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http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

Effect of Benzo[a]pyrene on Detoxification and the Activity of Antioxidant Enzymes of Marine Microalgae

SHEN Chen, MIAO Jingjing, LI Yun*, and PAN Luqing

Ministry of Education Key Laboratory of Mariculture, *Ocean University of China*, *Qingdao* 266003, *P. R. China*

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Abstract The objective of this study was to examine the effect of benzo[a]pyrene (BaP) on the detoxification and antioxidant systems of two microalgae, *Isochrysis zhanjiangensis* and *Platymonas subcordiformis*. In our study, these two algae were exposed to BaP for 4 days at three different concentrations including 0.5 μg L^{-1} (low), 3 μg L^{-1} (mid) and 18 μg L^{-1} (high). The activity of detoxification enzymes, ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST) increased in *P. subcordiformis* in all BaP-treated groups. In *I. zhanjiangensis*, the activity of these two enzymes increased at the beginning of exposure, and then decreased in the groups treated with mid- and high BaP. The activity of antioxidant enzyme superoxide dismutase (SOD) increased in *I. zhanjiangensis* in all BaP-treated groups, and then decreased in high BaP-treated group, while no significant change was observed in *P. subcordiformis*. The activity of antioxidant enzyme catalase (CAT) increased in *I. zhanjiangensis* and *P. subcordiformis* in all BaP-treated groups. The content of malondialdehyde (MDA) in *Isochrysis zhanjiangensis* increased first, and then decreased in high BaP-treated group, while no change occurred in *P. subcordiformis*. These results demonstrated that BaP significantly influenced the activity of detoxifying and antioxidant enzymes in microalgae. The metabolic related enzymes (EROD, GST and CAT) may serve as sensitive biomarkers of measuring the contamination level of BaP in marine water.

Key words benzo[a]pyrene; marine microalga; detoxifying enzyme; antioxidant enzyme; lipid peroxidation

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in marine environment (van Schanke *et al*., 2012). The major sources of PAH in aquatic environment include industrial and waste water effluents, atmospheric deposition such as the byproducts of incomplete combustion of organic compounds, and the accidental oil spills (Silva *et al*., 2013). In recent years, oil spill is a notable source of PAHs due to the increasing of oil spill accidents in China's coastal regions (Mu *et al*., 2012). Up to now, more than 400 carcinogenic PAHs and their derivatives have been described, among which benzo[a]pyrene (BaP) shows the strongest carcinogenicity (Ren *et al*., 2014). BaP is a well known genotoxicant. It affects not only mitochondrial and nuclear DNA, but also causes oxidative stress by inducing a high level of reactive oxygen species (ROS), which may relate to cellular damage (Bo *et al*., 2014).

In aquatic ecosystem, algae are important primary producers, providing food for diverse invertebrates and fish species (Mofeed and Mosleh, 2013). Any disturbance to phytoplankton due to the release and accumulation of toxic compounds is likely to have impact on others at higher trophic levels (Li *et al*., 2006). Microalgae have a key role in biomonitoring the changes that occur in aquatic habitats (Mofeed and Mosleh, 2013). Because of their short generation times, unicellular algae are an ideal group of studying the response to different environmental factors (Li *et al*., 2009). Algae are widely used as ecological indicators in polluted aquatic environments, due to the rapid and predictable responding to a wide range of pollutants and the ability of providing a useful early warning signal of deteriorating conditions.

The biotransformation of BaP is a complex process that involves the functioning of phase I and phase II xenobiotic-metabolizing enzymes (Brammell *et al*., 2010). In phase I, it is always performed by isoenzymes of the cytochrome P450 family, yielding either an epoxide or a phenolic metabolite *via* an initial radical cation or quinine (van Schanke *et al*., 2012). Cytochrome P450 1A (CYP1A) is one of the isoenzymes, and can be detected toward 7-ethoxy-different thiophene trazodone-deethylase (ER OD), which has been proposed as a biomarker of exposure to PAHs. In phase II, the intermediate metabolites are conjugated with polar endogenous constituents such as glucuronic acid, sulphate and glutathione by UDP-glucuronyl transferase, sulfotransferase and glutathione-S-transferase (GST), respectively, producing water soluble conjugates which are easily excreted (Ren *et al*., 2014). In

* Corresponding author. Tel: 0086-532-82032649
E-mail: sxsdlwl@ouc.edu.cn

addition to CYP1A, phase II xenobiotic metabolizing enzymes such as GST were as also used as biomarker (Brammell *et al*., 2010).

