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Photo-Enhanced Toxicity in Amphibians: Synergistic Interactions of Solar Ultraviolet Radiation and Aquatic Contaminants

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Amphibians experience a broad range of multiple environmental stressors that occur in natural systems. However, the impact of combinations of these stressors on amphibians are rarely examined. The effect of two stressors on amphibians, solar ultraviolet radiation (SUV) and environmental contamination, was investigated. To examine the interactive effects of SUV and environmental contaminants, *Hyla versicolor* and *Rana sphenocephala* were exposed in the laboratory to a carbamate insecticide and the water soluble fraction of a weathered petroleum in combination with various intensities of simulated solar radiation (SSR). The contaminants were tested at environmentally relevant concentrations. Synergistic interactions between SSR and these substances were observed during the exposures. Mortality of *H. versicolor* exposed to 2.51 mg/l carbaryl increased from 5% under control SSR conditions to 100% under low SSR irradiance (4 $\mu W/cm^2$). Exposure to a 10% solution of a water soluble fraction of petroleum under control SSR conditions was not lethal to *R. sphenocephala*; however under high SSR irradiance (17 $\mu W/cm^2$) a 5% water soluble fraction of petroleum was lethal. Relatively limited SSR irradiance is necessary to initiate photoenhanced toxicity, thus a range of amphibian habitats may be impacted by SUV. These studies indicate the importance of evaluating the interactive influence of environmental stressors present in amphibian habitats.

INDEX DESCRIPTORS: amphibian, ultraviolet radiation, aquatic contamination, interactive toxicity.

This investigation explores the potential impact that two environmental stressors, solar ultraviolet radiation (SUV) and environmental contamination, may have on amphibians. Many amphibians breed and develop through their early lifestages in shallow temporary pools. These habitats are vulnerable to chemical contamination from aerial transport, direct application, and runoff from point and non-point sources. For example, common amphibian habitats such as roadside ditches can receive significant amounts of lead, petroleum hydrocarbons, and other compounds from road drainage (Hatch and Burton 1998). Similarly, wetlands adjacent to agricultural areas could be subjected to agricultural chemicals through runoff, chemical drift, direct application, etc. (Zaga et al. 1998). Such contamination can result in immediate acute responses in amphibians and other organisms, but more likely, injury will occur as a result of sublethal effects manifested over extended periods.

Although guidelines and application factors have been developed to insure the safe use of many chemical products, the interaction of these products with other environmental stressors has not been thoroughly considered in regulatory guidelines for their use. One stressor of concern which can occur simultaneously with environmental contaminants is SUV. SUV can be directly harmful to developing amphibians, particularly if there is a change in water clarity or shade that would result in an increased duration or magnitude of exposure. Blaustein et al. (1994) found that ultraviolet-B radiation (UV-B) reduces egg hatching success of amphibians in a manner consistent with the species capability to utilize photorepair mechanisms. Indirectly, SUV can increase the hazards posed by chemical substances through either *in vitro* photomodification (Zepp and Schlotzhauer 1979, Ren et al. 1994, Zaga et al. 1998) or *in vivo* photosensitization (Newsted and Giesy 1987, Arfsten et al. 1996). For example, the toxicity of polyaromatic hydrocarbons (PAH) present in most petroleum products can increase by as much as 400 times in the presence of SUV (Bowling et al. 1985). In the presence of sunlight, 125 $\mu g/l$ of fluoranthrene, a common PAH in petroleum, delayed hatching success of northern leopard frog (*Rana pipiens*) embryos and caused mortality of newly hatched larvae within 24 hours of exposure (Hatch and Burton 1998). Such photomediated toxicity is often not considered in the regulated use of chemical products, and has not been considered a factor in amphibian habitats. The purpose of the present study is to examine the interactive toxicity of UV and contaminants to two amphibian species and to provide a better understanding of the impact such interactions may have on the status of amphibians.

METHODS

The larval amphibians used in this study included the gray tree frog (*Hyla versicolor*) collected as fertilized eggs from amplexing adults, and the southern leopard frog (*Rana sphenocephala*) collected as eggs by a commercial source. The eggs, embryos, and subsequent larvae were cultured in aerated well water (pH 7.0, hardness 283 mg/l as $CaCO_3$) of confirmed chemical purity at 21°C under static conditions. The well water was replenished daily to maintain water quality.

The contaminant exposures were conducted according to procedures described by Klemm et al. (1994) and ASTM (1993), as adapted by Zaga et al. (1998) for simulated solar radiation (SSR) treat-

Table 1. Simulated solar radiation treatments and their filter combinations used in the toxicity tests with *Hyla versicolor* and *Rana sphenocephala*.

