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Effects of levofloxacin hydrochlordie on photosystem II activity and heterogeneity of *Synechocystis* sp.

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ABSTRACT

Effects of LH on photosynthesis of *Synechocystis* sp. were investigated by a variety of in vivo chlorophyll fluorescence. O_2 evolution and the photosystem II (PSII) activity were clearly inhibited by LH. Exposure to LH increased the proportion of PSIIβ and this weakened the connectivity between PSII units and hindered excitation energy-transfer between PSII units. LH decreased the density of the active photosynthetic reaction centers, inhibited electron transport, and increased the dissipated energy flux per reaction center. The inhibitory effect of LH on Q_A^- reoxidation process could be divided into several stages. LH first inhibited the electron transfer from Q_A^- to Q_B by weakening the connectivity between Q_A^- and Q_B, and PQ binding began taking part in Q_A^- reoxidation. At the second stage, the connectivity between Q_A^- and PQ pool was broken and inhibition on PQ binding occurred. At this stage, some Q_A^- began to be oxidized by $S_2(Q_AQ_B)^-$. Finally, when the connectivity between Q_A^- and Q_B and PQ was completely broken, all Q_A^- was oxidized through charge recombination.

1. Introduction

Levofloxacin hydrochloride (LH), the l-form of the fluoroquinolone ofloxacin, is one of the most commonly used fluoroquinolone antibiotics that have excellent activity against a broad spectra of bacteria. LH inhibits bacterial DNA gyrase involved in unwinding the DNA helix for replication and transcription (Salem et al., 2005). The Levofloxacin excreted is largely unchanged as an active drug in the urine (Croom and Goa, 2003). LH is very chemically stable to hydrolysis and high temperatures (Thiele-Bruhn, 2003) and resistant to biodegradation. LH is discharged into the water environment via human excretion, improper disposal of expired medications and agricultural and aquacultural activities. LH has been detected in river waters in nanogram per liter to low microgram per liter (Andreozzi et al., 2003; Khetan and Collins, 2007). LH was found to be active phytotoxic agent at μg L^{-1} to mg L^{-1} (e.g., EC50 for *Lemna gibba*, was 0.185–1.41 mg L^{-1} (Brain et al., 2004). LH was also shown to be highly toxic to the microalga (Yamashita et al., 2006a,b).

Cyanobacteria occupy the lower trophic levels within food webs and changes in their community could have an indirect but significant effect on the rest of the freshwater community. In this context, they are usually used in environmental toxicity assessment. Recent studies showed that some antibiotics are highly toxic to cyanobacteria (Halling-Sorensen et al., 2000). Photosynthesis is the principal mode of energy metabolism of cyanobacteria. Photosystem II (PSII) is thought to be the primary and sensitive site of inhibition induced by a wide range of environmental pollution (Baker, 1991; Burda et al., 2003; Sigfridsson et al., 2004; Berden-Zrimec et al., 2007). A few studies reported the toxic effect of antibiotics on O_2 evolution of algae (Kviderova and Henley, 2005). However, the mechanism of inhibition of photosynthesis is largely unknown.

The aim of this study was to investigate the mechanisms involved in the toxic effect of amoxicillin on the PSII of *Synechocystis* sp., one species of common cyanobacteria, by in vivo chlorophyll fluorescence measurements.

2. Materials and methods

2.1. Chemicals

LH ($C_{18}H_{20}FN_3O_4$·HCl, molecular weight = 397.83) with a chemical purity of 99.5% was supplied from Jiangsu Yabang Pharma Group (Yancheng, China). A range of different nominal concentrations (from 0 to 100 mg L^{-1}) of exposure solutions of LH were prepared by dissolution of LH in deionized water. All the exposure solutions were filtered through a 0.22 μm filter membrane before use. Effects of LH in the range of μg L^{-1} to mg L^{-1} on the photosystem II of

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cyanobacterium were investigated. The measured concentration was 98.5–99.8% of the nominal concentration. Since nominal concentrations were close to the measured concentrations and the aim of our study is the biological reaction of a plant to LH but not the assessment of toxic doses, nominal concentrations were used.

