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The Effect of Anthracene and Phenanthrene on the Growth, Photosynthesis, and SOD Activity of the Green Alga *Scenedesmus armatus* Depends on the PAR Irradiance and CO_2 Level

A. Aksmann, Z. Tukaj

Department of Plant Physiology, University of Gdańsk, al. Marszałka Piłsudskiego 46, 81-378 Gdynia, Poland

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Abstract. Short-term (24-h) experiments were performed to examine the effect of phenanthrene (PHE) and anthracene (ANT) on algae grown in a batch culture system at irradiances of 12, 33, 48, and 64 W m^{-2} of the PAR range. Cultures were aerated (0.1 or 2% CO_2) or nonaerated. As a result of aeration the concentration of ANT dropped from 0.45 mg L^{-1} at the beginning of the experiment (t_0) to an undetectable value after 10 h. The PHE concentration dropped from 9.36 mg L^{-1} at t_0 to 0.17 mg L^{-1} after 24 h. ANT at nominal concentrations exceeding 0.05 mg L^{-1} inhibited the growth of the algae in a concentration- and irradiance-dependent manner. The algistatic effect of ANT observed at 64 and 48 W m^{-2} was independent of the CO_2 level, whereas the growth inhibition at 33 and 12 W m^{-2} was much greater in cultures aerated with 2% than with 0.1% CO_2. PHE inhibited the growth only at a concentration of 10 mg L^{-1} (about 50% of the control) regardless of the irradiance or the CO_2 concentration. The toxicity of both PHE and ANT was similar in aerated (0.1% CO_2) and nonaerated cultures. ANT and PHE had a distinct effect on the photosynthesis. At 0.1% CO_2, the irradiance-dependent inhibition (ANT) and stimulation (PHE) of the photosynthesis was observed. The inhibitory effect of ANT was enhanced when algae were grown at 2% CO_2, but at the same CO_2 concentration PHE did not affect the photosynthesis. ANT stimulated the total superoxide dismutase (SOD) activity in the cells, this effect being generally more pronounced at elevated CO_2 levels and increasing with the irradiance. The total SOD activity was 250–300% higher in cells treated with PHE aerated with 0.1% CO_2. At 2% CO_2 no effect of PHE on the enzyme's activity was noted. The results obtained indicate that ANT acts as a photosensitizer causing an oxidative damage of cells, while PHE seems to affect the macromolecular synthesis.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and persistent environmental contaminants produced mainly due to the incomplete combustion of coal, oil, petrol, and wood. Their transport to the aquatic environment occurs both *via* surface waters and atmosphere. In the water column, most PAHs are absorbed by particles and deposited in the underlying sediments (Law and Biscaya 1994). PAH concentrations at offshore sites are generally low or undetectable. Higher concentrations can be found in coastal and estuarine samples. In seawater around England and Wales, Law *et al.* (1997) found 15 individual parent PAH compounds the total concentration of which ranged from none detected to 10.7 mg L^{-1}, almost three orders of magnitude greater than the highest concentrations recorded for offshore waters. In some Mediterranean coastal sediments, 14 PAHs, mainly of pyrolytic origin, were detected at a concentration higher than 48 mg per g of the dry sediment. In Baltic Sea surface water the average concentration of 15 PAHs ranges from 2800 to 6500 pg L^{-1}; among them anthracene (ANT) reaches 27–84 pg L^{-1}, and phenanthrene (PHE) 381–1310 pg L^{-1} (Witt 2002).

Many studies indicate that the toxicity of PAH increases after the absorption of solar radiation, especially from the UV-A and UV-B regions. This photoinduced toxicity is caused by two photochemical processes: photosensitization and photomodification. During the photosensitization process, active oxygen species are generated, which can cause oxidative damages in biological systems. Photomodification of PAH results in the formation of a variety of oxygenated products, which in many cases are more toxic than the parent compounds. However, not all PAH are phototoxic. Anthracene and phenanthrene, used in this work, have similar structures (three fused aromatic rings) but different physicochemical properties and toxicity to organisms. ANT is assumed to be one of the most toxic and the fastest photomodified hydrocarbons (Krylov *et al.* 1997). It is a strong photosensitizer and its photomodification results in the formation of a complex mixture of more than 20 products (Huang *et al.* 1997b; Krylov *et al.* 1997), some of which are phototoxic (Mallakin *et al.* 1999). PHE represents a class of more persistent PAHs, which are poorly soluble in water and have a potential to accumulate in organisms. Its toxicity to plants is not as high as that of ANT. Photomodification of PHE is relatively slow and its toxicity is assumed to result

Correspondence to: A. Aksmann; *email:* anaks@sat.ocean.univ.gda.pl

equally from the photosensitization and photomodification processes (Huang *et al.* 1997a).

Although the key role of the UV irradiance in PAH toxicity seems incontestable, the contribution of visible light to photosensitization processes is not so clear. Moreover, many other experimental factors may affect the toxic response of algae (Mayer *et al.* 1998). Among them, the light intensity, sufficient supply of inorganic carbon, and controlled pH of the medium during the experiments appear to be especially important from the physiological viewpoint. It is known that when nonaerated algae are cultured in the laboratory at a low CO_2 concentration with nitrate as the nitrogen source, rapid alkalization of the medium occurs. As the medium alkalizes, the accessibility of inorganic carbon for cells changes, since the relative contribution of individual dissolved inorganic carbon (DIC) species strongly depends on pH. In addition, in such batch culture systems the density of cells may quickly reach a level at which the carbon demand by the growing algae exceeds the transfer of CO_2 from the gas phase to the liquid phase in the culture vessel, and carbon becomes the limiting growth factor (Halling-Sørensen *et al.* 1996). To avoid pH changes and carbon shortage, cultures of microalgae are usually enriched with 2–4% CO_2. However, some algal species grown at elevated CO_2 concentrations are not efficient in the acquisition of DIC, because the carbon concentrating mechanism (CCM) does not operate. If these same algae are grown at limiting CO_2, they become very efficient in CO_2 uptake and fixation. Thus, the growth conditions of the alga, changing the cell's affinity for CO_2, could be another factor influencing the results of toxicological experiments.

