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SOLAR RADIATION-INDUCED TOXICITY OF ANTHRACENE TO *DAPHNIA PULEX*

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Abstract—Photoinduced anthracene toxicity to *Daphnia pulex* was investigated. Organisms were exposed to three nominal anthracene concentrations (3.0, 9.6 and 30.0 $\mu g\ L^{-1}$) in static bioassays on clear, partly cloudy and cloudy days. A "shell coating" technique was used to achieve concentrations within the aqueous solubility range of anthracene and to obviate the need for a carrier solvent. Photoinduced anthracene toxicity was not observed under laboratory lighting conditions; it occurred only in the presence of solar radiation. A dose-response relationship existed for both anthracene concentration and solar radiation intensity. Anthracene was only slightly less toxic to organisms transferred into water containing no anthracene before exposure to solar radiation. This indicates that toxicity resulted from activation by solar radiation of material present on or within the animals and not in the water. Activation appeared to be of anthracene molecules and not anthracene degradation products, since similar concentrations of anthraquinone, the primary and most stable degradation product of anthracene, were not toxic at similar solar radiation intensities. Additionally, a series of filters was used to selectively remove UV wavelengths from solar radiation to determine the photoactive wavelengths. Mylar film absorbs in the UV-B region (285 to 315 nm) of solar radiation and Corning 0-52 glass absorbs essentially the entire spectrum of UV wavelengths (285 to 380 nm). Placement of Mylar film over bioassay beakers diminished photoinduced anthracene toxicity only slightly, whereas Corning 0-52 glass reduced toxicity proportionate to the reduction in UV intensity. Thus, wavelengths in the UV-A region (315 to 380) are primarily responsible for photoinduced anthracene toxicity.

Keywords—Anthracene *Daphnia* Photoinduced toxicity Bioassay Solar radiation

INTRODUCTION

Compounds of the class known as polycyclic aromatic hydrocarbons (PAH) are composed of two or more fused benzene rings, with occasional incorporations of cyclopentene rings and heteroatoms such as nitrogen and sulfur [1]. These compounds are ubiquitous in the environment from natural and anthropogenic sources [2]. Natural PAH sources include forest fires [2,3] and volcanic activity [2]. Some PAH are formed by plants and microbes [1]. However, most PAH inputs into the environment result from pyrolytic processes attributable to human activities [4] such as fossil fuel combustion.

Laboratory bioassays in which PAH have been found to be acutely toxic have often used carrier solvents that resulted in measured concentrations greatly in excess of aqueous solubility limits and environmental concentrations. Within aqueous solubility limits, many PAH are not acutely toxic [5]. For these reasons there has been greater concern over possible chronic, sublethal effects such as carcinogenicity and reproductive impairment in aquatic animals or bioaccumulation by animals as possible vectors of exposure to humans. In addition, laboratory bioassays of PAH are usually conducted under artificial lighting to minimize photodecomposition of the test compounds. Giesy et al. [6] and Bowling et al. [7] have demonstrated that, in the presence of solar radiation, anthracene, a linear, three-ring PAH (Fig. 1), is acutely toxic to bluegill sunfish (*Lepomis macrochirus*) at concentrations (12.7 $\mu g\ L^{-1}$) well within aqueous solubility limits.

The purpose of the present study was to further characterize the interaction between ecologically relevant solar radiation intensities and anthracene concentrations that are acutely toxic to *Daphnia pulex*. Specific objectives were to (a) determine the dose-response relationship for anthracene concen-

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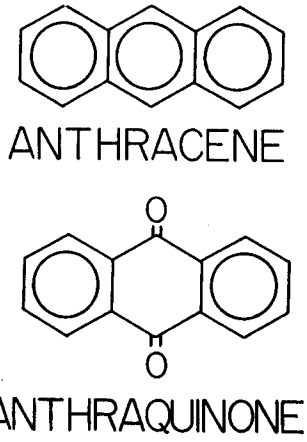

Fig. 1. Structures of anthracene and anthraquinone.

tration and solar radiation intensity, (b) determine if toxicity results from activation of anthracene molecules associated with *D. pulex* or molecules present in the water, (c) determine if toxicity is a result of anthracene or an anthracene degradation product and (d) determine the wavelengths of solar radiation responsible for photoinduced toxicity.

