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A study of the phototoxic effect of Benzo[a] Pyrene on bacteria

Research Article

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Abstract: Although Benzo[a]Pyrene (BaP) released to soils tends to bind very strongly to soil particles, however, small amounts have been shown to leach into groundwaters. This polycyclic aromatic hydrocarbon is stable and can stay (and travel) in the environment for an extended period of time. Release of BaP into the environment therefore causes concern at a global environmental level. In this paper we evaluated the effect of BaP on the cell growth of *Escherichia coli* and *Enterococcus faecalis* in aerobic conditions. Irradiation of BaP increased its antibacterial activity which suggests that this process produced a singlet oxygen. This way would be able to speed up the processes of photochemical degradation of BaP.

Keywords: *Escherichia coli • Enterococcus faecalis • Polycyclic aromatic hydrocarbons • Phototoxicity*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) in the environment are almost always derived from anthropogenic activities [1]. PAHs have a high melting and boiling point and low water solubility. Their solubility tends to decrease with increasing molecular weight and PAHs are highly lipophilic [2]. The volatility of the compounds from the aqueous phase is low, with half-lives of 500 and 1550 h for Benz[a]Anthracene (BaA) and Benzo[a]Pyrene (BaP), respectively [3]. Under aerobic conditions these compounds biodegrade very slowly in the aqueous compartment. Their biodegradation rates decrease significantly with increasing number of aromatic rings [4-7]. The most important degradation process for PAHs in air and water is indirect, hydroxyl radicals ($^{\bullet}$OH), mediated photolysis. Under laboratory conditions, the reaction of the PAH compounds with airborne $^{\bullet}$OH radicals shows maximum half-lives between about 3 and 11 h [8]. For pure water, the photodegradation half-lives appear to be in the range of hours [9,10], whereas the half-lives increase dramatically when there is sediment/water partitioning [11]. BaP is a potent environmental carcinogen that is metabolized into diolepoxides that react with exocyclic amines in DNA. Reports to date show that the highest molecular weight PAHs that can be mineralized by bacteria are four-ring PAHs, there are but few bacterial strains which can digest five-ring PAHs as the sole carbon and energy source [12-14]. These

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contaminants in the water may be present in various forms, for example immobilization on solid particles, the penetration into the cells or microorganisms in the form of suspensions. This is becoming a persistent and dangerous for the ecosystem [15]. The solubility is related to their hydrophobic character. PAHs with a lower number of aromatic rings are readily biodegradable, while a higher number of degradation is reduced [16] Therefore, it is needed to find new ways of degradation of PAHs can be used alone or in combination with biological processes. These methods can be classified advanced oxidation processes (AOP) and UV radiation [17-19]. Influences of the degradation products from oxidation processes for micro-organisms are not yet understood. BaP, metabolically activated is the ultimate carcinogenic product which is formed *via* a three-step process. The first step catalyzed by cytochrome P450 enzymes, involves the formation of (*7R,8S*)-epoxy-7,8-dihydrobenzo(a)pyrene. The second step is the conversion to (*7R,8R*)-dihydroxy-7,8-dihydrobenzo(a)pyrene, catalyzed by epoxide hydrolase. Finally step, cytochrome P450 enzymes catalyze the reaction, producing four possible isomers of 7,8-diol-9,10-epoxide [20].

Because BaP has very carcinogenic and mutagenic effects on humans, the European Commission has set a maximum permissible limit of BaP in drinking water at 10 ng/l [21]. PAHs are common contaminants of terrestrial and water ecosystems. Currently, there is evidence that exposure to of BaP to UV radiation and the nearby region of UV radiation may lead to increased toxicity [22]. The work was monitored with respect to the toxicity effects of light in the case of anthracene, pyrene, BaP, fluoanthene, naphthalene and phenanthrene [23-26]. In summary, various studies have demonstrated the phototoxicity of BaP. In algae, Cody *et al.* [27] produced experimental evidence suggesting that BaP is phototoxic to aquatic flora. Many bacteria have been proven to degrade PAHs *via* either metabolism or co-metabolism. The biochemical pathways of the bacterial metabolism of PAHs have been evaluated [28-30]. However, much less is known about the capabilities of bacteria to utilize PAHs containing five or more rings, such as BaP, as a carbon and energy source.