2.2. Culture of cyanobacterium

The cyanobacterium *Synechocystis* sp., supplied by Institute of Hydrobiology, Chinese Academy of Sciences, was precultured photoautotrophically at 25 °C and about 25 μmol m^{-2} S^{-1} in BG-11 growth medium (Rippka et al., 1979). Exponentially grown cells were diluted with fresh medium to achieve test samples at around 5 μg Chl *a* mL^{-1} for fluorescence measurement and cultured in 10 mm × 10 mm plastic cuvette at the volume of 3.4 mL each.

2.3. Measurement of O_2 evolution

After 12 h of LH treatment, 5-min photosynthetic O_2 evolution was measured using a Clark microelectrode (Unisense, Denmark) at 25 °C with illumination of 500 μmol m^{-2} S^{-1} (PAR) white light intensity in 2-mL cuvettes. The photosynthetically active radiation (400–700 nm) was measured at the surface of the culture vessels by Li-192 SA quantum sensor (Li-COR, USA).

2.4. Chlorophyll fluorescence tests

A fluorometer FL3500 (PSI,CZ) was employed to measure the polyphasic fast fluorescence induction, Q_A^- reoxidation kinetics, the heterogeneity of PSII antenna and the proportion of active and inactive reaction centers. All the samples were dark-adapted for 5 min before each test.

2.4.1. Measurement of Q_A^- reoxidation kinetics

The measurement of Q_A^- reoxidation kinetics was performed by a single turnover flash. The relaxation of the flash-induced increase in Chl *a* fluorescence yield reflects the reoxidation of Q_A^- via forward electron transport to Q_B and back reactions with the S_2 state of the oxygen evolving complexes (OEC) (Cao and Govindjee, 1990; Dau, 1994). In this study the Q_A^- reoxidation kinetics curves after a single turnover flash was measured in the 200 μs to 10 s time range. Both actinic (30 μs) flashes and measuring (2.5 μs) flashes were provided by red LEDs. The Q_A^- reoxidation kinetic data were recorded with eight datapoints per decade.

2.4.2. Measurement of the heterogeneity of PSII antenna

The determination of PS II antenna heterogeneity was done by the flash fluorescence induction which can cause transient closure of PSII centers (Nedbal et al., 1999). A strong 50 μs flash was applied. The proportion of PSIIα and PSIIβ was calculated by calculating the semi-log plot of complementary area over the fluorescence induction curve (Melis and Homann, 1976). Two kinetic components can be shown by the semi-log plot of the area growth with fast sigmoidal component ascribed as PSIIα and a slow exponential component ascribed as PSIIβ, respectively (Warren et al., 1983). The intercept of the linear phase in the semi-log plots was denoted as the proportion of PSIIβ.

2.4.3. Polyphasic fast fluorescence induction and JIP test

The chlorophyll fluorescence transients were recorded up to 1 s on a logarithmic time scale, with a data acquisition every 10 μs for the first 2 ms and every 1 ms thereafter. The polyphasic fluorescence induction kinetics was analyzed according to the JIP-test (Strasser et al., 1995). The polyphasic fast-phase fluorescence induction curve provides valuable information on the magnitude of stress effects on photosystem II (PSII) function (Strasser and Strasser, 1995). In the present study, the following data were directly obtained from the fast rise kinetic curves: F_0, the initial fluorescence, was measured at 50 μs, at this time all reaction centers (RCs) are open; F_J and F_I are the fluorescence intensity at J step (at 2 ms) and I step (at 30 ms); F_M, the maximal fluorescence, was the peak fluorescence at P step when all RCs were closed after illumination; F300 was the fluorescence at 300 μs. Selected parameters quantifying PSII behavior were calculated from the above original data as the formulae in Table 1.

Table 1
Formula and items of selected JIP-test parameters.