MATERIALS AND METHODS

Static acute toxicity bioassays with *D. pulex* [8] were used to characterize the actinic toxicity of anthracene. Established protocols were followed as closely as possible, but because of the unique nature of the phototoxic phenomenon, some modifications were required (see Discussion). Organisms used in the study were obtained from a research pond at the Michigan State University Limnological Research Facility and identified by D. J. Hall of Michigan State University. Cultures were maintained in well water (see Table 1 for water chemistry) and fed yeast (Red Star) and the green alga *Chlorella pyrenoidosa*. Organisms in the cultures readily reproduced, and regular harvesting was required to maintain the cultures in a growth phase.

The addition of organic carrier solvents directly to bioassay water is inappropriate for photochemical studies because many organic solvents are photosensitizers and can affect the bioavailability of PAH. A photosensitizer is a chemical that causes chemical reactions that do not occur in its absence [9]. For this reason, we employed a "shell coating" technique that obviated the need to add organic solvents directly to bioassay water. Anthracene (FW 178.23, Gold Label, purity 99.9%; Aldrich Chemical Co.) was combined with 9-^{14}C-labeled anthracene (3.3 μCi μM^{-1}, radiochemical purity 98%; California Bionuclear) in HPLC-grade acetone (Fisher Scientific Co.) to prepare a stock solution having 2.0 μg anthracene/ml acetone and an activity of 0.14 μCi ml^{-1} (70 nCi μg^{-1} anthracene). The aqueous solubility limit of anthracene has been reported in the literature to range from 30 μg L^{-1} [10] to 44.6 μg L^{-1} [11] at 25°C. A progressive bisection of the log scale [12] was used to select three nominal anthracene concentrations of 3.0, 9.6 and 30.0 μg L^{-1} from the anthracene solubility range. Anthracene stock solution was added to 300-ml beakers and the acetone was evaporated to dryness under a fume hood. Control beakers received only acetone. Two hundred milliliters of filtered (Whatman No. 42) well water was added to each beaker along with 20 adult *D. pulex* of approximately the same size and age. Each beaker, including controls, was gently swirled every 6 to 12 h for 24 h to allow the anthracene concentrations in the water to achieve steady state and the organisms to be "loaded" with anthracene.

Table 1. Water chemistry for water used in cultures and bioassays

Conductance (μmhos cm^{-1})	590
pH	7.20
Chloride (mg L^{-1})	11
Total phosphate (mg L^{-1})	0.022
Orthophosphate (mg L^{-1})	0.007
Nitrate (mg L^{-1})	0.010
Alkalinity (mg L^{-1})	306
Hardness (mg L^{-1} $CaCO_3$)	350
Dissolved oxygen (mg L^{-1})	8.8

Although the same shell-coating protocol was followed each time a bioassay was run, nominal concentrations were not always achieved. The efficiency with which the coated anthracene partitioned into the water varied with the amount of stock solution added to the beaker. Trial and error experiments were conducted prior to the bioassays to determine how much stock solution had to be shell-coated onto the beakers to attain measured concentrations that were near nominal concentrations.

For the experiments reported in this study, 0.3 ml of stock solution shell-coated on the bottom of a beaker resulted in an average anthracene concentration of 1.4 ± 0.27 μg L^{-1} (mean ± 1 SE; $n = 6$). Thus, 46.7% of the added anthracene went into solution. When 2.0 ml of the stock solution was shell-coated onto the beakers, the average anthra-

cene concentration was 9.7 ± 0.73 μg L^{-1} (mean $\pm$ 1 SE; $n = 12$) or 48.5% of added anthracene. Addition of 6.0 ml of stock resulted in an average concentration of 26.9 ± 1.35 μg L^{-1} (mean $\pm$ 1 SE; $n = 12$) or 44.8% of added anthracene. Even though the water temperature for all experiments was 22 ± 1°C during the 24-h period anthracene was permitted to go into solution, actual measured concentrations differed slightly from nominal concentrations.