It is well known that ROS such as superoxide, hydroxyl radicals and hydrogen peroxide are produced in cells once being exposed to environmental stresses (Li *et al*., 2006). Although ROS plays an important role in host defense, over production and residuals can cause oxidative damage (Ren *et al*., 2014). The antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), are important components in preventing oxidative damage in plants. SOD catalyzes the dismutation of two molecules of superoxide into H_2O_2 and oxygen. Subsequently, CAT degrades H_2O_2 to oxygen and water (Mofeed and Mosleh, 2013). These enzymes play important roles in protecting organisms from oxidative stress (Ren *et al*., 2014). The balance between the production of activated oxygen species and the quenching activity of antioxidant is disturbed by stress conditions, which often results in oxidative damage including protein degradation, DNA damage, and lipid peroxidation (LPO) (Wang *et al*., 2012). LPO is considered as a biomarker of the oxidative degradation of lipids caused by free radicals (Cima *et al*., 2013). Malondialdehyde (MDA) is a cytotoxic product of LPO, and is also an indicator of free radical production and consequent tissue damage (Wang *et al*., 2012).

So far, although there were a number of investigations on toxicity of many PAHs for fish (Lu *et al*., 2011), shellfish (Bebianno *et al*., 2009), and zooplankton (Correa-Reyes *et al*., 2007), as well as marine microalgae (Torres *et al*., 2008), the effect of BaP on the marine microalgal physiology have not been carried out yet. In the present study, the detoxification enzymatic activity of EROD and GST) and antioxidant enzymatic activity of SOD and CAT) and peroxidation product content (MDA) of *I. zhanjiangensis* and *P. subcordiformis* were measured to reveal the response of these microalgae to BaP stress, aiming to investigate the biochemical mechanism of the toxic effect of BaP on marine microalgae.

2 Materials and Methods

2.1 Experimental Organisms and Culture Conditions

Two marine microalgae *I. zhanjiangensis* and *P. subcordiformis* were provided by Prof. Ying Liang, Marine Alga Culture Collection of Ocean University of China. The microalgal species used in this study belong to two groups of marine phytoplankton, Bacillariophyta (*P. subcordiformis*) and Chrysophyta (*I. zhanjiangensis*), which are common live food used for aquaculture.

Batch cultures were grown in 250 mL flasks containing 100 mL f/2 medium in a chamber equipped with cool-white fluorescence tubes (about 3000 Lux) at a constant temperature of (22±1)℃ and a 12-h light:12-h dark cycle. Algal cells at the exponential phase were inoculated into the medium at an initial cell density of 4.0×10^5 cells mL^{-1}. To minimize cell deposition, cultures were shaken by hand three times a day.

2.2 Growth Rate Determination

Alga was cultured for 4 days, and 2 mL culture was aseptically sampled at day 0, 1, 2, 3 and 4 for cell counting. Cell density was measured in a microplate reader. In certain algal concentration range, the OD_{530} showed well linear relationship with cell numbers. The cell numbers were calculated by the standard curve.

2.3 Experimental Set-Up

BaP was first dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration was 0.01% in all tests except the control. In treatment condition, the algae were exposed to different concentrations (0.5, 3 and 18 μg L^{-1}) of BaP. The exposure concentrations of BaP were selected based on the concentration of BaP in the coastal seawater of Daya Bay (average about 0.633 μg L^{-1}) (Qiu *et al*., 2004), BaP solubility (22–25℃), as well as the algal EC_{50}. In the previous experiment we compared the 96 h-EC_{50} of BaP of *I. zhanjiangensis* (32.36 μg L^{-1}) and *P. subcordiformis* (>1 mg L^{-1}) (Shen *et al*., 2012). We used the EC_{25} of *I. zhanjiangensis* (about 18 μg L^{-1}) as the highest BaP concentration in the present experiment.

2.4 Preparation of Enzyme Extract

Eighty (80) mL of each culture was collected at day 1, 2, 3 and 4 by centrifuging at 6000 g for 10 min. The precipitate was re-suspended in 1 mL 0.1 mol L^{-1} phosphate buffer (pH 7.8) to extract crude enzymes with ultrasonic crushing method. Then the crude enzyme solution was centrifuged at 8000 g and 4℃ for 10 min, and the supernatant was collected and stored for further assay.

