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UVb-Induced Toxicity of PAHs: Effects of Substituents and Heteroatom Substitution

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Polycyclic aromatic hydrocarbons (PAHs) are not typically acutely toxic in standard laboratory tests because of their low aquatic solubility. However, a number of investigators have demonstrated that uptake of PAHs followed by exposure to solar radiation, in particular ultraviolet-B (UVb), results in acute toxic effects (Arfsten et al. 1996). Newsted and Giesy (1987) determined the photo-induced toxicity of a variety of PAHs on the microcrustacean *Daphnia magna.* They were able to develop quantitative structure-activity relationships (QSARs) based on several photophysical parameters: (a) lowest singlet and triplet energies, (b) singlet-triplet splitting energy, and (c) phosphorescence lifetime. More specifically, Newsted and Giesy (1987) found a parabolic relationship between photo-induced toxicity, measured as the median adjusted lethal time normalized to a constant concentration, and the energy of the triplet state of the PAHs. Recently, Mekenyan et al. (1994) reexamined these data and proposed a more mechanistic approach for the prediction of photo-induced toxicity of PAHs. They used a molecular predictor that could be calculated from chemical structure instead of empirical formula. Mekenyan et al. (1994) found that the $E_{HOMO-LUMO}$, the difference in the energies (E) of the highest-occupied-molecular-orbital ($_{HOMO}$) and lowest-unoccupied-molecular-orbital ($_{LUMO}$), was a useful descriptor to evaluate the molecular stability, light absorbance, and photo-induced PAH toxicity.

Photo-induced toxicity is the result of internal and external factors. Internal factors include light absorbance and chemical stability, while external factors include irradiation wavelength and irradiation intensity. Mekenyan et al. (1994) have shown that phototoxicity is the result of all these factors, resulting in a parabolic relationship between photo-induced toxicity and chemical structure (i.e., orbital energy difference).

The result is a "phototoxic window". Extrapolating from these findings, Veith et al. (1995) explored the effects of substituents on UVb photo-induced acute toxicity of PAHs. They concluded that alkyl- and hydroxyl-substitution has little impact on $E_{HOMO-LUMO}$ values and are unlikely to alter photo-induced toxicity from the parent compound. However, nitro- and alkene-substitution will have a greater effect on $E_{HOMO-LUMO}$ and may alter photo-induced toxicity.

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The purpose of this study was to use the common freshwater ciliate *Tetrahymena pyriformis* to develop a flexible, time- and cost-effective protocol to evaluate photo-induced acute toxicity. This system was used to evaluate a series of 9-position-substituted anthracenes and heteroatom substituted PAHs in an effort to examine the effect of substituents on UVb-induced acute toxicity.

METHODS AND MATERIALS

The chemicals tested (Table 1) include: (a) three positive controls, anthracene, pyrene, and acridine; (b) a negative control, phenanthrene; (c) eight 9-position-substituted anthracenes, and (d) five other heteroatom-substituted PAHs. Chemicals were purchased from either Aldrich Chemical Company, Inc., Milwaukee, WI, USA or Lancaster Synthesis Inc., Windam, NH, USA, and each had a purity of 95% or better. Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration varying from of 50 to 7,500 mg/L.

The 1-octanol/water partition coefficients (log K_{OW}) were computer estimated or retrieved as measured values from "ClogP for Windows"(BioByte Corp., Claremont, CA, USA). The molecular orbital quantum chemical parameter, $E_{HOMO-LUMO}$ was calculated using the AMI Hamiltonian from MOPAC6 quantum chemistry program (Stewart 1990). Low energy conformations of the molecules were produced by the COBRA conformational analysis package (version 3.0; Oxford Molecular Ltd., Oxford, UK). The lowest energy confirmation was then read into the PIMMS molecular modeling package (version 1.45) (Oxford Molecular Ltd., Oxford, UK) to produce an input file suitable for MOPAC. The geometry of the structure was optimized in MOPAC prior to molecular orbital calculations being performed.

Exposure concentration and chemical persistence were examined with the aid of a spectrophotometer. The wavelength of maximum absorbance (Table 1) was determined by scanning. Primary stock solutions were prepared in DMSO. Working stock solutions were prepared in sterile distilled water with no greater than 1% DMSO. Concentration absorbance curves at the maximum absorbance wavelength were determined for each PAH. Aliquots of working stock solution, no greater than 2.5 ml, were added to 10 ml volumetric flasks prior to filling with sterile 0.1X Hank's solution. A minimum of two replicates of no less than ten concentrations each were evaluated. Each replicate was made using fresh stock solutions. These curves were used to verify toxicity test concentrations (Table 1).

