concentrations well below aqueous solubility limits (<30 μg/l).

In this report we present the findings from a detailed examination of photo-induced toxicity of anthracene to juvenile sunfish. We have assessed this toxicity primarily in a laboratory system utilizing simulated sunlight. We report results from anthracene/light dose-response studies, and use observational and histological evidence to hypothesize sites of toxic action. Finally, a predictive statistical model to estimate the potential toxicity of anthracene to fish is presented.

MATERIALS AND METHODS

Laboratory system

Sunlight was approximated in the laboratory using a combination of Chroma F40C50 white (General Electric, Cleveland, OH) and FS40 ultraviolet (Westinghouse, Bloomfield, NJ) fluorescent bulbs. The lights were mounted on a 1.22×2.74 m frame on 15.24 cm centers, alternating every other bulb. A 5 mil thickness of cellulose triacetate (CTA) filter was used to eliminate wavelengths shorter than 285 nm. The ratio of UV-A:UV-B under the laboratory lighting system was 1.42, whereas the UV-A:UV-B ratio under natural sunlight was measured to be 8.21. Thus, the laboratory lighting system was weighted in the UV-B region. Light intensity was varied by changing the height of the light bank over the bioassay table or by changing the thickness, by adding or taking away layers, of the CTA filter.

Anthracene (M_r 178.23, Sigma grade III, no. A-3885) solutions were obtained from a once-through aqueous elution column, which avoided the use of carrier solvents in the bioassays. Columns were made by pouring anthracene dissolved in acetone onto a thin layer of silica sand at 0.2% wt/wt, and allowing the acetone to evaporate in the dark. When dry (24 h) the sand was packed into a 7.5×45 cm glass column and flushed with water for 48 h to remove loose anthracene crystals. Anthracene eluted from the column, as part of the laboratory water delivery system, at aqueous solubility (ca. 35 μg/l at 22°C) and was diluted to a desired concentration before use.

Bioassays

For most studies, a natural assemblage of juvenile sunfish (*Lepomis* spp.) was used. These fish were collected by seine from Park Lake, Clinton Co., Michigan. Other studies were conducted using juvenile bluegill sunfish (*L. macrochirus*) obtained from Osage Catfisheries (Osage Beach, MO) or from ByBrook Bass Hatcheries (Ashford, CT). All fish were 2–3 cm in length and 0.5–1 g in weight. Different populations of fish were kept segregated and were held in large flow-through fiberglass tanks with charcoal filtered, aerated tap water (temp., 22 ± 1°C; pH, 8.20 ± 0.27; D.O., 7.15 ± 0.24 mg/l; hardness, 328 mg/l $CaCO_3$; alkalinity, 346 mg/l $CaCO_3$). Fish were held for at least two weeks prior to bioassays on an 18:6 h light:dark (L:D) photoperiod under a low pressure sodium lamp (UV fluence

negligible) and were fed twice a day with Biodiet-Starter (BioProducts Inc., Worrenton, OR).

Fish were exposed to anthracene in 18.85-l glass aquariums in a flow-through system under the laboratory lighting system. Fish were transferred to dosing aquariums 48 h prior to bioassays in the absence of UV radiation for acclimation to the test system and to establish a nominal body burden of anthracene approximately 80% of the theoretical steady state (Spacie et al., 1983). Actual body concentrations of anthracene were 137.2 ± 47.34% (± SE) of the estimated bioconcentration factor of 998 calculated from Spacie et al. (1983). Ten fish per aquarium and two aquariums per anthracene concentration were used in all bioassays. During the acclimation period the photoperiod was changed from 18:6 h L:D to continuous light in 2 h per day increments. As part of a standard protocol (ASTM, 1984) fish were not fed for 48 h prior to, or for the first 96 h of a bioassay. After 96 h, fish were fed sparingly every other day for the duration of the test. Fecal and other particulate material was siphoned from the aquariums as needed. Mortality, gross physical damage, and behavioral changes were noted and recorded at least twice daily. A fish was considered dead when no opercular movements could be detected.

