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Effect of nutrient conditions on the toxicity of naphthalene to *Chlorella pyrenoidosa*

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Abstract

The toxicity of naphthalene to a freshwater microalga, *Chlorella pyrenoidosa*, and the subsequent recovery of algae from the damage were investigated under two nutrient conditions, either enriched with nitrogen (N) and phosphorus (P), or starved of N and P. Results showed that *C. pyrenoidosa* was more sensitive to naphthalene under N,P-enriched condition, and the inhibitory rate generally increased at first and then decreased gradually with the evaporation of naphthalene under both nutrient conditions. Enriched N, P reduced the inhibitory rate at initial naphthalene concentration of 5 and 10 mg/L, but enhanced it at 100 mg/L, at which more severe ultrastructure damages were found than those under N,P-starved condition. Observed damages included partly or totally disappearance of nucleolus, nuclear, and plasma membranes. According to the chlorophyll content and cell density measurements, *C. pyrenoidosa* could recover from naphthalene damage with initial concentrations $\leqslant$ 50 mg/L in 7 days under both nutrient conditions, while they could not recover if the initial concentration of naphthalene was at 100 mg/L. Under the N,P-starved condition, the inability of *C. pyrenoidosa* to recover from the naphthalene damage was consistent with the results of high inhibitory rate, low value of specific growth rate (SGR, 0.05 day^{-1}), and the severe destruction of cell structure. However, under the N,P-enriched conditions, the observed lower inhibitory rate, higher value of SGR (0.55 day^{-1}), and the intact cell structure of most cells suggested that algae could potentially recover from the naphthalene damage.

Key words: *Chlorella pyrenoidosa*; naphthalene; toxicity; nutrient conditions

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Introduction

The nutrient conditions of different water bodies have an effect on the toxicity of pollutants to aquatic organisms. Mounting evidence reveal nutrient conditions strongly influence the fate and effects of contaminants (Smith and Schindler, 2009; Lartigue et al, 2009), such as distribution of arsenic species (Hasegawa et al., 2009), bioaccumulation of hydrophobic organic compounds (HOCs) (Halling-Sørensen et al., 2000), the tolerance of algae to heavy metals (Miao and Wang, 2006). However, the information about the effects of nutrient conditions on the toxicity of persistent organic pollutants (POPs) to organisms, such as polycyclic aromatic hydrocarbons (PAHs), especially the recovery of organisms from PAHs damage, is not available. Because PAHs pollution and eutrophication are serious recently (Chen et al., 2007; Maskaoui et al., 2002), it is necessary to determine whether the nutrient conditions could affect the toxicity of PAHs to aquatic organisms and the subsequently recovery from the damage of PAHs.

PAHs, as the most common organic contaminants, are ubiquitous in aquatic environment (Chen et al., 2007). Pollution from PAHs in aquatic environment has received global concern due to their mutagenic and carcinogenic potential to the natural ecosystem (Cerniglia, 1992). As volatile organic compounds (VOCs), evaporation leads to decreases in naphthalene concentration with exposure time. Soto et al. (1975) reported that *Chlamydomonas angulosa*, inoculated to a medium saturated with naphthalene, could restore their growth when naphthalene gradually reduced through evaporation. However, the recovery processes still need to be discussed further. Naphthalene, one of the most major PAHs in fresh water (Chen et al., 2007), was used as representative to investigate the effects of nutrient conditions on toxicity of PAHs to aquatic organisms.

Microalgae are essential constituents of aquatic ecosystem since they are the first trophic level in the food chains and the major organism providing oxygen and organic

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substances to other life forms. Moreover, microalgae are the promising materials in bio-diesel production (Vyas et al., 2010) for their high-quality oil with low sulfur and nitrogen (Ginzburg, 1993). *Chlorella pyrenoidosa*, one of the dominant and widely distributed algal species in fresh water, was chosen as the target organism to investigate the effects of nutrient conditions on the toxicity of naphthalene and its following recovery from the damage.

The concentration of photosynthetic pigments, reflecting the changes in light utilizing abilities, was closely related to various types of plant stresses and senescence (Oguntimehin et al., 2008; Watts and Eley, 1981). Contaminants could destroy algal subcellular structures (Morlon et al., 2005; Qian et al., 2009b). To further explore the toxicity of PAHs to algae under different nutrient conditions, investigation on changes of subcellular structures and chlorophyll contents, were conducted

In this study, the inhibitory rate, chlorophyll content and subcellular structure for algae, were employed as indicators to determine the toxicity of naphthalene and the subsequent recovery of *C. pyrenoidosa* under nitrogen (N) and phosphorus (P)-enriched condition and N, P-starved condition, in aim to study the effect of nutrient conditions on the toxicity of PAHs to aquatic organism.