The percent lipid in *Tetrahymena pyriformis* was determined via solvent extraction. *Tetrahymena pyriformis* was reared under standard conditions (see following paragraph). Cell suspensions were concentrated by centrifugation and divided into four replicates prior to total dry weight determination. Dried cells were extracted three times with 12 ml of a 2:1 chloroform-methanol mixture.

Solvent extracts were pooled, dried, and lipid dry weight determined. Mean lipid content of *T. pyriformis* was determined to be 10.71 ± 0.22 (SE) %. The bioconcentration factors (BCFs) were predicted from the K_{OW} values (i.e., mean lipid content times K_{OW}).

Short-term static mortality tests were performed using the common freshwater ciliate *T. pyriformis* strain GL-C. From a 3-d-old axenic culture, reared at room temperature in 250 ml Erlenmeyer flasks containing 50 ml of a semi-defined medium (Schultz 1996), a 20 ml aliquot was diluted with 80 ml of 0.1X Hank's basic salt solution. Diluted cultures were allowed to stand at room temperature for at least 5 hrs to allow the cells to complete a final division prior to use. A known amount of stock solution was added to 10 ml of 0.1X Hank's basic salt solution in a sterile 16 X 125 mm Pyrex tube. In every case, the volume of stock solution added to each tube was limited so the final DMSO concentration did not exceed 0.5% (50 µ1 / 10 ml), an amount that preliminary tests had shown did not effect *Tetrahymena* viability. Following vortex stirring, 0.1 ml of diluted *Tetrahymena* culture was added to each tube. This made the final cell density approximately 10^5.

As noted previously, cultures prepared were exposed in the dark for 16 hrs at 26°C. Subsequently, *Tetrahymena* were examined for viability and motility with the aid of a dissecting microscope. The no observable effect concentrations and lethal concentration for 100% of the ciliates were determined. Cultures showing no toxic effects following exposure without light were illuminated with broad-band "blacklight" (UVb; 350 +/- 50 nm) radiation. Illumination was carried out with a Rayonet Model RMR-600 Photochemical Mini-Reactor in conjunction with a merry-go-round unit (The Southern New England Ultraviolet Co., Branford, CT, USA) operated in an environmental chamber at 26°C. At noted time intervals, the UVb light was shutoff and cell viability was evaluated, scored and subsequently the time (minutes) to 100% mortality was determined.

RESULTS AND DISCUSSION

Chemical Abstract Service registry numbers, selected molecular descriptors, and toxicity values are given in Tables 1 and 2. The log K_{ow} values spanned a range of over five log units from 1.78 for 1,10-phenanthroline to 6.01 for 9-phenylanthracene (Table 1). The log aquatic solubilities and BCF values show similar distributions (Table 1).

The $E_{HOMO-LUMO}$ values varied by almost 2 eV from -7.00 to -8.81 eV (Table 2). Of the seventeen chemicals tested, only the five with the greatest water solubility exhibited acute toxicity in the "dark" assay (Table 2). Of the evaluated compounds, five were determined to be non-phototoxic following 1,000 minutes of UVb exposure (Table 2). There was no correlation between aquatic solubility and non-phototoxicity.

Since photo-induced toxicity can only be elicited by activation of bioabsorbed molecules, a constant toxicant concentration within the protozoa is critical to comparing phototoxicity. To eliminate the effect of different internal concentrations resulting from different BCFs, results of the photo-induced mortality tests were compared at water concentrations approximating the same internal concentration, 75 µM/mg (Table 2). For the twelve phototoxic chemicals time to mortality varied from 52 to 770 min. A comparison of time to mortality for anthracene derivatives with unsaturated substituents resulted in the greatest