Control experiments were performed during preliminary testing and during the bioassays. Preliminary tests were conducted where fish were exposed to anthracene as long as 96 h under darkness, under cool-white fluorescent lamps (General Electric, F40CW-RS-WM; >400 nm), and under gold fluorescent lamps (General Electric, F40G0; >500 nm) to determine any effects due to anthracene in the absence of SUV. Also, in preliminary tests, fish were exposed to anthracene for 48 h in the dark and then transferred to clean water under SUV for 96 h to ascertain any effects due to the presence of anthracene in the water during SUV exposure. Similarly, fish were exposed to anthracene for 48 h in the dark and transferred to clean water in the dark for 144 h to allow for depuration of the compound. These fish were then transferred to clean water and exposed to SUV as long as 96 h. Concurrent with all anthracene-SUV bioassays, a no-anthracene treatment was performed as a SUV-only control. This treatment is reported as an anthracene concentration in water less than the analytical detection level of 0.1 μg anthracene/l.

Light measurement

UV-B (310 ± 34 nm) and UV-A (365 ± 36 nm) were quantified using a Macam Photometrics (Livingstone, Scotland) Model UV-103 radiometer equipped with Model SD104 cosine-corrected photodiodes fitted with water-tight, wavelength selective filters. Visible light (400–700 nm) was measured using a Techtum Instruments QSM-2500 (Sweden) scanning quantum spectrometer coupled to a LI-COR model LI188-B integrating quantum meter.

Analytical

Anthracene concentrations in water were determined directly by reverse-phase

HPLC. Twenty microliters of sample or standard were injected onto a Partisil ODS-3, 10 μm C18 column, at 30°C. An isocratic elution was performed with 9:1 acetonitrile:water at 1.0 ml/min. A Kratos FS-970 fluorescence detector was used at an excitation of 252 nm with a 370 nm emission filter. Peaks were recorded and quantified on a Hewlett-Packard 3390-A integrator. The limit of detection for anthracene, using this technique, was 2.0 pg total mass, or 0.1 μg/l.

Histology

At various times during bioassays, randomly selected fish were removed from each aquarium, swim bladders punctured, and were fixed in buffered formalin for 24 h. Whole fish were dehydrated in an ethyl alcohol series, cleared in xylene and embedded in Paraplast-Plus. Ten micron thick parasagittal sections were cut and then stained with hematoxylin and eosin. Sections were examined by light

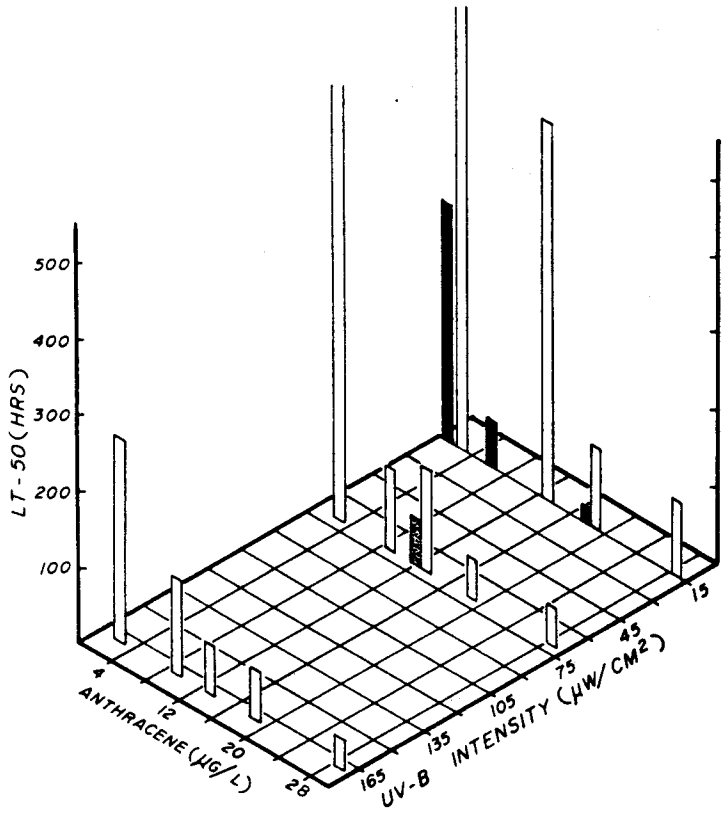

Fig. 1. Median lethal times (LT_{50}) of juvenile sunfish exposed to continuous simulated sunlight as a function of UV-B intensity (310 ± 34 nm) and anthracene concentration. Bars open at top indicate no mortality in 144 h. White bars = natural assemblage of juvenile sunfish collected from Park Lake, MI. Black bars and stippled bar = juvenile bluegill sunfish obtained from Osage Catfisheries, MO, and ByBrook Hatchery, CT, respectively. SUV-only controls are not shown.

microscopy for abnormalities or lesions and compared to fish from the SUV-only treatment.