Anthracene concentrations were determined by assaying duplicate 1-ml water samples taken from each beaker for radioactivity. Ten milliliters of scintillation fluor (PPO-POPOP plus Triton X-100) was added to each 1-ml water sample and a Nuclear Chicago Isocap/300 liquid scintillation counter (Program 2) was used to assay for radioactivity. All samples were corrected for background (45 to 50 cpm), quench and counting efficiency (98.5%). A quench curve and the sample channels ratio were used to determine quench and counting efficiency.

The test endpoint was organism immobilization: When an organism displayed no movement for 1 min, it was considered to be immobilized. The reported values are percentages of the test populations that were immobilized. Exposures were continued until approximately 50% of the organisms in the beakers having the intermediate anthracene concentrations were immobilized. Since anthracene was not toxic at the concentrations studied in the absence of UV radiation, the term "exposure" in this study means exposure to UV radiation. Each treatment was carried out in duplicate, and the reported anthracene and anthraquinone concentrations are the means of two determinations. Results of all bioassays are presented graphically as time to daphnid immobilization for each treatment.

In the experiment designed to determine if a dose–response relationship existed for anthracene, all three nominal concentrations were included. Exposures were conducted on clear (0% cloud cover), partly cloudy (50% cloud cover) and cloudy (100% cloud cover) days in an effort to determine if a dose–response relationship existed for solar radiation. Exposures were begun 24 h after the water and organisms were placed in the anthracene-coated beakers. Organisms remained in the beakers containing anthracene during solar radiation exposures, except for the experiment designed to determine whether toxicity resulted from anthracene associated with the animal or simply from the anthracene present in the water. For this experiment, organisms were loaded in water containing the various concentrations of anthracene and then transferred to water containing no anthracene prior to solar radiation exposure.

To determine if an anthracene degradation product was responsible for photoinduced toxicity, we conducted an experiment using anthraquinone (Fig. 1). Anthracene degrades to several compounds, but anthraquinone is the primary and most stable of the degradation products observed under our test conditions [13,14]. The experimental design was identical to that of the anthracene experiments, and similar concentrations and solar radiation intensities were used. Anthraquinone (FW 208.22, Baker Grade; J. T. Baker Co.) was dissolved in HPLC-grade acetone to prepare a stock solution having a concentration of 2.0 μg L^{-1}.

Anthraquinone concentrations at the end of the exposure were determined by extraction once with a 20-ml volume of toluene and twice with 20-ml volumes of ethyl acetate. Extracts were dried by passing through sodium sulfate and reduced in volume by rotary flash evaporation and evaporation under a stream of nitrogen. Fluorene internal standards were added and final volumes were adjusted to 200 μl. Gas chromatography (1.83 m, 4 mm i.d., packed column of 3% OV-101 on Supelcoat; Packard Model 804) was used to quantitate anthraquinone by measuring peak heights of peaks having the same retention time as anthraquinone standards. Anthraquinone concentrations are corrected for extraction efficiency (87.6 ± 1.57%, mean $\pm$ SE; $n = 4$), which was determined with samples spiked with known amounts of anthraquinone.

A series of filters was used to selectively remove UV wavelengths from natural solar radiation. Ozone in the earth's atmosphere removes essentially all wavelengths shorter than 285 nm. Mylar (E. I. du Pont de Nemours and Co.) film absorbs wavelengths in the UV-B (285 to 315 nm) region and Corning 0-52 (Corning Glass Works) glass absorbs wavelengths in both the UV-A (315 to 380 nm) and UV-B regions. Placement of Mylar and Corning 0-52 glass filters over a series of beakers allowed us to selectively remove bands of wavelengths. Measurements made in the field with a radiometer revealed that Mylar film and Corning glass did not completely remove all solar radiation in their respective absorption regions, and only a 70 to 75% reduction of intensity in the two wavebands was practical. Placement of the filters over the beakers also slightly reduced the intensity of unfiltered wavelengths. For this reason, cellulose triacetate (CTA) film (Kodacel; Eastman

