

Using chlorophyll *a* fluorescence to detect the onset of anthracene photoinduced toxicity in *Lemna gibba*, and the mitigating effects of a commercial humic acid

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Abstract

The influence of a commercial humic acid (Aldrich; AHA) on the development of anthracene photoinduced toxicity to the duckweed *Lemna gibba* was examined using both room- and low-temperature (77°K) chlorophyll fluorescence assays. Plants were exposed to 2 mg liter⁻¹ anthracene both with and without 6.2 mg liter⁻¹ of AHA and grown under either visible light or simulated solar radiation that mimics the natural abundance of UV radiation. Exposure periods ranged from 1 to 48 h to examine temporal changes in chlorophyll degradation and chlorophyll *a* fluorescence induction in response to these light and HA treatments. The onset of anthracene photoinduced toxicity followed a definite sequence; chlorophyll *a* fluorescence induction parameters (F_v/F_m , and $t_{1/2}$) responded earliest to anthracene exposure, with observable chlorophyll degradation requiring up to 24 to 48 h. Of these, $t_{1/2}$ was the most sensitive, with significant inhibition apparent within 1 h of exposure. Throughout the entire 48-h exposure, 6.2 mg liter⁻¹ AHA ameliorated the photoinduced toxicity of anthracene, in terms of both chlorophyll degradation and F_v/F_m inhibition. In contrast, AHA delayed the complete inhibition of $t_{1/2}$ by only 1 to 24 h rather than permanently protecting the plants from anthracene damage to PS2. This suggests that AHA may slow, but not prevent, the entrance of either intact anthracene or its photooxidized byproducts under these exposure conditions.

Predicting the negative impacts of anthropogenic contaminants is often complicated by various chemical and ecological factors that can modify the fate and effects of xenobiotics. For example, the toxicity of both inorganic and organic contaminants to freshwater organisms often is ameliorated in the presence of either natural or commercial forms of dissolved organic carbon (DOC) (Day 1991; Goodrich et al. 1991; Oikari et al. 1992; Steinberg et al. 1992; Hodge et al. 1993; Williamson et al. 1999). The mechanism likely involves chemical partitioning of the contaminant to DOC which diminishes its bioavailability and, hence, biological efficacy (Jaffé 1991; Knulst 1992; Loes et al. 1993; Kukkonen and Pellinen 1994; Kopinke et al. 1995). Polycyclic aromatic hydrocarbons (PAHs), for example, bind to natural forms of DOC (Johnsen and Gribbestad 1991; Schlautman and Morgan 1993; Kopinke et al. 1995), hence diminishing the toxicity and/or bioavailability of the unmodified parent compounds (Landrum et al. 1985; Kukkonen and Oikari 1991; Kukkonen and Pellinen 1994).

Further complicating this scenario, natural levels of ultraviolet radiation (UVR) in sunlight enhance the toxicity of PAHs to aquatic animals (Bowling et al. 1983; Oris and Giesy 1985; Holst and Giesy 1989; Monson et al. 1995) and plants (Greenberg et al. 1993; Huang et al. 1993; Gala and Giesy 1994; Gensemer et al. 1996). However, little work specifically has addressed the extent to which DOC controls PAH bioavailability or toxicity in the presence of natural

levels of UV radiation. UV radiation also induces a variety of photochemical changes in DOC, including photochemical degradation into more labile carbon forms (Zepp 1988; Schindler et al. 1996; Bushaw et al. 1996) and production of reactive oxygen species (e.g., singlet oxygen, peroxy radicals, etc.; Cooper et al. 1989; Hoigné et al. 1989; Canonica and Hoigné 1995). Therefore, UVR is likely to induce a variety of changes in the ability of DOC to control the fate and bioavailability of PAHs to aquatic organisms, as well as the direct effects of DOC on aquatic organisms (see also Williamson et al. 1999).

Oris et al. (1990) examined the amelioration of PAH toxicity by a commercial humic acid (Aldrich humic acid; AHA) under the influence of simulated solar radiation (SSR) containing both UV-A and UV-B. They found that AHA alleviated anthracene toxicity to the fathead minnow *Pimephales promelas* and the zooplankter *Daphnia magna* primarily by diminishing bioaccumulation. UV-light attenuation coefficients were also highly correlated with diminished toxicity, suggesting that AHA may selectively attenuate UV wavelengths known to induce toxicity. Similarly, AHA has been shown to ameliorate PAH effects on the aquatic duckweed *Lemna gibba* under both visible and SSR, although the presence of UVR quantitatively changed the protective effects of AHA (Gensemer et al. 1998).

Studies describing the phenomenon of PAH photoinduced toxicity in plants have mostly employed only individual or population-level endpoints (growth, mortality, reproduction). However, suborganismal endpoints (i.e., biomarkers or bioindicators) are now commonly used in aquatic toxicity testing and risk assessment (McCarthy and Shugart 1990; Benson and DiGiulio 1992; Huggett et al. 1992). For plants, an important suborganismal process that can be inhibited by chemical contaminants is photosynthesis (Simpson et al. 1988; Judy et al. 1990; Lewis 1995). Many assays measure photosynthetic performance, of which some of the potentially most sensitive involve chlorophyll *a* (Chl *a*) fluores-

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Acknowledgments

We thank Chris Marwood and Cheryl Duxbury for their assistance with laboratory techniques and experimental design.

This study was supported by National Sciences and Engineering Research Council of Canada grants to B.M.G. and D.G.D., and with additional support from a National Institute of Environmental Health Studies grant (No. ES-07381) to R.W.G.

cence induction kinetics (Hipkins and Baker 1986). For example, room-temperature fluorescence induction is a powerful *in vivo* indicator of contaminant-related damage to photosystem quantum efficiency in higher plants (Krupa et al. 1993; Prevot et al. 1993; Streb et al. 1993; Sgardelis et al. 1994; Gensemer et al. 1996; Huang et al. 1997a). While Chl *a* fluorescence induction assays have been used widely in terrestrial plant stress physiology (Hipkins and Baker 1986; Krause and Weis 1991) and in studies examining patterns of marine algal photosynthesis (Falkowski et al. 1991; Greene et al. 1992; Hofstraat et al. 1994; Olson and Zettler 1995; Olaizola et al. 1996; Olson et al. 1996), its use in freshwater plant contaminant studies is still in its infancy. Furthermore, low-temperature (77°K) chlorophyll fluorescence emission scans can present qualitative information regarding the structure and organization of photosystem reaction center pigments (Hipkins and Baker 1986; McCormac et al. 1996; Marwood and Greenberg 1996).

