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Responses of *Synechocystis* sp. PCC 6803 (cyanobacterium) photosystem II to pyrene stress

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Abstract

In order to explore the mechanism of acute toxicity for pyrene to cyanobacterial organisms, the responses of *Synechocystis* sp. PCC 6803 photosystem II (PS II) under pyrene stress were studied. The results showed there was no significant difference about the oxygen evolution under 0.125 mg/L pyrene stress when compared with control, but it was significantly lower than control at 0.625 mg/L pyrene. Polyphasic chlorophyll-*a* fluorescence transients in cells of *Synechocystis* sp. PCC 6803 exhibited a typical increase including O, J, I, and P phases. Fluorescence yield at phases J, I and P declined slightly at 0.125 and 0.625 mg/L pyrene, and significantly lower than control at 3.125 mg/L. According to the parameters deviated from JIP-test, no modification was induced by pyrene both at the donor side and at the acceptor side of PS II, and the reaction centre of PS II is the primary damaging target. Based on the expressing of four key genes (*psbA*, *psbB*, *psbC* and *psbO*) of PS II, only *psbA* showed significant difference at 3.125 mg/L pyrene when compared with control.

Key words: pyrene; *Synechocystis*; toxicity; photosystem II
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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more fused aromatic rings. They are ubiquitous, bioaccumulative, and persistent pollutants. Many of them are mutagens and carcinogens (Keith and Telliard, 1979). Pyrene is a four-ring containing PAH, and it is also one of most common PAHs in various environments. The toxicities of pyrene toward a wide range of organisms, including mussels (Okay et al., 2006), fish (Roling et al., 2004; Zapata-Pérez et al., 2002), copepods (Jensen et al., 2008), macrophytes (Yin et al., 2008) and eukaryotic algae (Djomo et al., 2004; Lei et al., 2006) have been studied. However, there is little information about the effects of pyrene on cyanobacteria which is one of most important prokaryotic organisms in aquatic ecosystem. Photosynthetic process is a universal feature of algae and higher plants, and PS II has been found to be sensitive to environmental changes (Berry and Björkman, 1980; Demmig-Adams and Adams, 1992). Untill now, there is no detailed report concerning toxicity of pyrene on cyanobacterial photosystems. Chl-*a* fluorescence can be used to indicate photosynthetic efficiency and provide information on the relationship between structure and function of PS II (Christen et al., 2007). Currently, the JIP test has been used in detection and evaluation of various stresses on plant (Pinior et al., 2005; Strauss et al., 2006; Christen et al., 2007) and cyanobacteria (Lu and Vonshak, 1999; Bueno et al., 2004; Zhao et al., 2008). Recently, molecular responses such as changes in gene expression have been detected to assess the biological effect of toxic chemicals on microbial organisms (Nadeau et al., 2001; De Jong et al., 2006; Operaña et al., 2007). The cyanobacterium *Synechocystis* sp. PCC 6803 is regarded as a model organism which has been widely studied for fundamental processes of photosynthetic metabolism. In order to explore acute toxicity of pyrene to cyanobacteria photosystem II (PS II), oxygen evolution, polyphasic chlorophyll-*a* (Chl-*a*) fluorescence transients, plus expression of key genes in *Synechocystis* sp. PCC 6803 PS II under pyrene stress were studied. The aim of this study is to provide toxicological information about pyrene on cyanobacteria.

1 Materials and methods

1.1 Strain and culture conditions

Synechocystis sp. PCC 6803 was originally obtained from the Pasteur Culture Collection of Cyanobacteria in France and kindly provided by the Freshwater Algae Culture Collection of the Chinese Academy of Sciences.

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The strain was grown in BG11 liquid medium (Rippka et al., 1979), under a 12 hr:12 hr (light:dark) cycle with an intensity of 30 μmol quanta/(sec·m^2) provided by cool white fluorescent tubes at (25±1)°C. Pyrene (purity > 98%) was purchased from Sigma Co. (USA). Experiments were carried out in 250 mL conical flasks containing 99.9 mL BG11 liquid medium, 100 μL of combined stock pyrene solutions (pyrene dissolved in dimethyl sulphoxide (DMSO)) and DMSO were spiked into conical flask to obtain pyrene concentrations as 0, 0.125, 0.625, and 3.125 mg/L, respectively. Prior to the present experiments, we had observed that 0.25% (*V/V*) DMSO had no obvious effect on the growth and photosynthetic processes of *Synechocystis* sp. PCC 6803. Each treatment was replicated three times, and the initial cyanobacterial concentration was 1.05×10^6 cells/mL. All treatments were cultured in the light at 30 μmol quanta/(sec·m^2) at (25 ± 1)°C for 12 hr.