Kodak Co.), which slightly reduces intensity but not spectral characteristics of solar radiation greater than 285 nm, was placed over a third set of beakers so that the intensity of unfiltered wavelengths was approximately the same among all treatments. The sides of all beakers were covered with aluminum foil so that solar radiation entered only through the filters. Only two nominal concentrations of anthracene (9.6 and 30.0 $\mu g\ L^{-1}$) were used in the experiment to determine active wavelength bands.

UV radiation was quantified with either a Macam Photometrics Model UV-103 radiometer equipped with Model SD104 UV-A or UV-B cosine-corrected photodiodes (Macam Photometrics, Ltd., Livingston, Scotland) or poly-(methylmethacrylate) actinometer films [15] containing *ortho*-nitrobenzaldehyde (ONBA). The ONBA in the actinometer films is quantitatively converted (efficiency 50%) to *ortho*-nitrobenzoic acid in the 290 to 400 nm range and conversion is quantified by monitoring the change in relative amplitude of the 1,530 cm^{-1} infrared absorption band.

RESULTS

D. pulex were not immobilized when exposed to anthracene under laboratory lighting during the 24-h equilibration period. Within aqueous solubility limits, anthracene is not phototoxic under fluorescent (wavelengths greater than 380 nm; F40CW; General Electric Corp.) laboratory lighting.

When organisms were exposed to anthracene and solar radiation under a clear sky (Fig. 2A), time to immobilization was a function of anthracene concentration. All organisms in beakers containing the greatest anthracene concentration were immobilized in less than 2 min. At the intermediate concentration, 100% of the organisms were immobilized in less than 10 min and in beakers containing the smallest concentration (1.2 $\mu g\ L^{-1}$), more than 50% of the organisms were immobilized in less than 15 min.

When *D. pulex* were transferred to water containing no anthracene before exposure to solar radiation, immobilization was not as rapid (Fig. 2B). However, 40 to 50% immobilization occurred within 2 min. After 30 min of exposure, 70% of organisms from beakers with the greatest anthracene concentration were immobilized, whereas 42% from the intermediate concentration and 30% from the lowest concentration were immobilized. Toxicity resulted from activation by solar radiation of material associated with the organisms.

A lesser solar radiation intensity resulted in

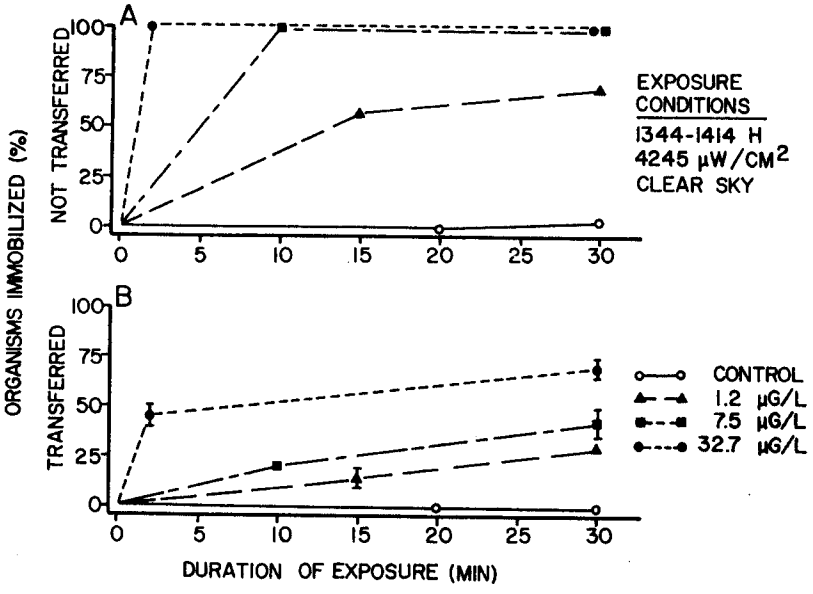

Fig. 2. Cumulative *Daphnia* immobilization as a function of anthracene concentration for clear sky conditions. Organisms were allowed 24 h to reach steady-state anthracene content and then either remained in water containing anthracene or were transferred to water with no anthracene before exposure to solar radiation. Symbols represent the means of two numbers. Brackets around a mean represent the range of the two numbers unless the range is too small to be shown.

