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The toxicity of naphthalene to marine *Chlorella vulgaris* under different nutrient conditions

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ABSTRACT

The toxicity of naphthalene to *Chlorella vulgaris* was studied under nitrogen (N)-, phosphorus (P)-enriched and N,P-starved condition. Results showed that naphthalene was less toxic under N,P-starved condition. The inhibitory rates were less than 15.3% to *C. vulgaris* during 7 days exposure with the initial concentrations of naphthalene at 5, 10, 50, 100 mg/L, respectively under N,P-starved condition, while they were 7.5–72.3% under N,P-enriched condition. The malondialdehyde (MDA) content indicated that there was no oxidative damage to algae when the initial concentration of naphthalene was less than 10 mg/L, and oxidative damage exhibited to algae at 50–100 mg/L of naphthalene under N,P-starved condition. Naphthalene induced oxidative damage to the algae at all tested concentrations (5–100 mg/L) under N,P-enriched condition. The results indicated that there was a negative relationship between the special growth rate (SGR) and naphthalene concentration in the medium. Under N,P-enriched condition SGR of the control decreased slowly from 0.669 to 0.186. However, SGR of the naphthalene treated group decreased sharply during the first 2–3 days when the dissolved concentration of naphthalene was above 0.1 mg/L, and then increased gradually with the evaporation of naphthalene.

1. Introduction

The nutrient conditions of different water bodies could have an effect on the toxicity of pollutants to aquatic organisms. Some researches have found that the nutrient conditions could affect the tolerance of algae to heavy metals [1]. Riedel et al. [2] found that Cu^{2+} had greater inhibition in the nutrient-enriched mesocosms. Rijstenbil et al. [3] gave the result that the diatom *Thalassiosira pseudonana* was more sensitive to Cu^{2+} under phosphorus limitation as metal exclusion/elimination systems might be impaired. Evidence is also mounting rapidly that nutrients strongly influence the fate and effects of other contaminants [4], such as distribution of arsenic species [5], bioconcentration of hydrophobic organic compounds (HOCs) [6]. However, the information about the effect of nutrient condition on the toxicity of persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), is not available.

PAHs represent a group of nonspecific combustion-related compounds with multiple aromatic rings. Many of them exhibit a potential for bioaccumulation and have a negative effect upon aquatic organisms [7–9]. Because the PAHs pollution and eutrophication are more serious recently [10,11], it is necessary to determine if the nutrient conditions could affect the toxicity of PAHs to aquatic organisms, and the results may give some references in setting the concentration criteria for the sea water quality standard in future.

Algae are essential in aquatic ecosystems since they are the first trophic level in the food chains and the major organism that can provide oxygen and organic substances to other life forms. *Chlorella vulgaris* was selected as it is one of the dominant algal species in sea water as well as having potential use either to degrade or adsorb a variety of organic pollutants. Naphthalene, one of the most major PAHs in sea water [12,13], was chosen as representative to investigate the effect of nutrient conditions on toxicity of PAHs to *C. vulgaris*.

Malondialdehyde (MDA) is a secondary product of lipid peroxidation widely used as a biomarker of oxidative reactions [14]. Lipid peroxidation is caused by reactive oxygen species (ROS) and enhanced when organisms are exposed to various contaminants [15], for example, heavy metals or PAHs [16]. After being peroxidized, the membranes became rigid, and permeability changed [17]. In order to determine the degree of oxidative stress under different nutrient conditions to the exposed algal cells, the MDA content of algal cells was mea-

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sured using the thiobarbituric acid (TBA) reactive substances test [14,18,19].

In this study, the toxicity of naphthalene to marine *C. vulgaris* was conducted under nitrogen (N)-, phosphorus (P)-enriched and N,P-starved condition. The inhibitory rate, MDA content and special growth rate (SGR) were employed as indicators of the toxicity of naphthalene to *C. vulgaris*.

2. Materials and methods

2.1. Chemicals

Artificial sea water was made by sea water crystal purchased from Binhai Seawater Crystal Plant of China, at a 31 psu salinity. Naphthalene was purchased from Aldrich Chemical Co. with a purity >98%, which was pre-dissolved in dimethyl sulfoxide (DMSO) at a concentration not exceeding 0.5 mL/L to ensure DMSO is not toxic [20].

