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THE PHOTO-INDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO LARVAE OF THE FATHEAD MINNOW (Pimephales promelas)

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ABSTRACT

The toxicity of 12 polycyclic aromatic hydrocarbons to larvae of the fathead minnow in the presence of simulated sunlight was examined. A measure of relative toxicities of the toxic compounds is described in which waveband radiation intensity, molar extinction coefficients, and molar body concentration of PAH are considered. In addition, a structure-lethality relationship has been developed, based on molecular structure and photochemical properties, that classifies compounds as being phototoxic or non-phototoxic.

INTRODUCTION

Previous studies (10,11,12) have demonstrated that anthracene, a fused, linear 3-ring polycyclic aromatic hydrocarbon (PAH), is acutely toxic to juvenile sunfish under laboratory and field conditions of solar ultraviolet radiation (SUVR), and that this toxicity can be predicted from knowledge of SUVR intensity, anthracene concentration and photoperiod duration. Because PAH belong to a large class of compounds, it is desirable to determine the extent and likelihood that PAH other than anthracene are capable of eliciting photo-induced toxicity to fish. Many PAH can be considered as potentially phototoxic (2), and there have been reports describing the photo-activity of PAH to mammals (4) and aquatic organisms (6). There are no known reports, however, concerning the range and extent of the photo-induced toxicities of PAH to fish. The present study was conducted to : 1) determine the relative photo-induced toxicities of a variety of PAH to fish, and 2) develop a structure-lethality relationship to estimate the photo-induced toxicity of a compound.

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MATERIALS AND METHODS

Compounds and Test Solutions

Anthracene (ANT), benzo(a)anthracene (BAA), benzo(b)anthracene (BBA), acridine (ACR), benzanthrone (BAN), dibenz(a,h)anthracene (DBA), perylene (PER), benzo(g,h,i)perylene (BGP), pyrene (PYR), benzo(a)pyrene (BAP), benzo(e)pyrene (BEP), and phenanthrene (PHE) were obtained at the highest purification available commercially. All compounds except BAN (8) were used without further purification. Saturated aqueous solutions, in charcoal filtered, aerated tap water (14), of each PAH were obtained using a shell coating technique to avoid the use of carrier solvents in the tests (9). Appropriate dilutions were made from these stock solutions to achieve the desired concentration in a test. Concentrations of PAH in all aqueous solutions were determined by reverse-phase HPLC and fluorescence detection (9). The study was designed such that the final PAH concentrations in the organisms would be equimolar. Therefore, PAH concentrations in water were selected on the basis of published bioconcentration values and molecular weights, normalized to the least water soluble compound, BGP. On this basis, a nominal PAH body burden of 100 nM/g was selected. Nominal and actual concentrations of PAH in water are presented in Table 1.

Organisms and Bioassay Procedure

Larvae of the fathead minnow (Pimephales promelas) were obtained four days post-hatching from the Michigan Department of Natural Resources Surface Water Quality Division. Larvae were transferred by pipette to a small flow-through aquarium filled with charcoal filtered, aerated tap water at 24 C. The larvae were maintained in the flow-through aquarium for two days after transfer and were fed newly hatched brine shrimp nauplii (Artemia salina; Metaframe Corp.) ad libitum twice a day. On the seventh day post-hatching, larvae were transferred by pipette to 300 ml Pyrex dishes containing 150 ml of PAH solution or dilution water. Treatments consisted of 20 to 25 larvae per dish and two dishes per PAH examined, including two dishes containing dilution water as SUVR-only controls. Dishes were covered with aluminum foil and the larvae allowed a 24 h pre-incubation period in the absence of SUVR. After the pre-incubation period, larvae were fed brine shrimp ad libitum for 0.5 h, all solutions were then replaced and the dishes with larvae were placed in random positions under a laboratory system light bank which simulated natural sunlight (10). Light was filtered with a 5 mil thickness of Mylar^{R} to eliminate >99% of the radiation of wavelengths shorter than 315 nm. SUVR intensities were monitored as a previous study (10) and for all exposures were UV-B (290-336 nm) = 20 uW/cm^2 and UV-A (336-400 nm) = 95 uW/cm^2. After the initial pre-incubation, solutions were changed at 12 h intervals. Larvae were fed brine shrimp ad libitum once a day for 0.5 h prior to changing solutions. Bioassay dishes were examined for larval mortality at least four times daily. Tests were conducted until 100% mortality was achieved or for a maximum of 96 h, whichever came first. PAH concentrations in water were

