Growth and adaptation of the green alga *Chlamydomonas reinhardtii* on diesel exhaust particle extracts

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We have examined the growth and photosynthesis of liquid cultures of the eukaryotic green alga *Chlamydomonas reinhardtii* incubated with iso-octane extracts of diesel particulate exhaust. At concentrations up to 0.125% (v/v), the extracts led to a dose-dependent growth retardation. Higher concentrations caused cell death. After a prolonged lag phase, air-grown illuminated algae, stressed with sublethal concentrations of diesel exhaust extracts, adapted to the pollutants. Further addition of toxic extract up to a concentration of 2.5% (v/v) affected neither growth nor photosynthesis. Media of adapted and non-adapted cells were analysed by HPLC and GC-MS and the concentrations of the remaining toxic compounds were determined. The results showed that the amounts of certain components decreased during incubation with adapted cells.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants either formed naturally or more particularly from anthropogenic sources (Blumer & Youngblood, 1975; Tritthart & Binder, 1979). The organic adsorbates on diesel particulate matter represent a major source of PAHs (World Health Organization, 1989). Diesel combustion engines emit a complex mixture of newly formed products and unburned fuel which is both gaseous and particle-associated (Rheinisch Westfälischer Überwachungsverein, 1981). The particle emission and composition of the organic adsorbates depend on the kind of diesel engine (Israel *et al.*, 1982; Springer & Stahman, 1977; Weidmann & Menrad, 1986), engine speed and load conditions (Cartellieri *et al.*, 1989; Pucker & Sperling, 1988).

Since some of the PAHs are reported carcinogens, a wealth of data has been obtained about the possible health effects on mammalian and prokaryotic cells (Cederlöf *et al.*, 1978; Higgenson & Muir, 1973; Schlipkötter *et al.*, 1987; Chechair *et al.*, 1981). There remains, however, a need for the validation of effects of PAHs on aquatic systems, mainly on micro-algae and phytoplankton. Algae are important components of aquatic communities and respond sensitively to many pollutants (Bate & Crafford, 1985; Cerniglia *et al.*, 1980;

Soto *et al.*, 1975). Oil pollution studies involving micro-algae demonstrated inhibitory effects by crude and fuel oils, chemical dispersants and hydrocarbons (Hilmer & Bate, 1983; Batterton *et al.*, 1978).

In this report we show the inhibitory effects of PAH derivatives on growth and photosynthesis of liquid cultures of the eukaryotic green alga *Chlamydomonas reinhardtii*. The algae adapt to sublethal concentrations of PAHs over a period of several days. Thereafter, toxic extracts at concentrations up to 2.5% (v/v) affect neither photosynthesis nor growth, and it appears that this adaptation involves uptake of some of the toxic compounds into the cells.

Methods

Solvents and chemicals. All salts for media were reagent grade and were obtained from Sigma. Solvents for Soxhlet extraction and column chromatography were purchased from Merck in p.A. quality. HPLC and GC analyses and the re-extraction experiments from media were performed with solvents for spectroscopy also obtained from Merck. Priority pollutant polynuclear hydrocarbons standards in acetonitrile were purchased as Standard Reference Material 1647 by the National Bureau of Standards from Promochem (Wesel, Germany). The purity of these 16 standards was 94–97.5% as determined by the manufacturer. Diesel exhaust particles were a generous gift of the Lehrstuhl für Kraft- und Arbeitsmaschinen, University of Kaiserslautern.