The aim of this work was to determine whether under laboratory conditions the toxic effect of two three-ring PAH on green microalgae depends on photosynthetic active radiation. This means that our interest was mainly focused on the photosensitization process related to the toxicity of both hydrocarbons. Photosensitization processes causing the evolution of reactive oxygen species (ROS) trigger some adaptative mechanisms, which are expressed by higher activity of antioxidant enzymes such as superoxide dismutase (SOD) (Larson and Berenbaum 1988; Bowler *et al.* 1992). SOD constitutes the first line of defense against ROS, thus examination of the enzyme's activity may contribute to a deeper knowledge of toxic effects of PAH on an algal cell and its ability to adapt to oxidative stress.

Our purpose was also to compare the influence of ANT and PHE upon *S. armatus* populations grown at such a concentration of carbon dioxide at which the CCM mechanism in the cells occurs (0.1%) and the pH of the medium alkalizes, as well as under conditions where this mechanism is absent (2%) and the pH of the medium during the experiments remains unchanged.

Materials and Methods

Chemicals

The chemicals used were two three-ring PAHs: anthracene (ANT) and phenanthrene (PHE) of high purity (Aldrich Chemical Co., USA). Hydrocarbons were dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Belgium) and supplied to the growth medium after dilution to the required concentrations. Chemicals of analytical grade used for medium preparation were purchased from POCH SA, Gliwice, Poland.

Organism

The *Scenedesmus armatus* strain (B1-76) used in this study was isolated from southern Baltic Sea phytoplankton at the Institute of Oceanography, Polish Academy of Sciences (Sopot, Poland) (Gędziorowska 1983).

Cultures

The algae were stored on slants containing Bristol's (BBM) medium, after Nichols and Bold (1965), solidified with 2% agar and enriched with 1% bactopeptone and 2% glucose. The algae were transferred from the slants into the liquid BBM and precultured for 3 days. When algae in the precultures reached the exponential growth phase, the material was used to set up batch cultures. The pH of the BBM at the beginning of the experiments was 6.9 ± 0.1 (pH-mV Meter Type 526; WTW, Germany) and the salinity was 0.2 psu (Multiline P4 salinometer; WTW). The initial cell density was 0.5×10^6 cell ml^{-1} in 50 ml of liquid culture media. Algae were grown in 100-ml glass test tubes submersed in a thermostated water bath at a constant temperature of 30°C, under continuous fluorescent light from TLD 58W/54 lamps (Philips), providing about 64, 48, 33, and 12 W m^{-2} of photosythetically active radiation (PAR) (380–690 nm) measured at the surface of the culture vessels. To adjust the irradiance to a given value, metal nets of appropriate density were inserted between the light source and the culture vessels. A quantitherm QRT1 light meter/thermometer (Hansatech Instruments, England) was used to measure the irradiance. One part of the experiment was performed in test tubes closed with microbiological stoppers (nonaerated cultures), shaken by hand several times a day. The second part (aerated cultures) was carried out using test tubes each closed with a glass stopper equipped with a glass pipe ending 3 mm above the bottom of the tube, providing an air flow of about 10.0 L h^{-1}. Cultures were aerated with a gas mixture containing 0.1% or 2.0% CO_2, which was passed through a bacteriological filter (Sartorius 2000; 0.2 mm PTFE). The CO_2 concentration in the inlet air mixtures was measured using an Air TECH 2500/2060 P (Telaire Systems Inc., USA).

All the experiments lasted 24 h. In growth experiments, the chemicals were delivered to the medium to final nominal concentrations within the range 0.05–1 mg L^{-1} for ANT and 0.1–10 mg L^{-1} for PHE, each in four (ANT) or six (PHE) replicates. Experiments on the photosynthesis and SOD activity were conducted at nominal concentrations of 0.5 mg L^{-1} for ANT and 10 mg L^{-1} for PHE, each in three replicates. A DMSO (0.1%, v/v) control sample was prepared for each experiment. DMSO had no significant effect on the growth, photosynthesis, or SOD activity in *S. armatus* cells.

Measurements

The number of cells was determined microscopically in a Bürker chamber following a standard procedure. The growth rate (μ) was calculated as $(\ln N_1 - \ln N_0)/(t_1 - t_0)$ [div. day^{-1}], where N_1 and N_0 are the numbers of cells determined at the end of the experiment (t_1) and at the beginning of the experiment (t_0), respectively.

The photosynthetic oxygen production was determined using a Clark-type (EO 96; WTW) oxygen electrode connected to a microprocessor (OXI 96; WTW). The electrode was equipped with a stirrer bar, a pressure corrector, and a temperature sensor. It was placed in a

homemade photosynthetic chamber with an outer jacket for thermostated water, all made of plexiglass. The cylindrical chamber had a 15-mm inside diameter and a capacity of 10 ml. Samples derived from the culture vessel were immediately placed in the chamber. Prior to the measurement, the samples were adapted for 3 min to reach a stable level of the oxygen evolution. Then an increase in the oxygen concentration during the next 3 min was observed. The cell suspension was continuously stirred during the measurement. Measurements were carried out under the same temperature and light conditions as those applied in the cultures.