$V_J = (F_{2\ ms} - F_O)/(F_M - F_O)$	Relative variable fluorescence intensity at the J-step
$M_O = 4(F_{300\ \mu s} - F_O)/(F_M - F_O)$	Approximated initial slope of the fluorescence transient
$\Psi_O = ET_O/TR_O = (1 - V_J)$	Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A (at $t = 0$)
$\phi_{Po} = TR_O/ABS = [1 - (F_O/F_M)] = F_V/F_M$	Maximum quantum yield for primary photochemistry (at $t = 0$)
$ABS/RC = M_O \cdot (1/V_J) \cdot (1/\phi_{Po})$	Absorption flux per RC
$ET_O/RC = M_O \cdot (1/V_J) \cdot \Psi_O$	Electron transport flux per RC (at $t = 0$)
$ABS/CS_O = F_0$	Absorption flux per CS (at $t = 0$)
$RC/CS = \phi_{Po} \cdot (V_J/Mo) \cdot ABS/CS$	Density of RCs (Q_A-reducing PSII reaction centers)
$DI_O/RC = (ABS/RC) - (TR_O/RC)$	Dissipated energy flux per RC (at $t = 0$)

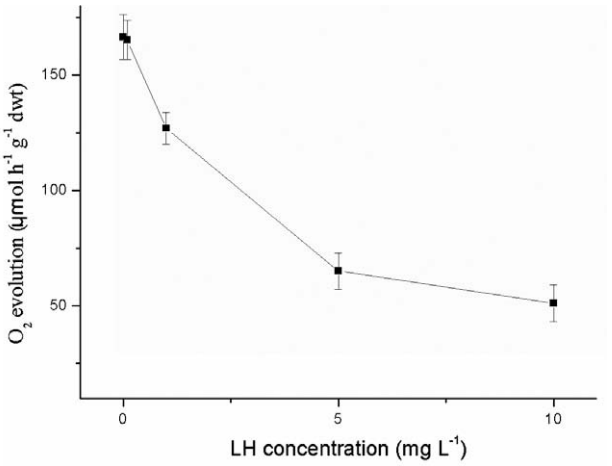

Fig. 1. Photosynthetic O_2 evolution of *Synechocystis* sp. treated with different concentration LH for 24 h. Values represent mean ± SE of five independent measurements. Bars indicate standard errors.

2.5. Statistical analysis

Each treatment was at least triplicated and the results were presented as mean ± SE (standard error). Student's *t*-test was employed for statistical analysis of experimental data. Statistical significance was accepted when *p*-value is less than 0.05.

3. Results

3.1. Effect of LH on O_2 evolution

It was observed that O_2 evolution of *Synechocystis* sp. was inhibited by LH and the inhibitory effect was concentration dependent (Fig. 1). Treatment with 5.0 mg L^{-1} LH for 24 h reduced O_2 evolution by 60.9%. It is clearly that the O_2-evolving complex (OEC) was a primary target site for LH toxicity.

