eJBio

Electronic Journal of Biology, 2007, Vol. 3(4): 80-86

Toxic response of dimethyl phthalate (DMP) to *Gracilaria lemaneiformis*

Jiang Yu*, Cuichan Yang, Ri'An Yu, Guya Fu

School of Public Health, Guangdong Pharmaceutical University, Guangzhou, Guangdong 510006, China

*Corresponding author: Tel: +86 (0)2034055924; Fax: +86 (0)2034055355; E-mail: rainblue02@163.com

Abstract

Physiological and biochemical effects of dimethyl phthalate (DMP) on *Gracilaria lemaneiformis* were studied under experimental and ecological conditions. The results showed that chlorophyll a (Chla) and phycoerythrin (PE) contents increased obviously with the prolonging of exposure time, soluble protein content and catalase (CAT) activity increased and propyldialdehyde (MDA) content decreased in short time under low DMP concentration (≤0.1mg/L), *G.lemaneiformis* grew well; And that Chla and PE contents decreased obviously with the rise of DMP concentration and the prolonging of exposure time, soluble protein content and CAT activity decreased and MDA content increased distinctly, the chloroplast swelled up and decreased in number, the thylakoids increased in number, arranged confusedly and dissolved partly, the number of starch grains decreased. *G.lemaneiformis* grew badly under DMP concentration exceeding 0.3mg/L; during the exposure of 20 days, peroxidase (POD) activity was inhibited continually, and there was time-effect relationship between glutathione peroxidase (GSH-PX) activity and the exposure time. The CAT and GSH-PX of *G.lemaneiformis* were sensitive to DMP, so they could be regarded as two referent index of biological monitoring exposed to DMP.

Keywords: *Gracilaria lemaneiformis*; dimethyl phthalate; catalase; ultrastructure

1. Introduction

Phthalic acid esters (PAEs), commonly known as phthalates, is the world's widespread use of synthetic organic compounds, but also has become a global pollutant. In which dimethyl phthalate (DMP), diethyl phthalate (DEP) are ranked by the United States, China, Japan and many other countries as a key to control pollutants in the environment [1]. Biological experiments indicated that PAEs had the general toxicity of poison, but also the toxicity of endocrine disruptors, reproductive and developmental toxicity and carcinogenic, teratogenic and mutagenic toxicity [2]. PAEs for the aquatic toxicity studies abroad have more reports, mainly on the effects of the PAEs on the growth, reproduction function of zooplankton, fish and other sensitive biology [3,4]. PAEs right on the ecosystem primary producers - microalgae whose toxicity and ecotoxicological research at home and abroad have also been reported [5], but for large algae, rarely reported in the literature, especially for *G.lemaneiformis*, physiological and biochemical effects of environmental factors and various chemical pollutants on it still have been little. *G.lemaneiformis* (Gracilaria, Rhodophyta), as an important major economic seaweed, has suffered from marine environmental pollution increasingly. Accordingly, in the experiment, the toxic response of *G.lemaneiformis* to DMP has been researched to explore the toxicity mechanisms phthalate esters on large algal, and provide the reference for the protection of marine resources and environmental biological monitoring.

In this paper, we report on the differential gene expression during the internode elongation of basal stem of wheat plant. Three differentially expressed cDNA fragments were cloned and sequenced, and possible roles in internode elongation were discussed.

2. Materials and Methods

2.1. Material sources and pre-cultivation

The *G.lemaneiformis* used in this study was taken from Nanao Cultivation Zone in Guangdong Province. After transferred to our laboratory, it was cultured temporarily in indoor tanks under the condition of temperature adaptation period of 20±1°C, light intensity of 22 μmol photons $m^{-2}s^{-1}$, salinity of 30‰, photon period of 12: 12h LD and pH value of 8.0.

2.2 Experimental methods

Using the filtration natural seawater and DMP as cultured medium, DMP was set three concentration gradient groups including low concentration group (0.1mg/L), middle concentration group (0.3mg/L) and high concentration group (1.0mg/L), another set up a control group (acetone + natural seawater), about 3g *G.lemaneiformis* was cultured continually

in 1000ml cultured medium. The solution was renewed every 3 days to maintain the DMP concentration and species during the culture period. The treatment was repeated triplicately. At the firth, 5th, 10th, 20th day, the specific growth rate (SGR), phycoerythrin (PE), chlorophyll a (Chla), soluble protein content, peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) of *G.lemaneiformis* were determined.