To determine the total SOD activity, centrifuged samples (2500 rpm, 15 min) of *S. armatus* were resuspended in 1 ml of phosphate buffer (pH 7.0, 0.05 *M*) and homogenized with glass beads (0.4 mm; Merck, England) for 3 min. Then the pellet was washed twice with 0.5 ml of phosphate buffer. The homogenisate obtained (total amount, 2 ml) was centrifuged (13,000 rpm, 30 min) and the extract was used for the SOD activity analysis. The SOD activity was assessed by nitroblue tetrazolium (NBT) photoreduction, according to the method of Beauchamp and Fridovich (1971). The results are given as units of SOD activity per milligram of total proteins (U mg^{-1} protein). The amount of protein in the extract was assessed using the Bradford (1976) method.

Determination of the PAH Concentration

To determine the changes in the PAH concentration during the experiments, samples of BBM (50 ml) containing 0.5 mg L^{-1} of ANT and 10 mg L^{-1} of PHE were prepared in 100-ml glass test tubes, each in three replicates. The vessels were kept under the same conditions as were the algal cultures (30°C, 64 W m^{-2} PAR, 0.1% CO_2). The 5-ml samples of PAH-containing Bristol's medium were withdrawn every 2 h from t_0 to t_{24} and transferred to a separatory funnel with a 100-ml capacity. Hydrocarbons were extracted with 10 ml spectrophotometric-grade hexane (Merck, Germany) for 15 min in a shaker (type 357; Olpan Lab. Instr., Poland). After shaking, hexane and water phases were allowed to separate for 5 min, the hexane phase was collected, and then the water phase was shaken again twice with 10 ml of hexane as described above. Absorbance of combined hexane extracts was measured at 251 nm (Hitachi U-2010). ANT and PHE concentrations were read from the standard curves. To prepare a reference solution, 5-ml aliquots of DMSO-containing Bristol's medium were taken every 2 h from t_0 to t_{24} and each sample was extracted three times with 10 ml of hexane as described above.

Statistical Analysis

Statistical analysis was performed using an MS Excel 2000 program (Microsoft). Standard deviation (SD) and standard error (SE) were calculated. To compare results obtained for control and treatment cultures Student's *t* test was calculated. Significance was set at $p < 0.05$.

Results

Changes in the PAH Concentration During the Experiments

The results showed that the nominal concentrations of both hydrocarbons in media prepared for experiments (0.5 mg L^{-1} for ANT and 10 mg L^{-1} for PHE) and chemically determined concentrations of ANT and PHE in these media were practically the same (0.45 ± 0.04 mg L^{-1} for ANT and 9.36 ± 0.9 mg L^{-1} for PHE) (Figure 1). Both hydrocarbons disappeared from the areated culture system—ANT much faster than PHE—and the most evident decrease in the concentration of both PAHs was observed during first 2 h of the experiments (Figure 1). The concentration of ANT dropped from 0.45 mg L^{-1} at the beginning of the experiment to 0.19 mg L^{-1} after 2 h and, next, progressively decreased to an undetectable value after 10 h. PHE concentration dropped from 9.36 mg L^{-1} at the beginning of the experiment to 6.69 mg L^{-1} after 2 h and, unlike ANT, was detected throughout the experiment, reaching 0.17 mg L^{-1} at the end of the experiment (Figure 1).

The Effect of Hydrocarbons on Growth

The growth of control cultures aerated with 0.1% CO_2 was similar for irradiances 33, 48, and 64 W m^{-2} (Table 3), with the growth rate ranging between 1.69 and 1.76 div. day^{-1}. The growth rate of cultures grown at 12 W m^{-2} was slightly lower (1.53 div. day^{-1}). At this CO_2 level, ANT inhibited the population growth in a concentration-dependent manner and these inhibitory effects were enhanced by higher irradiance (Table 1). At a concentration of 0.05 mg L^{-1}, ANT had no significant influence on the growth of *S. armatus* cells. At 12 and 33 W m^{-2}, statistically significant growth inhibition (about 60% of the control) was found at 0.5 mg L^{-1} ANT. Similar effects were caused by 0.1 and 0.075 mg L^{-1} concentrations of ANT when algae were cultured at 48 and 64 W m^{-2}, respectively. Among four PHE concentrations tested (0.1–10 mg L^{-1}), only the highest one significantly inhibited the population growth (about 50% of the control culture) (Table 2). In general, this influence was independent of the PAR irradiance intensity.

Due to bubbling of the cultures in our experiments, a continuous drop in the PAH concentration was observed (Figure 1), especially distinct in the case of ANT. To examine the influence of PAH evaporation on their toxicity, nonaerated cultures were performed. Under such conditions, the control cultures reached 1.2×10^6 cells ml^{-1} with the growth rate of 0.87 div. day^{-1}. We have found that the toxicity of PHE did not increase significantly in nonaerated cultures (Tables 3 and 4) compared to the aerated ones. The population density was 0.71×10^6 cells ml^{-1} (60% of control cultures) with the growth rate of 0.35 div. day^{-1}. The toxicity of ANT was also similar in aerated (0.1% CO_2) and nonaerated cultures (Tables 3 and 4). The EC_{50} concentration (0.25 mg L^{-1}) estimated for 0.1% CO_2-aerated cultures inhibited the growth of nonaerated cultures to 54% of the control.