determined at the beginning of a bioassay at zero and 12 h, and at least once more for initial and 12 h solutions during the test period. PAH concentrations in water are reported as the geometric mean between the measured concentrations in the initial and 12 h old solutions. In tests where mortality occurred, a 96 h dark exposure to the PAH was performed to assess any effect due to PAH in the absence of SUVR. No PAH tested in this study exhibited toxicity in the dark. Concentrations of PAH in all larvae were determined at the end of a test (9). Percent recoveries of PAH from spiked fish larvae samples were 72 +/- 12% (mean +/- SE).

Table 1. Nominal and actual concentrations of PAH in water and in organisms. Nominal PAH body-burdens were 100 nM/g for all compounds based on water solubility of BGP, bioconcentration factor, and molecular weights. N.D. indicates that no compound(s) was detected.

Compound	Water nominal (ug/L)	Water actual (ug/L)	Organism actual (nM/g)
ACR	526	525	120.6
ANT	14.7	5.4	111.8
PHE	14.5	10.0	293.2
BAA	1.9	1.8	6.7
BBA	1.1	1.9	N.D.
DBA	0.25	0.15	0.94
PYR	7.21	25.6	87.1
BAP	0.82	5.6	486.7
BEP	0.43	2.9	6.0
PER	0.79	1.7	7.5
BGP	0.20	0.15	3.7
BAN	31.6	49.5	52.9
SUVR-only control	0.0	N.D.	N.D.

Efficacy and Relative Potency Factor

The efficacy of each phototoxic compound was determined in a manner similar to that of Morgan and Warshawsky (6). Efficacy (Φ) is defined analagously to quantum yield in photochemistry and is a descriptor of the rate of larval mortality versus the rate of quanta absorbed by a compound in the larvae. The rate of mortality versus time can be described by equation 1.

$$\frac{d(\%\text{Mortality})}{dt} = \left[\frac{\sum_{\lambda}^{n} [(I_{o_\lambda} T_\lambda)\ (\epsilon_\lambda\ b\ C_a)]}{n} \right] \cdot \Phi \qquad = A \cdot \Phi \quad (1)$$

where: A = the average number of quanta absorbed per time
λ = waveband (UV-B=315-336 nm, UV-A=336-400 nm, VIS1=400-420 nm, and VIS2=420-450 nm).
$I_{o\lambda}$ = waveband radiation intensity (uW/cm^2)(9).
T_λ = optical transmittance of epidermis for waveband (15).
ϵ_λ = mean molar extinction coefficient of compound in octanol for waveband (L/mole/cm)(8).
b = depth of radiation penetration in organism (b = avg. diameter of larvae = 0.2 cm)
C_a = molar concentration of compound in organism (moles/kg).
n = number of wavebands
t = time (s)
Φ = efficacy of compound

Integration of equation 1 yields:

$$\%\text{Mortality} = A \cdot \Phi \cdot t + B \qquad (2)$$

which is in the form of a linear equation where, in plots of %Mortality versus time, B is the intercept and $A \cdot \Phi$ is the slope of the line. Efficacy, therefore, can be determined algebraically from knowledge of the calculated A and the slope of the %Mortality versus time curve for each individual compound. The Relative Potency Factor (RPF) is an index of the relative efficacy of a compound compared to the least efficacious of the compounds tested. Therefore, efficacy is a unique descriptor of the phototoxic activity of a compound and RPF gives a relative index of activity for the group of compounds used in this study.