Species	Light Regime	Simulated Solar Irradiance (mW/cm^2) UV-B	UV-A	Visible	Filter Combination
Hyla versicolor	Dark	0	0	0	side wraps: aluminum foil; top covers: cardboard
	Low	4	638	2099	side wraps: 0.005 inch gauge Mylar D; top covers: 0.030 inch gauge polycarbonate
	High	65	1097	2212	top covers: 0.005 inch gauge cellulose acetate
Rana sphenocephala	Standard	0.002	3.0	257	side wraps: 0.030 inch gauge polycarbonate and 0.005 inch gauge Mylar D; top covers: two 0.030 inch gauge polycarbonate and black meshed shade cloth
	Low	0.3	75.0	850	side wraps: 0.030 inch gauge polycarbonate and 0.005 inch gauge Mylar D; top covers: two 0.030 inch gauge UVF polystyrene, 0.005 inch gauge Mylar D and black, meshed shade (50%) cloth
	Medium	2.0	340	2180	side wraps: 0.030 inch gauge polycarbonate and 0.005 inch gauge Mylar D; top covers: 0.005 inch gauge Mylar D
	High	17.0	131	1081	side wraps: 0.79 mm gauge polycarbonate; top covers: one black, meshed shade (50%) cloth

ments. Toxicity tests were conducted in a solar simulator (Little and Fabacher 1996) approximately 1 m wide by 2 m long. The simulator was suspended over a water bath of similar dimensions and was enclosed with a highly reflective NIST specular aluminum. The simulator was equipped with cool white, UV-B (313 nm peak irradiance), UV-A (365 nm peak irradiance) fluorescent lamps and halogen flood lamps. The cool white and UV-A fluorescent lamps were controlled by a timer to operate for 14 h. The UV-B lamps were activated with a second timer to operate for 4 h. The UV-B photoperiod began five hours after the onset of the white light and UV-A photoperiod. These photoperiods were comparable to an August photoperiod at 38.5° north latitude and, based on preliminary studies with fish, were assumed to be of sufficient length to ensure that the exposed organisms received sufficient irradiance to utilize photorepair mechanisms. Water bath temperature was maintained by a recirculating water chiller and the solar simulator was checked daily to assure lamp function, photocycle intervals, water bath temperature, bath water level, and recirculating flow.

Various filtering materials were used to generate the laboratory irradiance treatments during the toxicity tests (Table 1). These treatments were based on solar irradiance values measured in amphibian habitats in central California (Barron et al. 2000) and central Missouri (Zaga et al. 1998) in August and were primarily intended to manipulate UV-B intensities since these wavelengths are the most harmful to aquatic organisms (Little and Fabacher 1996). The sides and the tops of the exposure chambers were covered with the filtering materials. The nominal simulated solar radiation treatments ranged from a low of 0.3 $\mu W/cm^2$ to a high of 65 $\mu W/cm^2$ (Table 1). The control irradiance treatment in the toxicity tests (UV-B—0.002 $\mu W/cm^2$; UV-A—3.2 $\mu W/cm^2$; visible—247 $\mu W/cm^2$) was the lowest possible irradiance that provided sufficient visible light within the chambers to allow feeding and was similar to average office-like lighting (UV-B—0.21 $\mu W/cm^2$; UV-A—3.2 $\mu W/cm^2$; visible—98 $\mu W/cm^2$).

All radiometric measurements during the tests were performed with an Optronic Laboratories Model OL-754 spectroradiometer over a wavelength range of 280–700 nm at 1 nm intervals to document the spectral quality and intensity of irradiance treatments. The radiometer was calibrated with an NIST-traceable lamp and radiometer voltage gain and wavelength accuracy were checked during the measurements. Prior to contaminant exposure tests, the light intensity across the simulator water bath was confirmed by measuring surface irradiance through each filter treatment at 12 locations in the water bath. Underwater irradiance was measured at fixed locations in the simulator with each filter combination used to generate the test light treatments to ensure that the output of the simulator lamps remained consistent.