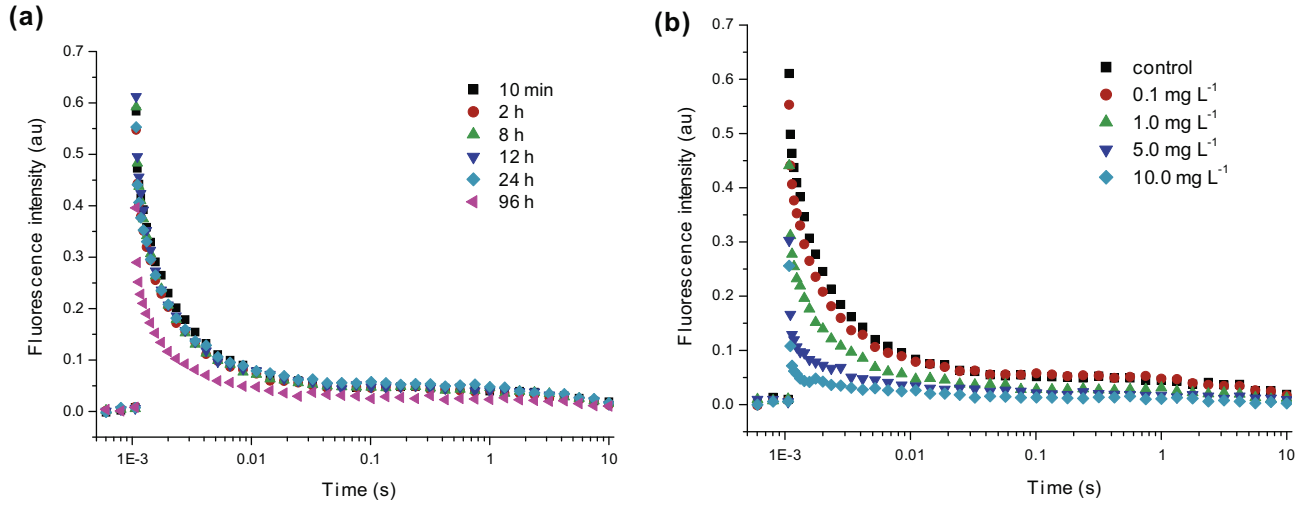

Fig. 2. Representative Q_A^- reoxidation kinetic curves for *Synechocystis* sp.: (a) Q_A^- reoxidation kinetic curves for *Synechocystis* sp. treated with 0.1 mg L^{-1} LH with different exposure time; (b) Q_A^- reoxidation kinetic curves for *Synechocystis* sp. treated with different concentration LH for 24 h.

3.2. Effect of LH on Q_A^- reoxidation kinetics

The Q_A^- reoxidation kinetics test (Fig. 2) was performed in order to understand the inhibitory effect of LH on the function of the acceptor side of PSII of *Synechocystis* sp. (Crofts and Wraight, 1983). The Q_A^- reoxidation kinetics curves were fitted by the three-component exponential Eq. (1):

$$F(t) - F_0 = A_1 \exp(-t/T_1) + A_2 \exp(-t/T_2) + A_3 \exp(-t/T_3) \quad (1)$$

where $F(t)$ is the variable fluorescence yield at time t; F_0 is the fluorescence level before the flash; A_1–A_3 are the amplitudes; T_1–T_3 are the time constants. The nonlinear correlation between the fluorescence yield and the redox state of Q_A was corrected for using the Joliot model (Joliot and Joliot, 1964) with a value of 0.5 for the energy-transfer parameter between PSII units. The fast component indicates electron transfer from Q_A^- to Q_B^- site occupied with Q_B (Q_B^-) before the actinic flash. The middle component is typical for PSII complexes where Q_A^- reoxidation is limited by diffusion of PQ molecules to an empty Q_B-site (Crofts and Wraight, 1983). The slowest component reflects the charge recombination from the $S_2Q_A^-$ state of the water oxidation to the S_1Q_A (Cao and Govindjee, 1990).

Several changes about the Q_A^- reoxidation curves were observed due to exposure to LH. The amplitude of the variable fluorescence (F_v) decreased with increasing LH concentration and exposure time and the decay phases were slowed down. The parameters of the Q_A^- reoxidation kinetics are summarized in Table 2. The fast phase component, with 113–184 μs time constant, absolutely dominated the Q_A^- reoxidation for the control. The fast phase accounted for all the Q_A^- reoxidation in the untreated *Synechocystis* sp. cells, indicating that almost all the Q_A^- was oxidized by electron transfer from Q_A^- to Q_B^- site occupied with Q_B (Q_B^-). Short-term (10 min) treatment with LH at all concentrations did not affect Q_A^- reoxidation kinetics. 0.1 mg L^{-1} LH had no inhibitory effect on Q_A^- reoxidation kinetics during the 96 h exposure.

The inhibition of the Q_A^- reoxidation kinetics induced by1.0 - mg L^{-1} or higher concentration LH occurred step by step as exposure time prolonged. LH exposure first resulted in drastic decrease and even complete loss of the fast component accompanied with increase of the amplitude of the middle phase component, indicating the electron transfer from Q_A^- to Q_B/Q_B^- was inhibited. The higher the LH concentration, the shorter the time needed for complete loss of the fast phase component. For example, the time needed for complete loss of the fast phase component for the cells treated with 1.0 mg L^{-1} and 5.0 mg L^{-1} LH were 12 h and 6 h, respectively. At this step, when the fast phase component was lost, most Q_A^- was oxidized through PQ binding with the empty Q_B site. As exposure time prolonged forward the middle phase component also decreased and even completely disappeared, accompanied by the increasing amplitude of the slow phase component. This change indicated that the PQ binding to the Q_B site was blocked and more and more Q_A^- was oxidized via charge recombination from the $S_2Q_A^-$ state of the water oxidation to the S_1Q_A. For example, exposure to 10 mg L^{-1} LH for 12 h induced complete loss of the fast phase component and all the fast phase component was converted into the middle phase component (100%). At this time, all the Q_A^- was reoxidized by PQ binding. As the cells were exposed to LH for another 12 h, the middle phase lost completely and the Q_A^- was dominated by the slow phase component (99.9%), indicating that almost all the Q_A^- was oxidized by charge recombination between Q_A^- and the positively charged species in the OEC in PSII centers, which are unable to perform the forward electron transfer.