Although Chl *a* fluorescence assays hold much promise for providing sensitive and mechanistically meaningful sub-organismal endpoints in toxicity bioassays, standard protocols have yet to be developed. One major issue to be resolved is the length of time that plants need to be exposed to contaminants before meaningful fluorescence data can be obtained and whether such data are predictive of inhibition of endpoints at higher levels of ecological organization (Gensemer et al. 1996). Given the rapid responses of biochemical processes in photosynthesis to environmental contaminants (Oettmeier et al. 1987; Huang et al. 1997c), Chl *a* fluorescence parameters potentially could respond within seconds to a chemical insult. Huang et al. (1997a) demonstrated that Chl *a* fluorescence induction in *L. gibba* was inhibited after only 4 h of exposure to anthracene under SSR. Chl *a* fluorescence induction in *L. gibba* also responded in a dose-dependent fashion during longer exposures (48 h) to a mixed PAH source (creosote), although changes in population growth were better predicted when fluorescence was measured at the end of the 8-d bioassay (Gensemer et al. 1996). However, none of these studies directly examined changes in the physiological responses of *L. gibba* ranging from times immediately following PAH exposure (one to several hours) to longer time periods of up to 2 d, which, for *Lemna*, is the maximum exposure time to a particular batch of media in static-replacement bioassays (Greenberg et al. 1992).

Here we examine the influence of HA on the development of anthracene photoinduced toxicity to *L. gibba* in greater detail using both room- and low-temperature Chl *a* fluorescence assays over relatively brief exposure times (1–48 h). Anthracene was chosen as a model PAH compound owing to its significant photoinduced toxicity relative to other PAHs (Greenberg et al. 1993) and to extensive prior experience in our laboratory. Our purpose is not only to describe the temporal onset of anthracene toxicity with regard to photosynthetic endpoints, but also to utilize the mechanistic significance of each photosynthetic parameter to help better understand the combined influences of UVR and AHA on anthracene photoinduced toxicity.

Materials and methods

Culture maintenance and general experimental conditions—Cultures of *L. gibba* L. G-3 were maintained aseptically on half-strength Hutner's medium and grown under 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of continuous "cool-white" fluorescent light at 24°C (Greenberg et al. 1992). During experiments, plants were incubated using either visible light (400–700 nm) provided by "cool-white" fluorescent lamps or a simulated solar radiation (SSR) source designed to mimic the relative amounts of visible light, UV-A (320–400 nm), and UV-B (290–320 nm) radiation (Am. Soc. Testing Mater. 1996; Greenberg et al. 1996). Our source consisted of two "cool-white" fluorescent lamps, one 350-nm photoreactor lamp, and one 300-nm photoreactor lamp (Southern New England Ultraviolet Co.) filtered through two layers of cheesecloth. For both visible and SSR exposure systems, the total visible fluence rate to which plants and media were exposed was 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants exposed to SSR were covered with polystyrene Petri dish tops (Phoenix Biomedical, Baxter-Canlab) to protect them from UV-C (200–290 nm). Fluence rates and spectral quality were periodically confirmed using a calibrated spectroradiometer (Oriel Instru.) and a quantum light meter (LiCor). Spectral distributions for our SSR light source are presented elsewhere (Am. Soc. Testing Mater. 1996; Greenberg et al. 1996, 1997).

Plants were exposed to PAHs and(or) AHA in 10 ml of half-strength Hutner's medium placed in 5-cm polystyrene Petri dishes. Although PAHs may selectively partition to the polystyrene, PAH assimilation by *Lemna* is known to be identical when using pyrex containers of the same size and volume (Duxbury et al. 1997). Anthracene stock solutions were prepared by dissolving reagent-grade crystals (Sigma Chem. Co.) in dimethylsulfoxide (DMSO; Sigma Chem. Co.) such that 10 μl of the stock solutions would produce a final nominal anthracene concentration of 2 mg liter⁻¹. Dose-response experiments confirmed that a 2 mg liter⁻¹ exposure represents about 15 and 75% growth inhibition of *L. gibba* under PAR and SSR, respectively (Huang et al. 1993; Gensemer et al. unpubl.). The final DMSO concentration (0.1% vol/vol) does not affect growth of *L. gibba* and has been confirmed analytically to provide accurate PAH delivery at this concentration without direct plant toxicity or photochemical interference (Huang et al. 1993). Identical DMSO concentrations were added to control (no PAH) treatments in the present experiments. AHA stock solutions were prepared by dissolving a commercially available sodium salt (Aldrich Chem. Co.) in deionized water. Final AHA concentrations (in mg liter⁻¹ as DOC) were confirmed analytically (see Gensemer et al. 1998).

Experimental design—Plants were exposed to 2 mg liter⁻¹ anthracene either with or without 6.2 mg liter⁻¹ AHA which was the maximum AHA level tested with PAHs in previous experiments using *L. gibba* (Gensemer et al. 1998). This AHA concentration is similar to those found in moderately humic rivers and streams, although concentrations can go much higher in wetlands (Wetzel 1983; Thurman 1985). Immediately following anthracene exposure, plants were placed under the appropriate light source (visible light or SSR) and

removed for analysis after 0, 1, 4, 24, and 48 h of exposure to light. Separate Petri dishes were used for each chemical treatment and time combination to ensure that plants were not removed from the light even temporarily during exposure. Changes in total plant chlorophyll content (Greenberg et al. 1992), room-temperature Chl *a* fluorescence induction, and low-temperature (77°K) chlorophyll fluorescence emission scans were all performed on plants taken from the same Petri dishes at the end of each exposure period. All treatments were run in triplicate, and each experiment was repeated to ensure accuracy.

Room-temperature Chl *a* fluorescence induction—Plants were dark-adapted for at least 10–20 min to ensure that all photosynthetic electron acceptors were fully oxidized. A plant sample (usually an entire plant containing four fronds) was then placed in a spectrofluorometer (Photon Technol. Int.) fitted with a photon-counting photomultiplier. A mechanical camera shutter then opened to emit a beam of light from a xenon arc lamp passed through a monochromator (435 nm) to expose plants to 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ actinic illumination. With this fluence rate we achieved control F_v/F_m values within 5% of those achieved with a fully saturating light source of 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (data not shown). Fluorescence was detected at an emission wavelength of 695 nm, and relative fluorescence intensity (cps) was recorded for 8–10 s with the resulting trace being saved for microcomputer analysis of two different Chl *a* fluorescence induction parameters: F_v/F_m , and $t_{1/2}$.