After treatment with different concentration of DMP for 7days, the same part selected was fixed with 3%(V/V) glutaraldehyde and 1%(W/V) osmium tetroxide, then were dehydrated through an ethanol series and embedded in Spur resin. The ultra-thin sections were cut using a diamond knife and a microtome (LKB-V). The sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (J EOL-100CX).

2.3 Determination of physiological and biochemical indicators

The SGR (%.d^{-1}) was calculated from the equation ln (Wt/Wo)/t×100%, where Wo was the initial fresh weight (g), Wt was t days later fresh weight (g). The contents of PE, Chla, soluble protein and MDA were determined using the light Duke method [6], Moran reference method [7], Coomassie Brilliant Blue method and thiobarbituric acid method [8] respectively. The activities of POD and CAT were determined according to Guaiacol, UV absorption spectrometry respectively [8].

2.4 Statistics and analysis

The data were expressed as the mean values F standard deviation (SD). Statistical significance of the data was tested with analysis of variance (ANOVA) or *t*-test. The significance level was set at 0.05 or 0.001.

3. Results

3.1. The effect of DMP on the SGR of *G.lemaneiformis*

The SGR of *G.lemaneiformis* was significantly different among different concentration of DMP (Figure 1). The SGR in low concentration of DMP group increased by 2.58% compared with the control group, but there was no significant difference (P>0.05) between the two ones; the SGR of mid/high concentration of DMP declined (P<0.01), and were much lower than those of the low concentration of DMP and the control groups, decreased by 300%, 453.78% respectively compared with the control group. From figure 1, it was also indicted there was negative correlation between the SGR and DMP concentration during the developing period ($y=-0.0465x^2+0.0369x+0.1505$, $R^2 = 0.9416$, P<0.05).

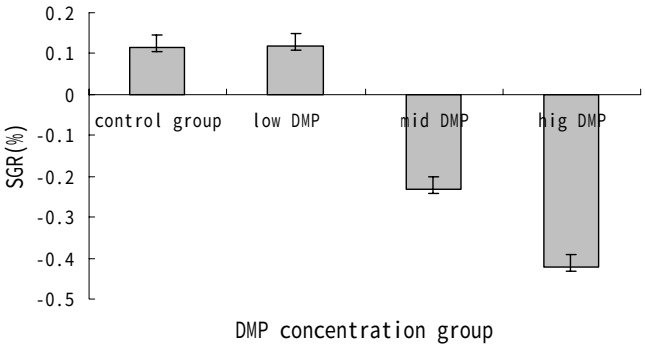

Figure 1. The effect of DMP on the SGR of *G.lemaneiformis.*

3.2 The effects of DMP on biochemical characteristics of *G.lemaneiformis*

The effects of different concentration of DMP on Chla of *G.lemaneiformis* were different (Table 1). Chla content increased gradually with the prolonged exposure time in low DMP concentration, and there was a significant positive correlation (y=0.0028x2-0.0083x+0.1823, R2=0.9932). Chla content showed downward trend with the cultivation time in mid/high concentration of DMP and there was a significant negative correlation (y=-0.0137x+0.193, R2=0.9569; y=-0.0193x+0.1945, R2=0.9768). When the cultivation time was more than 10 days, Chla content decreased significantly especially in high concentration of DMP (P<0.05, compared with the control group), but decreased obviously at 20th day (P<0.001, compared with the control group).

The PE content gradually increased with the prolong of exposure time in low DMP concentration, and reached 0.352mg/g at 20th day, there was a significant positive correlation (y=0.0065x+0.3265, R2=0.9929). PE content was on downward trend with the exposure time in mid/high concentration of DMP, and there was a significant negative correlation (y=-0.0261x+0.3505, R2=0.9808; y=-0.0246x+0.34, R^2=0.9189), while the exposure time was over 10 days, PE content was significantly dropped (P<0.001, compared with the control group) (Table 1).

The soluble protein content first increased and then dropped with the prolong of exposure time in low DMP concentration, the difference was not significant (P>0.05) compared with the control group (Table 1). Soluble protein content was on downward trend with the exposure time in mid/high concentration of DMP and there was a significant negative correlation (y=-0.1116x+1.5105, R^2=0.8421; y=-0.1083x+1.449, R^2 = 0.9487). When the cultured time was more than10 days, soluble protein content decreased significantly in high concentration of DMP (P<0.05, compared with the control group). The soluble protein content was distinctly decreased under the mid/high concentration of DMP at 20th day (P<0.05, compared with the control group).