1 Materials and methods

1.1 Chemicals and tested organisms

Naphthalene was obtained from Aldrich Chemical Co. (USA) with a purity > 98%, and was pre-dissolved in dimethyl sulfoxide (DMSO, China). Concentration of stock solution did not exceed 0.5 mL/L to ensure that DMSO is not toxic to algae (Djomo et al., 2004). *C. pyrenoidosa*, obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, was cultured in SE medium (Chen et al., 2009).

C. pyrenoidosa was cultured in a specialized incubator (RTOP-310D, China), with the light intensity of 80 μE/(m^2·sec) from the fluorescence tubes, at a constant temperature of (25 ± 1)°C under a diurnal light cycle of 14 hr light and 10 hr darkness. Culture medium and flasks were sterilized at 121°C, 1.05 kg/cm^2 for 30 min (Kong et al., 2010). A biological safety cabinet (BHC-1000 II A/B_3, China) was used to keep an axenic environment. Cell density was monitored spectrophotometrically at 660 nm (Lee et al., 2002). The regression equation between cell density (y, $\times 10^7$cells/mL) and A_{660} (x) was calculated as $y = 1.855x$ ($r^2 = 0.9991$).

The experiments were carried out in 250 mL erlenmeyer flasks containing 100 mL algal suspension at the same cell density (A_{660} = 0.072) of *C. pyrenoidosa*. Algal cells in logarithmic growth phase were separated by centrifuging at 3000 r/min, cultured in corresponding culture medium and then incubated for 24 hr and used under the inocula in the experiment. SE medium was used as N,P-enriched condition ($NaNO_3$, $K_2HPO_4{\cdot}3H_2O$ and KH_2PO_4) with the concentrations of N and P in the medium 40.2 and 50.1 mg/L, respectively, while SE medium without nitrogen and phosphorus was used as N,P-starved condition. Different volumes of the stock naphthalene solution were added into flasks to obtain a range of naphthalene concentrations (5, 10, 50 and 100 mg/L) in the culture medium. Three replicates were performed in the experiment and the flasks without the addition of naphthalene were used as the control.

1.2 Inhibitory rate and specific growth rate (SGR) determination

Cell density, calculated as absorbance of algal suspension, was measured every 24 hours for 7 days. The inhibitory rate (I) (Debenest et al., 2009) and specific growth rate (SGR) (μ, day^{-1}) (Miao and Wang, 2006) of the algae were calculated as follows:

$$I = \frac{C_0 - C_t}{C_0} \times 100\% \quad (1)$$

where, C_0 (cells/mL) and C_t (cells/mL) represent cell density in the control and in treated groups, respectively.

$$\mu = \frac{\ln C_{t_2} - \ln C_{t_1}}{t_2 - t_1} \quad (2)$$

where, C_{t1} (cells/mL) and C_{t2} (cells/mL) represent the cell density at the time t_1 and t_2, respectively.

1.3 Chlorophyll content analysis

Analysis of chlorophyll content was carried out according to Qian et al. (2009a). Algal cells were collected by centrifuging at 4000 r/min for 10 min. Samples were extracted by 90% acetone in the dark at 4°C for 24 hr. Then the extracts were centrifuged at 4000 r/min for 10 min at 4°C. Absorbance of the supernatant was measured at 647, 664 and 750 nm against 90% acetone. The absorbance at 750 nm, where chlorophyll does not absorb, did not exceed 0.005 to avoid interference of turbidity (Watts and Eley, 1981). The chlorophyll *a* content ($C_{\text{Chl-}a}$), chlorophyll *b* content ($C_{\text{Chl-}b}$), and the total chlorophyll content ($C_{\text{Chl-t}}$) were calculated as follows:

$$C_{\text{Chl-}a} = 12.7 \times A_{664} - 2.79 \times A_{647} \quad (3)$$

$$C_{\text{Chl-}b} = 20.7 \times A_{647} - 4.62 \times A_{664} \quad (4)$$

$$C_{\text{Chl-t}} = 17.9 \times A_{647} + 8.08 \times A_{664} \quad (5)$$

1.4 Electron microscopy analysis

Algal cells were harvested by centrifuging at 5000 r/min and were fixed for overnight in 2.5% glutaraldehyde at 4°C. After being embedded in agar and rinsed three times with phosphate buffer (pH 7.0), they were post-fixed for 1.5 hr in the same buffer containing 1% OsO_4. Samples were rinsed three times with the same buffer and subsequently dehydrated in 50%, 70%, 80%, 90%, 95%, 100% ethanol, 1:1 mixture of ethanol and acetone. After that, they were embedded in 1:1 mixture of acetone and spurr resin for 1 hr, 1:3 for 2 hr at room temperature, and then transferred to spurr resin for overnight. Samples were paced in capsule contained embedding medium and heated

at 70°C for about 9 hr (Estevez et al., 2001). Ultra-thin sections (70–90 nm) were obtained using a Reichert Ultracuts ultramicrotome (Leica, Germany), stained by uranyl acetate and alkaline lead citrate for 15 min, respectively, and then observed with a JEM-1230 microscope (JEOL Ltd., Japan).

1.5 Concentration determination of naphthalene

Algal suspension was centrifuged in sealed glass tube with a teflon cushion at 4000 r/min for 15 min at 4°C then the supernatant was transferred to be analyzed by the high-performance liquid chromatography (HPLC) coupled with ultraviolet detector (Agilent Corp., USA). Temperature of the column oven was kept at 30°C. Flow rate of the mobile phase was kept at 1.0 mL/sec with the ratio 1:9 of ultra pure water and methanol.

1.6 Data analysis

The data were analyzed with Origin 7.5 and SPSS 13.0. Values were considered significantly different when the probability was less than 0.05 by one-way analysis of variance (ANOVA).

2 Results and discussion

2.1 Relationship of naphthalene concentration and SGR

The concentrations of naphthalene were monitored for 4 days till naphthalene can not be detected in the medium (Fig. 1). The changes of naphthalene concentration were almost the same under two nutrient conditions because the decrease of naphthalene concentration mostly resulted from volatilization. There were not significant difference between two nutrient conditions for 5 mg/L (p = 0.969), 10 mg/L (p = 0.922), 50 mg/L (p = 0.934) and 100 mg/L (p = 0.990) groups.

SGR of a culture reflected the growth supporting ability of the medium (Ljunggren and Häggström, 1995). It changed with concentrations of naphthalene and nutrient conditions of the medium. SGR had a negative relationship with concentration of naphthalene in the medium and it was affected insignificantly in 5 and 10 mg/L groups compared to the control under both nutrient conditions (Fig. 2). Under N,P-enriched condition SGR fluctuated around 0.40 day^{-1} with naphthalene initial concentration at 5, 10, and 50 mg/L, and SGR of 100 mg/L group increased rapidly from –0.33 to 0.55 day^{-1} during the 7

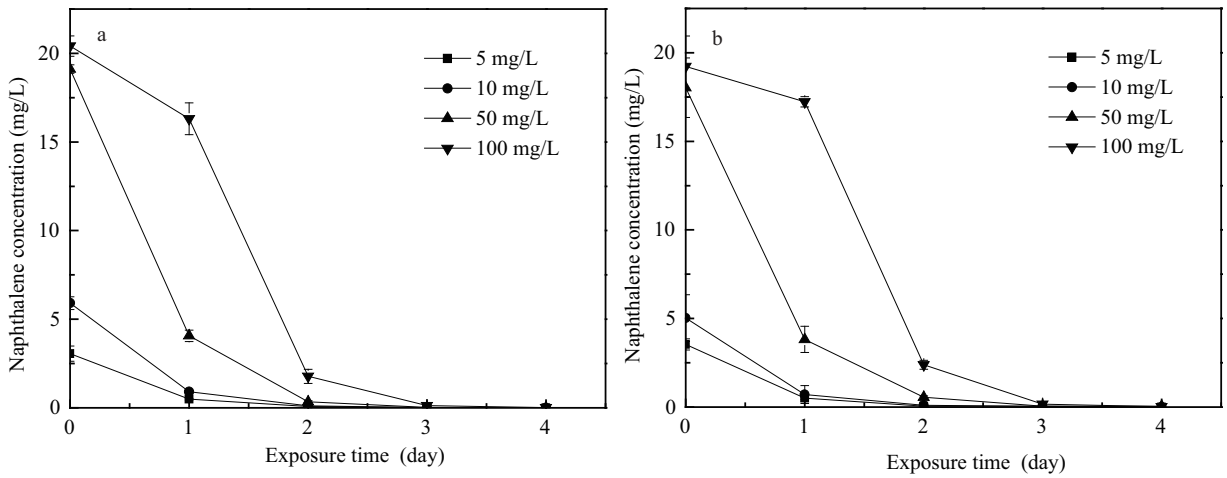

Fig. 1 Changes of naphthalene concentration with time under (a) N,P-enriched condition; (b) N,P-starved condition. Values are represented as means ± SD, n = 3.