F_v/F_m is the ratio of variable ($F_m - F_0$) to maximal fluorescence (F_m) after exposure of dark-adapted plants to actinic light and is an index of the quantum efficiency of photosystem 2 (PS2). Any physiological stress affecting PS2 quantum efficiency will diminish values of F_v/F_m from control values of ca. 0.8 in higher plants (Büchel and Wilhelm 1993). The parameter $t_{1/2}$ represents the time required to reach half of F_v during the saturating light pulse and is a measure of the size and accessibility of the plastoquinone pool which accept electrons from PS2 (Judy et al. 1990; Krause and Weis 1991; Sgardelis et al. 1994). More specific details on parameter estimates are given by Gensemer et al. (1996).

Low-temperature chlorophyll fluorescence emission scans—Low temperature (77°K) fluorescence emission spectra were measured on intact plants to obtain qualitative information regarding the organization of antennae pigment chlorophylls (McCormac et al. 1996) in response to anthracene treatments. At each time interval, whole plants were mounted on anodized aluminum probes and quick-frozen in liquid nitrogen prior to analysis on a scanning spectrofluorometer fitted with a liquid nitrogen reservoir and cuvette assembly (Photon Technol. Int. Inc.). Emission spectra were collected from 600 to 800 nm, scanned at a rate of 0.3 nm s^{-1} , using an excitation wavelength of 435 nm. Three separate scans using three separate plants were performed from each experimental replicate dish, and the triplicate scans were averaged at each wavelength after normalization to 600 nm to correct for slight differences in background fluorescence.

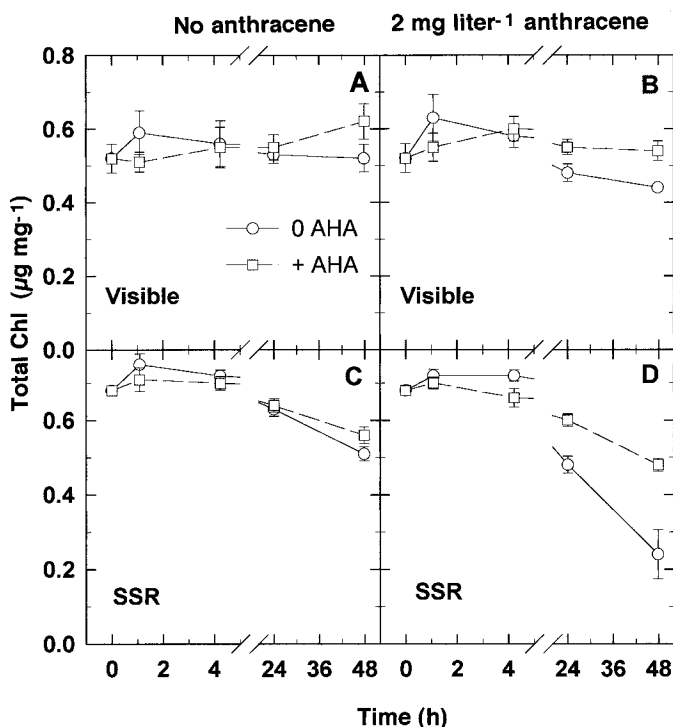


Fig. 1. Changes in total chlorophyll content ($\mu\text{g mg}^{-1}$) over time for plants exposed to (A) no PAH and visible light, (B) 2 mg liter $^{-1}$ nominal anthracene and visible light, (C) no PAH and SSR, and (D) 2 mg liter $^{-1}$ nominal anthracene and SSR. Treatments without humic acid: \circ ; treatments with 6.2 mg liter $^{-1}$ AHA: \square . All symbols represent treatment means ± 2 SE.

Statistics—The significance of the effects of AHA addition and light quality on plant responses over time (and their interactions) were determined using 2-way ANOVA. Because only a single set of time = 0 controls were performed prior to other treatments, these values were excluded from the 2-way ANOVAs. The significance of parameter responses over time compared to pre-exposure (time = 0) controls was analyzed for each treatment using 1-way ANOVA. Tukeys HSD post-hoc tests were used with significant factors to examine pairwise comparisons of significant differences between treatment means. All statistical analyses were performed using Systat (SPSS, Inc.) at a significance level of $P < 0.05$.

Results

When *L. gibba* was grown under visible light, 2 mg liter $^{-1}$ anthracene exerted little influence on chlorophyll content over time, except for a small, but significant ($r^2 = 0.40$, $P = 0.015$) increase at 1 h relative to 48 h after exposure (Fig. 1B; 0 AHA). Chlorophyll contents were higher in the presence of 6.2 mg liter $^{-1}$ AHA at virtually all time intervals, but this difference was not statistically significant. In anthracene-free controls (Fig. 1A), chlorophyll contents did not change significantly over time nor did AHA exert any significant effects.

In contrast to visible light, anthracene exposures to plants

grown under SSR induced significant decreases in chlorophyll content over time (Fig. 1D). This effect was most dramatic without AHA, where chlorophyll decreased significantly by 24 h ($r^2 = 0.86$, $P < 0.001$), with final values reaching $<50\%$ of control (time = 0) values by 48 h. Additions of $6.2 \text{ mg liter}^{-1}$ AHA under SSR significantly diminished anthracene-induced chlorosis over time with final chlorophyll contents improving up to 94% of 48-h control values ($r^2 = 0.85$, $P = 0.002$).

Without anthracene, AHA did not significantly influence chlorophyll contents in plants grown under SSR, although chlorophyll values in both treatments did decline significantly 24 and 48 h relative to 0 h after exposure (Fig. 1C; $r^2 = 0.71$, $P < 0.001$). However, chlorophyll values from plants grown under SSR (Fig. 1C) were significantly higher than those obtained under visible light (Fig. 1A) when AHA was present ($r^2 = 0.45$, $P < 0.001$).

Low-temperature fluorescence emission scans were consistent qualitatively with chlorophyll content results; a gradual degradation in the integrity of the photosynthetic antennae pigments was apparent over time when plants were exposed to anthracene under SSR (Fig. 2A). Low-temperature (77°K) Chl *a* fluorescence from healthy control plants showed a typical emission spectrum with peaks at 685 and 695 nm representing PS2 pigments, and a major peak at 730 nm representing the PS1 pigment-protein complex (Hipkins and Baker 1986; McCormac et al. 1996). After 1 h of exposure to 2 mg liter^{-1} anthracene under SSR, emission scans were essentially identical to the controls. By 4–24 h, however, the fluorescence emission peaks from both PS1 and PS2 complexes were substantially lower. By 48 h, the PS1 emission peak dropped further, and the characteristic PS2 peaks were no longer apparent; the 695-nm peak was detectable only as a shoulder on the 685-nm peak, the 685-nm peak decreased dramatically and shifted 1–2 nm to lower wavelengths, and a minor peak, not previously present, appeared at 649 nm. The timing of this steady degradation in the qualitative organization of the chlorophyll pigment systems is consistent with the steady declines in total chlorophyll content already described (Fig. 1D). Adding 2 mg liter^{-1} AHA almost completely ameliorated these anthracene-induced changes in the organization of photosystem pigments when plants were grown under SSR (Fig. 2B). Virtually no differences existed in 77°K fluorescence emission scans at any time after anthracene exposure relative to controls. Again, this is consistent with the slower degradation of total chlorophyll contents over time in the presence of AHA (Fig. 1D). Scans from all visible light treatments and anthracene-free treatments under SSR essentially were identical to the control scans presented here (data not shown).