2. Experimental Procedures

2.1 Measurement of BaP

The laboratory tested a model sample of BaP, as certification material (Fluka, 99% purity), dissolved in dimethyl sulfoxide (DMSO) at concentrations of 50–400 μmol L^{-1} BaP.

2.2 Chemical analysis

The absorption spectrum of BaP was obtained in a UV/VIS spectrophotometer Specord 250 (Analytik Jena, Germany). The spectrum of the lamp used was measured with an optical fibre high resolution of Red Tide USB650 Fiber Optic Spectrometer (Ocean Optics, USA).

Samples of bacteria and contaminants were processed at selected time intervals by liquid-liquid extraction. The extract was used as extractant dichloromethane (pa, Merck). After extracts were preconcentrated (Kuderna danish) to 1 ml the components were identified by chromatography. The identification of BaP was carried out by gas chromatography combined with mass spectroscopy GC/MS (Varian - Saturn 2100 T, USA), using an internal standard (Biphenyl D10, Fluka).

Samples were batched into an automatic autosampler (Varian CP-8410, USA), with Varian type 1177 injector, split/splitless with a constant temperature of 290°C gas chromatograph worked in on-line connection with the detector (Mass Spectrometric Detector - MSD), which was the mass spectrometer - ion trap. MSD worked with electron ionization in “Full Scan” (FS). In the mass range 45-650 m/z. Data were processed using MS Workstation. Separation was carried out in a chromatographic column EZ-guard VF-5 (Varian, USA) with dimensions L (30 m) × ID (0.25 mm) OD × (0.39 mm) + 10 m EZ - guard column. Guard column is connected directly to the main column without using the clutch. As a carrier gas we used helium (He 6.0, Messer, Slovak Republic) with a constant flow rate of 1 ml min^{-1}. The volume of 1 μl syringe (Hamilton 10 μl) was charged into the injector. Chromatographic separation was performed with a temperature program: 50°C (hold time 1 min), next by the rate of 10°C min^{-1} to 290°C (hold time of 10 min). The total length of the analysis was 45 min.

2.3 Sensitization process with BaP solution

The effect of BaP on bacterial growth (*Escherichia coli* CCM 3988 and *Enterococcus faecalis* CCM 4224) from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic) was determined using microdilution [31]. The experiments were carried out in two groups. One group was exposed to light and the other group was kept in the dark. The BaP stock solution was prepared in DMSO (Sigma-Aldrich, USA) and then diluted by the appropriate volume to obtain the test solutions. 150 μl of broth inoculated by bacteria cells was added to 50 μl of BaP solution at final concentrations ranging from 50–400 μmol L^{-1}. The light source was placed vertically at a distance of 12 cm above the microplates. The microplates were irradiated for 8 h with shaking at 37°C. The bacterial growth was quantified spectrophotometrically by the ELx808 Absorbance Microplate Reader (BioTek, US) at 630 nm and measured until confluent growth was reached. The antibacterial effect of BaP was characterized by the MIC values, which were read from toxicity curves.

2.4 Microscopic measurement

Microscopic observation (AXIO IMAGER-A microscope; CARL ZEIZZ, Germany) allows the comparison of biomass with and without BaP. For microscopic observation (light: X-CITE, SERIE 120) the following filters were used: filter (F1) - Filter set 02 - excitation G 365, Beam splitter FT 396, emission LP 420; filter (F2) - Filter set 05 - excitation BP 395-440, Beam splitter FT 460 and emission LP 470.

3. Results and Discussion

Advances in technological processing and related uncontrolled environmental pollution make PAH practically ubiquitous [15]. Photodegradation of PAHs may be due in addition to UVA rays as well as visible light, and this is especially true in the case of PAHs with a higher content of aromatic rings, which are photosensitized upon radiation with a wavelength >400 nm [32-34].