Instrumentation. GC-MS analyses were carried out with a Hewlett Packard model 5985 GC-MS system equipped with splitless injection. The capillary column was a Hewlett Packard Ultra 2, crosslinked 5% phenylmethylsilicon, 25 m × 0.20 mm × 0.33 µm film thickness, glass column. Electron impact ionization (70 eV) and internal

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Abbreviation: PAH, polycyclic aromatic hydrocarbon.

standardization mass spectrometry was used to identify the substances in comparison with standards and with the computer internal library, a Hewlett Packard 599 43A NBS model with a mass spectral database of over 43000 compounds. GC conditions were as follows. Injector temperature 250 °C, detector temperature 280 °C; the column temperature was held at 100 °C for 3 min and then programmed to rise to 150 °C at a rate of 10 °C min⁻¹. After holding the temperature at 150 °C for 1 min, the temperature was increased to 255 °C at a rate of 3 °C min⁻¹, and held for 7 min. The carrier gas (helium) rate was 2 ml min⁻¹ at room temperature.

HPLC separation was performed with a Merck 50943 LiChroCart 125-4, LiChrospher 100RP-18 (5 µm) column. A LKB 2140 Rapid Spectral Detector and a Knauer HPLC pump 64 were used. Each dry sample received was redissolved in pure acetonitrile. Solvent flow rates under isocratic conditions were maintained at 1 ml min⁻¹. Mobile phases were acetonitrile/distilled water, 70:60 (v/v) and 60:40 (v/v), respectively.

Preparation of diesel exhaust extract. Unless specified otherwise, the details of diesel exhaust extract preparations were identical with those described by Alsberg *et al.* (1984). In brief, 13.2 g of diesel exhaust

particles were extracted with dichloromethane for 24 h in a 500 ml Soxhlet extractor. The solvent was removed under reduced pressure at 30 °C. Final residues were fractionated on a gravity glass column (packed with Merck Kieselgel 60, 0.063–0.200 mm particle diameter) by step-by-step gradient elution with increasing solvent strength. This preparative silica column chromatography yielded five fractions which were evaporated to dryness with a rotary flash evaporator (30 °C); residues were redissolved in 3 ml iso-octane (or 3 ml acetonitrile for HPLC analyses) and stored at –20 °C in glass sample vials. GC–MS analyses have shown that fraction 3 consists mainly of polynuclear aromatic ketones and pure PAHs (Table 1), and all tests were performed with this fraction as an iso-octane solution. Compounds were identified or tentatively identified by comparison with HPLC retention times (*R_s*) or GC *R_t* and MS of reference compounds or by the computer library search.

Organism and growth conditions. *Chlamydomonas reinhardtii* strain 11-32b was purchased from the Sammlung für Algenkulturen (SAG) at Göttingen University, Germany. For starter cultures, 200 ml Erlenmeyer flasks containing 120 ml of media were inoculated with algae from agar plates and grown for 3 weeks at 22 ± 2 °C in M12-medium,

Table 1. Compounds identified or tentatively identified by GC–MS analysis in silica gel fraction 3 of diesel particulate exhaust