RESULTS

The toxic effects of anthracene were observed only in the presence of SUV. Fish exposed to anthracene under darkness, under cool-white fluorescent bulbs, or under gold fluorescent bulbs were not adversely affected. Fish that were exposed to anthracene for 48 h in the dark and then transferred to clean water under SUV exhibited signs of toxicity, but recovered slowly as anthracene was eliminated from the body. Sunfish that had attained steady-state body burdens of anthracene and were allowed sufficient depuration to eliminate anthracene in the dark were not adversely affected when subsequently exposed to SUV. These results are in agreement with the findings of Bowling et al. (1983). Mortality of fish exposed to simulated sunlight without anthracene as SUV-only controls was less than 10% in all bioassays.

Under continuous laboratory illumination, the time to reach 50% mortality (LT_{50}) for fish was dependent on both UV-B intensity and anthracene concentration (Fig. 1). LT_{50} values for Park Lake fish ranged from 36.5 h at 26.8 μg anthracene/l and 170 $\mu W/cm^2$ UV-B to intermediate values (40–500 h) at intermediate and low

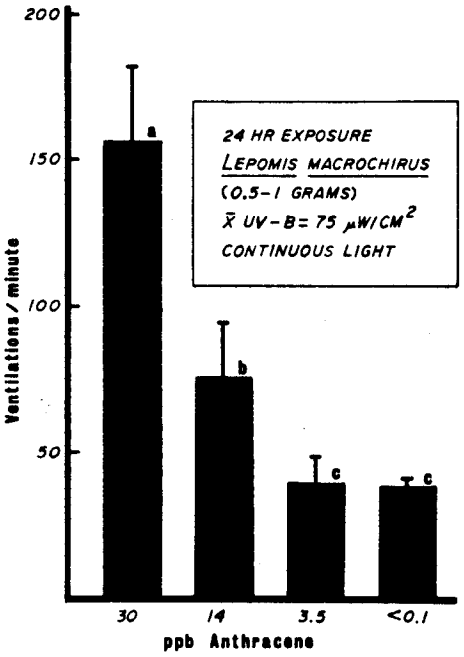

Fig. 2. Opercular ventilation rate as a function of anthracene concentration in fish exposed concurrently to simulated sunlight. Error bars represent 2 SE, and a >b>c (Student's *t*, alpha = 0.05).