The emission spectrum of a 23 W fluorescent light bulb and the absorption spectrum of BaP are shown in Figure 1. The final absorption band of BaP, centered at 405 nm, is almost coincident and overlaps with the light emission of fluorescent lamp. Therefore, BaP may be effectively excited by this light source and that ensuing photophysical processes of energy transfer to the present molecular oxygen will lead to formation of various photoactive species, e.g. singlet oxygen ($^1O_2$) [35,36] which may lead to higher efficiency of the photoactivation process of BaP. In a study by Krylov *et al*. [37], this chemical compound is an ideal photosensitizer with a conjugated aromatic system of π electrons abundance and is able to absorb environmentally relevant wavelengths of radiation. Larson and Berenbaum [38] assumed that the sensitized production of photoreactive and biologically damaging $^1O_2$ is an important and well-studied aspect of PAH phototoxicity. The $^1O_2$ generation by BaP is also confirmed in a study by Tobit *et al*. [39] at different concentrations ranging from 25 to 100 ppm under the exposure of UVA (5.76 J/cm), UVB (2.16 J/cm) and sunlight (60 min). UVA showed concentration dependent $^1O_2$ generation. However, concentration dependency was not seen with UVB and sunlight. The highest yield of $^1O_2$ was observed under sunlight [39]. In our study, the radiation flux density of the fluorescent lamp, which emits light in visible region of the spectrum, was 1.12 mW cm^{-2}.

In this study, we evaluated the efficiency of BaP as a lethal sensitizer on the *E. coli* and *E. faecalis* cell growth using the micro-dilution method. 50–400 μmol/l DMSO solutions of BaP were used for the inactivation experiments. Each experiment was carried out under irradiation of a fluorescent lamp or in darkness. In all experiments, the control samples were cultivated without BaP and every sample was prepared in three parallels. The irradiation in the absence of BaP had no effect on the viability of *E. coli* and *E. faecalis* (data not

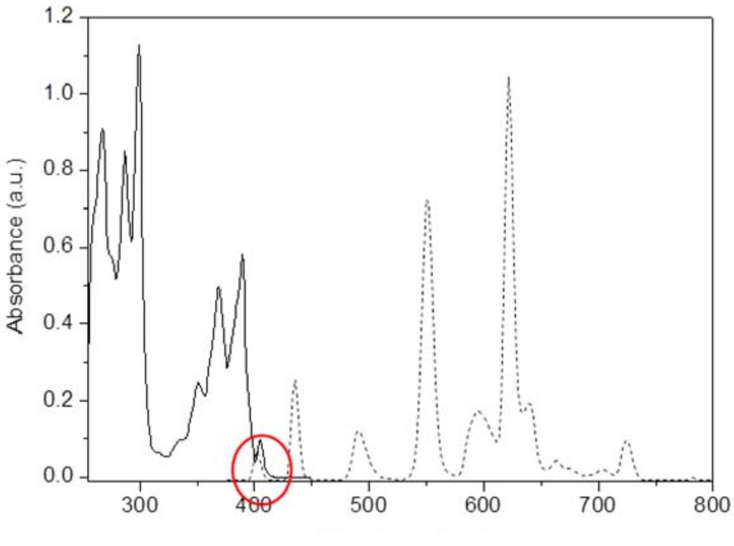

Figure 1. Absorption spectrum of BaP (solid line) and fluorescent lamp emission spectrum (dashed line); red circle – overlay of the BaP absorption spectrum and light source emission spectrum.

shown). Experiments revealed that treatment with BaP in both the dark and under light caused a reduction in bacterial viability (not shown). The degree of damage was dependent on the concentration of BaP. Nevertheless irradiation increased the antibacterial activity of BaP, suggesting that the ${}^1O_2$ formed during irradiation was responsible for this increase [40]. The results of Tobit *et al*. [39] showed that the ${}^1O_2$ generation by BaP produced phototoxicity under the exposure of UVA, UVB and sunlight (mentioned above). Botta *et al*. [41] investigated the effect of UVA/visible light role (320–800 nm) and visible light role (400–800 nm) on the phototoxicity and photogenotoxicity of BaP. Results revealed the phototoxic properties of BaP after irradiation with both light sources. BaP as one of the major carcinogens PAHs has a relatively high phototoxicity [41].