2.2. Algae culture and experimental set-up

The marine *C. vulgaris* was kindly provided by Culture Collection of Algae, Dalian Fisheries University. *C. vulgaris* was cultured in a specialized incubator (RTOP-310D, China), with the light intensity of 80 $\mu E\,m^{-2}\,s^{-1}$ from the fluorescence tubes, at a constant temperature of $25 \pm 1\,^{\circ}C$ on a diurnal cycle of 14 h light, 10 h dark. Konvey medium was used with the following components: $FeCl_3{\cdot}6H_2O$ (1.3 mg/L), $MnCl_2{\cdot}4H_2O$ (0.36 mg/L), H_3BO_3 (33.6 mg/L), EDTA-2Na·$2H_2O$ (45 mg/L), $NaH_2PO_4{\cdot}2H_2O$ (20 mg/L), $NaNO_3$ (100 mg/L), $ZnCl_2$ (2.1 μg/L), $CoCl_2{\cdot}6H_2O$ (2.1 μg/L), $(NH_4)_2Mo_7O_4{\cdot}4H_2O$ (2.1 μg/L), $CuSO_4{\cdot}5H_2O$ (2.0 μg/L).

The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL algal suspension at the same cell density of *C. vulgaris*. Algal cells in logarithmic growth phase were separated by centrifuging at 3000 rpm, cultured in corresponding culture medium and then incubated for 24 h to use in the experiment. Konvey medium was used under N,P-enriched condition, while Konvey medium without nitrogen ($NaNO_3$) and phosphorus ($NaH_2PO_4{\cdot}2H_2O$) was used under 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 made in the experiment and the flasks without the addition of naphthalene were used as the control. A biological safety cabinet (BHC-1000 II A/B_3) was used to keep an axenic environment.

Cell growth was measured every 24 h for 7 days. The absorbance was measured on a UV-2450 spectrophotometer at 660 nm [21] with the standard curve made with a haemocytometer slide under a light microscope (Olympus CHK, Japan). All the flasks and the culture medium were sterilized at 121 °C for 30 min, and all the operations were carried out in the axenic condition.

The inhibitory rate of the algae was calculated as follows [22]:

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

where I is the inhibitory rate (%), C_0 and C_t represent average cell density in the control and in treated groups, respectively.

The specific growth rate of the algae was calculated as follows [1]:

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

where μ is the specific growth rate (d^{-1}), C_{t_1} and C_{t_2} represent the cell density at the time $t1$ and $t2$, respectively.

2.3. MDA determination

After 7 days incubation, algae were harvested by filtering with 0.45 μm filter membrane. Algal cells were ground into pieces by a tissue grinder with 10% trichloroacetic acid (TCA) added in, and the mixture was subsequently centrifuged at 4000 rpm for 20 min. A mixture of the supernatant and equal volume 0.67% TBA (dissolved in 10% TCA) was heated in a 100 °C water bath for 30 min and then cooled down immediately to measure the absorbance at 450, 532 and 600 nm. The MDA content was expressed as mmol/cell, calculated as follows [14]:

$$C = \frac{[6.45 \times (A_{532} - A_{600}) - 0.56A_{450}] \times (V + V_e) \times (V_t/V_e)}{1000 \times V_a \times S_a} \quad (3)$$

where A_{450}, A_{532} and A_{600} are the absorbance of mixture at 450, 532 and 600 nm respectively, V is volume (L) of TBA, V_t and V_e are the total and the extract volume of TCA (L), V_a and S_a are the volume (mL) and cell density (cell/mL) of *C. vulgaris*.

2.4. The concentration determination of naphthalene

The algal suspension was centrifuged at 4 °C in sealed glass tube for 15 min at 4000 rpm, then the supernate was transferred to be analyzed by the HPLC coupled with ultraviolet detector (Agilent Corp., USA). The flow rate of the mobile phase was kept at 1.0 mL/s with the ratio 1:9 of ultra pure water and methanol. The temperature of the column oven was kept at 30 °C.