1.2 Measure oxygen evolution of PS II

Oxygen evolution of PS II was measured with a Clark-type oxygen electrode (Hansatech Instruments, UK) after 12 hr exposure. The cultures were incubated at 25°C under a light intensity of 500 μmol quanta/(sec·m^2) PAR in presence 0.9 mmol/L *p*-benzoquinone as an electron acceptor.

1.3 Measurement of polyphasic Chl-*a* fluorescence transients

The polyphasic rise in Chl-*a* fluorescence transients were measured by Handy-Plant Efficiency Analyser (Handy-PEA, Hansatech Instruments, UK) with an actinic light of 3000 μmol quanta/(sec·m^2). Before this experiment, we had observed that pyrene fluorescence was lower than detectable limits of Handy-PEA even at 3.125 mg/L. It showed that the fluorescence originating from pyrene do not interfere with the determination of polyphasic Chl-*a* fluorescence transients by Handy-PEA.

1.4 RNA extraction and reverse transcription

Synechocystis sp. PCC 6803 cells were harvested after 12-hr exposure to pyrene, by centrifuging them at 4.7×10^3 $\times g$ for 5 min. Pelleted cells were resuspended in TRIzol reagent (Invitrogen USA), and homogenized with a mini-beadbeater. Total RNAs were extracted following the manual of Trizol reagent (Invitrogen, USA). Total RNAs were digested with RQ1 RNase-free DNase (Promega, USA). These DNase treated RNAs were reverse transcripted to cDNA using random primers p(dN)9 and reverse transcriptase kit (Generay, China)

1.5 Primers design and real-time PCR

Out of the key genes of PS II , *psbA*, *psbB*, *psbC* and *psbO* of PS II were selected for this study. PCR primers designed for these four psb genes and 16S rRNA gene are listed in Table 1. The 16S rRNA gene was used as the control. Real-time PCR was performed with 10 μL Master Mix (SYBR Green, Japan), 0.2 μL (10 pmol/μL) forward primer and reverse primer respectively, plus 1 μL cDNA. The ddH_2O were added at a final volume of 20 μL. The amplification reactions were performed by an ABI Prism 7000 Sequence Detect System (Applied Biosystems, USA) with the following conditions: one cycle at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 59°C for 30 sec, 72°C for 30 sec. Gene expression data from Real-time PCR were evaluated using Ct value (Livak and Schmittgen 2001). The 16S rRNA gene was used as the housekeeping gene to normalize the expression levels of target genes, since the expression of 16S rRNA gene is stable under various conditions (Barbu and Dautry, 1989; Bustin, 2000). The induction ratio was calculated using $2^{-\Delta\Delta C_t}$, as following Eq. (1) according to the handbook of the ABI Prism 7000 SDS software.

$$\Delta\Delta C_t = (C_{t,\text{target gene}} - C_{t,16S\ rrn})_{\text{stress}} - (C_{t,\text{target gene}} - C_{t,16S\ rrn})_{\text{control}} \quad (1)$$

where, $\Delta\Delta C_t$ is comparative threshold cycle, $C_{t,\text{ target gene}}$, is the threshold cycle of target gene and $C_{t,16S\ rrn}$, is the threshold cycle of 16S rRNA gene.

1.6 Statistics

Significant differences were determined by one way ANOVA followed by LSD post-hoc test, differences were considered to be significant at $p < 0.05$.

2 Results

2.1 Oxygen evolution

The oxygen evolutions of *Synechocystis* sp. PCC 6803 in response to pyrene stress are shown in Fig. 1. Oxygen evolution of *Synechocystis* sp. PCC 6803 did not show a significantly difference from that of the control at a concentration of 0.125 mg/L pyrene in BG11 medium, but was significantly inhibited at the concentrations of 0.625 and 3.125 mg/L.