In Figure 2, it is shown that the increasing concentration of BaP under fluorescent light resulted in greater growth inhibition of *E. coli* and *E. faecalis* after 8 h cultivation. A 100% increase of inhibition (MIC) of cell growth was observed in *E. coli* and *E. faecalis* at 400 μmol/l of BaP under light conditions. From Figure 2, it can be deduced that the bacterial growth was less influenced in darkness. By comparing the relative increase of inhibition, irradiation had higher effect on cells growth. Analysis of our experiment showed that BaP was more effective in photodynamic inactivation of *E. coli* and *E. faecalis*.

The effect of light on BaP is a natural property of this hydrocarbon. BaP also penetrates into cells, as confirmed by studies of Sverdrup *et al*. [42] and Heitkamp and Cerniglia [13]. PAH are also generally highly hydrophobic, with high octanol-water partition coefficients (K_{OW}) and bioconcentration factors. Thus they easily partition from the aqueous phase into the organic matter or into organisms [43,44] causing them to become inactive.

There is upon radiation photodegradation of aromatic compounds to form decomposition intermediates which are more easily attacked by microorganisms than the original forms of these compounds [45]. The photodegradation of BaP in the presence of different bacterial species under exposure visible light (400-700 nm) during 8 h resulted in products that are presented in the GC-MS chromatograms. The obtained chromatogram of GC analysis with mass detection revealed the formation of a number of BaP degradation products, frequently co-eluting as presented in Figure 3. Peak (RT: 32.15 min) gives the precursor ion with m/z ~252. In accord with the previously published data of Bednáriková *et al*. [46], this fragmentation pattern corresponds to hydroxydione derivate of BaP, however, the detailed assignment of individual functional groups' position/structure is inadequate.

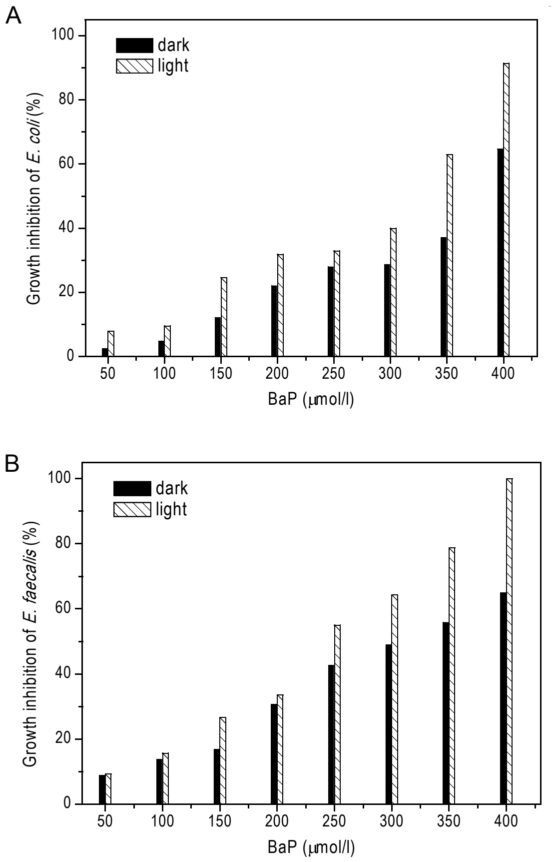

Figure 2. Effect of visible light and different concentration of BaP on the (A) *E. coli* and (B) *E. faecalis* cell growth. Bacteria were cultivated with BaP in range of 50 to 400 μmol/l.