In the control and PHE-treated cultures strong alkalization of the medium was observed. After 1 day of culture, the pH of the media changed from 6.9 ± 0.1 to 9.5 ± 0.4 (PHE) and to 10.3 ± 0.5 (control). On the contrary, in ANT-treated cultures, pH of the medium did not change significantly. To stabilize pH and, in consequence, to eliminate its possible contribution to the toxicity of both hydrocarbons, the cultures were bubbled with air enriched with 2% CO_2. Under such conditions, pH of all the media did not change and was 7.2 ± 0.2.

Data in Table 3 show the growth effects of ANT (0.5 mg L^{-1}) and PHE (10 mg L^{-1}) observed at low (0.1%) and elevated (2%) CO_2. In elevated CO_2, the control algae grew

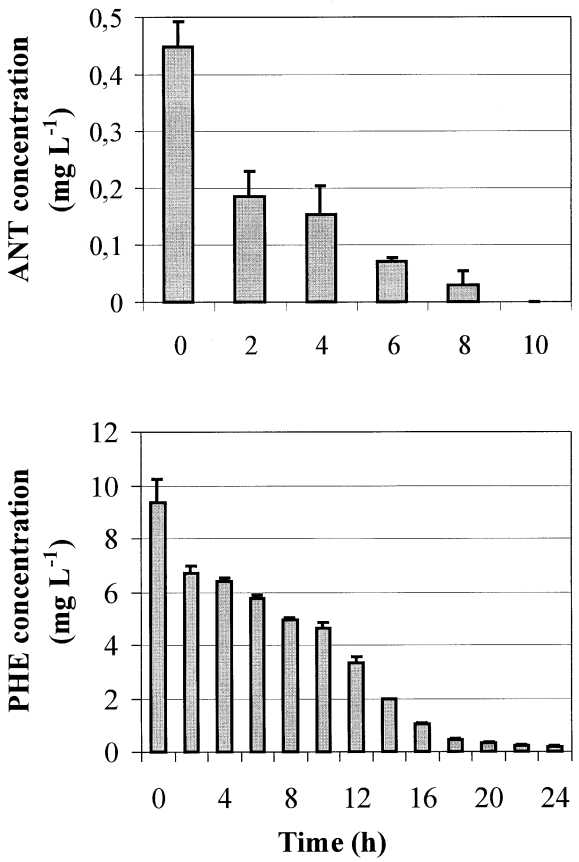

Fig. 1. Changes in anthracene (ANT) and phenanthrene (PHE) concentrations during the experiments. Absorbance of hexane extracts of both PAH was measured at 251 nm. ANT and PHE concentrations were read out from the standard curves. Each value is an arithmetic mean of three replicates ± SE (standard error bars)

about 2.5-fold faster than those cultured at 0.1% CO_2, and the differences were statistically significant at all irradiances applied. The growth rate of control cultures at 2% CO_2 was influenced by the PAR intensity. At 12 W m^{-2} the growth rate was 2.42 div. day^{-1}, at 33 W m^{-2} it increased to 2.45 div. day^{-1}, and it reached 2.56–2.57 div. day^{-1} at 48 and 64 W m^{-2} (Table 3).

The toxicity of ANT increased with the intensifying irradiance at both CO_2 levels, but this hydrocarbon was much more toxic at elevated than at low CO_2, which could be easily noticed at lower irradiance values (12 and 33 W m^{-2}). ANT almost completely inhibited the growth of the algae at higher irradiances (48 and 64 W m^{-2}) independent of the CO_2 level. In contrast to ANT, the inhibitory effect of PHE at elevated CO_2 was reduced by only 10–20% in comparison to low CO_2, regardless of the irradiance.

The Effect of Hydrocarbons on the Photosynthetic Rate

The exposure of the algae to ANT (0.5 mg L^{-1}) and PHE (10 mg L^{-1}) distinctly affected the photosynthesis in *S. armatus*, depending on the irradiance and CO_2 concentration (Figure 2). In the control cultures, the intensity of photosynthesis was 0.13 ± 0.02 μmol O_2 × mln $cells^{-1}$ × h^{-1} at all irradiances applied. In the ANT-treated cultures, inhibition of photosynthetic oxygen production was observed (Figure 2A). At 0.1% CO_2 this effect was enhanced by the increase in the irradiance. No statistically significant inhibition was observed at the lowest light intensity (12 W m^{-2}). At 33 W m^{-2}, oxygen evolved by algae constituted 77%, at 48 W m^{-2} it decreased to 64%, and at 64 W m^{-2} it was reduced to 52% of the control value. The harmful effect of ANT was more pronounced when the algae were aerated with 2% CO_2. In this case, the intensity of photosynthesis in control cultures ranged from 0.26 ± 0.01 to 0.29 ± 0.02 μmol O_2 × mln $cells^{-1}$ × h^{-1} (at 12 and 64 W m^{-2}, respectively) but differences among the four irradiances applied were not statistically significant. In the ANT-treated cultures the intensity of photosynthesis decreased from 78 to 31% of the control value, with the increase in the irradiance from 12 to 64 W m^{-2}. The effect of PHE on photosynthesis strongly depended on the CO_2 concentration (Figure 2B). When cultures were grown at 0.1% CO_2, the photosynthetic oxygen production was stimulated in a irradiance-dependent manner within the range from 134 to 290% of the control for 12 and 64 W m^{-2}, respectively. When the algae were grown at 2% CO_2, PHE had generally no statistically significant influence on photosynthesis of *S. armatus* with the exception of 27% inhibition noted in the algae cultured at 12 W m^{-2} (Figure 2B).