3.3. Effect of LH on the heterogeneity of PSII antenna size

The PSII antenna heterogeneity (PSIIα:PSIIβ) is related to the difference between PSII units in terms of energy transfer (Melis and Homann, 1976). The α part was attributed to interconnected groups of PSII units that could transfer excitation energy among themselves. The β part was ascribed to individual, separate PSII units that cannot transfer energy to other PSII units (Melis and Homann, 1976; Warren et al., 1983). The proportion of PSIIβ centers generally increased with increasing LH concentration (Fig. 3). The proportion of the PSIIβ of the control was about 12% and increased after exposure to LH. At low concentrations (here 0.1 and 1.0 mg L^{-1}), the proportion of PSIIβ increased to the peak at 12 h and then decreased with increasing exposure time, indicating that the effect of low concentration LH on PSII antenna size heterogeneity was partially or completely reversible. More PSIIα centers were converted into PSIIβ under higher LH stress and most of the PSIIα were converted into PSIIβ within the first 24 h. Further increase in the exposure time did not change this value markedly. The proportion of PSIIβ centers varied in the range of 45–55%, indicating that the adverse effect of LH on PSII antenna size at high concentration was irreversible.

Table 2
Parameters of Q_A^- reoxidation kinetics of the samples untreated and treated with different concentration LH and with different exposure time.

Exposure time	Total amplitude[a](%)	Fast phase A_1(%)/T_1(μs)	Middle phase A_2(%)/T_2(ms)	Slow phase A_3(%)/T_3(s)
The control				
10 min	100	99.9/145.7	0.1/2.39	0/8.59
2 h	100	100/113.1	0/2.01	0/6.47
6 h	100	99.7/179	0.3/2.49	0/8.17
12 h	100	99.9/136.7	0.1/1.92	0/5.73
24 h	100	99.5/184.4	0.5/2.76	0.1/6.80
96 h	100	100/167.8	0/1.50	0/6.31
With 0.1 mg L^{-1} LH				
10 min	101 ± 1	99.8/158.6	0.2/2.53	0/6.31
2 h	100 ± 1	100/108	0/1.97	0/5.39
6 h	101 ± 3	100/126.8	0/1.94	0/6.35
12 h	103 ± 3	99.8/163.4	0.2/2.19	0/5.73
24 h	90 ± 2	100/61.3	0/1.65	0/5.42
96 h	72 ± 1	0/139	97.5/0.57	2.5/2.69
With 1.0 mg L^{-1} LH				
10 min	102 ± 1	99.8/147.8	0.1/2.39	0/6.80
2 h	103 ± 3	100/100.5	0/1.82	0/6.97
6 h	91 ± 2	100/93.1	0/1.98	0/7.80
12 h	81 ± 1	0/182.7	96/0.77	4/2.53
24 h	72 ± 3	0.1/6.2	99.2/0.25	0.7/0.0169
96 h	37 ± 2	0/7.8	67.3 /0.013	32.7/0.0169
With 5.0 mg L^{-1} LH				
10 min	104 ± 3	99.7/166.9	0.3/2.48	0/6.94
2 h	98 ± 1	0/217.9	95.6/0.84	4.4/4.30
6 h	80 ± 2	0/148	95.5/0.81	4.5/2.35
12 h	66 ± 1	0/166.3	95.4/0.76	4.6/1.96
24 h	49 ± 3	0/18.7	36.1/0.074	63.9/0.001300
96 h	33 ± 3	0/30.3	12.4/0.101	87.6/0.000211
With 10.0 mg L^{-1} LH				
10 min	100 ± 2	100/66.4	0/1.85	0/5.39
2 h	90 ± 3	98/271.1	1.6/3.02	0.4/5.75
6 h	70 ± 3	0.1/65.7	95.4/0.753	4.5/3.83
12 h	54 ± 1	0/41.9	100/0.076	0/1.09
24 h	40 ± 2	0.1/39.1	0/0.039.1	99.9/0.000067
96 h	22 ± 2	–/–[b]	–/–[b]	–/–[b]