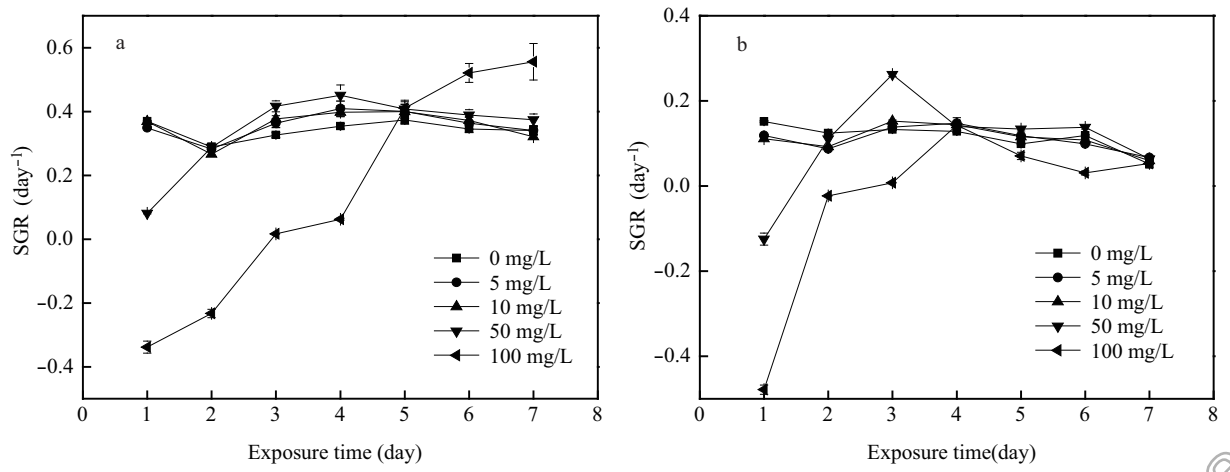

Fig. 2 Special growth rate (SGR) of *C. pyrenoidosa* with initial concentration of naphthalene at 5, 10, 50 and 100 mg/L, respectively under N,P-enriched condition (a) and N,P-starved condition (b). Values are represented as means ± SD, n = 3.

days, exceeding the other groups on day 5. Under N,P-starved condition, SGR was much lower than that under N,P-enriched condition, which indicated nutrient condition acted as an important limiting factor.

2.2 Effect of naphthalene on inhibitory rate

Toxicity of naphthalene to organisms mainly arises in the metabolic process. It is accepted that metabolites of these compounds are responsible for most of their biological effects (Schoeny et al., 1988). The metabolism of naphthalene by microorganism had been reviewed in detail by Haritash and Kaushik (2009), and it was much easier to be metabolized than other PAHs (Li et al., 1996). Green algae utilize dioxygenase to initiate the degradation of naphthalene like the bacteria (Warshawsky et al., 1988). Metabolites produced from naphthalene, such as 1-naphthol, 4-hydroxy-l-tetralone, naphthalene *cis*-1,2-dihydrodiol were found by different species of algae (Cerniglia et al., 1979, 1980a, 1980b). Under normal growth conditions reactive oxygen species (ROS) are formed at low rate as byproducts of metabolism, but many stresses can cause an increase in the production rate of ROS (Foyer and Noctor, 2000). The toxicity of PAHs was mainly due to the production of large amount of ROS when they were metabolized by algal enzymes.

Inhibitory rate of *C. pyrenoidosa* increased at first and then decreased due to the evaporation of naphthalene (Figs. 1 and 3). It was shown that under both studied conditions the inhibitory rates reached the maximum value on day 2 for 5, 10, 50 mg/L treated groups and began to decrease when naphthalene concentration was below 0.1 mg/L.