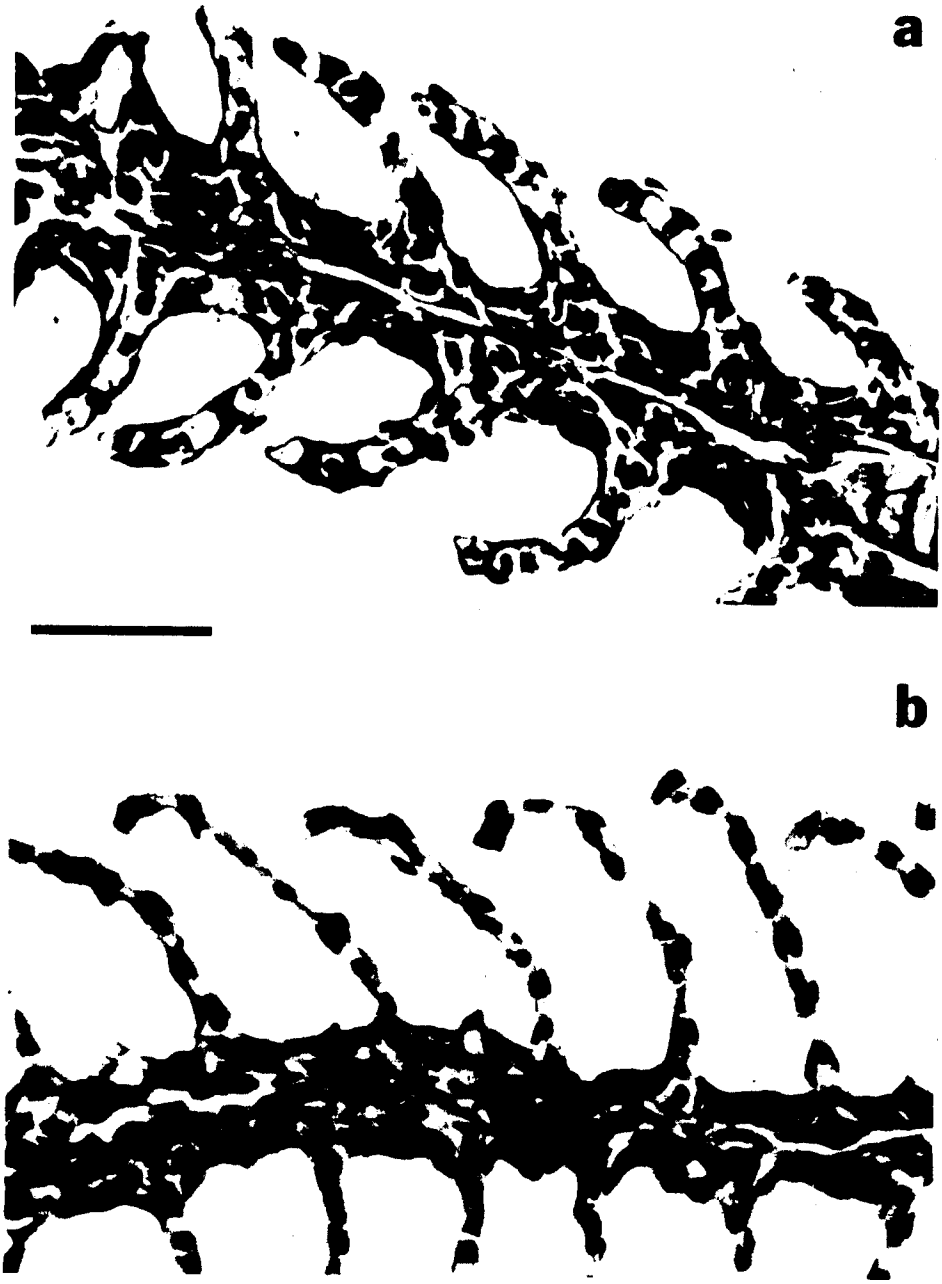

Fig. 3. Photomicrographs of gill filaments from juvenile bluegill sunfish exposed to <0.1 μg anthracene/l (SUV-only control) for 144 h (a) and to 9.98 μg anthracene/l for 24 h (b), at 14.8 $\mu W/cm^2$ UV-B (310 ± 34 nm) continuous simulated sunlight. Scale bar, 50 μm.