Table1. Comparison of Chla, PE and soluble protein contents in G. lemaneiformis under different DMP concentration (Means ± SD).

index	cultivation time	DMP(mg/L)			
		control group	low DMP group	middle DMP group	high DMP group
Chla (mg/g)	1d	0.178±0.006	0.177±0.004	0.176±0.001	0.173±0.005
	5d	0.176±0.004	0.175±0.003	0.169±0.007	0.161±0.006
	10d	0.165±0.008	0.163±0.004	0.155±0.006	0.133±0.002
	20d	0.151±0.004	0.152±0.006	0.135±0.008	0.118±0.001
PE (mg/g)	1d	0.337±0.005	0.333±0.008	0.321±0.002	0.315±0.004
	5d	0.33±0.003	0.339±0.006	0.305±0.001	0.298±0.002
	10d	0.326±0.008	0.347±0.001	0.269±0.003	0.253±0.001
	20d	0.316±0.011	0.352±0.01	0.246±0.004	0.248±0.008
soluble protein (%)	1d	1.435±0.015	1.489±0.016	1.362±0.015	1.319±0.033
	5d	1.411±0.014	1.516±0.013	1.302±0.009	1.245±0.014
	10d	1.349±0.016	1.354±0.014	1.257±0.008	1.164±0.015
	20d	1.305±0.015	1.169±0.016	1.005±0.014	0.985±0.015

3.3 The effects of DMP on antioxidant index of *G.lemaneiformis*

The CAT activity of different concentration of DMP showed first declined then rose with the prolonged time of exposure (Figure 2). CAT activity in low concentration of DMP reached the maximum at the 5th day (205.822μ/(g.Fw)/min), but there had no significant difference compared with the control group during the entire developing period (P>0.05). In mid/high concentration of DMP, CAT activity in the early developing stage (≤1d) reached the maximum (P<0.05, compared with the control group), then decreased gradually, especially dropped more significantly at the 20th day (P<0.05, compared with the control group).

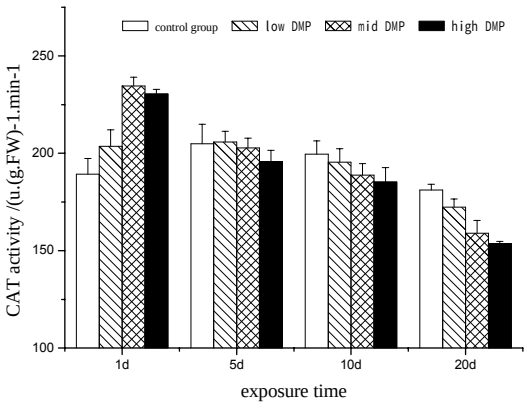

Figure 2. The effect of DMP on CAT activity of *G.lemaneiformis*.

When the cultivation time was less than 10 days, POD activity in low/mid concentration of DMP dropped slowly with the exposure time, but the difference was not significant (P>0.05) compared with the control group; while the cultivation time was more than 20 days, POD activity decreased obviously, particular in mid concentration of DMP (P<0.05). During the entirely developing period, POD activity decreased significantly with the prolong of exposure time in high concentration of DMP (P <0.05, compared with the control group), and there was a significant negative correlation (regression equation: $y=-2.8754x+50.609$, $R^2=0.9639$) (Figure 3).

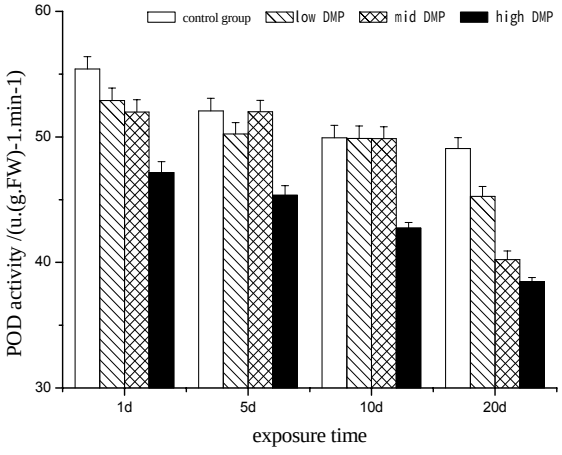

Figure 3. The effect of DMP on POD activity of *G.lemaneiformis*.