2.5. Statistical 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.

3. Results and discussion

3.1. The inhibitory rate for algae under different nutrient conditions

Under normal growth conditions reactive oxygen species (ROS) are formed at low rate as by-products of metabolism, but many stresses can produce a dramatic increase in the rate of ROS production [23]. Molecular oxygen is essential for the enzymatic attack on the PAHs rings — initial hydroxylation of polycyclic aromatic hydrocarbons by both prokaryotic and eukaryotic microorganisms [24]. Green algae, metabolizing PAHs more efficiently than brown and blue algae [25], utilize dioxygenase to initiate the degradation of naphthalene like the bacteria [26,27]. They utilize dioxygenase enzymes to incorporate both atoms of molecular oxygen into the aromatic nucleus to form *cis*-dihydrodiols [28]. *cis*-Dihydrodiols are stereoselectively dehydrogenated by *cis*-dihydrodiol dehydrogenases [29], which rearomatize the benzene nucleus to form dihydroxylated intermediates. Metabolite produced from naphthalene, such as 1-naphthol, 4-hydroxy-l-tetralone, naphthalene *cis*-l,2-dihydrodiol were found in different species of algae [30–32]. In a word, ROS are produced during the metabolic processes of PAHs and the toxicity of PAHs was mainly due to the production of large amount of ROS when they were metabolized by enzyme of the algae.

Nitrogen and phosphorus are the major elements to form protein, nucleic acid and phospholipid, and also the major component of bioplasm, nucleolus and biomembrane. As a result, they can affect the growth and the cleavage of algae. The growth rate will decrease when nitrogen and phosphorus are deficient. In our experiment the algae cultured in N,P-enriched medium grew much faster

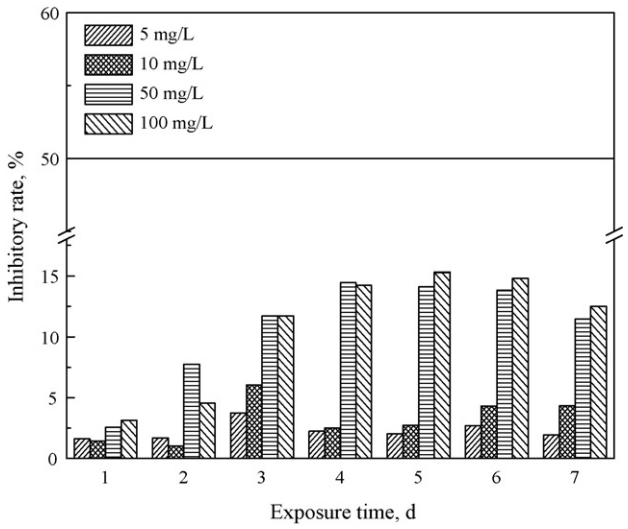

Fig. 1. The inhibitory rate of *C. vulgaris* by naphthalene under N,P-starved condition. The bar showed the EC_{50}.

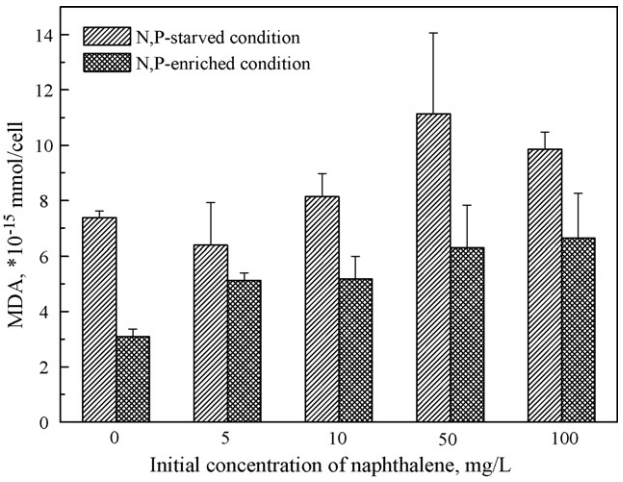

Fig. 3. The MDA contents of *C. vulgaris* under different nutrient conditions after 7 days incubation.

than those in the N,P-starved medium because of nutrient deficiency of the latter and the cell density was about 3:1 after 7 days' incubation.

Fig. 1 shows that the inhibitory rates were less than 15.3% for all treated groups under N,P-starved condition and there was a dose–toxicity relationship in the experiment. The maximum value appeared after 3–5 days exposure for the 5, 10, 50 and 100 mg/L naphthalene, respectively because of the volatilization of naphthalene.