2.5 Enzyme Assay

7-ethoxy-different thiophene trazodone-deethylase (EROD) activity was measured with modified rapid termination fluorometry method (Pohl and Fouts, 1980). The concentrations of resorufin in the samples were calculated by comparing with the resorufin standards. One unit of EROD activity was expressed as 1 nmol resorufin per min per mg protein. Glutathione S-transferase (GST) activity was measured with the method of Habig *et al*. (1974). The GST activity was measured at 340 nm in a microplate reader. One unit of GST activity was expressed as 1 nmol 2,4-dinitrophenyl glutathione per min per mg protein.

Superoxide dismutase (SOD) activity was measured using improved pyrogallol autoxidation method (Zou, 1986). 1 U of SOD activity was defined as 50% inhibition of the autoxidation process in 1 min (U mg^{-1} protein). Catalase (CAT) activity was measured using ultraviolet absorption method (Hao, 2004). 1 U of CAT activity was defined as 50% decomposition of H_2O_2 in 1 min (U mg^{-1} protein).

Microalgal lipid peroxidation was measured by determining the secondary oxidation products malondialdehyde (MDA) content according to the improved thiobarbituric method (Wills, 1987). MDA content was expressed as

nmol per mg protein. Protein content was determined by Coomassie brilliant blue G-250 method (Bradford, 1976). The concentrations of protein in sample were calculated by comparing with the bovine serum albumin standards.

2.6 Data Processing

Difference between treatments and controls was tested using a one-way analysis of variance (ANOVA). The LSD test for *post-hoc* comparison of means was used to determine whether the difference among the treatments was statistically significant ($P < 0.05$). Data were log-transformed ahead of analysis. All statistical analyses were performed using SPSS 17.0.

3 Results

3.1 Effect of BaP on the Growth of Microalgae

Exposure of BaP at different concentrations and 0.01% of DMSO showed no inhibiting effect on the growth (measured as a cell density) of *I. zhanjiangensis* compared to the control in the first 2 days of incubation (Fig.1A). Two days later, the cell density of DMSO group decreased to values with no significant difference with the control. The cell density of mid and high BaP ($3\ \mu g\ L^{-1}$ and $18\ \mu g\ L^{-1}$) treated groups decreased to values with no significant difference with those of DMSO group. Interestingly, low BaP-treated group ($0.5\ \mu g\ L^{-1}$) showed a significant increase in cell density compared with control. In other words, no significant change on the growth of *P. subcordiformis* was observed in 4 days of exposure between control and DMSO and BaP-treated groups (Fig.1B).

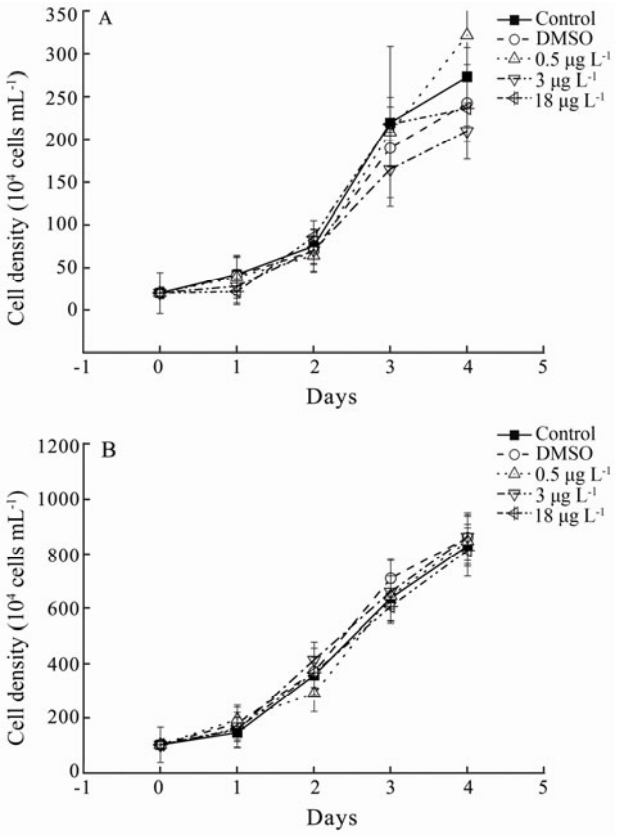

Fig.1 The growth curve of *I. zhanjiangensis* (A) and *P. subcordiformis* (B) in low concentrations of BaP. Bars represent the mean ± SD.