The GSH-PX activity of different concentration of DMP first declined then rose and dropped finally compared with the control group (Figure 4). There was a significant parabolic type time-effect relationship between GSH-PX activity and the exposure time (the regression equation of low/mid/high concentration of DMP as followed respectively:

$y=-127.81x^2+659.49x+255.82$, $R^2=0.8363$;

$y=-124.53x^2+654.84x+179.83$, $R^2=0.9851$;

$y=-181.07x^2+923.59x-164.45$, $R^2=0.9739$. GSH-PX activities all dropped obviously, decreased by 29.53%, 34.97%, 47.76% respectively compared with the control group at the early exposure period (≤1d), but increased significantly again at 5th day and 10th day , and the difference was not significant compared with the control group ($P>0.05$); GSH-PX activity decreased significantly in all concentrations of DMP particularly in high concentration of DMP ($P<0.05$) when the exposed time was more than 20 days, but still were slightly higher than that of the initial culture.

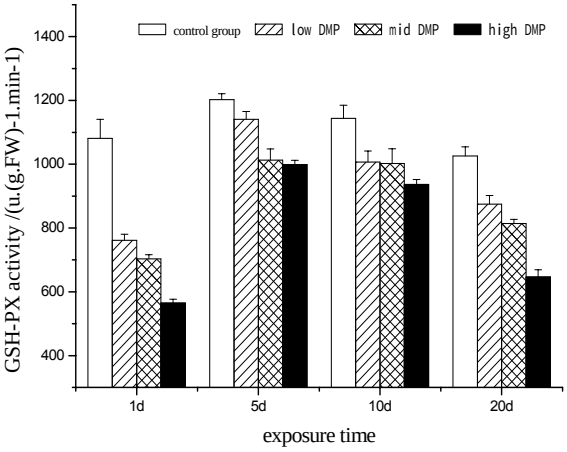

Figure 4. The effect of DMP on GSH-PX activity of *G.lemaneiformis.*

The MDA content decreased first and then increased slowly in low concentration of DMP, there had no significant difference compared with the control group ($P>0.05$). MDA content generally showed an upward trend in mid/high concentration of DMP (≤5 days) increased marginally at the early cultured period, as 2.23%, 5.75% of the control group respectively, But more than 10 days, the MDA content significantly increased ($P<0.001$, compared with the control group). There was a significant positive correlation between DMA content and cultivation time in mid/high concentration of DMP during the whole culture period ($y=2.5087x+15.556$, $R^2=0.9893$; $y=2.566x+16.11$, $R^2=0.9972$) (Figure 5). It was also showed from figure 8 that the changed trend of DMA content in different concentration of DMP contrary to the change of growth rate, PE / Chla.

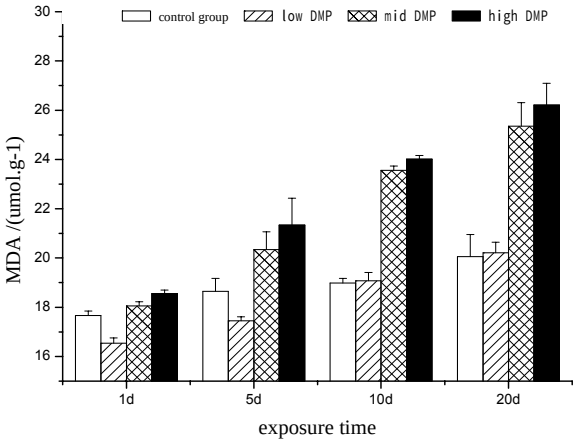

Figure 5. The effect of DMP on MDA content of *G.lemaneiformis*

3.4 The effect of DMP on the chloroplast ultrastructure of *G.lemaneiformis*

Under normal condition, the chloroplasts of *G.lemaneiformis* showed regular shape of rotundity or long ellipse, dense stroma, clear membrane, and its grama thylakoids were concentrically parallel to chloroplast envelope (Figure 6A). After treatment with different DMP concentration, the chloroplast ultrastructure changed obviously. The chloroplast began to swelled up and decreased in number, the thylakoids increased in number and arranged confusedly slightly at DMP of 0.3mg/L (Figure 6B), while the number of chloroplasts decreased obviously, the chloroplast envelope disappeared partly, the thylakoids swelled up and dissolved partly, the number of starch grains decreased at DMP of 1.0mg/L (Figure 6C).