No.	Compound	Mol. mass (Da)	Retention time (min)	Identification method†	Concn‡ [ng (µl exhaust) ⁻¹]
1.	4-Methyl-2-nitrophenol	153	9.64	<i>a</i>	3.1
2.	1-Methylnaphthalene	142	10.75	<i>a</i>	8.45
3.	2-Nitroethenylbenzene	149	13.14	<i>a</i>	6.71
4.	1,4-Naphthalenedione	158	13.40	<i>a</i>	1.95
5.	2-Naphthalenecarboxaldehyde	156	16.06	<i>a</i>	27.11
6.	2-Methyl-1,4-naphthalenedione	172	16.29	<i>a</i>	5.23
7.	2,6-Dimethyl-4-nitrophenol*	167	17.39	<i>a</i>	1.91
8.	1-Naphthalenylethanol	170	19.22	<i>a</i>	5.42
9.	(1,1'-Biphenyl)-2-ol	170	19.76	<i>a</i>	8.72
10.	(1,1'-Biphenyl)-3-ol	170	20.11	<i>a</i>	10.87
11.	(1,1'-Biphenyl)-dimethyl*	182	22.79	<i>a</i>	5.46
12.	9H-Fluoren-9-one	180	23.25	<i>a,b,c</i>	13.87
13.	(1,1'-Biphenyl)-4-methanol	184	23.65	<i>a</i>	10.37
14.	Anthracene	178	24.59	<i>a,b,c</i>	27.79
15.	Phenanthrene	178	24.92	<i>a,b,c</i>	59.4
16.	1-Methyl-9H-fluorene*	194	26.26	<i>a</i>	10.0
17.	Dibenzothiophene*	184	27.86	<i>a</i>	12.2
18.	9-Phenanthrenol	194	28.11	<i>a</i>	16.62
19.	4,7-Dimethylbenzo(c)cinnoline*	208	28.69	<i>a</i>	8.6
20.	2-Methylanthracene	192	28.90	<i>a,b,c</i>	40.7
21.	1-Methylanthracene	192	29.69	<i>a,b,c</i>	2.3
22.	9(IOH)-Anthracenone*	194	30.09	<i>a</i>	38.15
23.	9-Ethylfluorene* (or tetrahydro-1-phenylnaphthalene)	194	31.22	<i>a</i>	15.8
24.	1,2,3,6,7,8-Hexahydropyrene	208	31.52	<i>a</i>	12.11
25.	4-Methylbenzo(c)cinnoline*	194	32.19	<i>a</i>	8.76
26.	Methylanthracene (or -phenanthrene)*	192	32.39	<i>a</i>	14.7
27.	Diethylphenanthrene (or -anthracene)*	192	32.59	<i>a</i>	17.7
28.	Dimethylphenanthrene (or -anthracene)	206	32.72	<i>a</i>	11.77
29.	4,7-Dimethylbenzo(c)cinnoline	208	32.79	<i>a</i>	11.9
30.	Methylphenanthrene (or -anthracene)*	192	33.21	<i>a</i>	13.12
31.	Methylphenanthrene (or -anthracene)*	192	33.39	<i>a</i>	13.13

Table 1. (continued).

No.	Compound	Mol. mass (Da)	Retention time (min)	Identification method†	Concn‡ [ng (µl exhaust) ⁻¹]
32.	9,10-Anthracenedione*	208	34.79	<i>a</i>	25.6
33.	9-Anthracenecarboxaldehyde	206	35.29	<i>a</i>	15.6
34.	9-Phenanthrenecarboxaldehyde	206	35.47	<i>a</i>	7.8
35.	Dimethylphenanthrene (or -anthracene)*	206	36.21	<i>a</i>	10.1
36.	Dimethylphenanthrene (or -anthracene)*	206	36.50	<i>a</i>	3.7
37.	Dimethylphenanthrene (or -anthracene)*	206	36.80	<i>a</i>	14.2
38.	Ethylanthracene*	206	37.39	<i>a</i>	4.3
39.	Pyrene*	202	37.74	<i>a,b,c</i>	8.9
40.	Ethyl-9,10-anthracenedione	236	37.88	<i>a</i>	8.3
41.	Ethylanthracene*	206	38.48	<i>a</i>	9.82
42.	Ethyl-9,10-anthracenone (or dimethyl-9,10-anthracenedione)	236	37.88	<i>a</i>	13.3
43.	10-Methylanthracene-9-carboxaldehyde	220	39.44	<i>a</i>	8.4
44.	Trimethylphenanthrene*	220	41.23	<i>a</i>	9.4
45.	Tetrahydrobenz(a)anthracene*	232	41.86	<i>a</i>	3.4
46.	1-Pyrenecarboxaldehyde	230	42.28	<i>a</i>	16.4
47.	Trimethylphenanthrene*	220	42.94	<i>a</i>	7.73
48.	1,4-Dimethyl-9,10-anthracenedione	236	43.20	<i>a</i>	5.80
49.	Methylphenylcinnoline*	220	43.33	<i>a</i>	2.04
50.	7H-Benz[de]anthracene-7-one	230	44.00	<i>a</i>	6.8
51.	Triphenylene	228	45.62	<i>a</i>	6.7

* Although slight resolution of double peaks was observed under temperature conditions described above, the resolution did not allow positive identification and made experimental variations in temperature programming necessary. The retention times given are obtained by temperature programming as follows: from 100 °C to 270 °C at 3 °C min⁻¹, then hold for 10 min.