3.2 Effect of BaP on the Activities of Detoxification Enzymes

Phase I (EROD) and phase II (GST) detoxification activities in *I. zhanjiangensis* and *P. subcordiformis* are shown in Fig.2. No significant change of EROD activity in *I. zhanjiangensis* was observed in 4 days exposure between control and DMSO treatment. In low BaP-treated group, the EROD activity showed gradual increasing compared to that of group during exposure. In mid and high BaP-treated groups, the EROD activity reached the highest on day 2, and then dropped to levels even lower then DMSO-treated group on day 4. On the 2nd day, the EROD activity in mid and high BaP-treated groups was 1.43 and 1.2 times higher than that in DMSO group, respectively. And on the 4th day, the EROD activity of mid and high BaP-treated groups decreased to 83% and 72% of DMSO group.

EROD activity of DMSO-treated group in *P. subcordiformis* was increased slightly compared to control, which showed no significant difference during 4 days exposure. Similarly, in all BaP-treated groups, the EROD activity increased during the whole exposure, and peaked on day 4 compared to control. And on the 4th day, the EROD activity of low, mid and high BaP-treated groups was 1.43, 1.38 and 1.22 times higher than that of DMSO group.

GST activity in DMSO- and low BaP-treated groups in *I. zhanjiangensis* changed slightly, and remained at the similar level of control, which showed no significant difference during the exposure period. However, GST activity in *I. zhanjiangensis* exposed to mid and high BaP was higher than that of control on day 2, and reached the highest on day 3, and then dropped to that of DMSO-treated group on day 4. On the 3rd day, the GST activity of mid and high BaP-treated groups was peaked, which was 1.44 and 1.65 times higher than that of DMSO group, respectively.

GST activity in *P. subcordiformis* of DMSO-treated group increased slightly compared to control, which showed no significant difference during 4 days of exposure. Similar to the EROD activity in *P. subcordiformis*, the GST activity of all BaP-treated groups in *P. subcordiformis* increased during the whole exposure period, and peaked on day 4 compared to control. On the 4th day, the GST activity in low, mid and high BaP-treated groups was 1.14, 1.46 and 1.51 times higher than that of DMSO-treated group.

3.3 Effect of BaP on the Activities of Antioxidant Enzymes

The antioxidant enzyme (SOD and CAT) activities in *I. zhanjiangensis* and *P. subcordiformis* are shown in Fig.3. SOD activity in *I. zhanjiangensis* of DMSO-treated group changed slightly compared to control, which showed no