2.2 Polyphasic Chl-*a* fluorescence transients

Polyphasic Chl-*a* fluorescence transients in cells of *Synechocystis* sp. PCC 6803 exhibited a typical increase as shown in Fig. 2, with the phases of O-J-I-P being

Table 1 Primers designed for real-time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
16S rrn	GGACGGGTGAGTAACGCGTA*	CCCATTGCGGAAAATTCCCC**
psbO	GGATAAATTCCGTACCAAC	CCAAATCAGTGTCAGAGGG
psbA	GGTCAAGARGAAGAAACCTACAAT	GTTG AAACCGTTGAGGTTGAA
psbB	GTGCTATGAACAGTGGTGATGGC	TTTAGACTCGGAACGACGGAAGG
psbC	GGCTCTGATTTGGTCTGGGGA	GGAAGGTGAAGGCTTGGGATT

* Modified according to Urbach et al. (1992); ** modified according to Nübel et al. (1997).

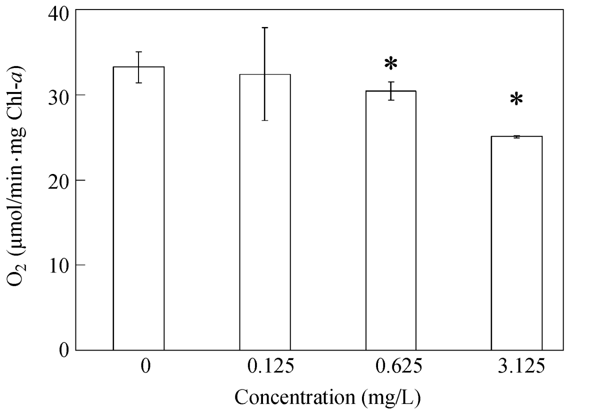

Fig. 1 Oxygen evolution of *Synechocystis* sp. PCC 6803 under pyrene stress. The horizontal axis is the concentrations of pyrene in BG11 medium. The error bar is means ± standard deviation. * $p < 0.05$ (LSD).

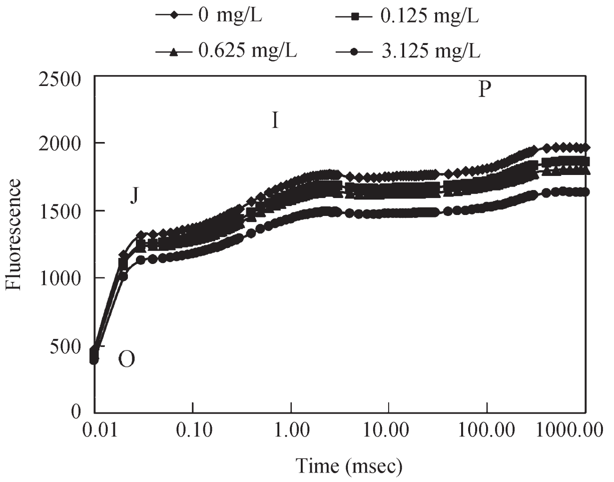

Fig. 2 Chl-*a* fluorescence transients of *Synechocystis* sp. PCC 6803 under pyrene stress.

presented. The minimal fluorescence level (O) did not change significantly under all pyrene stress treatments. Fluorescence yield at phases J, I and P declined slightly at pyrene concentrations of 0.125 and 0.625 mg/L, but they were significantly lower than control at a concentration of 3.125 mg/L.

JIP-test provided considerable information about the photosynthetic apparatus. Six important parameters dV/dt_0 (the slope at the origin of the normalized fluorescence rise), φD_0 (maximum quantum yield of non photochemical deexcitation), φP_0 (maximum quantum yield of primary photochemistry), φE_0 (quantum yield of electron transport beyond Q_A^-), ψ_0 (the probability that a trapped exciton moves an electron further than Q_A^-) and PI_{ABS} (performance index) deviated from Chl-*a* fluorescence induction curves as shown in Fig. 3. These results were based on the following Eqs. (2)–(7) (Christen et al., 2007):

$$dV/dt_0 = (F_{300\mu s} - F_0)/(F_M - F_0) \quad (2)$$

$$\varphi P_0 = (F_M - F_0)/F_M \quad (3)$$

$$\varphi D_0 = F_0/F_M \quad (4)$$

$$\psi_0 = 1 - V_J \quad (5)$$

$$\varphi E_0 = (1 - F_0/F_M)(1 - V_J) \quad (6)$$

$$PI_{ABS} = (V_J/M_0)(F_V/F_M)[\varphi P_0/(1 - \varphi P_0)][\psi_0/(1 - \psi_0)] \quad (7)$$