Carbaryl Tests

Larvae of *H. versicolor*, selected from an intermixed culture of larvae from two egg masses were exposed at Gosner stage 25 (Gosner 1960) to carbaryl for 48 h under static conditions to carbaryl and UV combination treatments (Zaga et al. 1998). The larvae were exposed to a control and 1.24, 1.76, and 2.57 mg/l carbaryl. Carbaryl is a carbamate insecticide used to control insects for citrus, fruit, vegetable, forage, turf, and forest crops. Stock solutions of carbaryl dissolved in acetone were prepared with technical grade carbaryl containing 99.7% active ingredient (Rhone-Poulenc, Research Triangle Park). Appropriate volumes of exposure water were added to 1-l square, glass chambers and then calculated volumes of stock solution required to obtain each treatment were added to each chamber and mixed. Similar amounts of acetone were added to each treatment. Each carbaryl treatment was tested in combination with three UV-B treatments, 4.0 $\mu W/cm^2$, 65 $\mu W/cm^2$, and a control treatment

of 0.002 μW/cm^2. A randomization scheme was used for assigning UV irradiance treatments and for the placement of exposure chambers within the solar simulator. Each UV/carbaryl treatment combination consisted of two replicates containing ten organisms. The experimental unit was the replicate, with 3 levels of irradiance and 4 carbaryl concentrations for a total sample size (n) of 24. These tests were static non-renewal exposures.

Petroleum Tests

Samples of a weathered middle-distillate petroleum were collected from an abandoned oil field in California and shipped to Columbia Environmental Research Center in chilled 1-l amber glass bottles. The samples were refrigerated at 4°C prior to use to minimize volatilization. A slow-stir apparatus was used to prepare the water soluble fraction (WSF) of the oil. A Teflon stir-bar and a 20 mm glass tube was placed into a 1-l screw-top glass jar. Eighty ml of well water was added to the jar then 80 ml of oil was added gently to the surface of the water. The jar was sealed with the screw cap and the mixture was stirred slowly to avoid formation of an emulsion (100 ± 20 RPMs) for 24 ± 2 h in a fume hood at room temperature. A Teflon tube was inserted through the glass tube to siphon off the WSF without disturbing the overlying layer of oil.

A range of SSR irradiance and dilutions of WSF were tested simultaneously in a factoral design using 0% WSF/control light treatment as the experiment-wise control. Randomized experimental designs were used to expose the larvae to diluted solutions of the WSF in the presence of the simulated solar radiation intensities. Two static-renewal tests were conducted with *R. sphenocephala* according to procedures described by Klemm et al. (1994) and ASTM (1993). The larvae used in this study were selected from an intermixed culture of four egg masses. In Test 1, larvae were exposed at Gosner stage 25 for 96 h to 0, 5.0, and 10.0% dilutions of WSF, under a control irradiance of 0.002 μW/cm^2 and an SSR irradiance of 17 μW/cm^2 UV-B. Three replicates of each treatment were tested. In Test 2, the same WSF concentrations were tested but the SSR irradiance was reduced from 17 to 2 μW/cm^2. Ten *R. sphenocephala* larvae were exposed in each replicate to 500 ml of the WSF dilution prepared with well water in 600-ml glass beakers. A randomization scheme was used for assigning UV irradiance treatments and for the placement of exposure chambers within the solar simulator. The beakers were covered with appropriate light filters to obtain the desired light intensity. The light filters were changed daily. Renewals of WSF dilutions were performed daily by siphoning off 95% of the old dilutions and adding fresh dilutions. After the renewals, tadpoles were fed 1 ml of a solution containing 15 g of flaked fish food (Tetramin®) homogenized in 300 ml of well water. The amount of food provided was reduced proportionately as mortality occurred during the test. Mortality in each treatment was recorded daily.

Exposure water samples were collected during the tests and analyzed for total petroleum hydrocarbon using gas chromatography/mass spectrometry methods (Barron et al. 1999). The analytical detection limit ranged from 0.05–0.2 mg/l TPH, depending on the collected sample volume.

Statistical Analysis

Analysis of Variance (ANOVA) was used to analyze data from the UV exposures. The analyses were performed with Statistical Analysis System (SAS 1989) computer programs. *Hyla versicolor* data collected at the end of the 48-h exposure were analyzed as a factoral UV X carbaryl design. The experimental unit was the replicate, with three levels of irradiance and four carbaryl concentrations for a total sample size of 24 (Zaga et al. 1998). ANOVA was performed to determine treatment effects. The data were arcsine square root transformed prior to analysis.