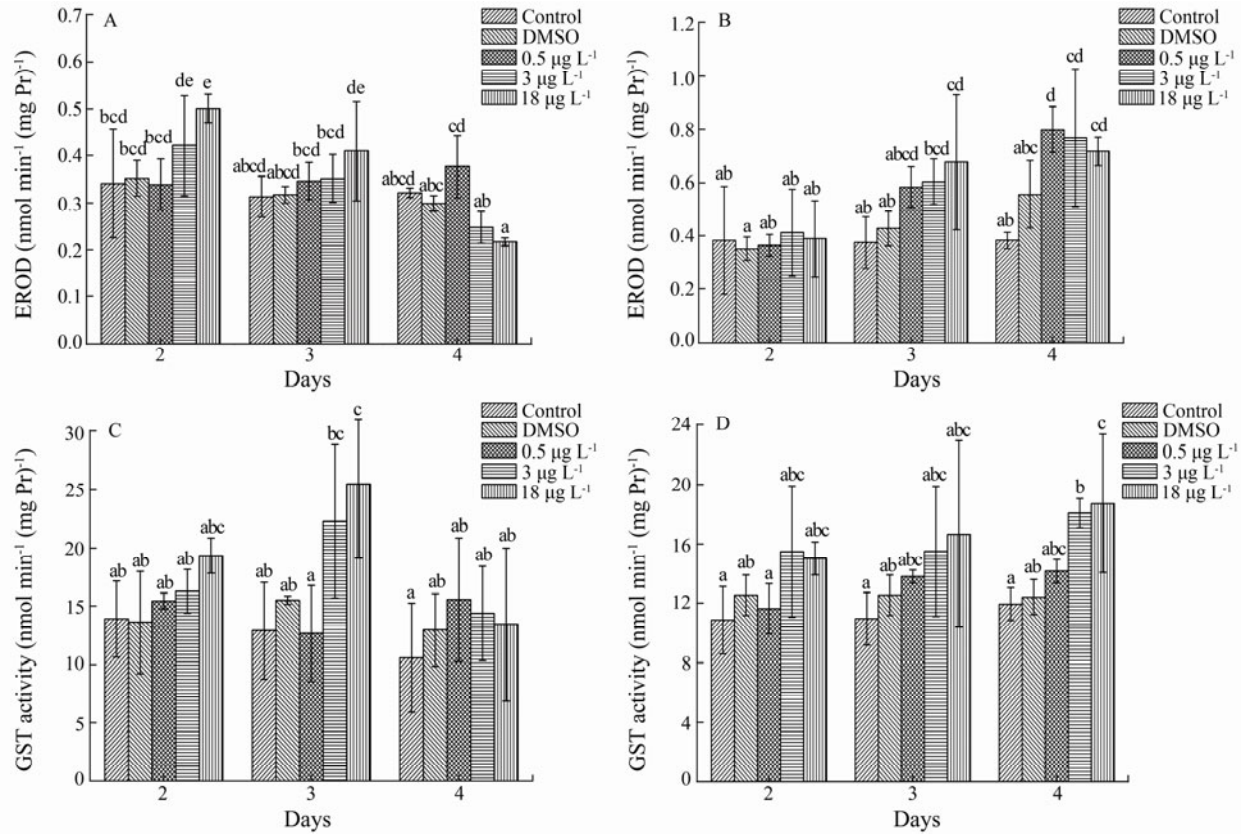

Fig.2 Effects of BaP on the activities of EROD and GST in *I. zhanjiangensis* (A, C) and *P. subcordiformis* (B, D). Bars represent the mean±SD. Significant differences exist among a, b and c ($P<0.05$).

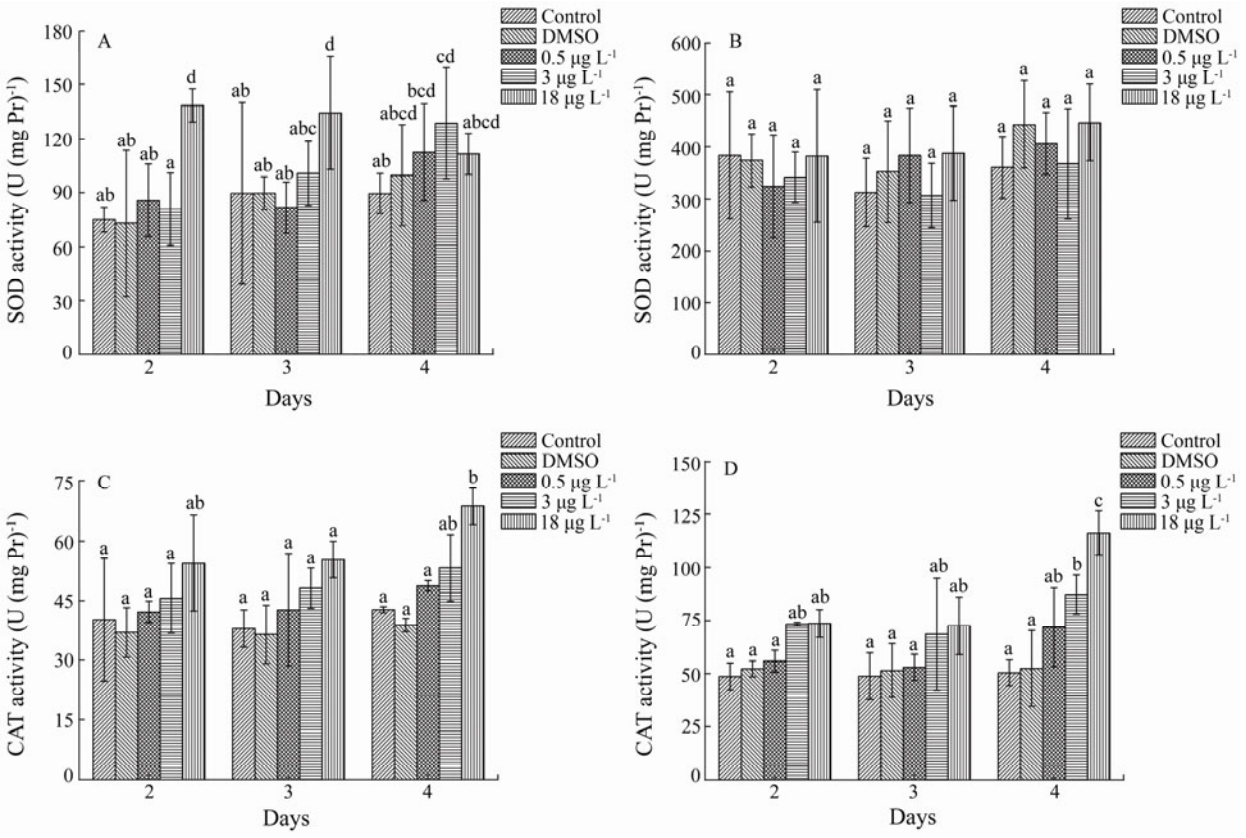

Fig.3 Effect ofBaP on the activities of SOD and CAT in *I. zhanjiangensis* (A, C) and *P. subcordiformis* (B, D). Bars represent the mean±SD. Significant differences exist among a, b and c ($P<0.05$).