RESULTS

Six of the 12 compounds tested exhibited acute photo-induced toxicity (Table 2). Median-lethal-time (LT50) values ranged from 0.83 h for BAN to 65.1 h for BAA. On the basis of RPF, BAN exhibited the greatest and BAP exhibited the least level of concentration and absorption-specific photo-induced toxicity among the compounds that were phototoxic (Table 2). Of the remaining six compounds, four compounds exhibited no effect compared to SUVR-only controls (BEP, DBA, PER, PHE) and the other two compounds exhibited a marginal level (<20% mortality in 96 h) of photo-induced toxicity (BBA, BGP). Mortality in SUVR-only controls was less than 5% in all tests. Contrary to the original design of this experiment, equimolar body-burdens of PAH were not obtained (Table 1), even though PAH concentrations in water were relatively close to the selected nominal concentrations. The equation used to calculate

A and Φ takes into account the concentrations of compound in the animal, so even though the achievement of equimolar body-burdens was desirable, it was not entirely necessary. Since BBA was not detected in fish tissue (Table 1) this compound was not used for further analysis.

Table 2. Tabulated values of median lethal times (LT50), average quanta absorbed (A), efficacy (Φ), and Relative Potency Factor (RPF) for all phototoxic compounds. Compounds are listed in decreasing order of relative potency.

Compound	LT50 (h)	A	Φ	RPF
BAN	0.83	0.183	5.46 E-2	337.1
PYR	3.20	0.372	1.45 E-2	100.1
ACR	4.30	0.397	7.00 E-3	48.3
ANT	15.75	0.218	3.12 E-3	21.5
BAA	65.09	0.100	2.38 E-3	16.4
BAP	40.05	2.913	1.45 E-4	1.0

Correlation analyses were performed with the measures of mortality and the chemical characteristics of the compounds to determine a structure-activity relationship. The factors considered included octanol-water partition coefficients, first and second order molecular connectivity indicies, energies of lowest singlet excited state splitting, energies of lowest triplet excited state splitting, the difference between singlet and triplet splitting energies, phosphorescence lifetimes, average molar extinction coefficients in octanol for each of the four wavebands examined, and the summed total of all molar extinction coefficients across all wavebands (315-450 nm). No significant univariate correlations were observed between any of the measures of mortality (LT50, RPF, $A \cdot \Phi$) and any of the above chemical characteristics.

Because no useful univariate predictive relationships were observed, discriminate analyses (13) were used to classify the compounds as being either phototoxic or non-phototoxic. All compounds tested were designated as being toxic (TOXIC) or non-toxic (NOTOX) on the basis of bioassay results, and a stepwise discriminant analysis was performed to determine which variables could be used to best classify the compounds into the two groups. Stepwise discriminant analysis determined that the best canonical discriminant model for classification of the compounds consisted of phosphorescence lifetime (PLT) and first order molecular connectivity index (MCl). Phosphorescence lifetime exhibited the main effect in the classification with a partial r^2 in

the model of 0.69 ($P > F = 0.003$) compared to MCl with a parital r^2 in the model of 0.39 ($P > F = 0.056$). Discriminant analyses were then conducted to calibrate a classification model to predict photo-induced PAH toxicity. All compounds were correctly classified when both phosphorescence lifetime and MCl were entered in the discriminant function (Table 3).

To determine the accuracy of the classification criterion, a test classification was performed using the 11 compounds tested in the present study plus an independent set of 17 PAH for which MCl was calculated and for which information on PLT was available (Table 4). Of compounds examined in the test classification, 12 (43%) were designated toxic and 16 (57%) were designated non-toxic (Table 4). Ten of the 12 compounds designated as toxic had posterior probabilities of greater than 90% for membership in the toxic classification and 13 of the 16 compounds designated as non-toxic had posterior probablities of greater than 90% for membership in the non-toxic classification (Table 4).