Inhibitory rates were lower under N,P-enriched condition than those under N,P-starved condition with initial naphthalene concentration at 5 and 10 mg/L. Calabrese (2005) showed that low dose of a stressful stimulus activated an adaptive response to enhance the resistance of organisms to different stress levels (it was also called hormesis). Consistent with above results, naphthalene-induced hormesis at low dose was observed under N,P-enriched condition in our study. Activities of ROS scavenging enzymes could not be stimulated by naphthalene for the deficiency of nutrition elements (Furbank et al., 1987), so that algae growth was inhibited by ROS accumulation in all treated groups under N,P-starved conditions. Naphthalene induced the same inhibitory rate to algae under both nutrient conditions with 50 mg/L initial naphthalene initial concentration. Under N,P-enrcihed condition, either polyunsaturated fatty acids, which were the main targets of free radicals in lipid peroxidation (Gibson and Subramanian, 1984) or the activities of metabolic enzyme increased compared to those in N,P-starvation medium (Furbank et al., 1987). More ROS would be accumulated, resulting in a higher inhibitory rate at higher initial naphthalene concentration of 100 mg/L under N,P-enriched condition.

2.3 Effect of naphthalene on chlorophyll content and *a*:*b* ratio

Nutrition starvation reduced chlorophyll *a*, *b* and total chlorophyll contents (Fig. 4). Under N,P-starved condition maximum values of chlorophyll *a*, *b* and total chlorophyll contents were 0.10, 0.09 and 0.19 mg/L in the control, and 1.69, 0.93 and 2.63 mg/L under N,P-enriched condition, respectively. Nitrogen and phosphorus are major elements in the formation of protein, nucleic acid and phospholipids, their deficiency resulted in the decrease in chloroplasts, then photosynthesis. As a result, they can affect the growth and cleavage of algae. These results corresponded with the ultrastructure and biomass of algal cells in this study.

Less than 100 mg/L naphthalene supplementation affected chlorophyll *a*, *b* and total chlorophyll contents insignificantly under both nutrient conditions ($p > 0.05$), while these indexes decreased in 100 mg/L treated groups significantly ($p < 0.05$). Algae could recover from the damage of naphthalene under both nutrient conditions (except 100 mg/L group under N,P-starved condition) judging from cell density, chlorophyll *a*, *b* and total chlorophyll contents. Soto et al. (1975) reported that high percentages of *Chlamydomonas angulosa,* a green microalgal species, inoculated in a medium saturated with naphthalene were killed but the remaining live cells could restore their growth when naphthalene in the medium

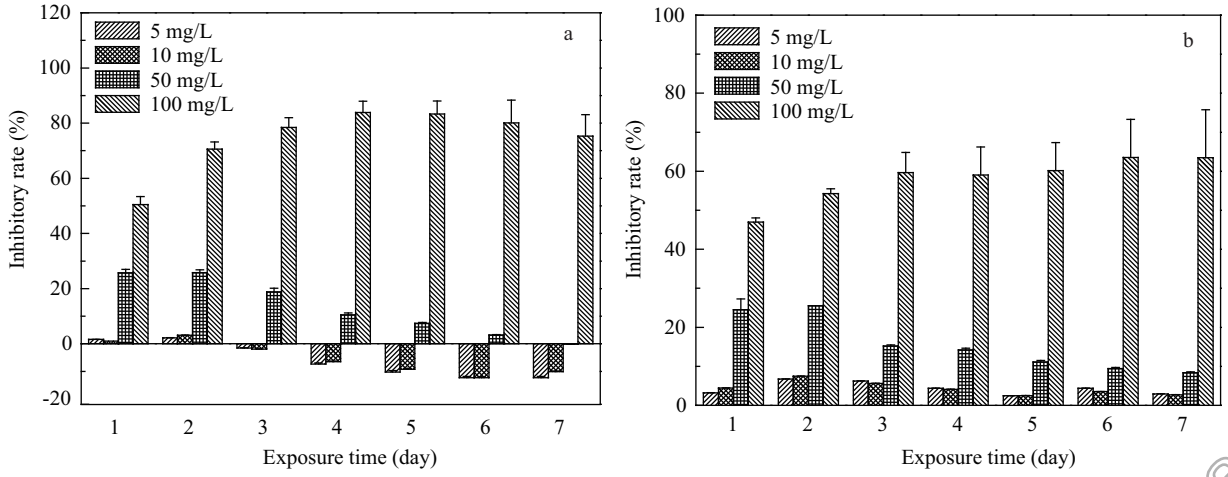

Fig. 3 The inhibitory rate of *C. pyrenoidosa* with different initial naphthalene concentrations under (a) N,P-enriched condition; (b) N,P-starved condition. Values are represented as means ± SD, $n = 3$.