significant difference during the 4 days of exposure. In low and mid BaP-treated groups, the SOD activity increased gradually and peaked on day 4 compared to control. While in high BaP-treated group the SOD activity reached the highest on day 2, and then dropped to a higher level on day 4 compared with DMSO-treated group. On the 2nd day, the SOD activity of high BaP-treated group was 1.90 times as high as that of DMSO group. And on the 4th day the SOD activity of low, mid and high BaP-treated groups was 1.13, 1.29 and 1.12 times higher than those of the DMSO-treated group, respectively.

No significant change of SOD activity in *P. subcordiformis* was observed in 4 days exposure between control and BaP-treated groups. However, SOD basal activity in *P. subcordiformis* was 4.29 times higher than in *I. zhanjiangensis*.

CAT activity of DMSO-treated group in *I. zhanjiangensis* decreased slightly compared to control, which showed no significant difference during the whole exposure. The CAT activity in all BaP-treated groups of *I. zhanjiangensis* increased during the whole exposure and peaked on day 4 compared to control. On the 4th day, the CAT activity of low, mid and high BaP-treated groups was 1.25, 1.37 and 1.77 times higher than that of DMSO group.

Unlike in *I. zhanjiangensis*, the CAT activity in *P. subcordiformis* of DMSO-treated group increased slightly compared to control, which showed no significant difference during the whole exposure. Interestingly, similar to the CAT activity in *I. zhanjiangensis*, the CAT activity in *P. subcordiformis* of all BaP-treated groups increased during 4 days exposure, and peaked on day 4 compared to control. On the 4th day, the CAT activity of low, mid and high BaP-treated groups was 1.37, 1.67 and 2.22 times higher than that of DMSO-treated group.

3.4 Effect of BaP on MDA Content

MDA content of *I. zhanjiangensis* of DMSO-treated group changed slightly compared to control, which showed no significant difference during 4 days exposure. No significant differences were observed in 0.5 and 3 μg L^{-1} BaP treatment groups during the exposure ($P>0.05$). The MDA content of the highest BaP-treated group significantly increased on day 3 and day 4 compared to control group ($P<0.05$).

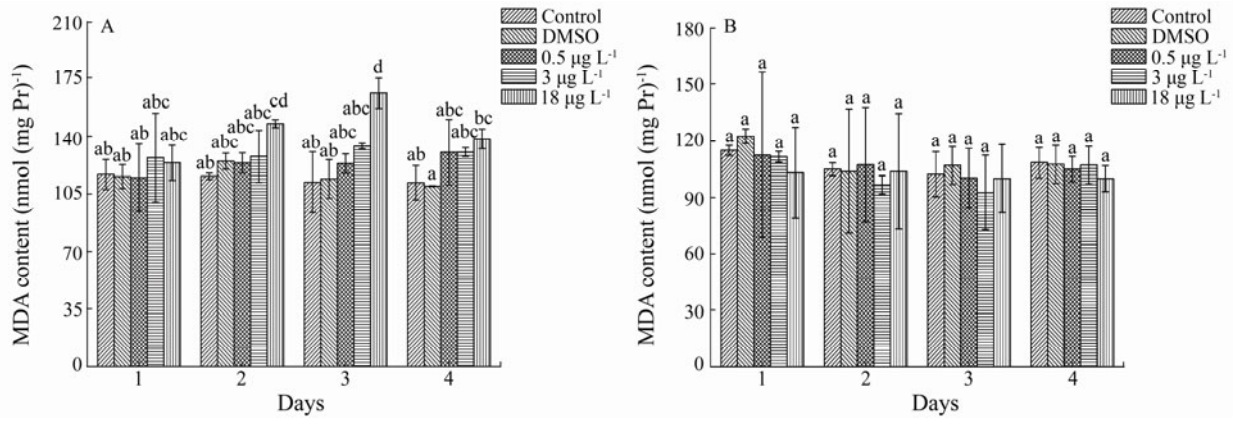

Fig.4 Effect of BaP on the content of MDA in *I. zhanjiangensis* (A) and *P. subcordiformis* (B). Bars represent the mean± SD. Significant differences exist among a, b and c ($P<0.05$).