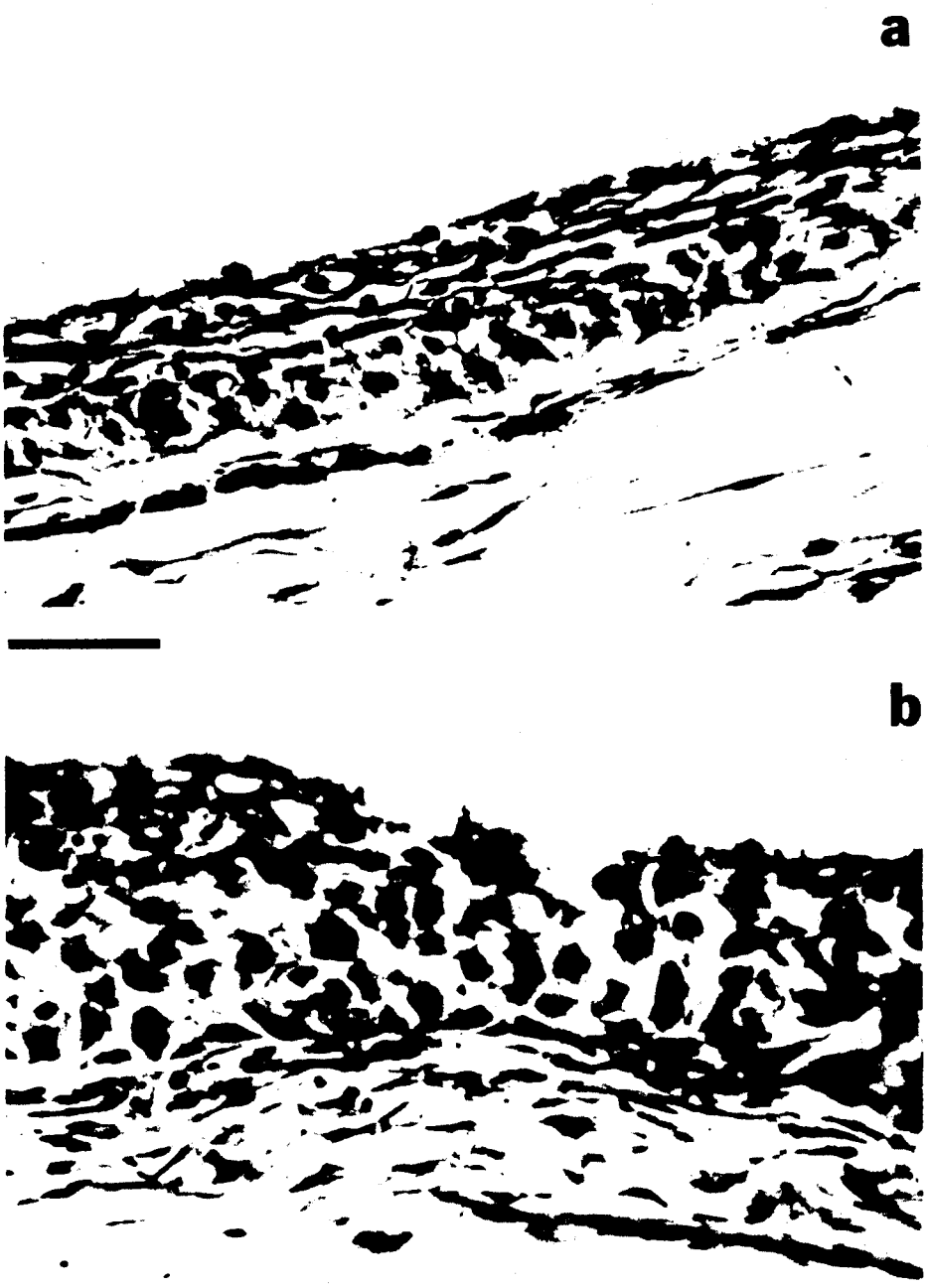

Fig. 4. Photomicrographs of dorsal epidermis, anterior to the dorsal fin, from juvenile bluegill sunfish exposed to <0.1 μg anthracene/l (SUV-only control) for 144 h (a) and to 9.98 μg anthracene/l for 24 h (b), at 14.8 $\mu W/cm^2$ UV-B (310 ± 34 nm) continuous simulated sunlight. Scale bar, 20 μm.

light intensities and anthracene concentrations, with no mortality in 144 h at the lowest measured anthracene concentration and UV-B intensity.

Affected fish showed signs of irritation and hypoxia. An increase in opercular ventilation rate was observed in an anthracene dose-related fashion (Fig. 2), suggesting a respiratory involvement in the toxic response. Dead fish exhibited signs of asphyxia: open mouth, splayed opercula, and pale gill filaments (Reichenbach-Klinke and Landolt, 1973). Compared to SUV-only controls, gills of SUV-anthracene treated fish lacked a defined epithelial cell layer, and in most cases, only the structural supporting pillar cells appeared to remain intact (Fig. 3). Dorsal surfaces became thickened and acquired a creamy white appearance similar to the description of sunburn in fish presented by Bullock (1982). Sections of dorsal epidermis, anterior to the dorsal fin, from SUV-anthracene treated fish were structurally disorganized and extensively eroded compared to SUV-only controls (Fig. 4). A severe necrotic condition and rapid loss of the upper epidermal cell layers was evident in these fish.