DISCUSSION

The results of these experiments demonstrate that PAH other than anthracene, which has been used in previous studies as a representative phototoxic PAH (10,11,12), are acutely phototoxic to fish. Based on RPF, anthracene ranked fourth out of 12 among compounds tested, exhibiting a median level of toxicity among the compounds that were toxic. From the point of view of relative toxicities, therefore, anthracene appears to be an adequate model compound for the examination of photo-induced PAH toxicity to fish. Anthracene also exhibits a median level of photo-induced toxicity to *Daphnia magna* (8) and with few exceptions, the relative toxicities of the various compounds were very similar between fish larvae and zooplankton. The relative photodynamic activities of PAH that were tested for toxicity against brine shrimp nauplii (6) do not correlate well with the RPF values obtained for the same compounds in the present study. This disagreement in the relative toxicities can be explained in part by the fact that, in the previous study (6), the concentration of PAH in organisms were based on nominal values. The importance of obtaining direct measurements of tissue PAH concentrations is illustrated by the present study because the selected nominal concentrations of PAH in fish were not all accurately obtained (Table 1).

The development of a classification scheme that can determine whether or not a PAH has the potential to cause photo-induced toxicity is significant. While the discriminant analysis presented here can only classify a compound as being toxic or non-toxic and has no predictive capabilities with regard to relative levels of toxicity, it may prove to possess power in assessing the potential for environmental impact and in identifying geographic areas of environmental concern. Validation of the current classification scheme will be difficult. However, comparisons among other studies show that most compounds examined in the test classification were designated correctly. All compounds considered in Morgan and Warshawsky (6) which were common to the

Table 3. Phosphorescence lifetimes (7) [PLT], first order molecular connectivity values (3) [MCl], classification of compound based on bioassay results, classification of compound by discriminant analysis with probability of membership, and linear discriminant function. TOXIC = phototoxic within 96 h, NOTOX = non-phototoxic within 96 h.

Compound	PLT (s)	MCl	Bioassay Designation:	Classified Into:	Posterior Probability Of Membership In: NOTOX	TOXIC
ACR	0.150	4.5856	TOXIC	TOXIC	0.0002	0.9998
ANT	0.090	4.8094	TOXIC	TOXIC	0.0003	0.9997
BAN	0.020	6.2635	TOXIC	TOXIC	0.0108	0.9892
BAA	0.359	6.2201	TOXIC	TOXIC	0.0185	0.9815
PYR	0.630	5.5594	TOXIC	TOXIC	0.0295	0.9705
BAP	0.105	6.9701	TOXIC	TOXIC	0.0621	0.9379
BGP	0.438	7.7201	NOTOX	NOTOX	0.5451	0.4549
DBA	1.600	7.6308	NOTOX	NOTOX	1.0000	0.0000
BEP	2.120	6.9761	NOTOX	NOTOX	1.0000	0.0000
PHE	2.940	4.8154	NOTOX	NOTOX	1.0000	0.0000
PER	3.500	6.9761	NOTOX	NOTOX	1.0000	0.0000

GENERALIZED SQUARED DISTANCE FUNCTION

$$D^2(I|J) = (X_i - X_j)\ COV^{-1}\ (X_i - X_j)$$

	NOTOX	TOXIC
NOTOX	0.000000	10.897554
TOXIC	10.897554	0.000000

LINEAR DISCRIMINANT FUNCTION

Constant = $-0.5\ X_j\ COV^{-1}\ X_j$ Coefficient Vector = $COV^{-1}\ X_j$

	NOTOX	TOXIC
Constant	-40.631639	-19.831543
MCl	9.257358	6.750125
PLT	8.536444	4.224812

Table 4. Phosphorescence lifetimes (7) [PLT], first order molecular connectivity values (3) [MCl], and results of discriminant analysis test classification for some selected polycyclic aromatic hydrocarbons. TOXIC = Predicted to be phototoxic, NOTOX = predicted to be non-phototoxic, * = Compounds tested in bioassays.