4 Discussion

In recent years, there has been an increasing concern on the widely spread contaminants, PAHs, due to their high toxic potential to living organisms. In China, PAHs pollution has been a serious problem since they were detected in many sites of Chinese coastal at a concentration range from 0.001 to 4.799 μg L^{-1} (Qiu *et al*., 2004). Marine phytoplankton forms the basis of the marine food chain and is essential for the normal functioning of ecosystems (Li *et al*., 2006). Algae are increasingly used as ecological indicators of polluted aquatic environments due to their pivotal importance as primary producers (Li *et al*., 2009). It is well known that the sensitivity of microalgal species to various toxicants was highly species-specific (Gao and Tam, 2011). In previous studies, we compared 96 h-EC_{50} of BaP to *I. zhanjiangensis* (32.36 μg L^{-1}) and *P. subcordiformis* (>1 mg L^{-1}). We used the EC_{25} of *I. zhanjiangensis* (about 18 μg L^{-1}) as the highest BaP concentration in present experiment. In the present study, lower concentrations of BaP exposure (<18 μg L^{-1}) did not significantly inhibited the growth of two algae.

It is well known that the biotransformation of BaP is a complex process that needs phase I and phase II enzymes (Brammell *et al*., 2010). The cytochrome P450 enzyme system (such as EROD) acts in phase I metabolism by adding functional groups of non-reactive xenobiotics, providing them with reactive sites capable of conjugating to water-soluble groups through the phase II metabolism (such as GST) in order to be excreted (da Silva Rocha *et al*., 2012). The increase of EROD activity usually indicates the activation of phase I enzymes, while the activity of GST can be activated when exposed to lipophilic xenobiotics (van Oosterom *et al*., 2010). David *et al*. (2009) reported that the EROD activity of *Chlamydomonas reinhardti* was increased 20% when exposed to genotoxicants for 48 hours over DMSO group. Ren *et al*. (2014)

showed that tissue-specific function and metabolic rate can influence EROD and GST activities. Mofeed *et al*. (2013) found that the GST activity of *Scenedesmus obliquus* increased after 24, 48 and 96 h of exposure to both fenhexamid and atrazine. Lei *et al*. (2003) also found that the GST activity in *Scenedesmus platydiscus* and *Selenastrum capricornutum* increased significantly, but that in *Chlorella vulgaris* showed no significant change, and that in *Scenedesmus quadricauda* remarkably decreased at high pyrene concentrations. In present study, the EROD and GST activities in *P. subcordiformis* increased in all BaP-treated groups during the whole exposure, and peaked on day 4. However, the EROD activity in *I. zhanjiangensis* gradually increased in low BaP-treated group, while in mid and high BaP-treated groups the EROD activity reached the highest on day 2, and then dropped down to levels even lower than that of DMSO-treated group on day 4. Similar changes were found in GST activity in *I. zhanjiangensis*. In low BaP-treated group, it changed slightly, while in mid and high BaP-treated groups it peaked on day 3, and then dropped down to that of DMSO-treated level on day 4. These results suggested that there seems to be an induction mechanism in these two algae, and further studies are needed.

ROS plays an important role in host defense, but the residuals of ROS can cause oxidative damage (Ren *et al*., 2014). SOD and CAT are two important enzymes for ROS scavenging. The activity of these enzymes may be enhanced following exposure to moderate environmental stresses (Wang *et al*., 2012). Qian *et al*. (2010) reported that both SOD and CAT activities were increased in *Chlorella vulgaris* and *Microcystis aeruginosa* after streptomycin exposure for 24 hours. Li *et al*. (2006) found that the SOD activity of *Pavlova viridis* enhanced after copper treatments, while it was reduced after zinc exposure, and both copper and zinc stimulated the activity of CAT. Gao *et al*. (2011) also found that the SOD activity of *Euglena gracilis* was not significantly different among all treatments, expect for the organic pollutants extractions from western Taihu Lake. Sáenz *et al*. (2012) found that the CAT activity increased 23%–33% in low concentrations of cyfluthrin treatments, and suggested that the CAT activity can act as a potential biomarker of examining pollution. Our results were in accordance with the findings of this study. In our study, the CAT activity in *I. zhanjiangensis* and *P. subcordiformis* increased during the 4 days exposure, and peaked on day 4 in all BaP-treated groups. However, for the SOD activity, the patterns of change varied from species to species. Interestingly, the SOD basal activity of *P. subcordiformis* was 4.29 times higher than that of *I. zhanjiangensis*. In *I. zhanjiangensis*, the SOD activity increased gradually and peaked on day 4 in low and mid BaP-treated group, while in high BaP-treated group, it reached the highest on day 2, and then dropped down to the higher on day 4 compared with DMSO-treated group. However, there was no significant change observed in 4 days exposure between control and BaP-treated groups in *P. subcordiformis*. These studies indicated that the antioxidants enzymes can increase in low concentrations of pollutants, but can be restrained in high concentrations.