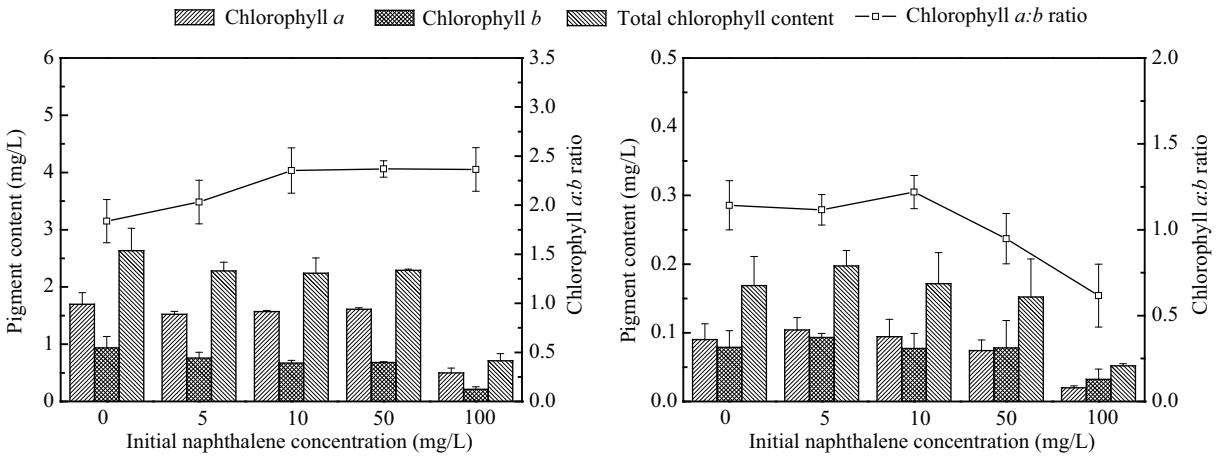

Fig. 4 The chlorophyll *a*, *b* and total chlorophyll contents of *C. pyrenoidosa* with different initial naphthalene concentrations under (a) N,P-enriched condition; (b) N,P-starved condition. Values are represented as means ± SD, $n = 3$.

gradually reduced through evaporation.

Nutrition deficiency reduced chlorophyll *a*:*b* ratio of the control from 1.81 (under N,P-enriched condition) to 1.13 (under N,P-starved condition) (Fig. 4). Chlorophyll *a*:*b* ratios for *Chlorella capsulata*, *Chlorosarcinopsis halophila*, *Nannochloris atomus* and *Micromonas pusilla* were found to be 1.74, 2.46, 1.89, and 1.53, respectively (Wood, 1979). In our study chlorophyll *a*:*b* ratio of *C. pyrenoidosa* was 1.81 under N,P-enriched condition which was similar to *C. capsulate*, since they belong to the same genus. Contaminants like heavy metals can reduce the chlorophyll content and the chlorophyll *a*:*b* ratio (Appenroth et al., 2003). Chlorophyll *a*:*b* ratios were affected insignificantly by naphthalene supplementation under N,P-enriched condition ($p > 0.05$), and this agreed with Oguntimehin et al. (2008), who found that chlorophyll *a*:*b* ratios of Japanese red pine (*Pinus densiflora*) were unaffected by fluoranthene and phenanthrene. chlorophyll *a* was found to be more susceptible to oxidative stress than chlorophyll *b* (Puckett et al., 1973; Xia and Tian, 2009). Under N,P-starved condition chlorophyll *a*:*b* ratio decreased insignificantly in 5, 10 and 50 mg/L treated groups ($p > 0.05$). The decline in chlorophyll *a*:*b* ratio for 100 mg/L treated group ($p < 0.05$) might be attributed to the earlier structural loss of the chloroplast stroma lamellae (containing photosystem I and most of the chlorophyll *a*), compared to the grana lamellae (containing photosystem II) (Bricker and Newman, 1982). Part of decrease in chlorophyll *a* might be account for conversion to chlorophyll *b*, which apparently formed from chlorophyll *a* by oxidation of the methyl group ring II to the aldehyde (Bidwell, 1979).