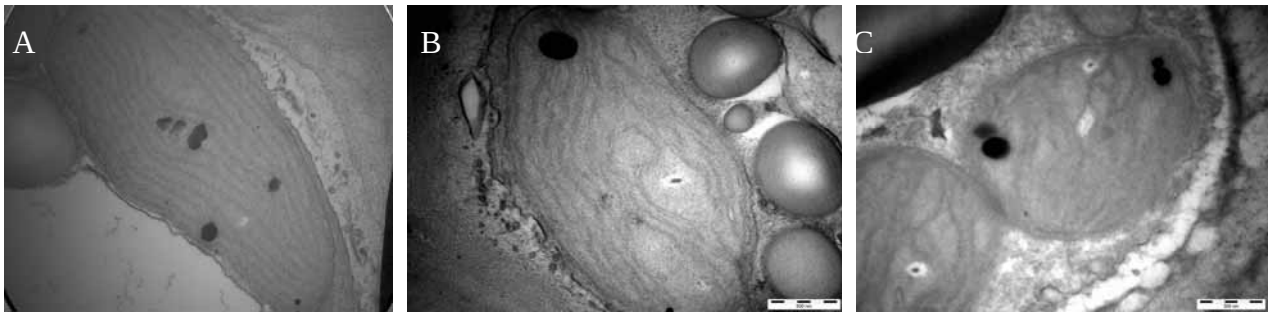

Figure 6. A: Ultrastructure of the chloroplast of control group, ×65000. B: Ultrastructure of the chloroplast after 0.3mg/L of DMP treatment for 7d, ×39000. C: Ultrastructure of the chloroplast after 1.0mg/L of DMP treatment for 7d, ×39000.

4. Discussion

4.1 The effect of DMP on the Growth of *G.lemaneiformis*

The results of this study showed that the growth rate of *G.lemaneiformis* was affected by DMP concentration and exposure time under the same conditions including temperature, salinity, light and nutrients, pH and other environmental factors. Low concentration of DMP could promote *G.lemaneiformis* grow to some extent, which had come to consistent conclusions as many researchers [9-10] found that low concentration of organic pollutants on the algae was not poisonous but acted as the role of promoting. Stebbing [11] considered that it was a biological plus phenomenon gained as "the excitatory effects of poison", and also a self-protection mechanism. That organic pollutants promoted algae produce "excitement effect" was because poison and degradation processes existed at the same time in low concentration, in which degradation process dominated, so that pollutants did small harm to algae. Generally high concentration of pollutant on the algae was often a limiting factor; it also accorded Shelford's law of tolerance [12]. Once exceeding the tolerance limit, it would have toxicological effects on the algae, such as inhibiting photosynthesis, respiration and nitrogen fixation, which inhibited the growth of algae in the end. Our study found that when the concentration of DMP was more than 0.3mg/L, *G.lemaneiformis* growth rate decreased significantly, this also confirmed the view.

4.2 The effects of DMP on biochemical index of *G.lemaneiformis*

Phycobiliprotein is found in abundance in the light-harvesting chromoprotein (including phycoerythrin, phycocyanin and allphycocyanin) of red algae (Rhodophy-ta). Phycobiliprotein could transfer the captured light energy to Chla efficiently, which makes the photosynthesis to occur [13]. Chla content as biological indicators of pollution is very common in the toxicology studies of organic pollutants affecting on microalgae [10]. Organic pollutants could change the Chla content of algae, which implied to affect photosynthesis. When *G.lemaneiformis* exposed to 0.1 mg/L of DMP within 20 days, Chla content was on the rise. While DMP concentration was higher than 0.1 mg/L, Chla content reduced increasingly with the stress intensity and the prolonged time of exposure, whose reasons might have three aspects: Firstly, excessive concentration of DMP interfered pigment synthesis, secondly, it aroused pigment degradation, thirdly, high concentration of DMP induced the color mutant of algae to emergence [14], but which one played the specific performance of a mechanism was still need further study. The experimental results proved that there were obvious time-effect and dose-response relationships between DMP concentration and Chla content, which showed Chla could be one physiological monitoring indicator as *G.lemaneiformis* exposed to DMP.