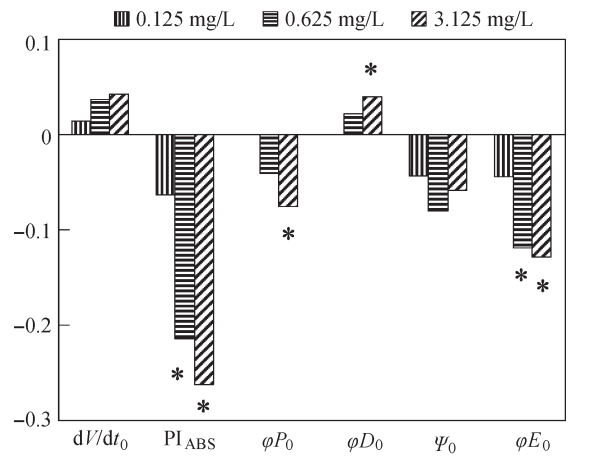

Fig. 3 Deviation of the JIP-test parameters from Chl-*a* fluorescence induction curves, expressed as relative values ((stress–control)/control). * $p < 0.05$ (LSD).

where, F_0, $F_{300\mu s}$, F_M represent fluorescence intensity at 50 μsec, 300 μsec and maximal fluorescence intensity, respectively. V_J represents relative variable fluorescence at 2 msec. As compared with control, there was no significant difference in parameters φP_0, φD_0, ψ_0, and dV/dt_0 at concentrations of 0.125 and 0.625 mg/L, but φE_0 and PI_{ABS} significantly decreased at 0.625 mg/L, φP_0, φE_0, PI_{ABS} are all significantly lower than control at a concentration of 3.125 mg/L, but φD_0 was significantly higher than the control.

2.3 Gene expressions of *psbO*, *psbA*, *psbB* and *psbC* in pyrene stress

Among the four *psb* genes, *psbO* encodes the 33-kDa polypeptide of the oxygen-evolving complex for PS II, *psbA* encodes the D1 protein, *psbB* encodes chlorophyll-binding core antenna proteins-CP47, and *psbC* encodes the chlorophyll-binding core antenna proteins-CP43. Figure 4 shows the expressions for the four *psb* genes in response to pyrene stress. Compared with control, the expression of *psbA* showed no significant change at concentrations of 0.125 and 0.625 mg/L, but was up-regulated at a

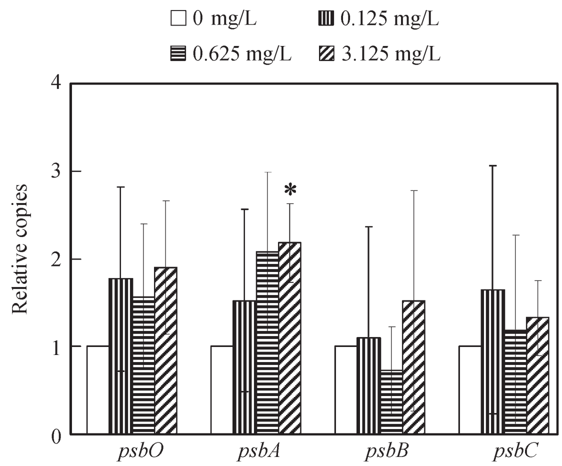

Fig. 4 Relative normalized expression of *psbO*, *psbA*, *psbB* and *psbC* of *Synechocystis* sp. PCC 6803 under pyrene stress. The error bar is standard deviation. * $p < 0.05$ (LSD).

concentration of 3.125 mg/L. The expressions of *psbO*, *psbB* and *psbC* in all pyrene stress treatments showed no significant difference when compared with the control.