2.4 Effect of naphthalene on subcellular structure

Under N,P-enriched condition cells of the control contained typically intact plasma membrane, mitochondria and chloroplast. Plasma membrane was a little detached from cell wall probably caused by dehydration during sample preparation (Fig. 5a). Starch nodules occupied most of the cell volume and chloroplast deformed after 7 days incubation in medium without nitrogen and phosphorus (Fig. 5d). Ball et al. (1990) reported the starch accumulation after nutrient starvation. A modification of starch abundance in algal cells upon exposure to contaminants, either an underproduction or an overproduction (Nishikawa et al., 2003), has often been reported, reflecting important changes in metabolism. Morlon et al. (2005) reported that the unicellular green alga, *Chlamydomonas reinhardtii*, was full of starch treated by 500 μmol/L selenite after 48 hr. In contrast, naphthalene did not enhance the accumulation of starch grains obviously in *C. pyrenoidosa* in this study (Fig. 5b). Nutrition deficiency reduced the chloroplasts and the pyrenoid region under N,P-starved condition, which agreed to damages caused by selenate (Wong and Oliveira, 1991) .

After 24 hours exposure, cells under N,P-starved condition were less damaged than that under N,P-enriched condition due to the intact nucleoli and nuclear membrane. However, some disorder involved, such as dilated and fingerprint-like appearance of thylakoids, plasma membrane being detached from the cell wall severely and less stroma (Fig. 5e). Under N,P-enriched condition, the highly reactive radicals, caused by higher activities of metabolic enzyme, attacked unsaturated membrane fatty acids, rapidly opening up and disintegrating the cell membranes and tissues (Oguntimehin et al., 2008). Thereby, cells were more disorganized than those under N,P-starved condition after 24 hours exposure. The main ultrastructural changes involved the disorder of the chloroplasts, partly or totally disappearance of thylakoids, nucleolus, nuclear and plasma membrane, a granulous, and less-dense stroma (Fig. 5b). Nutrition enrichment enhanced damage in subcellular structure of algae, which could not be found by inhibitory rate, because the destroyed structures or indistinguishable normal cell organelles might not change absorbance of algal suspension obviously.

Under both nutrient conditions, 100 mg/L naphthalene had done severe damage to algal cells, and a lot of debris of cell could be found after 7 days exposure. Under N,P-enriched condition, most cells showed typical characters, intact nucleoli and nuclear membrane, lamella structure in the thylakoids (Fig. 5c), which showed their recovery from the damage of naphthalene in few days, in accordance with the decreasing inhibitory rate and high value of SGR