greater time to immobilization. The greatest measured anthracene concentration during the exposure conducted under partly cloudy conditions (Fig. 3) was only 18.9 $\mu g\ L^{-1}$. This was well below the nominal 30.0 $\mu g\ L^{-1}$ concentration, but 100% immobilization still occurred within 30 min. At the intermediate concentration, 67% immobilization occurred within 60 min. With complete cloud cover (Fig. 4), 100% immobilization occurred in 60 min at the greatest anthracene concentration. No immobilization occurred in 60 min at the two lesser concentrations.

D. pulex were exposed to anthraquinone under a clear sky (0% cloud cover), with a solar radiation intensity similar to the intensity for the anthracene–clear sky exposure (Fig. 2A). Mean measured anthraquinone concentrations in the bioassay beakers were 24.9, 4.6 and 0.7 $\mu g\ L^{-1}$. No immobilization occurred in any of the beakers in 30 min; therefore, the results are not presented graphically. Anthraquinone was not phototoxic at the concentrations and solar radiation intensities studied.

In the experiment designed to determine the photoactive wave-length bands of solar radiation, all organisms in the beakers covered with the CTA filters (all UV wavelengths present) and having the greatest anthracene concentration were immobilized within 45 min (Fig. 5). At the lesser concentration, 50% were immobilized within 45 min. Mylar filters (absorbing UV-B wavelengths) reduced times to immobilization slightly, but 100% immobilization still occurred within 45 minutes at the greater concentration (Fig. 5). Corning 0-52 filters (absorbing both UV-A and UV-B wavelengths) reduced time to immobilization markedly (Fig. 5), although the UV light not removed by the filter (29% of incident) was quite effective in immobilizing organisms. The reductions in immobilization were proportionate to reductions

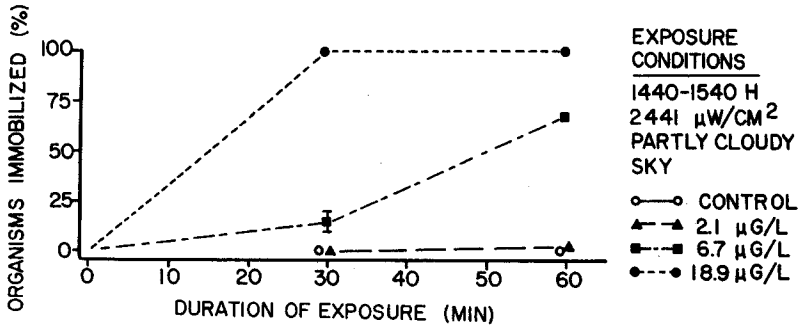

Fig. 3. Cumulative *Daphnia* immobilization as a function of anthracene concentration for partial cloud cover. Symbols represent the means of two numbers. Brackets around a mean represent the range of the numbers unless the range is too small to be shown.

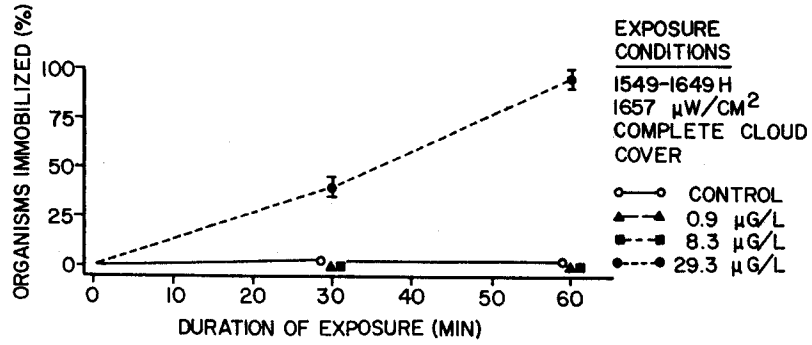

Fig. 4. Cumulative *Daphnia* immobilization as a function of anthracene concentration for complete cloud cover. Symbols represent the means of two numbers. Brackets around a mean represent the range of the numbers unless the range is too small to be shown.