F_v/F_m values for *L. gibba* were somewhat more sensitive to anthracene than was chlorophyll content (Fig. 3). In visible light, F_v/F_m values dropped significantly, by 0.12 and 0.08 units at 1 and 4 h, respectively, relative to time 0, and again to nearly 50% of time 0 values at 24 and 48 h (Fig. 3B; $r^2 = 0.72$, $P < 0.001$). However, in the presence of AHA, significant decreases in F_v/F_m were prevented up through 4 h after exposure ($r^2 = 0.64$, $P < 0.001$). When AHA treatments were compared to AHA-free treatments (Fig. 3B), AHA significantly increased F_v/F_m values by about

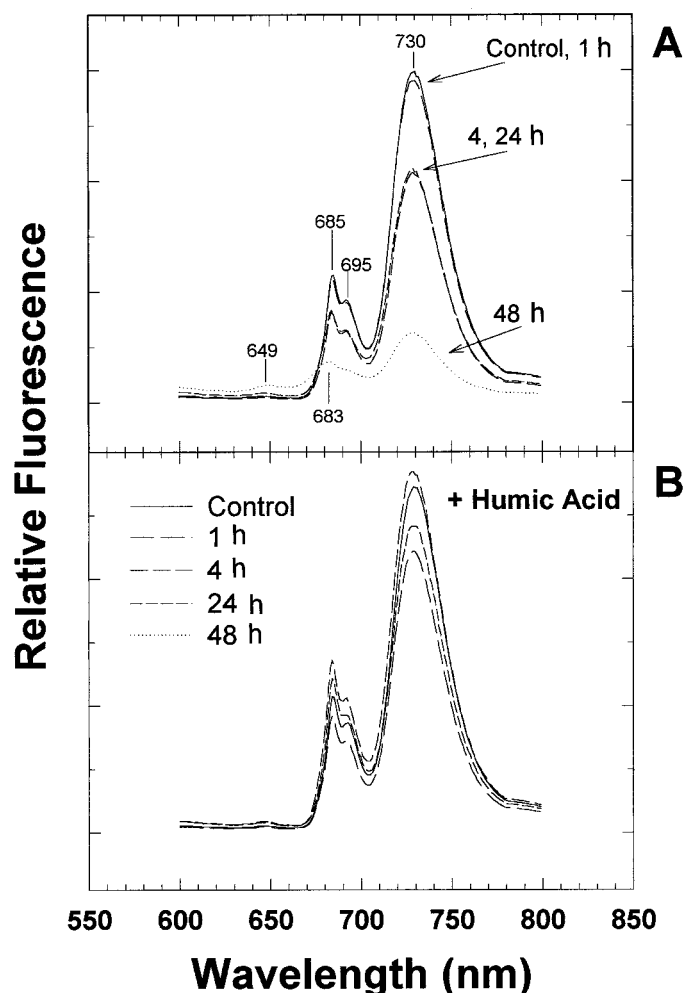


Fig. 2. Low-temperature (77°K) fluorescence emission scans from plants exposed at all time intervals to (A) 2 mg liter^{-1} nominal anthracene and SSR, and (B) the same treatment but with $6.2 \text{ mg liter}^{-1}$ AHA. Details concerning scan normalization given in text.

0.1 units at all time intervals, although this occurred only 1 and 4 h after exposure ($r^2 = 0.65$, $P < 0.001$). Without anthracene, F_v/F_m values in plants exposed to visible light varied little over time, except for significant decreases at 1 h as compared to all other time intervals (Fig. 3A; $r^2 = 0.285$, $P = 0.001$). AHA had a significant overall positive influence on F_v/F_m under visible light when no anthracene was present (Fig. 3A; $r^2 = 0.22$, $P = 0.012$).

As expected, decreases in PS2 quantum efficiency were more severe when plants were exposed to SSR lighting (Fig. 3D). F_v/F_m values dropped nearly 50% only 1 h after anthracene exposure ($r^2 = 0.73$, $P < 0.001$), followed by steady, nearly linear, decreases over time leading to near zero values by 48 h. AHA had a significant protective effect overall ($r^2 = 0.57$, $P < 0.001$), except at 24 h when values were actually lower than corresponding treatments without AHA. This unexpected result at 24 h was not observed either with chlorophyll (Fig. 1D) or $t_{1/2}$ (Fig. 4D) data nor have similar trends ever been observed in *L. gibba* in our lab (Gensemer et al. 1998). Further experimentation will be required to con-

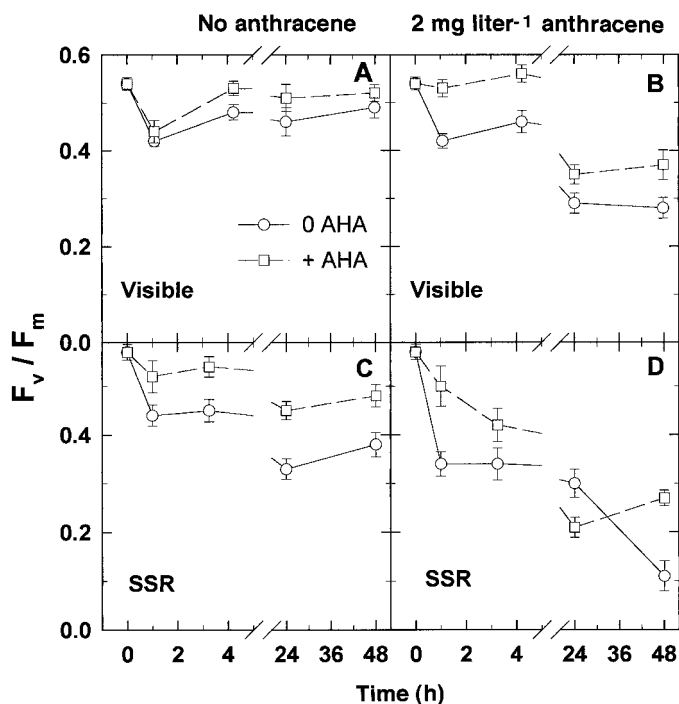


Fig. 3. Changes in F_v/F_m ratios over time for plants exposed to (A) no PAH and visible light, (B) 2 mg liter⁻¹ nominal anthracene and visible light, (C) no PAH and SSR, and (D) 2 mg liter⁻¹ nominal anthracene and SSR. Symbols as Fig. 1.

firm whether this is a unique response of F_v/F_m to humic acid.