Compound	PLT (s)	MCl	Classified Into:	Posterior Probability of Membership in: NOTOX	TOXIC
Benzo(b)anthracene	0.01	6.2141	TOXIC	0.0000	1.0000
Phenazine	0.08	4.6546	TOXIC	0.0000	1.0000
* Acridine	0.15	4.5856	TOXIC	0.0002	0.9998
* Anthracene	0.09	4.8094	TOXIC	0.0003	0.9997
Benz(c)acridine	0.28	5.8541	TOXIC	0.0050	0.9950
Benz(a)acridine	0.41	5.8541	TOXIC	0.0100	0.9900
* Benzanthrone	0.02	6.2635	TOXIC	0.0110	0.9890
* Benzo(a)anthracene	0.36	6.2201	TOXIC	0.0185	0.9815
* Pyrene	0.63	5.5594	TOXIC	0.0295	0.9705
* Benzo(a)pyrene	0.11	6.9701	TOXIC	0.0621	0.9379
Benzo(b)chrysene	0.18	7.6308	TOXIC	0.2800	0.7200
Dibenz(a,c)phenazine	0.29	7.4819	TOXIC	0.2520	0.7480
* Benzo(g,h,i)perylene	0.44	7.7201	NOTOX	0.5451	0.4549
Fluoranthene	0.99	5.5654	NOTOX	0.7940	0.2060
Benzo(k)fluoranthene	0.83	6.9701	NOTOX	0.8900	0.1100
Benz(b)triphenylene	0.80	7.3867	NOTOX	0.9350	0.0650
Benzo(a)fluorene	2.61	6.0225	NOTOX	1.0000	0.0000
Benzo(b)fluorene	2.24	6.0166	NOTOX	1.0000	0.0000
Chrysene	2.54	6.2261	NOTOX	1.0000	0.0000
Fluorene	5.00	4.5118	NOTOX	1.0000	0.0000
Dibenz(a,h)acridine	2.31	7.5535	NOTOX	1.0000	0.0000
Carbazole	8.04	4.4046	NOTOX	1.0000	0.0000
Coronene	9.50	8.2142	NOTOX	1.0000	0.0000
Dibenz(a,j)anthracene	2.51	7.3421	NOTOX	1.0000	0.0000
* Dibenz(a,h)anthracene	1.60	7.6308	NOTOX	1.0000	0.0000
* Benzo(e)pyrene	2.12	6.9761	NOTOX	1.0000	0.0000
* Phenanthrene	2.94	4.8154	NOTOX	1.0000	0.0000
* Perylene	3.50	6.9761	NOTOX	1.0000	0.0000

test classification were designated correctly as being toxic or non-toxic. All but two compounds tested against D. magna by Newsted and Giesy (8) (fluoranthene and benzo(k)fluoranthene) matched the designated categories from the present study (Table 4). These compounds were of intermediate toxicity to D. magna with LT50 values of 10.8 and 13.0 h for fluoranthene and benzo(k)fluoranthene, respectively. Although these compounds appear to have been misclassified, comparisons of other phototoxic compounds studied by Newsted and Giesy (8) indicate that PAH causing photo-induced toxicity to D. magna with estimated LT50 values greater than 8 to 9 h are not phototoxic to fish within 96 h. More work is needed in this area of study before a more general assessment of PAH photo-induced toxicity to aquatic organisms can be accomplished.