† Identification method: (a) tentatively identified by computer library search of sample spectra; (b) identified by standard injected into GC-MS; (c) identified by standard injection into HPLC.

‡ Estimated from the chromatographic intensity within 20% error limits.

continuously shaken on a rotary shaker operating at 80 r.p.m. under continuous illumination (25 µmol m⁻² s⁻¹). M12-medium was prepared according to Kuhl (1962).

The pH was adjusted to 7.0 with 3 M-NaOH, and the medium was autoclaved in 40 ml portions in incubation flasks (i.d. 3.5 cm, length 20 cm, volume 60 ml, Glasbläserei Kaiserslautern) or in 200 ml Erlenmeyer flasks containing 120 ml of media for starter cultures. At the start of an experiment, mineral medium in test flasks was combined with 20 µl of internal standard (decanoyl acid, methyl ester, 1 µg µl⁻¹) and 0.12% (v/v) diesel particulate exhaust fraction 3 (carrier iso-octane).

Under sterile conditions, the test flasks were inoculated with 1 ml of starter culture (3 × 10⁶ cells). Aerobic conditions were maintained by bubbling air (0.035% CO₂) through sterile glass-wool filters into the flasks. All tests were performed at room temperature (22 ± 2 °C) and continuous illumination (130 µmol m⁻² s⁻¹). Sterilized blanks containing PAH stock solution but no algae, and sterile controls containing internal standard and algae, were run concurrently. Growth of algae was estimated each day in duplicate by counting the cells in a Thoma cell chamber after fixing cells with an equal volume of 0.25% (v/v) glutaraldehyde.

Total chlorophyll was determined daily in duplicate according to Arnon (1949). Protein determination was carried out using the Coomassie Blue method according to Sedmark & Grossberg (1977).

Net rates of photosynthesis were obtained by measuring O₂ evolution in the light with a Clark electrode. Portions (2 ml) of the stressed suspensions with a chlorophyll content of about 6–20 µg ml⁻¹ were incubated in 25 mM-Hepes buffer in the dark for 20 min. After a constant respiration rate was obtained, cells were illuminated (200 µmol m⁻² s⁻¹) and the net O₂ evolution under steady-state conditions was measured at a saturating bicarbonate concentration (350 µM).

Media analysis. Each day during incubation (up to 6 d), algal suspensions were centrifuged for 10 min at 2000 g, and the aqueous supernatant was analysed for PAH compounds. After addition of 10 µl of external standard (undecanoic acid, methyl ester, 1 µg µl⁻¹), 2 × 10 ml of dichloromethane were added and samples were extracted on a rotary shaker (225 r.p.m.) for 20 min. The organic phase was removed and evaporated to dryness under a gentle stream of N₂. Residues were redissolved in 100 µl iso-octane for GC-MS analysis or 100 µl acetonitrile for HPLC analysis and briefly mixed by ultrasonic treatment. Samples were stored in glass vials closed by Teflon septa,

Fig. 1. Effect of 0.12% (v/v) diesel exhaust fraction 3 (○) on growth of *Chlamydomonas reinhardtii*. The alga was grown at $22 \pm 2^\circ\text{C}$ under continuous illumination ($130 \mu\text{mol m}^{-2} \text{s}^{-1}$) in 50 ml flasks containing 40 ml M12-medium. Aerobic conditions were maintained by aeration (0.03%, v/v, CO_2) through sterile glass-wool filters. The carrier was iso-octane, and controls (●) contained 0.12% (v/v) iso-octane. Cell densities were determined each day in duplicate by counting in a Thoma cell chamber. Initial inoculation with cells of starter cultures was equal. Values represent the means of a series of 18 experiments; the standard deviation of the mean was ± 1.2 .