Plants grown under SSR without anthracene exhibited a significant F_v/F_m decrease after 1 h, a further decrease by 24 h, and a minor recovery by 48 h (Fig. 3C; $r^2 = 0.56$, $P < 0.001$). AHA additions significantly increased PS2 performance to an even greater extent in SSR than in visible light (Fig. 3A), with differences of up to 0.12 units observed at 24 h (Fig. 3C; $r^2 = 0.40$, $P < 0.001$). Interestingly, F_v/F_m values from plants exposed to SSR longer than 4 h (Fig. 3C, 0 AHA) were significantly lower than those grown under visible light (Fig. 3A, 0 AHA; $r^2 = 0.34$, $P < 0.001$). This direct negative effect of UV light was not observed in any of the present experiments using any other endpoints (Figs. 1, 4) nor in related experiments in plants exposed for 8 d (Gensemer et al. 1998). No significant visible light-related differences in F_v/F_m values from plants grown in media containing AHA were observed (Fig. 3A and B).

Anthracene-induced decreases in F_v/F_m (Fig. 3) were usually driven by decreases in maximal (F_m) rather than minimal (F_0) fluorescence (Table 1). Except for diminished F_0 values in many cases at 4 h relative to $t = 0$ controls, few other trends are apparent. In contrast, where F_v/F_m values were most inhibited (i.e., in the presence of anthracene under SSR; Fig. 3D), F_m values clearly declined over all time periods tested (Table 1). Furthermore, in the presence of anthracene, F_m data were linearly correlated with F_v/F_m values ($y = 0.002x + 0.216$, $P < 0.001$, $r^2 = 0.26$), whereas F_0 values were not (non-significant regression). Although this F_m vs. F_v/F_m relationship was not particularly strong (low r^2), it

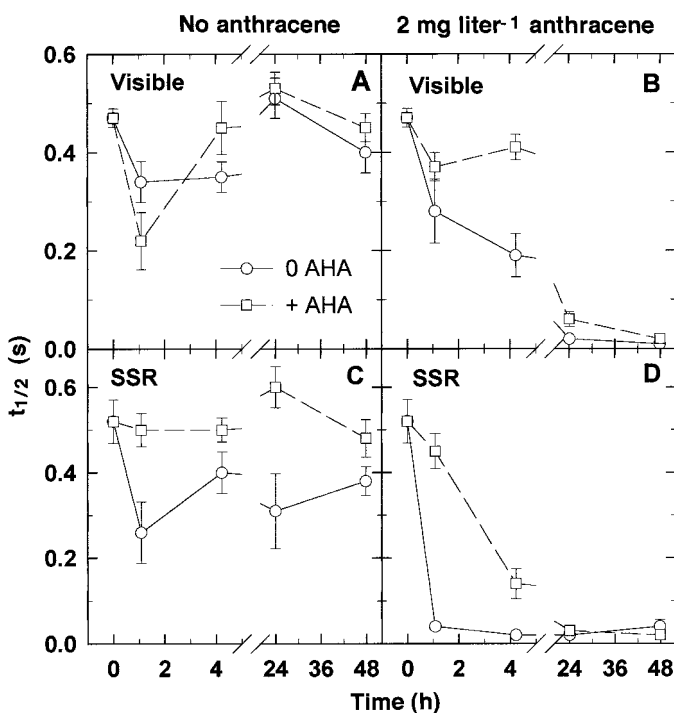


Fig. 4. Changes in $t_{1/2}$ (seconds) over time for plants exposed to (A) no PAH and visible light, (B) 2 mg liter⁻¹ nominal anthracene and visible light, (C) no PAH and SSR, and (D) 2 mg liter⁻¹ nominal anthracene and SSR. Symbols as Fig. 1.

underscores that anthracene-induced decreases in F_v/F_m are more likely to be caused by decreases in F_m , rather than increases in F_0 .

Even when grown under visible light, $t_{1/2}$ values were inhibited strongly soon after exposure to anthracene (Fig. 4B). After only 1 h, $t_{1/2}$ dropped nearly 50% compared to pre-exposure values (time = 0) and continued to decay to near zero by 24 h ($r^2 = 0.69$, $P < 0.001$). AHA still exerted a significant positive effect in plants grown under visible light up to 4 h after anthracene exposure ($r^2 = 0.89$, $P < 0.001$), after which it ceased to be protective (Fig. 4B). Even without anthracene present, $t_{1/2}$ values in plants grown under visible light decreased significantly at 1 and 4 h after exposure (Fig. 4A; $r^2 = 0.27$, $P = 0.003$), but not nearly to the same extent as in anthracene-treated plants (Fig. 4B). By 24 h after exposure, $t_{1/2}$ values recovered to pre-exposure levels (Fig. 4A). With the exception of 1 h after experiment initiation, treatments containing AHA exhibited higher $t_{1/2}$ values (Fig. 4A), but these differences were not statistically significant.

Again, $t_{1/2}$ values dropped immediately after anthracene exposure in SSR-exposed plants, but in contrast to visible light treatments, values approached zero after only 1 h (Fig. 4D; $r^2 = 0.86$, $P < 0.001$). $t_{1/2}$ values were 0.24 and 0.17 s lower than corresponding visible light treatments (Fig. 4B; 0 AHA) 1 and 4 h after exposure, respectively ($r^2 = 0.51$, $P < 0.001$). AHA was protective insofar as values did not decrease significantly from pre-exposure values at 1 h, but dropped significantly 4 h after anthracene exposure and then dropped to zero by 24 h ($r^2 = 0.80$, $P < 0.001$). Thus, AHA effectively delayed the onset of anthracene toxicity, yet still

Table 1. Mean fluorescence values (relative units) used to calculate F_v/F_m (Fig. 3).