Based on the comparison of 96 h LC_{50} values, the natural assemblage of juvenile sunfish was not as sensitive to the light-anthracene combination as were the hatchery bluegill sunfish (Table I). Bluegill sunfish from Osage Catfisheries were 10-times more sensitive than sunfish from Park Lake. Although no LC_{50} value could be calculated for ByBrook bluegill sunfish, for at least one anthracene concentration and UV-B intensity these fish were approximately twice as sensitive as fish from Park Lake. LT_{50} values were 63 and 135 h for fish from ByBrook and Park Lake, respectively, at comparable anthracene concentrations and at the same UV-B intensity (Fig. 1). No direct comparisons between bluegill sunfish from Osage and ByBrook could be made.

DISCUSSION

The incorporation of an important environmental parameter in aquatic ecosystems (i.e., SUV) into laboratory toxicity tests has demonstrated that at least one common PAH can be extremely toxic to fish. Previously, anthracene had not been

TABLE I

96-h LC_{50} values for juvenile sunfish exposed to anthracene at different UV-B (310 ± 34 nm) intensities in the laboratory.

Fish	UV-B intensity ($\mu W/cm^2$)	LC_{50} ($\mu g/l$)	95% Fiducial limits	
			Lower	Upper
Park Lake	14.8	26.47	22.62	34.48
	70.0	18.23	16.14	21.11
	170.0	11.92	10.15	13.40
Osage Hatchery	14.8	2.78	1.94	3.92

observed to be acutely toxic to fish in laboratory experiments when SUV was not a consideration. Spacie et al. (1983) studied the pharmacokinetics of anthracene in *L. macrochirus* and observed no mortality after 48 h exposures as great as 32 μg/l. In similar studies, no adverse effects were reported after dosing young coho salmon with 14 or 28 μg anthracene by injection or feeding, respectively (Roubal et al., 1977). Applegate et al. (1957) reported a 24 h no-effect anthracene concentration of 5 mg/l for juvenile bluegill sunfish. This no-effect value is 190, 274, and 420-times greater than our calculalted 96 h LC_{50} values at UV-B intensities of 14.8, 70, and 170 $\mu W/cm^2$, respectively, for the natural assemblage of juvenile sunfish (Table I). On the basis of LC_{50} values, bluegill sunfish from the Osage hatchery were 1800-times more sensitive than the no-effect concentration at a UV-B intensity of 14.8 $\mu W/cm^2$ (Table I).

While studying the fate of anthracene in outdoor artificial stream microcosms at the Savannah River facility in S. Carolina, Bowling et al. (1983) observed unexpected acute mortality among juvenile bluegill sunfish held in cages exposed to full sunlight in the channels. It was concluded that the SUV portion of the electromagnetic spectrum significantly enhanced the toxicity of anthracene to fish. Calculated LT_{50} values from the data presented by Bowling et al. (1983) for 12 μg anthracene/l are 38 and 23.6 h for total exposure time and cumulative light exposure, respectively. Cumulative light exposure LT_{50} values were calculated based on the total number of hours of light during the anthracene-light dosing period. This manipulation was necessary in order to compare results from studies conducted outdoors, under fluctuating conditions of SUV, to our results under conditions of continuous light. In downstream reaches of the artificial channels, where anthracene concentrations fluctuated from 12 to 5 μg/l on a daily basis due to photolytic degradation, calculated LT_{50} values are 45.5 and 28 h for total exposure time and cumulative light exposure, respectively. These values are comparable to the results obtained in this study (cf. Fig. 1), making the necessary assumption that SUV intensities were greater in the outdoor experiments, where no radiometry was reported, than in our laboratory system. This assumption has validity since the maximum light intensity achieved in our laboratory system was approximately 5–10-times less than what has been measured at the water surface during the summer months on Lake Michigan (unpublished data).

Site of toxic action

Signs of hypoxia and death from apparent asphyxiation were used as initial indications that SUV-anthracene treated fish were being adversely affected at the gills. Ventilations per minute were increased from 2 to greater than 3-times the rate observed for the SUV-only treatment in one experiment (Fig. 2), except in the case of the 3.5 μg anthracene/l treatment where no fish died during the experimental period of 144 h. In all bioassays, mortality reached 100% within 144 h in treatments with fish exhibiting a statistically significant (Student's t, alpha = 0.05) increase in