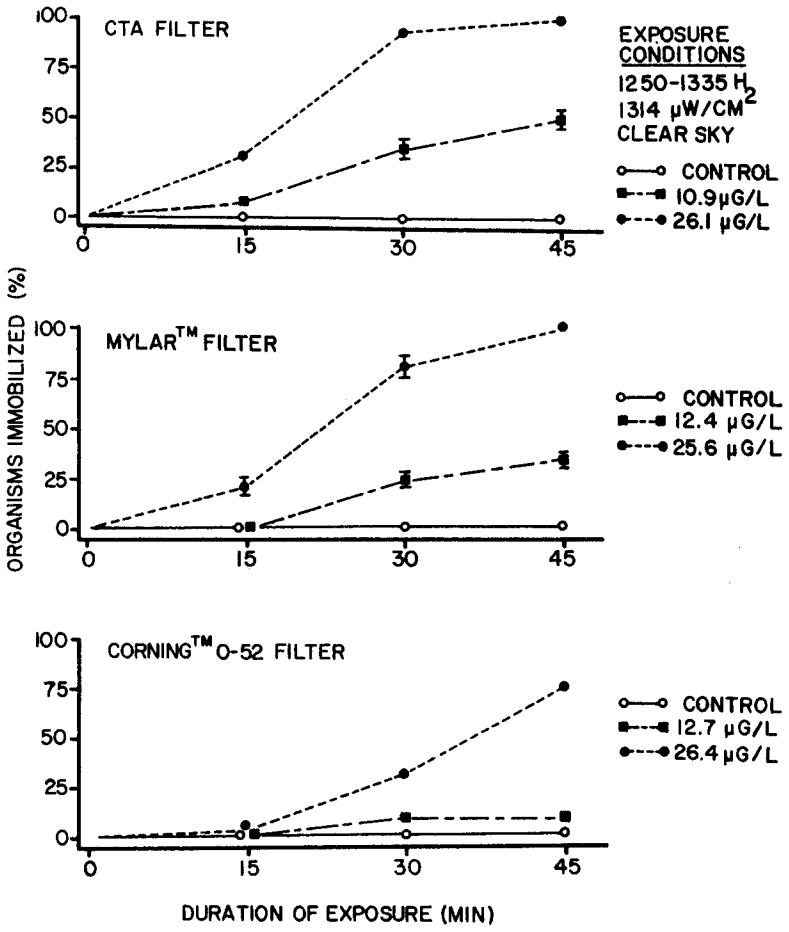

Fig. 5. Cumulative *Daphnia* immobilization as a function of anthracene concentration for clear sky conditions; bioassay beakers were covered with cellulose triacetate (CTA), Mylar or Corning 0-52 filters. Symbols represent the means of two numbers. Brackets around a mean represent the range of the numbers unless the range is too small to be shown.

in UV intensity. At the greater anthracene concentration, 74% of the organisms were immobilized in 45 min and, at the lesser concentration, 7% were immobilized in 45 min.

DISCUSSION

Organism immobilization is the endpoint commonly used in daphnid bioassays to calculate a 48-h EC50 [8,12]. Organism immobilization from photoinduced anthracene toxicity occurred in minutes rather than hours and immobilization at the lesser anthracene concentrations and solar radiation intensities was at times difficult to assess. Organisms would occasionally cease swimming, lie on the bottom of the beaker and then resume swimming. Conversely, at the greater concentrations and intensities, immobilization was so rapid that inspection of all the beakers for immobilized organisms was difficult. Use of the larger adult organisms instead of neonates made visual examination and assessment of immobilization simpler. Organisms with impaired locomotion did not recover when transferred back to the laboratory.