Stress conditions can disturb the balance between the production of ROS and the quenching activity by antioxidants, which may result in LPO (Wang *et al*., 2012). MDA quantification is the way of evaluating LPO level (Ren *et al*., 2014). Yang *et al*. (2012) reported that the MDA content of *Palmellococcus miniatus* increased after treated with volatile oil for 24 or 48 h. And Chen *et al*. (2012) found that the MDA content of *Chlamydomonas reinhardtii* treated with nano-TiO_2 showed a typical bell-like curve according to processing time. Jiang (2010) reported that the MDA content of *Scenedesmus obliquus* and *Microcystis aeruginosa* treated with naphthalene showed no significant difference. Cima *et al*. (2013) found that the LPO activity was not significantly affected by Sea-Nine 211 in *Sarcophyton cf. glaucum*. In present study, the MDA content in *I. zhanjiangensis* increased gradually and remained at a high level on day 4 in low and mid BaP-treated groups, while in high BaP-treated group it peaked on day 3, and then dropped down to the higher on day 4 compared with control. However, in *P. subcordiformis* there was no significant difference in MDA content between control and BaP-treated groups (Cima *et al*., 2013). Enhanced lipid peroxidation in aquatic animals was responsive to toxicants and was a direct indicatior of oxidative stress and hence quantification of MDA is the way to evaluate the LPO level. In our results, no difference in MDA content indicated that the exposure period and/or the concentrations of BaP were not sufficient to trigger membrane oxidative damage.

In this study, under the same condition, the EROD, GST and SOD activities and MDA content varied between *I. zhanjiangensis* and *P. subcordiformis*. These results suggested that BaP can be metabolized, and can cause oxidative stress in these two algae, while their response was species-specific. In previous study, we found that *I. zhanjiangensis* (96 h-EC_{50} =32.36 μg L^{-1}) was the most sensitive to BaP toxicity, while *P. subcordiformis* (96 h-EC_{50} >1 μg L^{-1}) was more resistant to BaP. In present study, the increasing activities of EROD and GST suggested that they may play important roles in detoxification of BaP in both algae. Furthermore, the EROD and GST activities in *I. zhanjiangensis* with higher BaP treatment decreased, probably due to BaP toxicity. It was hypothesized that the algal species with more tolerance would exhibit higher detoxification enzyme activities. Similar to detoxification enzyme, the activity of antioxidant enzyme (CAT) increased with BaP concentration, indicating that they were efficiently removing excessive ROS. But, the SOD activity in *P. subcordiformis* did not show any change. No difference in MDA content was observed, indicating that the exposure period and/or the concentration of BaP were not sufficient to trigger membrane oxidative damage, which may due to the rapid and efficient scavenging of ROS in *P. subcordiformis*. The MDA content in *I. zhanjiangensis* increased in all BaP-treated groups, but in high BaP-treated group, it dropped to the higher level at day 4 compared with control. The

increasing MDA content indicated the damaging of membrane in *I. zhanjiangensis*. The decreasing MDA content in high BaP-treated group may be explained by the destruction of cells. All the results confirmed that the more tolerant algal species exhibit higher detoxification and antioxidant enzyme activities.

In summary, in the present study, the activities of metabolic relative enzymes (including detoxification and antioxidant enzymes) in microalgae were changed in exposure to BaP, while no significant change of growth rate was observed in lower BaP concentration (<18 μg L^{-1}). The algal species that are more tolerant to BaP exhibit higher detoxification and antioxidant enzyme activities. We considered that the metabolic related enzymes (EROD, GST and CAT) in both of pollution-sensitive and pollution-resistant algae increased under lower BaP concentrations. All of these parameters can potentially be sensitive biomarkers indicating the contamination level of BaP in marine water.

Acknowledgements

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