Soluble protein in seaweed was one of the most important indicators to explore algae metabolism. Increasing its content contributed to the maintenance of normal cell metabolism and improved the resistance of the seaweed. However, certain concentration of organic pollutants would change the pattern of protein synthesis, such as inducing algae endonuclease and protease activity, then decrease protein content [10]. In this experiment the soluble protein content in low concentration of DMP reached a peak value at the 5th day, the reason might be that *G.lemaneiformis* started the self-protection mechanism to increase the cellular concentration and the amount of functional protein through increasing soluble protein content, thus contributed to the maintenance of the normal algae cell metabolism. But when exposed to pollution for 10days, soluble protein content decreased again, which due to cell damage had reached a critical level, and protein synthesis had come to damage [10]. In mid/high concentration of DMP group soluble protein content continued to drop as the exposure time extended, which showed algae cell would be jeopardized in a relatively short period, thereby reduced the algae resistance and inhibited the growth as DMP concentration exceeded 0.1 mg/L.

4.3 The effect of DMP on antioxidant index of *G.lemaneiformis*

Plant antioxidant system had the capability of protecting the cells from oxidative stress [15], and through its inhibition of activity-oxygen generation or mitigation damage of reactive oxygen species could alleviate the body oxidative stress.

POD, as a protein containing Fe, could catalyze peroxidase phenol reaction and play an important role in plant respiratory metabolism and physiological resistance. CAT could be effective in catalytic decomposition of H_2O_2, and protect the body from damage. By taking POD, CAT as pollution monitoring markers had been searched in some literature [16], but those reports about the effects of the pollutants on seaweed, especially for large algae were still few [17]. Our study found that when the exposure time was less than 10 days, The POD activity of *G.lemaneiformis* in low concentration of DMP declined slowly, but when the exposure time was greater than 10 days, the POD activity decreased significantly. Different concentrations of DMP made the CAT activity of *G.lemaneiformis* first rise and then drop with the extension of exposure time. For low concentration of DMP, it could induce CAT activity in a short period of time, but inhibit CAT activity with longer exposure. The reason might be that the algae had some

tolerance stress on the environment, which made antioxidant system produce the adaptive induced response in less pollution stress in order to reduce or eliminate the pollution caused by oxidative stress, but serious pollution stress would inhibit antioxidant system work well, which resulted in decreasing POD, CAT activities, thus affecting algae cell metabolism and physiological processes, reducing cytochrome, stopping cell division, destroying cell contents, distorting cell morphology, finally, it caused the algae to loss the anti-stress capability.

Under stress conditions, reactive oxygen species metabolism of the plant was imbalance, and excessive reactive oxygen species could occur membrane lipid peroxidation, then injury and destruct the cell membrane. Malondialdehyde (MDA), as a cell lipid peroxidation product, could measure membrane peroxidation level by its content [13]; the results also confirmed this point. When DMP concentration was below 0.1mg/L , MDA content dropped at the beginning of culture then increased with the prolonged exposure time and the concentration of DMP, which contrary to the changed trend of the growth rate.

As the photosynthesis cell organ of *G.lemaneiformis*, the chloroplast transformed the absorbed light energy to chemical energy, and deposited in carbohydrate. The thylakoid membranes contained the enzyme system demanded by photosynthesis pigment and dark reaction, and its arranged mode of thylakoid ensured absorbed light area to the greatest extent, which prompted the photosynthesis go through [18]. After treatment with DMP, and with the increase of DMP concentration, the thylakoid changed in number, even ruptured and dissolved, which decreased photosynthesis area, meanwhile, DMP affected the activity of photosynthesis enzyme system, and caused photosynthesis to be habited, further resulted in cell dead in the end.

5. Conclusion

(1) Under exposed to low concentration of DMP (≤0.1mg/L), *G.lemaneiformis* grew well, Chla, PE contents increased with the prolonged exposure time, but MDA content showed a downward trend. When DMP concentration was more than 0.1mg/L, Chla, PE contents were obvious downward trend with DMP concentration and the exposure time, on the contrary there was a clear upward trend at MDA, *G.lemaneiformis* growth was significantly inhibited.

(2) Low concentration of DMP (≤0.1mg/L) in a short time could increase the soluble protein content of *G.lemaneiformis*, subsequently converted to inhibition; high concentration of DMP (≥0.3mg/L) declined the soluble protein content with the prolonged exposure time within 20 days.

(3) POD activity almost continued to be restrained with the increase of DMP concentration and the extended exposure time. Low concentration of DMP (≤0.1mg/L) could induce the formation of CAT activity in a short period of time, but inhibit CAT activity under exposing for a long period and high concentration of DMP, which suggested The CAT of *G.lemaneiformis* was sensitive to DMP, so it could be regarded as one referent index of biological monitoring exposed to DMP.