[a] Values represent the amplitude of total variable fluorescence as a percentage of the control.
[b] The variable fluorescence was very weak and the decay curves were poor, therefore no reliable parameters were derived.

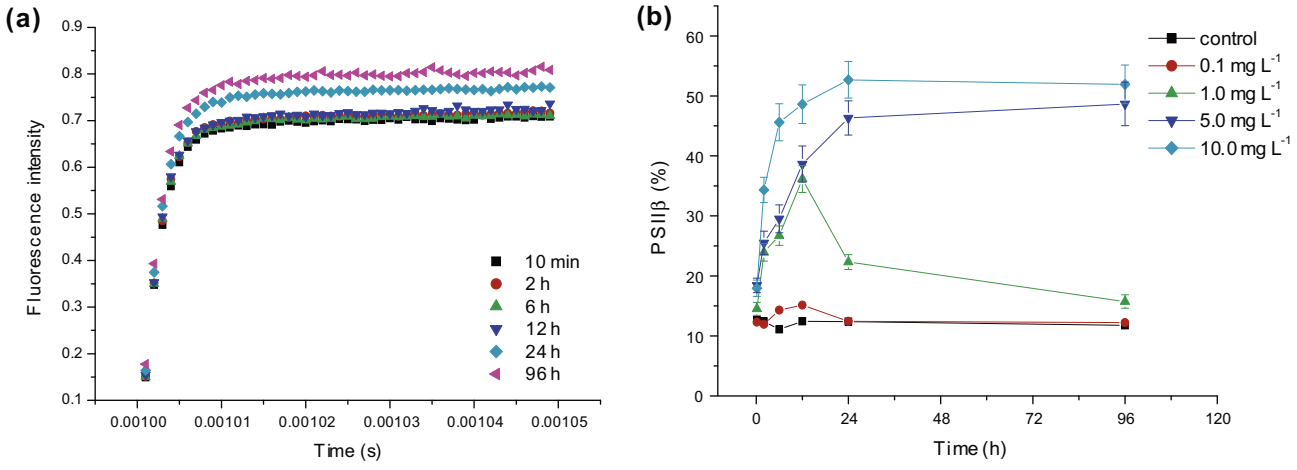

Fig. 3. (a) Representative flash induction curves for 0.1 mg L^{-1} LH treated *Synechocystis* sp. at different exposure time. (b) Proportion of PSIIβ reaction centers derived from the flash induction curves; value represent mean of five measurements; bars indicated SE. When the SE was less than the symbol size, the error bar was not given.

3.4. Effect of LH on polyphasic fast fluorescence induction

The fast kinetic induction curves of the control and the samples treated with LH was measured (Fig. 4a). It was found that LH had concentration-dependent inhibitory effect on the fast rise fluorescence transient of *Synechocystis* sp. F_0 generally showed an increasing trend with increasing LH concentration. JIP-test showed LH treatment decreased the density of the active photosynthetic reaction centers (RC/CS_O) (Fig. 4b) and inhibited electron transport (ET_O/TR_O) (Fig. 4c), which resulted in an increase in absorption flux

per RC (ABS/RC) (Fig. 4d) and the dissipated energy flux per reaction center (DI_O/RC) (Fig. 4e). Finally, the maximum quantum yield for primary photochemistry (F_V/F_M) was reduced (Fig. 4f).

4. Discussion

In the present work, we demonstrated that LH in μg L^{-1} and mg L^{-1} range had an adverse effect on the PSII of *Synechocystis* sp., supported by the evidences from O_2 evolution and a variety of in vivo chlorophyll fluorescence tests. The inhibitory effect of LH on PSII increased with increases of its concentration.