With respect to the limited information on BaP photodegradation mechanisms, as well as unavailability of corresponding standards of its oxidized forms (oxy-BaPs), four compounds, *i.e.* Benzo[a]Pyrene-9,10-dihydrodiol, 3-hydroxyBenzo[a]Pyrene, Benzo[a]Pyrene-6,12-dione and 9,10-dihydroBenzo[a]Pyrene-7(8H)-one were chosen as standards [46]. In this work, due to the lack of reliable supporting data and standards for oxidized forms of BaP in Table 1, only the concentration of BaP was quantified after photodegradation. Table 1 shows BaP photodegradation fluctuating when the concentration of BaP in the culture medium was varied from 100 μmol/l to 200 μmol/l and 400 μmol/l in the presence of *E. coli* and *E. faecalis*. The results demonstrated (Table 1) the influence of light/dark conditions on the degradation of BaP in the presence of bacteria. It can be seen that at 400 μmol/l concentration, which induced 100% growth inhibition, the light supported degradation. In less concentrated suspensions (200 μmol/l), bacteria supported degradation as well.

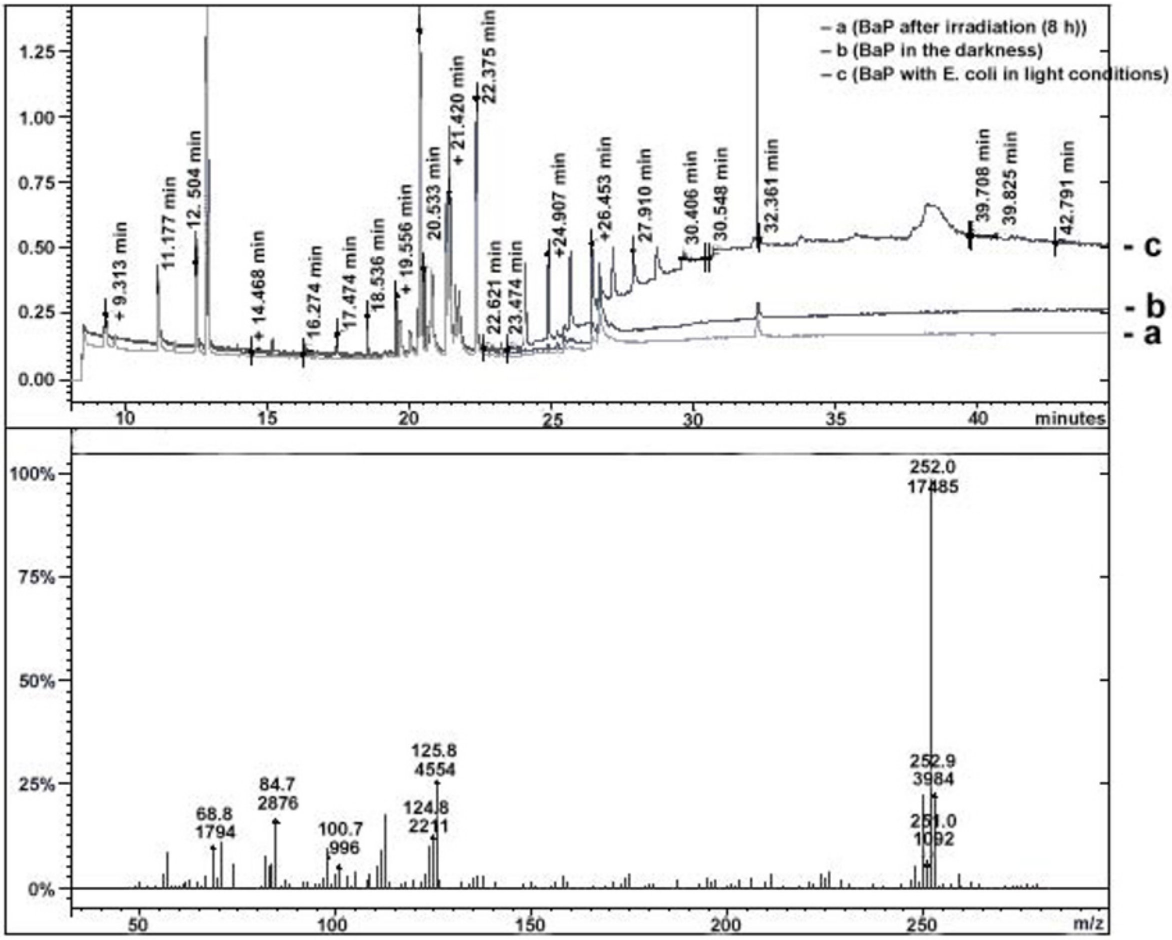

Figure 3. Chromatogram records of GC analysis with mass detection. RT value for BaP was 32.15 min (200 *μ*mol/l); (a) BaP after irradiation (8 h), (b) BaP in the darkness, (c) BaP with *E. coli* in light conditions.

Amount of BaP added (*μ*mol L^{-1})	Total amounts of BaP after 8 h in the presence of light (*μ*mol L^{-1})		
	without bacteria	with *E. coli*	with *E. faecalis*
100	85 ± 3	75 ± 4	66 ± 4
200	172 ± 5	154 ± 7	146 ± 8
400	357 ± 10	380 ± 18	382 ± 15

Table 1. The degradation of BaP in the presence of *E. coli* and *E. faecalis*.

The values are the average masses ± standard deviations of BaP in triplicate samples.

Many studies have focused on the photodegradation of PAHs in real water samples. Zepp and Schlotzhauer [11] in their work present relatively rapid photodegradation of PAHs in real water samples, which ranged from several minutes to hours. The relatively significant observed made the authors Mill *et al.* [9], who in his study discovered relatively rapid photodegradation of BaP in real samples in the presence of sunlight. The resulted intermediate of photodegradation, benzo[a]pyrene-r-7,t-8-dihydrodiolt-9,10-epoxide (+/-, against, BPDE), can cause genotoxicity of biological macromolecules, including DNA [47]. In the work Gao *et al.