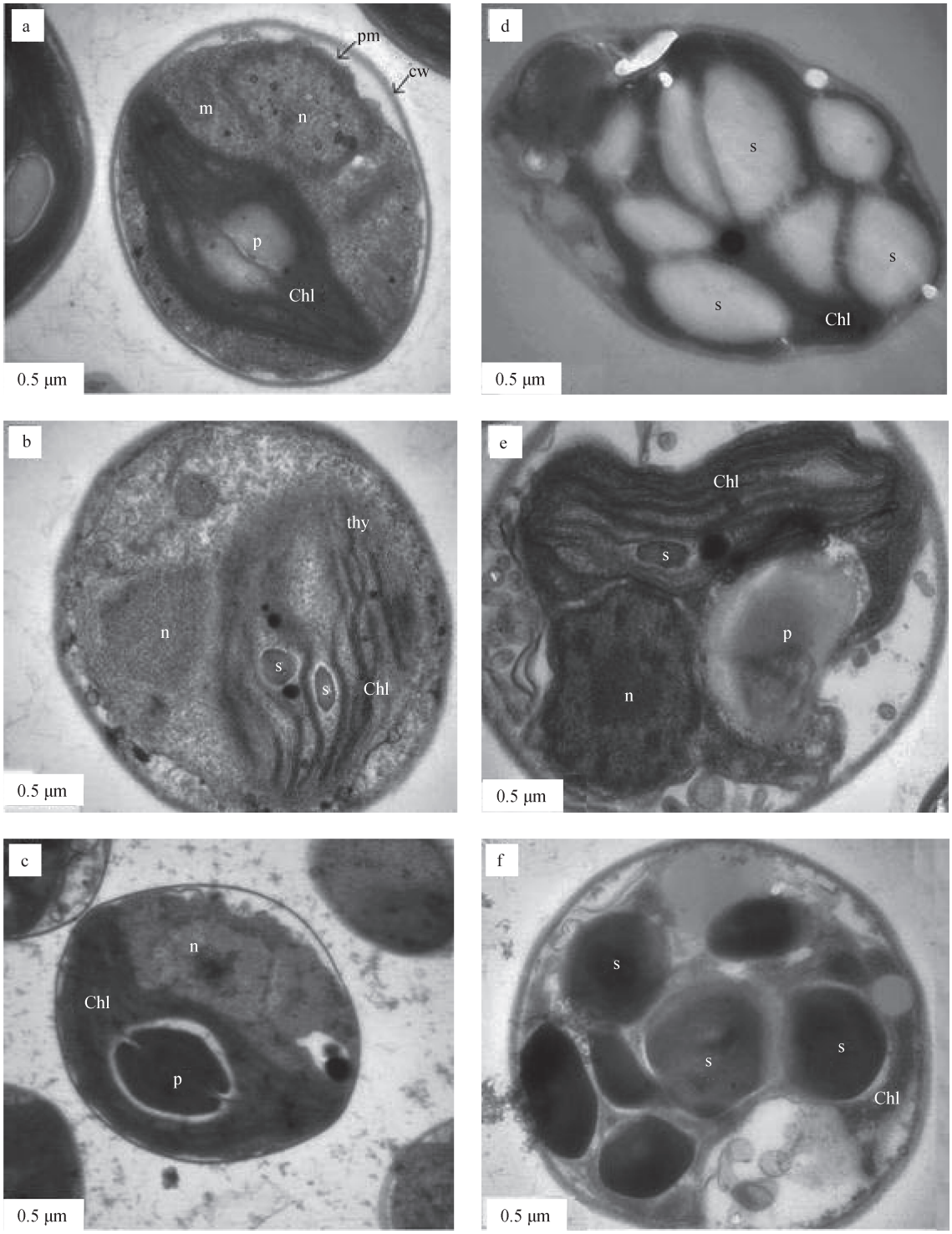

Fig. 5 Ultrastructure of *C. pyrenoidosa* after exposure to 100 mg/L naphthalene under different nutrient conditions. N,P-enriched condition: (a) control; (b) exposure for 24 hr; (c) exposure for 7 days. N,P-starved condition: (d) control; (e) exposure for 24 hr; (f) exposure for 7 days. p: prenoid; n: nucleus; Chl: chloroplast; m: mitochondria; thy: thylakoids; s: starch; v: vacuole; pm: plasma membrane; cw: cell wall.

(0.55 day^{-1}) (Soto et al., 1975). Supplementation of 100 mg/L naphthalene destroyed cell structure severely after 7 days exposure under N,P-starved condition. Most of the cells lost typical structure like nucleoli and nuclear membrane, with only part of stroma and thylakoids left. They were still full of starch with appearance of electron-dense deposits compared to the control (Fig. 5f). They could not recover from the naphthalene damage in the future, according to the increasing inhibitory rate and low value of special growth rate (0.05 day^{-1}).

3 Conclusions

Nutrition deficiency reduced chlorophyll contents and chlorophyll *a*:*b* ratio, and enhanced the accumulation of starches. With naphthalene initial concentrations at 5 and 10 mg/L, N,P enrichment reduced the toxicity of naphthalene to *C. pyrenoidosa*, and naphthalene-induced hormesis

was observed. Algae could recover from naphthalene damages in 7 days under both nutrient conditions according to chlorophyll contents and cell density. Naphthalene induced the same inhibitory rate to algae under both nutrient conditions with 50 mg/L initial naphthalene concentration and algae could recover from the naphthalene damages in 7 days according to chlorophyll contents and cell density. With higher initial concentration of 100 mg/L, naphthalene showed more toxicity to algae under N,P-enriched condition, which could be deduced from higher inhibitory rate compared to that under N,P-starved condition. Electron microscopic analysis showed algae had more severe ultrastructural changes under N,P-enriched condition, such as partly or totally disappearance of thylakoids, nuclear and plasma membrane in comparison with those under N,P-starved condition. In short, eutrophication enhanced the toxicity of naphthalene to *C. pyrenoidosa* at high dose. Under N,P-starved condition, algal cells could not recover from the naphthalene damage in the future, corresponding to the increasing inhibitory rate, low value of SGR (0.05 day^{-1}) and the destruction of cell structure. However, the decreasing inhibitory rate, high value of SGR (0.55 day^{-1}) and intact cell structure of most cells indicated that algae could recover from the naphthalene damage in few days under N,P-enriched condition.

Acknowledgments

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