Treatment	F_0			F_m		
	Mean	SE	<i>N</i>	Mean	SE	<i>N</i>
PAR						
0 AHA, 0 ANT						
<i>t</i> 0	84.2	6.0	12	182.5	9.1	12
1 h	77.6	6.2	12	134.3	10.3	12
4 h	49.2	3.1	12	95.1	7.1	12
24 h	75.1	13.9	11	134.0	20.1	11
48 h	76.6	5.5	12	150.8	9.2	12
+ AHA, 0 ANT						
<i>t</i> 0	84.2	6.0	12	182.5	9.1	12
1 h	90.3	8.4	12	162.8	15.1	12
4 h	48.8	2.8	12	104.3	4.8	12
24 h	68.3	9.8	12	135.6	14.9	12
48 h	69.2	3.4	12	146.1	7.9	12
0 AHA, + ANT						
<i>t</i> 0	84.2	6.0	12	182.5	9.1	12
1 h	87.3	6.3	12	151.7	11.9	12
4 h	45.9	2.9	12	85.0	4.6	12
24 h	84.1	13.4	11	116.2	17.1	11
48 h	75.6	4.6	12	104.9	5.3	12
+ AHA, + ANT						
<i>t</i> 0	84.2	6.0	12	182.5	9.1	12
1 h	71.1	5.9	12	149.7	10.3	12
4 h	45.9	4.7	11	105.6	10.8	11
24 h	69.8	8.7	12	105.9	11.8	12
48 h	68.2	4.4	12	109.8	7.2	12
SSR						
0 AHA, 0 ANT						
<i>t</i> 0	65.2	5.6	12	151.5	12.8	12
1 h	69.5	6.5	12	126.5	13.3	12
4 h	46.9	5.5	12	86.2	9.6	12
24 h	64.5	3.9	12	96.3	4.7	12
48 h	59.8	4.9	11	97.8	7.4	11
+ AHA, 0 ANT						
<i>t</i> 0	65.2	5.6	12	151.5	12.8	12
1 h	51.6	4.2	11	109.7	10.7	11
4 h	51.7	6.7	11	107.6	10.7	12
24 h	55.6	4.6	11	100.7	7.4	12
48 h	56.4	2.9	11	109.8	5.8	12
0 AHA, + ANT						
<i>t</i> 0	65.2	5.6	12	151.5	12.8	12
1 h	64.5	5.2	12	100.5	10.1	12
4 h	49.3	6.2	12	72.1	6.3	12
24 h	55.1	3.0	11	80.1	4.7	11
48 h	50.2	3.7	11	55.6	3.1	11
+ AHA, + ANT						
<i>t</i> 0	65.2	5.6	12	151.5	12.8	12
1 h	61.5	5.8	12	131.0	15.1	12
4 h	53.0	5.7	12	89.7	7.2	12
24 h	67.9	3.7	12	86.3	3.9	12
48 h	56.9	3.8	12	78.1	4.8	12

resulted in near-zero $t_{1/2}$ values 24 h after anthracene exposure.

Even without anthracene present, $t_{1/2}$ values dropped significantly 1 h after exposure to SSR followed by a partial recovery (Fig. 4C; $r^2 = 0.18$, $P < 0.037$). Although $t_{1/2}$ values were lower than corresponding visible light treatments after

4 h, thereby suggesting direct UV inhibition, these differences were not statistically significant. Adding AHA significantly enhanced $t_{1/2}$ measurements at all time intervals by a maximum of 0.183 s at 4 h following light exposure ($r^2 = 0.28$, $P < 0.001$).

Discussion

The onset of anthracene toxicity in *L. gibba* followed a sequence whereby the Chl *a* fluorescence induction parameters (F_v/F_m , and $t_{1/2}$) responded earliest to anthracene exposure (Figs. 3 and 4), with observable chlorophyll degradation requiring 24–48 h (Figs. 1 and 2). Of the Chl *a* fluorescence measurements, $t_{1/2}$ was the most sensitive both in terms of the timing and magnitude of its response to anthracene toxicity. Both fluorescence induction parameters usually responded after 1 h, but unlike F_v/F_m , $t_{1/2}$ was significantly inhibited by anthracene even under visible light with AHA present (Fig. 4B). Therefore, $t_{1/2}$ clearly is the earliest and most sensitive indicator of anthracene stress in *L. gibba* when grown under either visible light or SSR.

Chl *a* fluorescence induction parameters have been shown to detect even subtle biochemical damage within photosynthetic reaction centers in a wide variety of both terrestrial and marine plants. The room-temperature Chl *a* fluorescence induction kinetics discussed here focus specifically on the primary photochemistry of PS2 (Krause and Weis 1991; Krupa et al. 1992). F_v/F_m is an overall index of the quantum efficiency of PS2; physiological stress affecting PS2 quantum efficiency usually will decrease F_v/F_m from control values of ca. 0.7–0.8 in higher plants and chlorophyte algae (Büchel and Wilhelm 1993). F_v/F_m decreases in response to several stresses including photoinhibition and UV light (Streb et al. 1993; Hofstraat et al. 1994; Herrmann et al. 1995), as well as chemical contaminants (Krupa et al. 1993; Prevot et al. 1993; Streb et al. 1993; Sgardelis et al. 1994; Gensemer et al. 1996). The parameter $t_{1/2}$ represents the size and accessibility of the plastoquinone pool which accept electrons from PS2 (Judy et al. 1990; Krause and Weis 1991; Sgardelis et al. 1994). Contaminant stress diminishes this time from ~180–300 ms in healthy plants to <100 ms in severely affected individuals (Sgardelis et al. 1994; Gensemer et al. 1996).

In the present study, decreases in F_v/F_m represented decreases in maximal (F_m) rather than minimal (F_0) fluorescence (Table 1). This preferential drop in F_m is consistent with known mechanisms for the inhibition of photosynthesis by anthracene in *L. gibba* whereby PS2 electron transport rates diminish owing to photooxidative damage following inhibition of PS1 (Huang et al. 1997c). The impact of photooxidation-induced damage on photosynthesis directly confirmed that decreases in F_m values were responsible for declines in F_v/F_m (Herrmann et al. 1995). Furthermore, electron transport inhibitors such as parathion and DCMU have also been shown to diminish F_m rather than F_0 (Prevot et al. 1993). In contrast, chemicals which do not inhibit PS2 specifically, such as trace metals, increased both F_0 and F_m in the terrestrial plants *Taraxacum* and *Sonchus* exposed to metal-contaminated soils (Sgardelis et al. 1994).