It is clear that high concentration LH increased the proportion of the PSIIβ. As a result of accumulation of PSIIβ, the interconnectivity between PSII units became worse, the excitation energy transfer among themselves was inhibited, and the density of the active photosynthetic reaction centers (RC/CS_O) decreased (Fig. 4b). This

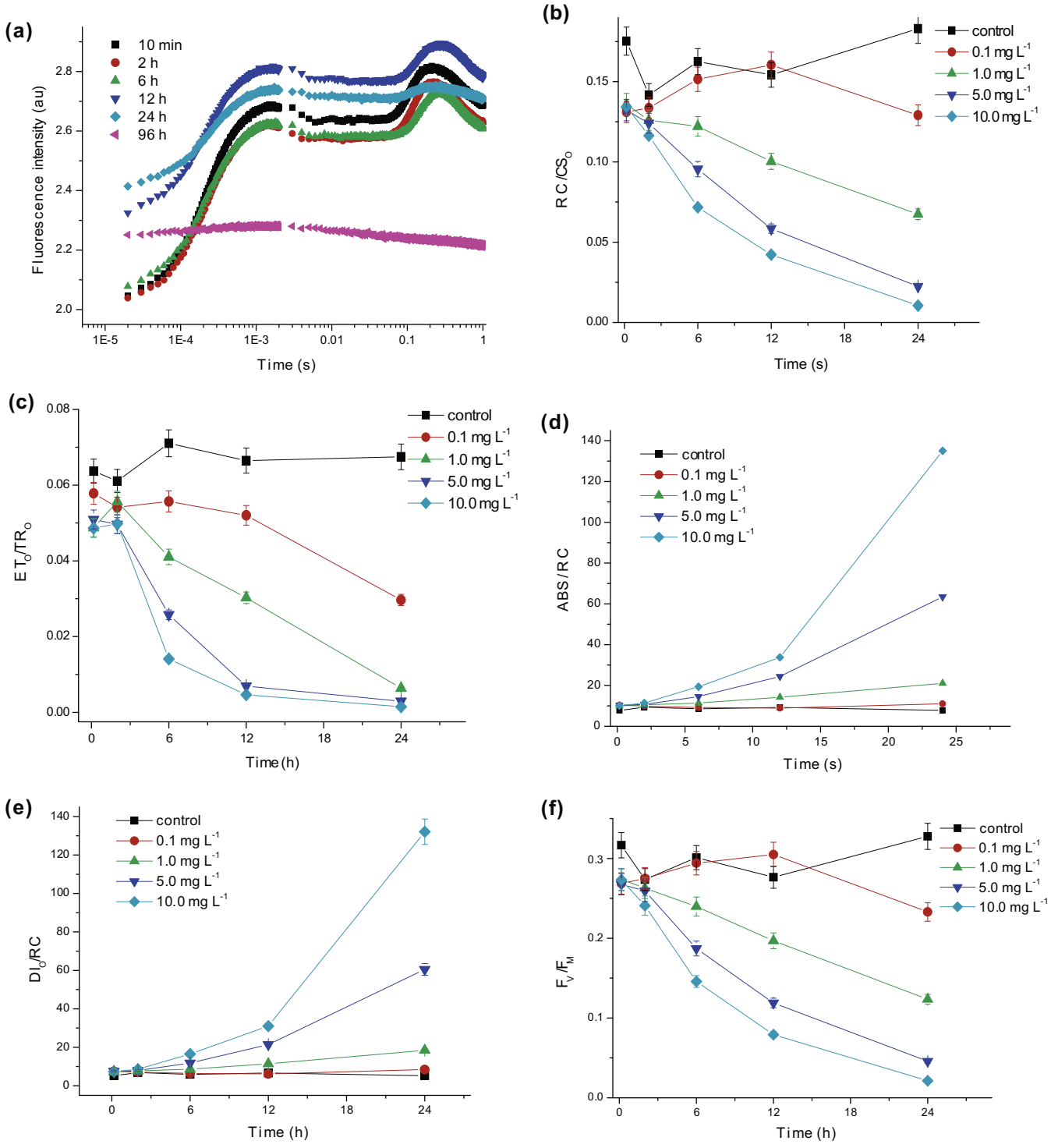

Fig. 4. Effect of LH on dissipated energy flux per RC for cells untreated and treated with LH. Since the OJIP curve became flat, the JIP-test parameters were not given. Value represent mean of five measurements; Bars indicated SE. When the SE was less than the symbol size, the error bar was not given.