Table 1. Molecular descriptors of selected polycyclic aromatic compounds

Compound	CAS number[a]	Log K_{ow}[b]	Melting point (MP; °C)	Log aquat. sol.[c] (M)	BCF[d]	Absorbance wavelength (nm)	Molecular weight	Tested conc. (μM/mg)
xanthene	92-83-1	4.23[m]	102	-4.20	1.8×10^3	210.0	182.22	71.6
phenanthridine	229-87-8	3.40	109	-3.44	2.7×10^2	206.5	179.22	68.2
1,10-phenanthroline	66-71-7	1.78[m]	117	-1.90	6.4×10^1	322.0	180.21	74.4
phenanthrene	85-01-8	4.47[m]	101	-5.15[m]	3.2×10^3	203.5	178.23	75.4
phenothiazine	92-84-2	4.15[m]	185	-4.95	1.5×10^3	209.0	199.28	74.5
acridine	260-94-6	3.40[m]	110	-3.45	2.7×10^2	243.0	179.22	75.3
anthracene	120-12-7	4.45[m]	218	-6.68[m]	3.2×10^3	209.0	178.23	79.0
pyrene	129-00-0	5.00[m]	156	-6.18[m]	1.1×10^4	212.0	202.26	78.9
9-acetylanthracene	784-04-3	3.93	76	-3.64	9.1×10^2	246.5	220.27	74.8
9-anthraldehyde oxime	34810-13-4	4.11	165	-4.71	1.4×10^3	209.0	221.26	75.9
9-methylanthracene	779-02-2	5.07[m]	79	-5.87[m]	1.3×10^4	212.0	192.26	77.8
9-phenylanthracene	602-55-1	6.01[m]	154	-6.50	1.1×10^5	212.0	254.33	73.5
9-anthraldehyde	642-31-9	3.84	105	-3.84	7.4×10^2	209.0	206.24	75.0
9-vinylanthracene	2444-68-0	5.21	65	-4.81	1.7×10^4	210.0	204.27	74.9
9-nitroanthracene	602-60-8	4.78[m]	146	-5.19	6.4×10^3	210.0	223.23	74.5
9-cyanoanthracene	1210-12-4	4.26[m]	177	-4.98	1.9×10^3	210.0	203.24	73.2
thianthrene	92-85-3	4.47[m]	159	-5.01	3.2×10^3	210.0	216.32	74.0

[a]Chemical Abstract Service registry number; [b]Calculated values from the "ClogP for Windows" program; [c]Log aquatic solubility (M)≈ log K_{ow}-0.01 MP + 1.05 (Yalkowsky and Valvani 1980); [d]BCF = 0.107 (K_{ow}); [m]measured value

Table 2. Toxicity to *Tetrahymena pyriformis* and molecular stability of selected polycyclic aromatic compounds

Compound	16-hr dark NOEC (mM)	16-hr dark LC_{100} (mM)	UVb Phototoxicity (min)	Log (phototox^{-1})	ΔE^a
xanthene	NTAS[b]	NTAS	NPT[c]	NPT	-8.81
phenanthridine	6.7×10^{-2}	1.7×10^{-1}	NPT	NPT	-8.40
1,10-phenanthroline	8.0×10^{-3}	1.1×10^{-2}	NPT	NPT	-8.31
phenanthrene	5.0×10^{-4}	7.0×10^{-3}	NPT	NPT	-8.20
phenothiazine	NTAS	NTAS	368	-2.57	-7.78
acridine	5.9×10^{-2}	2.0×10^{-1}	228	-2.36	-7.52
anthracene	NTAS	NTAS	52	-1.72	-7.29
pyrene	NTAS	NTAS	56	-1.75	-7.24
9-acetylanthracene	1.8×10^{-2}	4.5×10^{-2}	116	-2.06	-7.21
9-anthraldehydeoxime	NTAS	NTAS	300	-2.48	-7.18
9-methylanthracene	NTAS	NTAS	420	-2.62	-7.17
9-phenylanthracene	NTAS	NTAS	660	-2.82	-7.17
9-anthraldehyde	NTAS	NTAS	375	-2.57	-7.14
9-vinylanthracene	NTAS	NTAS	720	-2.86	-7.12
9-nitroanthracene	NTAS	NTAS	720	-2.86	-7.11
9-cyanoanthracene	NTAS	NTAS	770	-2.89	-7.07
thianthrene	NTAS	NTAS	NPT	NPT	-7.00

[a]The difference between E_{HOMO} and E_{LUMO}; [b]not toxic at saturation; [c]not phototoxic after 1,000 minutes.