The Effect of Hydrocarbons on the SOD Activity

The SOD activity in the control cultures aerated with 0.1% CO_2 ranged from 3.24 ± 0.7 to 2.39 ± 0.6 U mg^{-1} protein (at 12 and 64 W m^{-2}, respectively), with no statistically significant differences among the irradiations applied. In the control cultures aerated with 2% CO_2, the SOD activity ranged from 3.58 ± 1.0 to 3.71 ± 1.2 U mg^{-1} protein (at 12 and 64 W m^{-2}, respectively), and the differences among the irradiations applied were not significant.

Figure 3 shows the results of the total superoxide dismutase (SOD) activity assessed in *S. armatus* cells cultured at low and high CO_2 concentrations and exposed to ANT (0.5 mg L^{-1}) and PHE (10 mg L^{-1}). The stimulation of the SOD activity was observed in ANT-treated algae (Figure 3A). In 0.1% CO_2-grown cultures, the activity of SOD significantly increased from 127% at 12 W m^{-2} to 144% of the control at 33 W m^{-2} and reached about 178% of the control at higher irradiance values (48 and 64 W m^{-2}). In high-CO_2-grown treatments, ANT roughly doubled the SOD activity, with the exception of the lowest irradiance (12 W m^{-2}), at which no statistically significant influence of ANT was observed.

PHE caused the stimulation in the SOD activity only when the cells were grown at 0.1% CO_2. Depending on the irradiance, this stimulation ranged from 234 to 293% of the control (Figure 3B). The SOD activity in cultures enriched with 2% CO_2 showed a tendency to decrease with the increase in the irradiance (122 and 87% of the control for 12 and 64 W m^{-2}, respectively). However, none of these results was statistically different from the control.

Table 1. Concentration-dependent influence of anthracene on the growth of *Scenedesmus armatus* cultured for 24 h at 12, 33, 48, and 64 W m^{-2} of PAR irradiance, a 0.1% concentration of CO_2, and a temperature of 30°C

Irradiance (W m^{-2})	Anthracene concentration (mg L^{-1})										
	0	0.05		0.075		0.1		0.5		1	
	N	*N*	% C	*N*	% C	*N*	% C	*N*	% C	*N*	% C
12	3.152 (0.31)	3.089 (0.28)	98	2.610 (0.10)	83	2.655 (0.19)	84	1.751 (0.23)	56*	0.988 (0.14)	31*
33	3.069 (0.24)	3.088 (0.25)	101	3.000 (0.16)	98	3.039 (0.30)	99	1.376 (0.43)	45*	0.593 (0.14)	19*
48	3.356 (0.22)	3.361 (0.23)	100	2.429 (0.50)	72	2.129 (0.36)	63*	0.534 (0.03)	16*	0.546 (0.07)	16*
64	3.031 (0.15)	2.613 (0.19)	86	2.035 (0.42)	67*	1.957 (0.36)	65*	0.507 (0.07)	17*	0.478 (0.05)	16*

Note. Anthracene was delivered to the medium *via* DMSO (0.1%, v/v); the same amounts of DMSO were added to the controls. C, control; *N*, number of cells per ml ($\times 10^6$).
* Values statistically different from the control ($\alpha = 0.05$); the values in parentheses represent the standard error of four experiments. Anthracene concentration values are nominal.

Table 2. Concentration-dependent influence of phenanthrene on the growth of *Scenedesmus armatus* cultured for 24 h at 12, 33, 48, and 64 W m^{-2} of PAR irradiance, a 0.1% concentration of CO_2, and a temperature of 30°C

Irradiance (W m^{-2})	Phenanthrene concentration (mg L^{-1})								
	0	0.1		1		5		10	
	N	*N*	% C	*N*	% C	*N*	% C	*N*	% C
12	2.916 (0.46)	2.308 (0.24)	79	2.389 (0.27)	82	2.012 (0.32)	69	1.614 (0.26)	55*
33	3.365 (0.46)	2.974 (0.43)	88	3.065 (0.42)	91	2.357 (0.17)	70	1.471 (0.34)	44*
48	3.144 (0.43)	3.144 (0.28)	100	3.214 (0.35)	102	2.949 (0.52)	94	1.694 (0.15)	54*
64	3.093 (0.10)	3.061 (0.12)	99	2.968 (0.15)	96	2.769 (0.16)	90	2.109 (0.53)	68*

Note. Phenanthrene was delivered to the medium *via* DMSO (0.1%, v/v); the same amounts of DMSO were added to the controls. C, control; *N*, number of cells per ml ($\times 10^6$).
* Values statistically different from the control ($\alpha = 0.05$); the values in parentheses represent the standard error of six experiments. The phenanthrene concentration values are nominal.

Table 3. Effect of nominal concentrations of anthracene (0.5 mg L^{-1}) and phenanthrene (10 mg L^{-1}) on the growth of *Scenedesmus armatus* cultured for 24 h at low (0.1%) and elevated (2%) CO_2 concentrations and at 12, 33, 48, and 64 W m^{-2} of PAR irradiance