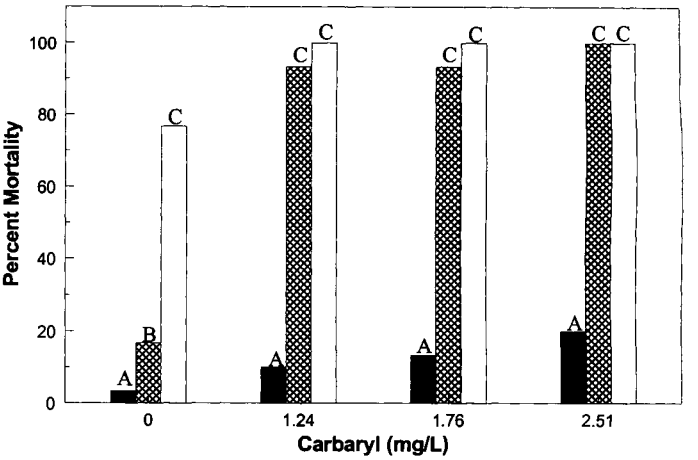

Fig. 1. Percent mortality of *Hyla versicolor* at 48 h of exposure to carbaryl and three light treatments including 0 μW/cm^2 (Black), 4 μW/cm^2 (Cross-hatched) and 65 μW/cm^2 (White) UV-B. Histograms accompanied by different letters are significantly different($P < 0.05$). Redrawn from Zaga et al. (1998).

Data collected at the end of the 96-h *R. sphenocephala* tests were analyzed as a UV X WSF factorial design. The experimental unit was the replicate, with two tests, three replicates, three levels of irradiance and three WSF concentrations for a total sample size of 27. All mortality data were arcsine square root transformed prior to analysis. There was no significant difference between tests, so the data were combined. If there was a significant interaction term, ANOVAs were performed for each UV treatment using the 0 mg/l WSF treatment as a control. The one-tailed Dunnett's test (Dunnett 1955) was used to compare all treatment means.

RESULTS AND DISCUSSION

The light intensities selected for these studies were based on UV-B intensities measured in amphibian habitats in the field, and included treatments that were below tolerance limits for the species tested. The combination of these low irradiance intensities with various contaminants resulted in significant increases in the toxicity of those contaminants.

Hyla versicolor/Carbaryl Exposures

The carbaryl exposure concentrations applied during this study were within the range of concentrations expected from runoff after field applications (Peterson et al. 1994). The synergistic interaction of SSR and carbaryl was evident in the toxicity to *H. versicolor* larvae with a significant ($P < 0.0001$) 3-way interaction between carbaryl/SSR/day (Zaga et al. 1998). In the absence of UV, 1.24, 1.76, and 2.51 mg/l of carbaryl induced 10%, 14%, and 21% mortalities after 48 h of exposure. In the presence of low SSR (4 μW/cm^2 UV-B), carbaryl treatments induced significant mortalities by day 2 with 93.3%, 93.3%, and 100% mortalities for the treatments with 1.24, 1.76, and 2.51 mg/l of carbaryl, respectively (Fig. 1). This contrasts with 16.7% mortality among larvae exposed to low SSR alone, and the 10–22 % mortality among larvae exposed to carbaryl treatments in the absence of SSR. In the presence of high SSR (65 μW/cm^2 UV-B) the carbaryl treatments caused significant mortality somewhat earlier than the low SSR combinations with 100% mortalities for all treatments on day 1. Exposure to UV alone induced 78% mortality

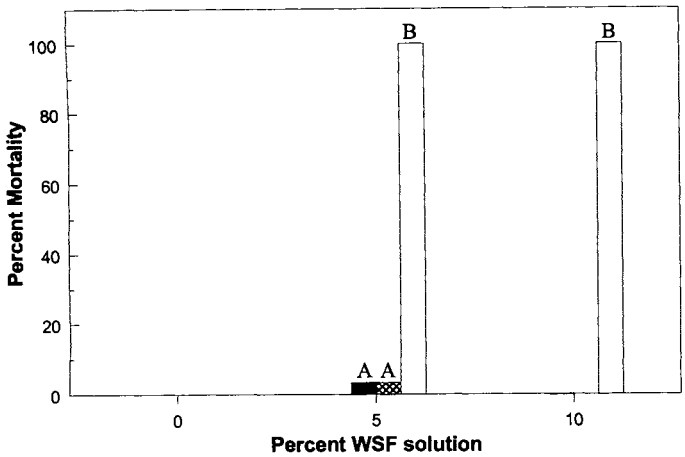

Fig. 2. Percent mortality of *Rana sphenocephala* exposed to WSF for 96 h under four light treatments including 0 $\mu W/cm^2$ (Black), 0.3 $\mu W/cm^2$ (Dotted), 2.0 $\mu W/cm^2$ (Cross-hatched), and 17 $\mu W/cm^2$ (White). Histograms accompanied by different letters are significantly different ($P < 0.05$).

at 65 $\mu W/cm^2$ UV-B. The low irradiance level of 4 $\mu W/cm^2$ UV-B is considerably lower than values of 41–22 $\mu W/cm^2$ UV-B measured in the water column at depths of 5–10 cm in *H. versicolor* habitats. In addition to mortality, Zaga et al. (1998) report that exposure to carbaryl in the presence of UV caused a reduction in locomotory activity to less than 50% of the control response. Such reductions would impair feeding (Picker 1985, Seale 1982), increase time to metamorphosis (Smith 1987, Wassersug 1989), and decrease survival (Morin 1987).