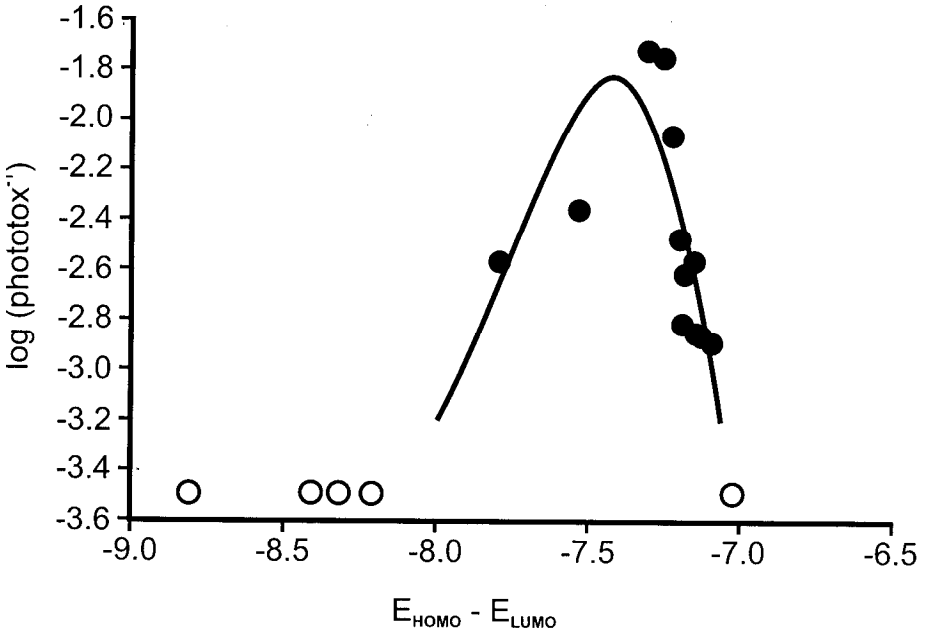

Figure 1. A Semi-log plot of inverse of time to 100% *Tetrahymena pyriformis* mortality versus $E_{HOMO-LUMO}$ (open circles represent chemicals that were not phototoxic following 1,000 minutes of exposure).

resistance to photo-induced toxicity. While nitrogen heterocyclic compounds (e.g., acridine) were phototoxic, sulfur or oxygen heterocyclic compounds were resistant to photo-induced toxicity.

Mekenyan et al. (1994) presented a mechanistic explanation for photo-induced toxicity of PAHs. They noted that photo-inducted toxicity is due to two types of factors: (a) internal factors, relates to molecular structure that determines the ability to absorb light and stability, and (b) external factors, relates to radiation energy and intensity. The $E_{HOMO-LUMO}$ is an index of the electronic structure that relates to both light absorbance and molecular stability with threshold values defining toxic and non-Toxic PAHs (Mekenyan et al. 1994). In the case of the experimental conditions imposed by Newsted and Giesy (1987) the photo-inducible region was about 7.2 ± 0.4 eV (Mekenyan et al. 1994). A plot of time to mortality versus $E_{HOMO-LUMO}$ for the data in the present study is shown in Figure 1. The present results compare very favorably with those of Mekenyan et al. (1994) as only PAHs within the $E_{HOMO-LUMO}$ window of -7.3±03 eV were UVb phototoxic.

Photo-induced toxic potency varies depending on specific exposure parameters, in particular, intensity (Arfsten et al. 1996). With an increase in intensity, more photons are available for toxicant activation (Mekenyan et al. 1994). Therefore, irradiation intensity should be directly related to photo-induced toxicity. In an effort to evaluate the effect of intensity on time to mortality, anthracene, acridine,

and pyrene were tested at identical concentrations (1.35 mM/mg) and wavelength but with varying intensities. Intensity was varied by altering the number of 4.5 watt bulbs (i.e., 1,2,4, or 8) energized during the exposure period. The time to 100% mortality was found to be inversely related to intensity (Table 3). However, when intensity was increased eight-fold (i.e., 8 bulbs energized), neither thianthrene nor phenanthrene, compounds with $E_{HOMO-LUMO}$ values bracketing the photo-induced toxicity window, became phototoxic (Table 3).

Table 3. Effects of UVb light intensity on time (minutes) to 100% *Tetrahymena pyriformis* mortality

	Intensity (number of 4.5 watt bulbs)			
Compound	8	4	2	1
anthracene	<1	1.5	3	5.5
acridine	2.5	5.5	11	22
pyrene	<1	1.3	2.5	5
thianthrene	NPT[a]	NPT	NPT	NPT
phenathrene	NPT	NPT	NPT	NPT

[a]not phototoxic after 1,000 minutes.

In an effort to evaluate the effect of concentration on time to mortality, anthracene acridine, and thianthrene were tested at different concentrations. Results showed that for those chemicals that were phototoxic (i.e., anthracene and acridine) as concentration increased time to 100% *T. pyriformis* mortality decreased in a linear fashion (Table 4). However, when the concentration was increased, thianthrene did not become phototoxic.

Table 4. Effects of toxicant concentration on time (minutes) to 100% *Tetrahymena pyriformis* mortality

	Concentration (μM/mg)				
Compound	75	150	225	300	1350
anthracene	52	30	22	15	5.5
acridine	228	125	90	70	22
thianthrene	NPT[a]	NPT	NPT	NPT	NPT

[a]not phototoxic after 1,000 minutes.

In conclusion, *T. pyriformis* used in conjunction with a Rayonet Photochemical Mini-Reactor provides a rapid and inexpensive means of determining photo-induced toxicity. The results of using such an apparatus compare favorably with the *Daphnia magna* assay of Newsted and Giesy (1987). Additionally, the $E_{HOMO-LUMO}$ photo-induced toxicity "window" described by Mekenyan et al. (1994) holds

for substituted and heteroatom-containing PAHs. Lastly, while intensity effects time to 100% mortality it does not alter the range of the photo-induced toxicity window. Thus, a short-term, high intensity exposure regime is a surrogate for the more natural long-term, low intensity exposure.

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