CO_2 concentration	Irradiance (W m^{-2})	Control		Anthracene			Phenanthrene		
		N	μ	*N*	% C	μ	*N*	% C	μ
0.1%	12	2.33 (0.09)	1.53 (0.04)	1.61 (0.18)	69	1.15 (0.09)	1.21 (0.23)	52	0.99 (0.13)
	33	2.86 (0.24)	1.72 (0.07)	1.46 (0.39)	51	1.05 (0.1)	1.47 (0.41)	51	1.13 (0.19)
	48	2.76 (0.19)	1.69 (0.07)	0.53 (0.03)	19	0.04 (0.01)	1.75 (0.38)	63	1.33 (0.19)
	64	2.95 (0.18)	1.76 (0.06)	0.43 (0.07)	15	−0.15 (0.09)	1.95 (0.29)	66	1.46 (0.16)
2%	12	5.62 (0.08)	2.42 (0.01)	2.12 (0.18)	38	1.44 (0.05)	3.54 (0.23)	63	1.96 (0.04)
	33	5.81 (0.06)	2.45 (0.01)	1.11 (0.09)	19	0.79 (0.05)	4.47 (0.05)	78	2.19 (0.006)
	48	6.46 (0.15)	2.56 (0.02)	0.75 (0.18)	12	0.38 (0.14)	5.03 (0.02)	78	2.31 (0.002)
	64	6.53 (0.15)	2.57 (0.02)	0.74 (0.16)	11	0.37 (0.13)	4.88 (0.04)	75	2.28 (0.005)

Note. Hydrocarbons were delivered to the medium *via* DMSO (0.1%, v/v); the same amounts of DMSO were added to the controls. The CO_2 concentration was measured in the inlet gas mixtures used for culture aeration. C, control; *N*, number of cells per ml ($\times 10^6$); μ, growth rate $[(\ln N_1 - \ln N_0)/(t_1 - t_0)$; N_1 and N_0, numbers of cells at t_1 and t_0 time, respectively. The values in parentheses represent the standard error of four (ANT) or six (PHE) experiments. All hydrocarbon treatments were statistically different from the control ($\alpha = 0.05$). The differences in the control cultures aerated with 0.1% versus 2% CO_2 were statistically significant at all irradiances applied ($\alpha = 0.05$).

Discussion

The results reported herein show a much higher toxicity of ANT than PHE to *S. armatus*, thus confirming the literature data and conclusions of other authors (Huang *et al.* 1993; Duxbury *et al.* 1997) that ANT is one of the most toxic aromatic hydrocarbons, while PHE exhibits a "medium" level of toxicity. McConkey *et al.* (1997) have shown that, under simulated solar radiation (VIS:UV-A:UV-B ratio of 100:10:1), the EC_{50} of PHE for *Photobacterium phosphoreum* (bacterium) and *Lemna gibba* (aquatic plant) was 0.53 and 3.5 mg L^{-1}, respectively. Gala and Giesy (1994) have found *Selenastrum capricornutum* (green alga) to be an organism extremly sensitive to ANT, (EC_{50}, 3.6–16.1 μg L^{-1}) but their experiments were conducted under both visible and UV-A radiation. In our experiments, however, concentrations of ANT (0.5 mg L^{-1})

Table 4. Effect of nominal concentrations of anthracene (0.25 mg L^{-1}) and phenanthrene (10 mg L^{-1}) on the growth of *Scenedesmus armatus* grown for 24 h in nonaerated cultures at 64 W m^{-2} of PAR irradiance

	Control	Anthracene	Phenanthrene
N	1.20 (0.04)	0.64 (0.04)	0.71 (0.02)
% cont.	100	54	60
μ	0.87 (0.04)	0.24 (0.06)	0.35 (0.03)

Note. Hydrocarbons were delivered to the medium *via* DMSO (0.1%, v/v); the same amounts of DMSO were added to the controls. *N*, number of cells per ml ($\times 10^6$); μ, growth rate $[(\ln N_1 - \ln N_0)/(t_1 - t_0)$; N_1 and N_0, numbers of cells at t_1 and t_0 time, respectively]. The values in parentheses represent the standard error of three experiments, each in three replicates. All hydrocarbon treatments were statistically different from the control ($\alpha = 0.05$).

and PHE (10 mg L^{-1}) required for a significant growth inhibition were high compared to data obtained by the authors mentioned above. One of the reasons for these differences may be the source of light used, devoid of the UV range, as well as the more favorable conditions of our experiments, such as the relatively higher temperature and the cultures being aerated and enriched with CO_2.

Hydrocarbons characterized by Henry's constant higher than 1.01 Pa m^3 mol^{-1} are considered to be volatile. At 30°C, the Henry's law constants (H) calculated for ANT and PHE are 5.60 and 5.04 Pa m^3 mol^{-1}, respectively (Alaee *et al.* 1996). Thus, due to continuous bubbling of the cultures in our experiments, large amounts of both hydrocarbons evaporated. This was especially noticeable for ANT, which disappeared within 10 h of the test duration. Laboratory toxicological tests are usually conducted at 20–25°C and cultures are not aerated. Under such conditions, due to the lower rate of evaporation, the expected toxicity of volatile hydrocarbons should be higher. For instance, Halling-Sørensen *et al.* (1996) found that the EC_{50} for PHE, tested at of 22°C using *Selenastrum capricornutum*, ranged from 302 to 2021 μg L^{-1}, depending on whether the tests were carried out in open or sealed E-flasks. On the other hand, we have found that the toxicity of ANT and PHE does not differ in nonaerated and aerated cultures. Moreover, anthracene, evaporating faster than phenanthrene, is more toxic. Therefore, it seems that the most important for ANT and PHE toxicity are a few initial hours of exposition, when absorption of the hydrocarbons by cells is very fast (Duxbury *et al.* 1997). Thus, the rate of weathering of the PAH solutions during our experiment could not be the physical reason for the lower toxicity of ANT and PHE compared to the results reported by other authors (Gala and Giesy 1994; McConkey *et al.* 1997), and it cannot explain the differences in toxicity between the two hydrocarbons.