Both F_v/F_m and $t_{1/2}$ were sufficiently sensitive to respond negatively to the presence of UV light even without anthracene, although this was statistically significant only for F_v/F_m . This is consistent with known mechanisms of UV-B damage of PS2 reaction centers which diminish their quantum efficiency (Greenberg et al. 1997). However, diminished F_v/F_m values in the presence of UVR in the present study probably do not represent a long-term negative effect of UV light on photosynthesis. No such inhibition relative to visible light was observed after 8 d of exposure to SSR lighting in any other PAH-free experiments using *L. gibba* (Gensemer et al. 1996, 1998, unpubl.). Responses of the fluorescence induction parameters to UVR exposure alone thus may represent only a short-term negative response to SSR, with plants recovering fully by the end of a standard 8-d growth experiment. This is consistent with synthesis rates of flavonoids that begin protecting chloroplasts in higher plants from UV-B damage after about 2 d (Wilson and Greenberg 1993; Greenberg et al. 1997).

Based on chlorophyll degradation and F_v/F_m inhibition, AHA diminished the photoinduced toxicity of anthracene throughout the 48-h exposure period when plants were grown in either visible light or SSR (Figs. 1, 3). Qualitative changes in the organization of chlorophyll reaction centers were also consistent with this pattern. Without AHA, peaks representing low-temperature fluorescence emission from both PS1 (730 nm) and PS2 (685 and 695 nm) reaction centers (Hipkins and Baker 1986; McCormac et al. 1996) were diminished at 4 and 24 h after exposure (Fig. 2A). Further degradation occurred at 48 h where the characteristic PS2 peaks were lost in favor of smaller peaks emitting maximally at shorter wavelengths. This loss of organization in the photosynthetic reaction center pigments by anthracene was virtually prevented in the presence of 6.2 mg liter⁻¹ AHA (Fig. 2B); chlorophyll degradation (measured as either total content or from changes in 77°K emission spectra), as well as overall PS2 quantum efficiency (F_v/F_m), thus was less inhibited in the presence of AHA.

The magnitudes of these protective effects were similar to those observed in a related set of studies using population-level endpoints during an 8-d exposure to 2 mg liter⁻¹ anthracene (Gensemer et al. 1998). Such long-term amelioration of toxicity is consistent with the observation that AHA partitions PAHs from the freely dissolved aqueous phase, thereby diminishing bioavailability (Leversee et al. 1983; Landrum et al. 1985; Kukkonen and Pellinen 1994). However, because humic substances also absorb UVR, it can be photodegraded into a variety of more labile compounds (Zepp 1988; Schindler et al. 1996; Bushaw et al. 1996). Given that both commercial and natural sources of aquatic DOC photodegrade into relatively water-soluble compounds such as low-molecular-weight organic acids and formaldehydes (Palenik et al. 1991; Allard et al. 1994), one might predict that their ability to partition hydrophobic organic compounds would decrease under the influence of UVR. However, this photodegradation does not appear to be sufficient to eliminate the capability of AHA to ameliorate PAH photoinduced toxicity. Although UV light changes the apparent ability of AHA to protect against PAH toxicity (Gensemer et al. 1998), UV degradation of the AHA itself does not seem to eliminate

its protective effects when present at sufficient concentration (Oris et al. 1990).

In contrast to chlorosis and F_v/F_m , AHA only delayed the complete inhibition of $t_{1/2}$ by anthracene within 24 h (Fig. 4), rather than permanently protecting the plants from anthracene damage to PS2 electron transport. This suggests that AHA may slow but not prevent the entrance of either intact anthracene or its photooxidized byproducts. Thus, AHA may protect against complete inhibition of PS2 quantum efficiency or even population growth, but only delay some of the initial damage to photosynthetic electron transport. For example, population growth rates in *L. gibba* were not completely inhibited at these same combinations of AHA and anthracene exposure under SSR (Gensemer et al. 1998). Therefore, PS2 electron transport either recovered somewhat after 48 h or the plants produced sufficient new biomass from residual energy stores to exhibit small but positive population growth in an 8-d exposure assay. Given that $t_{1/2}$ inhibition recorded at 48 h recovered somewhat by the end of an 8-d exposure to creosote levels which were moderately toxic to *L. gibba* grown under SSR (Gensemer et al. 1996), the former hypothesis seems more likely at this time.

Because primary photochemical reactions can be inhibited rapidly by chemical contaminants, bioindicators based on such reactions should exhibit effects more quickly than total chlorophyll degradation. The greater sensitivity of $t_{1/2}$ relative to F_v/F_m (Figs. 3 and 4) is likely to be a function of the mechanisms by which PAHs inhibit PS2 primary photochemistry. Whereas F_v/F_m is a rather general indicator of PS2 quantum efficiency, $t_{1/2}$ is a much more specific indicator of reactions that inhibit electron flow from the primary to secondary plastoquinone electron acceptors. Because electron transport is known to be inhibited by PAHs both in PS1 (Huang et al. 1997c) and PS2 (Oettmeier et al. 1987), $t_{1/2}$ should be one of the first fluorescence induction parameters to respond to anthracene toxicity. Our work supports that view; near complete inhibition of $t_{1/2}$ values was apparent after 1 h of anthracene exposure under SSR. Although relatively minor F_v/F_m inhibition also occurred this quickly, much more time was required for nearly complete inhibition to occur (~48 h). In contrast to either Chl *a* fluorescence induction parameter, 24–48 h were required for chlorophyll to degrade to any significant degree. The degradation of chlorophyll is a relatively long process owing to the time required for pigment photooxidation following inhibition of photochemical activity in PS1 or PS2 (Damanakis 1970; Powles 1984).

One of the critical steps in verifying that Chl *a* fluorescence assays are useful as indicators of contaminant stress in freshwater plants is to examine their quantitative relationships to higher level ecological responses. In *L. gibba*, both $t_{1/2}$ and F_v/F_m were more sensitive than was population growth rate to the photoinduced toxicity of anthracene when recorded at the end of a typical 8-d test (Gensemer et al. in prep.). This was also true when *L. gibba* was exposed to a PAH mixture (creosote) and SSR after 48 h, yet by the end of the 8-d growth test, they exhibited sensitivity virtually equal to population growth inhibition (Gensemer et al. 1996). In contrast to higher plants, photosynthetic responses to the photoinduced toxicity of anthracene in microalgae may be less

sensitive than population growth. For example, ^{14}C incorporation per cell was less sensitive to anthracene and UV exposure than either population growth or total primary productivity in *Selenastrum capricornutum* (Gala and Giesy 1992). Using the same species, fluorescence induction parameters were also found to be less sensitive to anthracene than population growth rate (Marsh et al. in prep.). Whether these represent taxonomic-related differences in relative sensitivity or a functional consequence of using population vs. individual-level bioassays remains to be determined.