Fig. 2. Soluble protein content (relative to cell number) of liquid cultures of *Chlamydomonas reinhardtii* grown with 0.12% (v/v) diesel exhaust fraction 3 (□). ■, Control. Protein was determined each day in triplicate according to Sedmark & Grossberg (1977). Values represent means from a series of 18 experiments; the standard deviation of the mean was ± 1.9 .

separated from the external environment and analysed in duplicate as soon as possible. The sterile blanks, used to confirm that no loss of PAH compounds occurred through abiotic degradation, volatilization or photodegradation, and the controls, used to identify those compounds derived from algae or media, were treated similarly.

Results and Discussion

Liquid cultures of the eukaryotic green alga *Chlamydomonas reinhardtii* strain 11-32b were tested for their ability to grow in media contaminated with PAH derivatives isolated from diesel particulate exhaust. The organic extract of diesel particulate matter, containing mainly oxygenated PAH derivatives, led to a dose-dependent growth retardation of the illuminated, aerated cultures, up to a concentration of 0.125% (v/v); higher concentrations caused cell death (data not shown). Cells incubated with small quantities of PAH fractions showed an extremely prolonged lag phase of about 96 h (Fig. 1).

During this period, the protein content relative to the biomass increased slightly compared to unstressed controls (Fig. 2). Rates of net photosynthesis, determined after 96 h incubation with toxic extracts in sublethal concentrations, were diminished (Fig. 3), indicating multiple effects from a variety of compounds. However, we are aware that chromatographic properties alone are insufficient for positive identification of compounds, and the complete composition of the fraction of the organic soluble diesel exhaust extract used

Fig. 3. Rates of dark respiration and net O_2 evolution in the light of cells incubated for 96 h with 0.08% (■), 0.1% (▨) and 0.125% (□) diesel exhaust fraction 3 (all concentrations v/v). Untreated control (100%) values were $95 \mu\text{mol O}_2 (\text{mg chlorophyll})^{-1} \text{h}^{-1}$ for dark respiration and $169 \mu\text{mol O}_2 (\text{mg chlorophyll})^{-1} \text{h}^{-1}$ for net O_2 evolution in the light. Dark respiration rates were determined after 20 min dark incubation, rates of net photosynthesis at steady-state, both under saturating bicarbonate ($350 \mu\text{M}$) conditions. The standard error of the mean was about 4%.

in the present study remains tentative. Nevertheless, after 96 h growth with 0.12% (v/v) of fraction 3, it is obvious that the growth and metabolism of *Chlamydomonas reinhardtii* were strongly inhibited by the mixture of toxic compounds and, despite the generally low aqueous solubility of many PAHs, biological availability was apparent.

Table 2. Decrease of some diesel exhaust fraction compounds remaining in incubation media after 3 d growth with adapted or non-adapted cells of *Chlamydomonas reinhardtii*

The percentage decrease is expressed relative to concentrations in incubation media of non-adapted cells. Data are mean values from a series of eight experiments; the standard deviation was less than 4% of the mean. The concentration in the medium of diesel exhaust fraction 3 was 0.125% (v/v).

Retention time (min)	Compound	Concentration of compounds in blanks ($\mu\text{g } \mu\text{l}^{-1}$)	Concentration of compounds in incubation media ($\mu\text{g } \mu\text{l}^{-1}$)		Percentage decrease
			Non-adapted cells	Adapted cells	
23.5	9H-Fluorene-9-one	6.91	6.72	0.32	95.2
24.5	Phenanthrene	33.3	30.1	21.4	28.9
25.9	4-Methylbenzo(c)cinnoline	6.76	6.3	6.03	4.3
28.2	1-Methylanthracene	1.15	1.36	0.62	54.3
30.9	2-Methylanthracene	20.5	20.5	16.7	18.5
31.7	Dimethylphenanthrene	5.1	4.81	0.71	85.2
32.6	Dimethylphenanthrene	10.15	9.88	6.83	30.9