It is well documented that the harmful effect of aromatic hydrocarbons strongly depends on photosensitization and photoconversion processes (Huang *et al.* 1997a, 1997b; Krylov *et al.* 1997). ANT and PHE strongly absorb in the UV-B and UV-A (290–400 nm) regions. In our experiments this spectral region was extremely limited owing to the light source spectrum used in the cultures (380–690 nm). Hence, photosensitization rather than photomodification processes would be related with the toxicity of both hydrocarbons. Experiments with

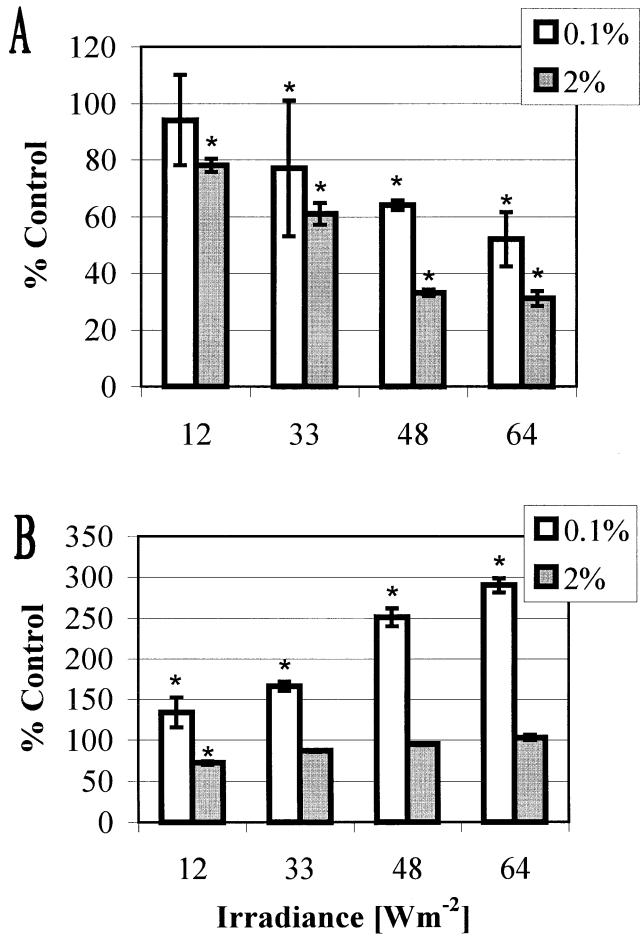

Fig. 2. Effect of anthracene (A) and phenanthrene (B) at nominal concentrations of 0.5 and 10 mg L^{-1}, respectively, on the photosynthesis intensity in *Scenedesmus armatus* cells grown at two (0.1 and 2%, v/v) CO_2 concentrations and four (12, 33, 48, and 64 W m^{-2}) PAR irradiance values. The results are presented as percentage oxygen evolution relative to the control cultures. Each value is the arithmetic mean of three experiments ± SE (standard error bars). *Values statistically different from the control ($\alpha = 0.05$)

Lemna gibba (Huang *et al.* 1997a) provided evidence that the main reason for the ANT toxicity is photosensitization reactions, while the toxicity of PHE results equally from photosensitization and photoconversion processes, when plants were cultured in simulated solar radiation. This may partly explain why our results for ANT-treated cultures are generally in agreement with those reports in which the toxicity of PAH to aquatic organisms has been shown to increase upon exposure to light (Gala and Giesy 1992; McConkey *et al.* 1997), while in the case of PHE-exposed cells the above relationship was not observed. Since ANT is an efficient photosensitizer (Huang *et al.* 1997a, 1997b; Mallakin *et al.* 1999), its acute toxicity required the presence of light. The primary mode of ANT action accumulated in a tissue is the overproduction of singlet oxygen and reactive oxygen species (ROS), leading to the so-called oxidative stress phenomenon (Girotti 1983; Huang *et al.* 1997a). The marked increase in the total SOD activity observed especially at high irradiance (48 and 64 W m^{-2})