Rana sphenocephala:Petroleum Exposures

The concentration of total petroleum hydrocarbons (TPH) analytically determined for each WSF treatment was 2.82 mg/l for 10% WSF, 1.52 mg/l for 5% WSF, and 0 mg/l for 0% WSF (control). ANOVA conducted on mortality data from the two different exposures revealed no statistically significant differences among controls in either test, so the results of these two studies were combined and the ANOVAs were conducted on the pooled data. There was a significant interaction between WSF treatment, duration of exposure, and the 17 $\mu W/cm^2$ UV treatment (Fig. 2). No mortality occurred among larvae exposed to 5% and 10% WSF under the control SSR treatment. However, under the 17 $\mu W/cm^2$ UVB treatment, photoenhanced toxicity was evident with 100% mortality induced among larvae exposed to the 5 and 10% WSF. The UV exposure of 17 $\mu W/cm^2$ was not harmful to *R. sphenocephala* and was slightly lower than subsurface environmental irradiances which ranged from 20–97 $\mu W/cm^2$. Under SSR irradiance conditions of 2 $\mu W/cm^2$ UV-B there was no mortality among any of the treatments. The occurrance of photoenhanced toxicity at elevated UV irradiance levels suggests that lower irradiances may have been blocked by the skin surface. In tests with fish (Little et al. 2000), we found that 0.12 $\mu W/cm^2$ UV-B caused significant photo-enhanced toxicity at WSF concentrations as low as 1.25%. Possibly the epidermal melanin of *R.. sphenocephala* provided a measure of photoprotection, and prevented the UV from reaching the membrane-bound oil residues at the lower irradiance levels but were unable to shield the organism from 17 $\mu W/cm^2$. In addition to absorbing UV radiation, melanin precursors can provide antioxidant activity and reduce the formation of lipid peroxidation products that are generated by the UV oxidation of membrane-bound petroleum residues (Schmitz et al. 1995).

Exposure to SUV alone can cause injury to early lifestages of amphibian species (Nagl and Hofer 1997, Ankley et al. 1998). The extent of injury from SUV exposure may differ among species because they exhibit differential sensitivities (Corn et al. 1998). The variation in SUV sensitivity among amphibian species is probably related to differences in their abilities to utilize photo-repair mechanisms (Blaustein et al. 1998) as well as differences in epidermal characteristics such as melanin content (Licht and Grant 1997). The high melanin content in amphibian species such as *Rana sphenocephala* probably mitigate the effects of SUV by preventing penetration to reactive sites below the epidermis. The harmful effects of SUV on early life-stage amphibians may be exacerbated by recent increases in levels of SUV reaching the earth (Herman et al. 1996) due to ozone depletion. Additionally, changes in water quality, especially decreased dissolved organic carbon concentration, increased water clarity, or loss of shade can be expected to substantially increase SUV in amphibian habitats. The irradiance conditions applied during the present study were actually below the expected range of increase.

The sensitivity of early lifestage amphibians to SUV coupled with increased natural exposure and the interactive effects of environmental contaminants may further jeopardize wild populations. Synergistic effects on amphibians can result from exposure to a broad range of environmental contaminant classes in the presence of SUV intensities that occur in natural habitats. Of the numerous hypotheses that attempt to explain the global decline in amphibian populations, ultraviolet radiation and environmental contamination appear to be likely candidates in certain habitats. SUV and environmental contamination are pervasive and occur together on a global scale in many habitats that support amphibian life and the synergistic effects elicited by these two environmental stressors may be the underlying cause of the decline in amphibian populations. Both contamination and SUV are known to impair immune function and as individual or combined stressors could also increase susceptibility to pathogens such as chytrid fungus (Berger et al. 1998). Further, it is apparent from the present study as well as others that contaminant regulatory processes that do not incorporate the interactive effects of contaminants and other environmental stressors are not adequate to protect biological resources.

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