The derivation of a classification criterion based on MCl and PLT lends important insight into the molecular mechanism of action in the photo-induced toxicity of PAH. The major factor determining whether or not a compound is classified as being phototoxic is PLT (cf. Results). PLT is a direct measurement of the radiative energy dissipation of a molecule from the excited triplet state to the singlet ground state (16). Molecules such as PAH in excited states may return to the ground state in a number of pathways, including radiative processes in which energy is dissipated in the form of light and heat. Non-radiative processes may also occur, where energy from a photosensitized molecule is passed to other molecules leading to the formation of excited states in, and reactions with, these other molecules. Radiative and non-radiative processes operate simultaneously and the net dissipation of energy from an excited state molecule is an integration of these competing processes. The probability of photosensitized reactions with molecules for which radiative processes dominate is directly proportional to the lifetime of the excited state (16). The results of these experiments demonstrate, however, that PAH with short phosphorescence lifetimes are more phototoxic to fish larvae, indicating that the photo-induced toxicity of PAH is inversely proportional to the length of existance of the excited triplet state. The rate of energy transfer in non-radiative processes has been shown to be inversely proportional to the radiative lifetime of excited states (1). These facts lead to the speculation that the photo-induced toxicity of PAH to fish is determined by the rate of non-radiative energy transfer from the excited state of a particular compound. Therefore, mechanisms which depend on the lifetime of excited states, such as direct interaction (5), most likely do not predominate, and reactions which depend on the rate and efficiency of energy transfer, such as the formation of reactive singlet oxygen (1), are more probable.

CONCLUSIONS

This study has demonstrated that the potential exists for many PAH to cause photo-induced toxicity to fish, and that anthracene has been an adequate

model compound in the previous studies of this phenomenon (10, 11, 12). The development of a classification criterion, based on easily obtained information about the structure and photochemistry of a compound, to predict whether or not a PAH has the potential to cause photo-induced toxicity to fish is a significant advancement in the assessment of the possible environmental impact of these compounds.

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REFERENCES

1. Bennett, R.G., and R.E. Kellogg. Photochem. Photobiol. 7: 571 - 581 (1968).
2. Dworkin, M. J. Gen. Physiol. 41: 1099 - 1112 (1958).
3. Koch, R. Toxicol. Environ. Chem. 6: 87 - 96 (1983).
4. Kochevar, I.E., R.B. Armstrong, J. Einbinder, R.R. Walther, and L.C. Harber. Photochem. Photobiol. 36: 65 - 69 (1982).
5. Landrum, P.F., J.P. Giesy, J.T. Oris, and P.M. Allred. In J.H. Vandermeulen and S. Hrudey, eds., Oil and Freshwater: Chemistry, Biology, Technology, Pergamon Press, NY, (in press) (1986).
6. Morgan, D.D., and D. Warshawsky. Photochem Photobiol. 25: 39 - 46 (1977).
7. Morgan, D.D., D. Warshawsky, and T. Atkinson. Photochem. Photobiol. 25: 31 - 38 (1977).
8. Newsted, J.L., and J.P. Giesy. Environ. Toxicol. Chem. (in press) (1986).
9. Oris, J.T. PhD Dissertation. Michigan State University, East Lansing, MI (1985).
10. Oris, J.T., and J.P. Giesy. Aquat. Toxicol. 6: 133-146 (1985).
11. Oris, J.T., and J.P. Giesy. Environ. Toxicol. Chem. 5: 761 - 768 (1986).
12. Oris, J.T., J.P. Giesy, P.M. Allred, D.F. Grant, and P.F. Landrum. In T.N. Veziroglu, ed., The Biosphere: Problems and Solutions, Elsevier Science Publ. B.V., Amsterdam, pp. 639-658 (1984).
13. SAS Institute Inc. 1982. Statistical Analysis System, Cary, NC.
14. Versteeg, D.J., and J.P. Giesy. Arch. Environ. Contam. Toxicol. 14: 631 - 640 (1985).
15. Wan, S. R.R. Anderson, and J.A. Parrish. Photochem. Photobiol. 34: 493 - 499 (1981).
16. Wells, C.H.J. Introduction to Molecular Photochemistry. Halsted Press, NY, 146 pp. (1972).

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