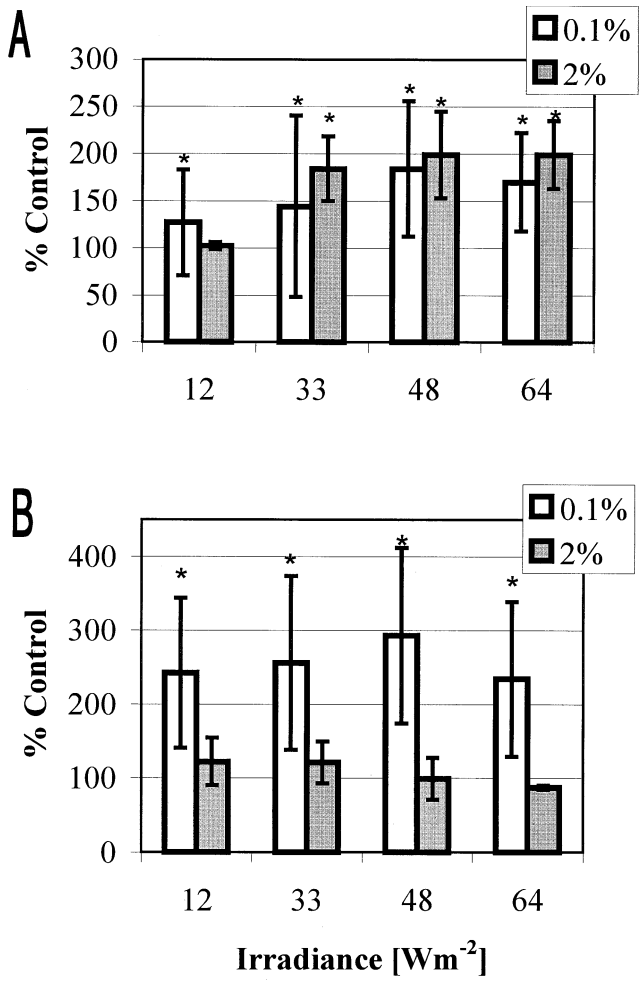

Fig. 3. Superoxide dismutase (SOD) activity in *Scenedesmus armatus* cells treated with anthracene (A) and phenanthrene (B) at nominal concentrations of 0.5 and 10 mg L^{-1}, respectively. The cultures were grown at 12, 33, 48, and 64 W m^{-2} PAR and aerated with 0.1 or 2% CO_2. After 24 h of exposure the algae samples were homogenized, SOD activity was determined spectrophotometrically and it is expressed as specific activity per 1 mg of protein. The data are presented as percentage SOD activity relative to the control. The bars represent standard errors of the mean from three experiments. *Values statistically different from the control ($\alpha = 0.05$)

suggests the occurrence of the oxidative stress in the *Scenedesmus* cells treated with ANT. This suggestion is in line with the observation that the photosynthetic oxygen production was inhibited by ANT in an irradiance-dependent manner. Babu *et al.* (2001) found that one of the ANT photoproducts, 1,2-dihydroantraquinone, inhibited the photosynthetic electron transport at the cytochrome b_6/f complex, leading to the overproduction of $O_2^{-\cdot}$ and, in consequence, to the oxidation of proteins, membranes, and pigments. Hydrocarbon-treated cells of *S. armatus* were bleached regardless of irradiance. Similar effects were observed in *Lemna gibba* and *Myriophyllum spicatum* plants after a 2-day exposition to PAH (Marwood *et al.* 2001).

Cells kept at 2% CO_2 grew much faster and produced much more oxygen at the same irradiance compared to cells cultured at low CO_2. ANT stronger affected the growth and photosynthesis of *S. armatus* when the cultures were bubbled with 2% CO_2. Under such conditions, singlet oxygen and ROS could be generated more intensively, because greater amounts of O_2 and electrons interacted with PAH. Therefore, faster damage of cells should be expected. PHE exhibited a different pattern of toxicity, as the population growth was inhibited by this hydrocarbon regardless of irradiance and only slightly depended on the CO_2 concentration. Moreover, in contrast to ANT, the effect of PHE on photosynthesis strongly depended on the CO_2 concentration; in 0.1% CO_2 the stimulation of photosynthetic oxygen evolution and a very high SOD activity were observed, whereas the SOD activity and photosynthesis in high-CO_2-grown cultures was not influenced by PHE. It is known that the metabolism of green algae, including *Scenedesmus* cells, changed with the CO_2 concentration: in low-CO_2-grown cells a carbon concentrating mechanism (CCM) operates, causing the accumulation of CO_2 in chloroplasts, whereas at concentrations of CO_2 higher than 2–4%, this mechanism does not work (Palmqvist *et al.* 1994). In the absence of stress factors, CO_2 is fixed in the Calvin cycle but many chemicals, including some PAHs, can affect this fixation (Kusk 1981; Abarzua *et al.* 1984). We assume that CO_2 not fixed in the photosynthesis process caused the acidification of the stroma, which, in turn, would lead to the enhanced O_2 reduction, because the optimum pH for this process is about 5 (Reising and Schreiber 1992). There is some evidence that the photoreduction of O_2 is enhanced under environmental conditions when CO_2 fixation is suppressed. The example are *Scenedesmus* cells, in which all the electrons from PS II are used for the photoreduction of O_2 after the inhibition of the CO_2-fixation cycle (Asada 1999).

It was established that in PHE-treated low-CO_2-grown cultures, the pH increased from a neutral value to about 9, which indicates that dissolved inorganic carbon (DIC) in this treatment was efficiently uptaken. The growth of the population was inhibited by only about 50%, demonstrating that part of the CO_2 uptake had to be fixed in the Calvin cycle to enable the growth and division of cells. However, as the SOD activity in this treatment was also high, it is likely that some amount of the measured oxygen not only originated from water splitting in PS II, but also was derived from $O_2^{-\bullet}$ disproportionation, which appeared to be a very efficient process (Asada 1999). Thus, the observed stimulation of oxygen production could be the summary effect of photosynthesis and SOD activity. In high-CO_2-grown cultures, the CCM does not operate, so the photosynthesis rate is closely connected with external DIC (CO_2 or HCO_3^-) availability, and excessive stroma acidification and $O_2^{-\bullet}$ generation under such conditions should not proceed. Indeed, the SOD activity and oxygen evolution in high-CO_2-grown cultures were not influenced by PHE. Therefore, it seems reasonable to assume that the mechanism of the growth inhibition by PHE is not connected with a harmful influence of ROS but presumably results from the partial inhibition of macromolecular synthesis as a consequence of suppressing the CO_2 fixation.

Concentrations of ANT and PHE detected in the natural environment are about four orders of magnitude lower than those used in this work. One should, however, take into account that in nature the toxicity of PAH is strongly enhanced by their co-pollutants, especially redox active heavy metals (Babu *et al.* 2001) as well as solar UV radiation (Marwood *et al.*

1999), which was absent in this study. The results reported herein indicate that for aquatic organisms, even a short-term exposition to PAH leads to significant physiological and biochemical changes within the cells, which is all the more alarming when one considers a continuous inflow of polycyclic aromatic hydrocarbons to the aquatic environment.

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