* [48] has been studied BaP and its active metabolites, represented by BPDE. The addition of UVA radiation to these compounds generates ROS that can cause oxidative damage of DNA. Constantly increase in the concentrations of BaP in the environment and loss of the ozone layer associated with the increased penetration of UV radiation is necessary due to findings concerning the synergistic effect of BaP and UVA radiation, worry about increased carcinogenicity [21]. Photolysis plays an important role in BaP degradation in both environment and food industry. Usually, it results in the formation of less non-polar compounds and, on the other hand, significantly increases formation of the polar ones, especially of oxidized intermediate compounds. The last mentioned are more susceptible to biodegradation than the parent, non-oxidized molecules. In addition, the oxidized compounds have revealed even more toxic effects on living organisms then the original BaP molecule itself [46]. The obtained results show that photodegradation of BaP even under model conditions represents a complex problem, leading to the formation of numerous oxidation products. The bacteria are unable to utilize PAHs as the sole sources of carbon and energy, but they do completely degrade PAHs to CO_2 [13].

4. Conclusions

Many chemicals have been reported as pollutants in drinking water. The constantly increasing industrial production causes high PAHs contamination across the ecosystem, but the greatest environmental load is mainly in aquatic environment. Due to their lipophilic nature and chemical stability, a microbial degradation combined with the photodegradation is one of the possible form of environmental decontamination. Our results show that phototoxicity of Benzo[a]Pyrene (BaP) represents a complex problem, owing to the possible formation of numerous oxidation products. Simultaneously, when exposed to colloidal BaP (400 μmol/l) in the presence of light *Escherichia coli* and *Enterococcus faecalis* showed growth inhibition. Our results suggest that inhibition is enhanced by the photochemical degradation of BaP. The irradiation with simulated visible light leads to BaP degradation and simultanously to the increased inhibition of bacterial growth a process that can supress the formation of toxic bacterial metabolic products formed from BaP. The use of bacteria may thus be an attractive alternative to existing physicochemical methods for the remediation of polycyclic aromatic hydrocarbons release into environment.

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