ventilation rate after 24 h SUV-anthracene exposure. Ventilation rate of SUV-only controls, and SUV-anthracene treated fish that were not adversely affected, were not significantly different from the rates reported for free swimming normal controls of juvenile *L. macrochirus* (Carlson, 1982). Ventilation rates of fish significantly increased by SUV-anthracene treatment ranged from the same to 2-times greater than the rates reported for free swimming juvenile bluegill sunfish exposed to an acutely toxic concentration of $ZnSO_4$, a known gill toxicant, for 24 h (Carlson, 1982). This evidence, along with the observed damage to the gill epithelium (Fig. 3), implicates the respiratory apparatus as being a major site of SUV-anthracene toxic action. However, destruction of dorsal epidermal cells (Fig. 4) indicates that the actions of anthracene phototoxicity are not specifically located at the gills. Instead, a more general mode of action is presumed. This mechanism of action is not known but evidence from a broad base of phototoxicity literature indicates that damage to cell membranes might be involved. Studies are currently in progress to further elucidate these mechanisms.

Population sensitivity differences

At present, it is not known why the different populations of sunfish varied so widely in sensitivity to the light-anthracene combination. One possibility for the observed differences in sensitivities is the fact that the natural population of sunfish was a mixed and unavoidably hybridized population. Hybrid sunfish are known to be less sensitive to low oxygen conditions compared to *L. macrochirus* due to differences in the oxygen affinities of the respective hemoglobins (Hochachka and Somero, 1973). Since the observational and histological findings reported here indicate that adversely affected fish are under respiratory stress, undergo damage to gill surfaces, and die of asphyxiation, we expect that the natural population of sunfish would be less sensitive to anthracene phototoxicity than the hatchery fish. In addition, other biochemical and genetic differences or subtle differences in the health status of the different populations are possible explanations for the observed ranges of sensitivities. More detailed study is needed in this area before any definitive conclusions can be made with regard to population differences in sensitivity to anthracene phototoxicity.

Predictive model

While we recognize the importance of investigating the site and mode of toxic action of anthracene phototoxicity, we are also concerned with the potential environmental consequences involved with this phenomenon at the organism and population levels of organization. In order to establish guidelines and to protect fish and other aquatic organisms it is necessary to develop the ability to predict the toxicity of anthracene in the environment given a particular intensity of solar UV radiation and aqueous concentration of anthracene. We have developed a preliminary predictive relationship based on the Bunsen-Roscoe photochemical law of reciproci-

ty. This law states that for any photochemical reaction, the product of the light intensity and the reaction time is constant for a fixed concentration of sensitizer if there are no complicating side reactions (Dworkin, 1958). In other words, given a fixed anthracene concentration in water, if an LT_{50} of 50 h is calculated at a UV-B light intensity of 100 $\mu W/cm^2$, then at 200 $\mu W/cm^2$ the law of reciprocity predicts an LT_{50} of 25 h. The relationship between UV-B intensity multiplied by the time to 50% mortality, and anthracene concentration from laboratory experiments conducted under conditions of continuous light was investigated (Fig. 5). The regression is linear ($r^2 = 0.78$) and significant ($P = 0.004$). However, when making predictions in the environment it is desirable to obtain a fairly high degree of certainty in the accuracy of the prediction. The necessary level of accuracy is dependent on the margin of safety which is utilized when standards or guidelines are developed from laboratory toxicity tests. It is evident from the continuous confidence bands for the 90th and 95th percentile drawn around the regression (Fig. 5) and noting the log-scale on the Y-axis, that the degree of confidence in our predictions is not very high. Additionally, the model consistently overpredicts toxicity at the greater light intensities and underpredicts toxicity at lesser light intensities. These discrepancies indicate that the toxic phenomenon does not entirely follow the reciprocity law, and that complicating processes such as biological repair may be occurring.