A dose-response relationship existed for both anthracene concentration and solar radiation intensity. As concentration and intensity increased, time to daphnid immobilization decreased. UV radiation alone is injurious to most aquatic organisms including bacteria, phytoplankton, zooplankton and fish larvae [16], though many organisms possess repair capabilities [17]. Processes responsible for UV-induced damage include disruption of cell membranes [18], inactivation of enzyme systems [19] and damage to chromatin material [20].

Bowling et al. [7] placed anthracene-contaminated fish in uncontaminated water for increasing periods of time prior to solar radiation exposure. Time to mortality increased as the fish eliminated anthracene. This indicates that anthracene reversibly partitions into fish. Bowling et al. also observed that fish exposed to anthracene photoproducts in the absence of solar radiation did not die. This indicates that anthracene photoproducts are not acutely toxic by themselves. In our studies with *D. pulex*, activation of anthracene occurred at some site on or within the organisms, and anthraquinone did not display actinic toxicity at the concentrations studied. Anthracene-contaminated *D. pulex* rapidly eliminated anthracene upon being placed into uncontaminated water, thereby increasing time to immobilization.

The best-known example of the actinic effects of radiant energy is photosynthesis. The nonpathological, photosensitized oxidation–reduction reactions in photosynthesis [refs. 21–23, cited in ref. 24] are closely related to photosensitized reactions responsible for phototoxicity. Raab reported in 1900 [ref. 25, cited in ref. 24] that, in the presence of sensitizing dyes and oxygen, microorganisms are killed by light. Through the years, numerous classes of natural and synthetic compounds have been found to be photoactive [26] including, but not limited to, sensitizing dyes [26,27], drugs [28], pesticides [29], thiophene compounds [30] and PAH [6,7,31,32].

Mechanisms of direct photooxidation and photosensitized oxidations have been reviewed by Foote [24,33]. Upon absorption of radiant energy, an organic molecule may have an electron excited to a singlet or triplet state. The singlet state is short-lived; therefore, most photosensitized oxidations are thought to proceed via the triplet state. The triplet sensitizer may interact with oxygen to form singlet molecular oxygen [33], which reacts further with various acceptors.

Singlet oxygen has been suggested to participate in anthracene endoperoxide formation and subsequent anthraquinone formation [1,14]. The addition of the electron-withdrawing substituents on the molecule alters absorption characteristics. Anthracene has absorption peaks at 295, 310, 324, 357 and 376 nm, whereas anthraquinone has only one absorption peak, at 330 nm [34]. Reduced photoinduced toxicity of anthraquinone and anthracene in the UV-B region probably results from the diminished absorption of UV radiation. UV-A wavelengths appear to be responsible for most of the photoinduced anthracene toxicity.

Photosensitized oxidations bring about many alterations in cellular structure and function that could cause photoinduced toxicity. Although the mechanisms responsible for the actinic toxicity of anthracene remain unknown, several possibilities exist. Damage may occur to DNA [32], structural proteins may be altered [32], enzymes may be inactivated [35] or lipid peroxidation may take place [32].

Organic compounds absorb at various wavelengths in the solar radiation spectrum. Those that absorb strongly in the UV range have a greater potential for photosensitization because UV wavelengths possess greater energy capable of causing molecular rearrangements [36]. Undoubtedly, organic compounds other than anthracene will prove to be phototoxic to aquatic animals. Although environmental concentrations of these compounds may be low, they are highly lipophilic, and aquatic organisms, particularly larvae with high lipid content, may acquire considerable body burdens of them. Juvenile life forms of many aquatic species show a high sensitivity to UV exposure [37] and they often reside in shallow habitats [38]. UV radiation penetrates to significant depths in natural waters [39] and conditions may presently exist in the environment under which organic pollutants cause photoinduced toxicity. The ecological significance of photoinduced toxicity has not been investigated.

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