(4) Upper DMP concentration damaged the chloroplast ultrastructure of *G.lemaneiformis* evidently, habited the activity of photosynthesis enzyme system, which restrained *G.lemaneiformis* grow.

Acknowledgements

This research was supported in part by the National Natural Science Foundation of China (Grant No. 40473046).

References

[1] Emiko, K., Eisuke, F. (2002) Role of oxidative stress in germ cell apoptosisbydi (2-ethylhexyl) phthalate. *Biochem J*, **365**: 849-856.

[2] Van, W.A., Van, V., Posthumus, R., et al. (2000) Environmental risk limits for two phthalates with special emphasis on endocrine disruptive properties. *Ecotoxico Environ Safety*, **2**: 46-52.

[3] Kuang, Q.J., Zhang, W.Y., Deng, P. (2003) Studies on the toxic efficiency of dibutylphthalate to scenedesmus obliquus and natural algae. *Acta Hydrobiologica Sinica*, **1**: 103-105.

[4] Chen, W.L., Sung, H. (2004) The toxic effect of phthalate esters on immune responses of giant fresh water prawn (Macrobrachium rosenbergii) via oral treatment. *Aquatic Toxicology*, **74**: 160-171.

[5] Qiu, H.Y., Wang, X. (2005) Toxic effects of phthalic esters on algae. *Journal of Xiamen University (Natural Science)*, **44**(2): 294-296.

[6] Duke, C.S., Litaker, W., Ramus, J. (1989) Effect of temperature, N supply, and tissue N on ammonium uptake rates oft he Ulva curuata and Codium decorticatum. *J. Phycol*, **25**: 113-120.

[7] Moran, R. (1982) Formulae for determination of chlorphyllous pigments extracted with N, N-dimethylformamide. *Plant physiol*, **69**:1376-1381.

[8] Hao, Z.B., Chang, J., Xu, Z. (2004) *Laboratory of plant physiology*. Harbin Institute of Technology Press, China, pp. 30-53.

[9] Feng, S., Lu, X.Z., Yu, W.G. (2004) Biochemical and physiological effects of adversity stress on porphyra yezoensis. *Transaction of Oceanology and Limnology*, **3**: 22-26.

[10] Li, Q., Wei, F.Q., Chen, J.X., et al. (2004) Physiological effects of methmidophos and phoxim on Porphyra haitanensis. *Journal of Fisheries of China*, **2**: 110-113.

[11] Stebbing, A.R.D. (1982) Hormesis the stimulation of growth by low levels of inhibitors. *Sci. Tol, Environ*. **22**: 213-234.

[12] Yan, H., Ye, C.M., Yin, C.Q. (1995) Kinetics of phthalate ester biodegradation by chlorella pyrenoidosa. *Environ. Toxicol. Chem*. **14**(6): 931-935.

[13] Sui, Z.H., Zhang, X.C. (2000) Cloning and analysis of phycoerythrin genes in Gracilaria lemaneiformis (Rhodophyceae). *Chinese Journal of Oceanology and Limnology*, **18**(1): 42-46.

[14] Lin, G.H., Zhang, X.Q., Wu, C.Y. (1990) The mutagenesis of benzo (a) pyrene in porphyra yezoensis ueda. *Marine Sciences*, **2**(2): 57-59.

[15] Fujita, S., Nazamid, S. (1995) Purification and properties of polyphenol oxidase from cabbage (B rassica olerace L). *J Agric Food Chem*, **43**(50): 1138-1142.

[16] Li, Q., Zheng, W.Y., Wang, C.G., et al. (2003) Study on effect of perioxidase in porphyra haitanensis exposed to organ phosphorus pesticides. *Journal of Xiamen University (Natural Science)*, **42**(2): 201-210.

[17] Yu, J., Yang, Y.Y., Nie, X.P. (2007) Response of seaweed Gracilaria lemaneiformis to cadmium stress. *Journal of Sichuan University (Engineering Science Edition)*, **39**(3): 83-90.

[18] Ma, S.Y., Huang, Y.C., Yang, X.H., et al. (1999) Effects of high K+ and alkaline pH on ultrastructure of dunaliella salina chloroplasts. *Acta Botanica Sinica*, **41**(12): 1342-1344.