3 Discussion

Oxygen evolution is a general index in evaluating the efficiency of the photosynthetic process. As shown in Fig. 1, the oxygen evolution did not change significantly at a concentration of 0.125 mg/L pyrene, but decreased significantly at 0.625 mg/L, suggesting that the photosynthetic apparatus of *Synechocystis* sp. PCC 6803 encountered damage under pyrene stress at 0.625 mg/L but not at 0.125 mg/L pyrene. The changes of PI_{ABS} under pyrene stress also proved this. It is known that phases O, J, I, P fluorescence transient reflect the redox state of PS II (Q_A, Q_B and PQ pool) (Papageorgiou, 1975; Strasser and Govindjee, 1992). Phase O corresponds to the situation when all molecules of Q_A are in the oxidized state, phase P corresponds to situation in which all molecules of Q_A are in reduced state, Q_A was reduced to Q_A^- in the transition from phase O to J, and PQ pool was reduced when phase J transformed to phase P (Strasser et al., 1995). The parameters that deviated from Chl-*a* fluorescence induction curves can reflect the photosynthetic characters of PS II. dV/dt_0 represents the relative rate of Q_A reduction (dQ_A^-/dt_0 per Q_A total). The dV/dt_0 showed no significant variation at 0.625 and 3.125 mg/L pyrene when compared with control, suggesting that no modification was induced by pyrene at the donor side of the PS II electron transport system under these concentrations. ψ_0 represents the probability that a trapped exciton moves an electron further than Q_A^-. From Fig. 3, it also could be deduced that electron transport at the acceptor side is not the main site of damage by pyrene since ψ_0 showed no significant difference at 0.625 and 3.125 mg/L pyrene when compared with control. φP_0 represents the maximum quantum yield of primary photochemistry and φE_0 represents quantum yield of electron transport beyond Q_A^-. The decrease of φE_0 at 0.625 and 3.125 mg/L pyrene and of φP_0 at 3.125 mg/L pyrene indicated that the reaction centre of PS II is the primary damaging target. φD_0 represents the maximum quantum yield of non photochemical deexcitation. The increase of φD_0 under pyrene stress also indirectly proved this.

There are four important proteins in the reaction centre of PS II: PsbO protein, D1 protein, CP47 protein and CP43 protein. The PsbO protein and D1 protein are reported as most susceptible to environmental stress (Gong et al., 2007; Zhao et al., 2008). Photosynthetic organisms have developed many strategies to acclimate to a broad range of environmental conditions. An intriguing feature of the D1 protein is that the damaged D1 protein in PS II is replaced by a newly synthesized precursor to the D1 protein encoded by *psbA* (Nishiyama, et al., 2005). There are three *psbA* genes (*psbA*-I, *psbA*-II and *psbA*-III) in *Synechocystis* sp. PCC6803, but only one type of D1 protein encoded by both the *psbA*-II and *psbA*-III genes could be detected under normal growth conditions as well as under stresses conditions (Mate et al., 1998). The transcript from *psbA*-II was reported to account for 90% of the total *psbA* transcript pool under normal growth conditions (Mohamed et al., 1993). The *psbA*-I gene encodes a divergent D1 protein which has not been detected in wild type cells (Mohamed et al., 1993; Salih and Jansson, 1997). The primers used in the present study covered the conservative region of *psbA*-II and *psbA*-III, thereby the result in this study represented the transcript pool of *psbA*. The expression of *psbA* showed no significant difference at a concentration of 0.625 mg/L pyrene when compared with control, but up-regulated at 3.125 mg/L. This suggested the D1 protein of PS II did not undergo damage at 0.625 mg/L pyrene stress, but the results from oxygen evolution as well as JIP-test indicated that the reaction centre of PS II undergoes damage under 0.625 mg/L pyrene stress. These results suggest the PsbO protein rather than the D1 protein maybe is the primary target under pyrene stress. Of course, unambiguous conclusion for this suggestion requires further study in Proteomics.

The reported pyrene concentrations in waters of China were always less than 2 μg/L, and in sediments, it is always less than 200 μg/kg. This is far lower than the concentration found effective (0.625 mg/L) in this study. It indicates that in most water bodies, no damage of *Synechocystis* PS II will be induced by pyrene only. But in some serious contaminated areas, pyrene concentration does exceed 1 g/kg in sediment (Simpson et al., 1996). We can deduce that the algal photosystems will be damaged in such areas. Wang et al. (2008) reported the growth of the eukaryotic alga diatom *Phaeodactylum tricornutum* was significantly inhibited under 0.1 mg/L pyrene stress. Photosynthesis of the eukaryotic flagellate *Isochrysis galbana* was also significantly inhibited at 0.12 mg/L pyrene (Pérez et al., 2009). In our study, there was no inhibition effect on photosynthesis and growth (data not shown) at 0.125 mg/L pyrene. This suggests that cyanobacteria are more tolerant to pyrene stress when compared with eukaryotic algae.

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