The relative sensitivity of Chl *a* fluorescence induction responses also lends support to the importance of photo-modified end-products in addition to photosensitization reactions as mechanisms responsible for the photoinduced toxicity of PAHs in aquatic organisms. Some have attributed the photoinduced toxicity of PAHs in the presence of UVR almost entirely to photosensitization reactions (Newsted and Giesy 1987; Veith et al. 1995). QSAR analyses, however, have demonstrated that PAH photoinduced toxicity can be predicted more accurately when both photosensitization and photooxidation reactions are considered as mechanistic parameters in the model (Greenberg et al. 1993; Huang et al. 1997b; Krylov et al. 1997). In the present experiments, the sensitivity of $t_{1/2}$ inhibition by anthracene, relative to F_v/F_m and chlorophyll degradation, supports the importance of photooxidized anthracene as a significant contributor to photoinduced toxicity. While intact anthracene inhibited $t_{1/2}$ by 24 h at this concentration (Fig. 4A), <1 h was required to induce the same magnitude of effect in the presence of UVR (Fig. 4B). Significant photomodification of PAHs can occur in this amount of time (Huang et al. 1993; McConkey et al. 1997), and these products have been shown to diminish Chl *a* fluorescence in *L. gibba* (Huang et al. 1997c). Therefore, it is likely that $t_{1/2}$ was responding most strongly to the presence of the photomodified degradation products of anthracene. Photosensitization reactions also would be induced virtually instantaneously by UVR, but their damage to photosynthetic pigments and their associated biochemical reactions would require at least several hours. This is because the reactive oxygen species generated by photosensitization will randomly damage the photosynthetic membrane, and thus photosynthetic inhibition is likely to take additional time to be manifested (Damanakis 1970; Powles 1984). Therefore, the earliest events of anthracene photoinduced toxicity in plants probably involve damage to photosynthetic electron transport by photomodified anthracene within 1 h, followed by chlorophyll degradation and loss of PS2 quantum efficiency possibly resulting from photosensitized oxidation from anthracene and the inactive chlorophylls themselves.

Our study clearly supports the utility of chlorophyll *a* fluorescence assays in understanding how humic acid ameliorates anthracene photoinduced toxicity in the freshwater macrophyte *L. gibba*. However, it should be noted that further study will be required to extrapolate from our laboratory results to a thorough understanding of how natural humic acids might control PAH photoinduced toxicity under field conditions. For example, it remains to be established whether natural humic substances will control PAH photoinduced toxicity in similar fashion to commercial humic substances.

The commercial AHA used in both the present study and in most other laboratory bioassays (i.e., Oris et al. 1990; Day 1991; Gensemer et al. 1998) differs chemically from those found in natural aquatic habitats. Commercial humic materials contain different relative abundances of organic functional groups than naturally derived materials; in particular, commercial sources possess a 41% greater degree of aromaticity (Malcolm and MacCarthy 1986). Aldrich humic acid is a terrestrially derived humic material and appears to partition organic contaminants 4–20-fold more strongly than natural aquatic humic and fulvic acids (Landrum et al. 1985; Chiou et al. 1987).

Although this would seem to limit the usefulness of using AHA as a model compound, some have proposed that chemical properties common to all types of natural and commercial humic acid may determine how they control organic contaminant bioavailability (Kukkonen and Oikari 1991). Kukkonen and Oikari (1991) concluded that the degree of aromaticity measured by HA absorbance at 270 nm (Traina et al. 1990) and the hydrophilic acid composition of HA were both highly correlated to chemical partition coefficients and bioavailability of several organic contaminants (including PAHs) to *D. magna*. Both parameters were equally predictive of xenobiotic bioavailability regardless of whether the DOC was commercial or naturally derived. Furthermore, the greater degree of aromaticity in commercial HAs (Malcolm and MacCarthy 1986) is consistent with their stronger contaminant partitioning capabilities relative to natural sources (Landrum et al. 1985; Chiou et al. 1987).

Thus, the mechanisms by which commercial HAs partition organic contaminants may be similar to those for natural HAs, even if the magnitude of the chemical partitioning differs. A commercial AHA was chosen for our study primarily owing to the prevalence of their use in laboratory toxicity testing (e.g., Day 1991; Goodrich et al. 1991; Oikari et al. 1992; Steinberg et al. 1992). In lieu of using natural humic materials, future studies using commercial HAs should at least quantify functional properties common to all types of HA in order to improve extrapolations from the laboratory to natural systems.

Another important factor which could affect our ability to extrapolate from this laboratory exposure system to more natural conditions includes differences in light quality and quantity. The SSR light source used here has been used extensively in laboratory studies of both UV-B stress and PAH photoinduced toxicity in plants (Greenberg et al. 1997). Although not a perfect mimic of natural sunlight spectral quality, the SSR ratios of visible:UV-A:UV-B (100:10:1) closely match those of natural light at midtemperate latitudes (Greenberg et al. 1996). Several researchers have found that ratios of visible:UV-A:UV-B are among the most critical factors determining plant responses and adaptation to UVR (Singh 1994; Greenberg et al. 1997). Our light intensities ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) also were low compared to ambient sunlight ($\sim 2,000 \mu\text{mol m}^{-2} \text{s}^{-1}$), but this should not have negatively affected the ability of the plants to respond or adapt to the presence of UV-B. Plant responses to UV-B under SSR lighting have been shown to be similar to those grown in full sunlight as long as the visible:UV-B ratio is maintained at about 100:1 (Greenberg et al. 1997). How-

ever, given that studies of humic acid amelioration of PAH photoinduced toxicity have only been performed using SSR to date (Oris et al. 1990; Gensemer et al. 1998, present study), it remains to be seen how the humic acid-PAH interaction might change under more natural lighting intensities or spectral quality.

Finally, the degree and type of nutrient limitation is also likely to interact with results obtained from chlorophyll fluorescence assays from natural populations. For example, both iron- and nitrogen-limited marine phytoplankton exhibited inhibition of PS2 quantum yield concomitant with the loss of chlorophyll reaction centers and electron transport complexes (Kolber et al. 1988; Greene et al. 1992). We are unaware of any similar studies using freshwater plants except for a single study of phosphorus limitation using the phytoplankton *Selenastrum capricornutum* (Gensemer et al. in prep.). In this later case, neither F_v/F_m nor $t_{1/2}$ were affected by the degree of phosphorus limitation, whereas steady-state fluorescence quenching was strongly inhibited under the most severe P-limited conditions. In the present study, plant growth was nutrient replete, so further study will be required to detect meaningful PAH-induced stress using chlorophyll *a* fluorescence assays under the full influence of interacting stressors in nature.

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