After a prolonged lag phase, algae incubated with 0.12% (v/v) of fraction 3 grew exponentially and were not affected by further addition of PAHs at concentrations which were lethal to non-adapted cells. The growth of these adapted cells treated again after 120 h of incubation was similar to the exponential phase of the lower curve in Fig. 1. These adapted cells were resistant to quantities of 2.5% (v/v) fraction 3, the highest concentration tested (data not shown). On the other hand, there were no significant differences in growth between adapted cells in fresh media with or without the toxic extract. Therefore, the PAH derivatives were probably not consumed as nutrients.

The acquired resistance to the adverse effects of the PAH derivatives led us to examine the fate of these compounds during incubation. A basic approach was initiated for determining the concentration of the toxic compounds in aerated and illuminated incubation media that contained toxic fractions with and without adapted or non-adapted cells. Whereas the blanks containing PAH derivatives but no algae, and all samples with non-adapted cells, showed no or little loss of toxic compounds, there were significant changes in media incubated with adapted cells. After 3 d incubation, the concentration of seven compounds was greatly decreased: phenanthrene, 9H-fluorene-9-one, 4-methylbenzo(c)cinnoline, 1-methylanthracene, 2-methylanthracene and two isomers of dimethylphenanthrenes (Table 2). The extent of removal of the individual compounds ranged from 4.3% for 4-methylbenzo(c)cinnoline, a heteroaromatic compound containing two nitrogen atoms, to 95.2% for 9H-fluorene-9-one.

The mechanism responsible for the removal of these compounds by adapted cells is at present unknown and needs further investigation. The apparent acclimatiz-

ation period may result from the time required for a small number of cells capable of tolerating PAH derivatives to attain sufficient densities to reach a detectable range for counting in a cell chamber. The selective growth of existing or induced mutants seems more likely than the use of a preferential substrate. Alsberg *et al.* (1984) described the fraction of organic soluble diesel particulate matter containing mainly oxygenated PAH derivatives as that of the highest mutagenicity; therefore, mutation may contribute to adaptation. Otherwise no consensus exists as to the mutagenicity of the individual PAH compounds present in the fraction. The complexity of the extract used prevents us from drawing any conclusion concerning the effects of individual compounds.

Another important aspect to be considered is bio-adsorption by the cell wall of the alga. Adsorption of organic compounds by micro-organisms is mainly a passive process driven by chemical partitioning into the hydrophobic biomass. Because a metabolic reaction is not involved in this accumulation, adsorption is usually the same for living and dead cells. The time necessary to reach equilibrium for adsorption appears to be very short and is reported to vary from a few minutes for algae to a few hours for bacteria (Smets & Rittmann, 1989). For estimation of adsorption events, we incubated cultures of adapted and non-adapted cells during stationary phase of growth for 6 h with 0.125% of diesel exhaust fraction 3. There were no losses of the seven compounds listed above (data not shown), so that a selective decrease in some compounds brought about by adapted cells cannot be explained by passive adsorption equilibria.

The capacity of algae to degrade petroleum compounds and other arenes has long been known (Walker *et al.*, 1975; Cerniglia *et al.*, 1980; Ellis, 1977), and the use

of naphthalenesulphonic acids as sulphur sources, or the use of nitro- and amino-substituents of aromatic compounds, has been reported (Luther, 1990). The considerable decrease in concentration of some growth-inhibiting compounds brought about by adapted cells may, therefore, be caused by biodegradation, which would explain both the lag phase and acquired tolerance.

We believe that the results presented in this paper could have profound implications for the fate and effects of PAH compounds in the environment and further investigation of the resistance mechanism is evidently required.

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