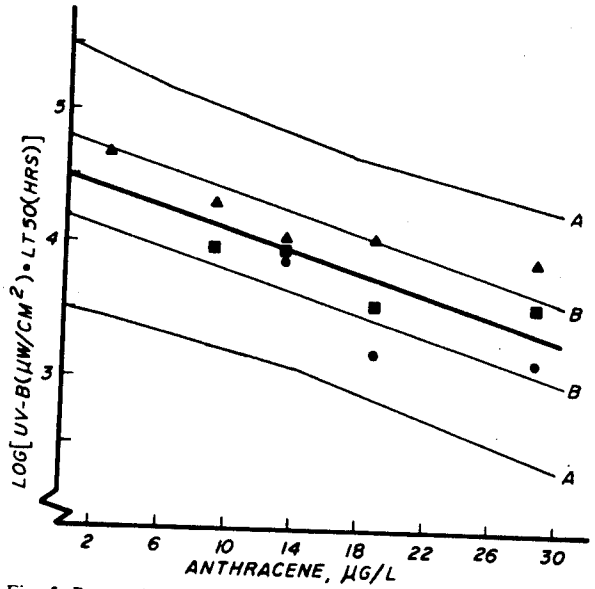

Fig. 5. Regression, based on the Bunsen-Roscoe law of reciprocity, of the product of light intensity and median lethal time versus anthracene concentration in water for continuous simulated sunlight exposures. Experiments conducted at: ▲, 170 $\mu W/cm^2$; ■, 70 $\mu W/cm^2$; and ●, 14.8 $\mu W/cm^2$ UV-B. Lines A and B are continuous confidence bands around the regression for the 95th and 90th percentile, respectively.

TABLE II

Comparison of laboratory predictions to field results[a].

Anthracene concentration (μg/l)	Lab LT_{50} prediction (h)	90% Confidence range (h)	Field LT_{50} result (light h)
1.5	70.72	39.17–123.86	66.57
7	43.05	23.88– 77.26	37.83
22	11.12	6.14– 19.86	10.45

[a]Laboratory predictions are for 24 h continuous light at 400 μW/cm^2 UV-B. The field experiment had a photoperiod of 11.75:12.25 h L:D and a measured mean light intensity of 400 μW/cm^2 UV-B. Field results are expressed in total hours of light exposure, making the assumption that no biological repair processes took place during the dark hours.

To test the applicability of the predictive model, we conducted an acute static-renewal anthracene phototoxicity bioassay under natural sunlight, and compared the results to laboratory bioassays (Table II). The LT_{50} values for the field experiment fall well within the range of accepted statistical confidence from the laboratory predictions. LT_{50} values calculated from Bowling et al. (1983) also correspond to laboratory predictions. The closeness of agreement among the laboratory predictions and the field results indicates that although there are many potential complicating factors, we can still make accurate predictions concerning the toxicity of anthracene under natural sunlight. These factors include the constantly changing intensity of UV radiation under natural conditions, and biological repair processes. The predictions in Table II are based on the measured mean UV-B intensity over a 48-h period while the predictive relationship was developed under continuous light and intensity conditions. In addition, there is a minimum latent period, the length of which is dependent on light intensity and photoperiod, before any mortality occurs (unpublished data). That we can make relatively accurate predictions from controlled laboratory conditions to fluctuating environmental conditions is interesting given all of the possible confounding properties of the toxic phenomenon.

Relevance to the environment

Current environmental concentrations of anthracene are less than our measured acute toxicity values. However, considerable fluxes of anthracene from non-point source inputs into the Great Lakes ecosystem occur. These inputs have been measured as being on the order of 10^5–10^6 kg/yr, resulting in enrichment of anthracene as well as total PAH concentrations in southern Lake Michigan (Strand and Andren, 1980). Total PAH concentrations in surface waters around the world range from not detectable to 3 μg/l (Neff, 1979). In addition to anthracene, many PAH can be considered to be potential environmental phototoxins (Krinsky, 1976). Therefore, since the additive or synergistic phototoxicologic properties of natural mixtures of PAH are unknown, and since chronic effects have not been assessed in

this study, it is feasible that small increases of PAH concentrations in surface waters could cause dramatic impacts in aquatic ecosystems. For further reference, Oris et al. (1984) have presented a detailed examination of potential effects of phototoxic PAH in the aquatic environment.

CONCLUSION

The findings presented in this report demonstrate the need to consider SUV when assessing the toxicity of PAH to aquatic organisms. Toxicity values ranging from 190 to 1800-times less than previously observed no-effect anthracene concentrations in the absence of SUV indicate a lack of environmental realism in laboratory toxicity testing. Major environmental parameters, including SUV, need to be identified and incorporated into standard laboratory tests before an adequate assessment of PAH toxicity in the aquatic environment can be made.

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