further inhibited electron transport (ET_O/TR_O) (Fig. 4c) and increased the absorption flux per RC (ABS/RC) (Fig. 4d). Finally the dissipated energy flux per reaction center (DI_O/RC) increased (Fig. 4e) due to the low interconnectivity between PSII units.

Accumulation of PSIIβ under stress was also reported elsewhere (Sundby et al., 1986; Hill and Ralph, 2006). The reported values of PSIIβ centers of untreated and stressed photosynthetic organisms varied with different species. In untreated higher plants leaves about 30% of the total PSII centers are PSIIβ centers (Sundby et al., 1986; Bukhov and Carpentier, 2000). Heat stress increased the proportion of PSIIβ centers up to 60% (Bukhov and Carpentier, 2000) or over 80% (Sundby et al., 1986).

LH altered the Q_A^- reoxidation kinetics step by step as exposure time prolonged forward. At the first step, the fast phase significantly decreased and was even complete lost under LH stress, accompanied with an increase of the amplitude of the middle phase component, indicating electron transfer from Q_A^- to Q_B was partially or completely inhibited. At this step, Q_A^- was oxidized through the binding of plastoquinone (PQ) to vacant Q_B-binding site before the Q_A^- to Q_B electron transfer can take place (Dau, 1994). At the second step, with the cells continued to be exposed to LH, this Q_A^- reoxidation way began to be modified, as indicated by a drastic decrease in the amplitude of middle phase and a significant increase of the slow phase. The connectivity between the Q_A^- and Q_B and the PQ pool was severely inhibited. Therefore, Q_A^- was forced to be oxidized with back electron transfer from Q_A^- to S_2 state of the OEC, i.e., $S_2(Q_AQ_B)^-$ charge recombination (Cao and Govindjee, 1990). In short, LH inhibited the forward electron transfer via breaking the connectivity between Q_A^- and Q_B and the PQ pool step by step. Since that PSIIβ was ascribed to individual, separate PSII units that cannot transfer energy to other PSII units (Melis and Homann, 1976; Warren et al., 1983), the decreasing connectivity between Q_A^- and Q_B and the PQ pool might be attributed to the accumulation PSIIβ centers.

LH in $\mu g\ L^{-1}$ and $mg\ L^{-1}$ range may have similar inhibitory effects on PSII activity in green algae and higher plants since they have similar photosynthetic apparatus to cyanobacteria. LH, therefore, might have adverse effect on the photosynthetic primary producer. In addition, the in vivo chlorophyll fluorescence parameters such as Q_A^- reoxidation kinetic constants and JIP-test parameters can be useful in detecting toxicity of antibiotics to cyanobacteria, algae and higher plants. Other biophysical and biochemical methods should be employed to study other possible targets of antibiotic substances in photosynthetic microorganisms.

5. Conclusions

(1) LH clearly has inhibitory effects on O_2 evolution and PSII activity of *Synechocystis* sp.
(2) The PSII heterogeneity was severely modified under LH stress. LH resulted in increases increase in the proportion of PSIIβ centers and poor connectivity and poor excitation energy-transfer between PSII units, and thus increased the dissipated energy flux per reaction center.
(3) LH firstly inhibited the electron by weakening the connectivity between Q_A^- and Q_B. At this stage, PQ binding began taking part in Q_A^- reoxidation. At the second stage, as the LH exposure continued, the connectivity between Q_A^- and PQ pool was reduced and inhibition on PQ binding occurred. At this stage, some Q_A^- began to be oxidized by $S_2(Q_AQ_B)^-$. Finally, if the connectivity between Q_A^- and Q_B and PQ was completely broken, all Q_A^- was oxidized through charge recombination.

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