N,P enrichment enhanced the inhibitory rates for all treated groups during 7 days exposure (Fig. 2). The maximum value was 73.2% after 4 days exposure for 100 mg/L group. Similar to the algae cultured in N,P-starved medium, inhibitory rate increased with time and the concentration of naphthalene. The inhibitory rates increased at first and then decreased gradually with time, with the maximum values appearing after 3 or 4 days exposure for different treated groups.

Exponential growth ceases when the nitrogen supplies is exhausted, however, when the growth rate decreases, assimilation of carbon continues at a reduced rate for some time [33]. Phosphorus limitation caused significant changes in the fatty acid and lipid composition of *Monodus subterraneus*. With decreasing phosphate availability the proportion of the major very long chain polyunsaturated fatty acids (VLC-PUFA), eicosapentaenoic acid (EPA) decreased [34]. It has been demonstrated that polyunsaturated fatty acids are the main targets of free radicals in lipid peroxidation [35]. Under N,P-starved condition, the content and activities of enzyme of algae decreased compared to those in N,P-enriched medium [36], polyunsaturated fatty acids which were the main targets of free radicals in lipid peroxidation also decreased. The metabolic ability of naphthalene would decrease for lower enzyme activities, thus less ROS would be accumulated, accompanied by the decrease of PUFA, the naphthalene was less toxic to algae under N,P-starved condition.

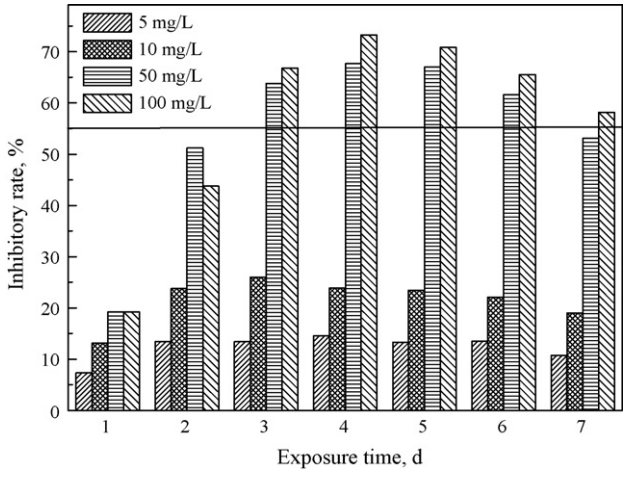

Fig. 2. The inhibitory rate of *C. vulgaris* by naphthalene under N,P-enriched condition. The bar showed the EC_{50}.

The inhibitory rates increased when concentration of naphthalene in the medium was above 0.1 mg/L, and then decreased during last few days for evaporation of naphthalene in the medium. The algae recovered for the ROS' elimination by the enzyme like superoxide dismutase (SOD) and catalase (CAT). The inhibitory rates of 10 mg/L were smaller than that of 5 mg/L during the first 2 days exposure under N,P-starved condition for the dilution of the algae suspension (at about 1:10 ratio) at the beginning of the experiment, because the abrupt transference of cells to new medium could produce a different physiological response [37].

3.2. Influence on the content of MDA

MDA, measured after 7 days exposure, was used as a toxicity endpoint. As a product of lipid peroxidation caused by ROS, the accumulation of MDA was enhanced when algae were exposed to naphthalene. To the algae cultured in N,P-starved medium, MDA contents of 5 and 10 mg/L groups had no significant difference compared to the control ($p > 0.05$). However, MDA contents of 50 and 100 mg/L groups had significant differences ($p < 0.05$) (Fig. 3) compared to the control, indicating that there was no oxidative damage to algae when naphthalene initial concentration was less than 10 mg/L after 7 days exposure under N,P-starved condition. To algae cultured in the N,P-enriched medium, the MDA content increased for all treated groups ($p < 0.05$), which showed that naphthalene had oxidative damage to the algae with naphthalene initial concentration above 5 mg/L after 7 days exposure. Algal cells cultured in N,P-enriched medium had more active enzyme to metabolize naphthalene and produced plenty of ROS [34,36], and they also had more PUFA [24] being easily attacked by ROS, which resulted in the

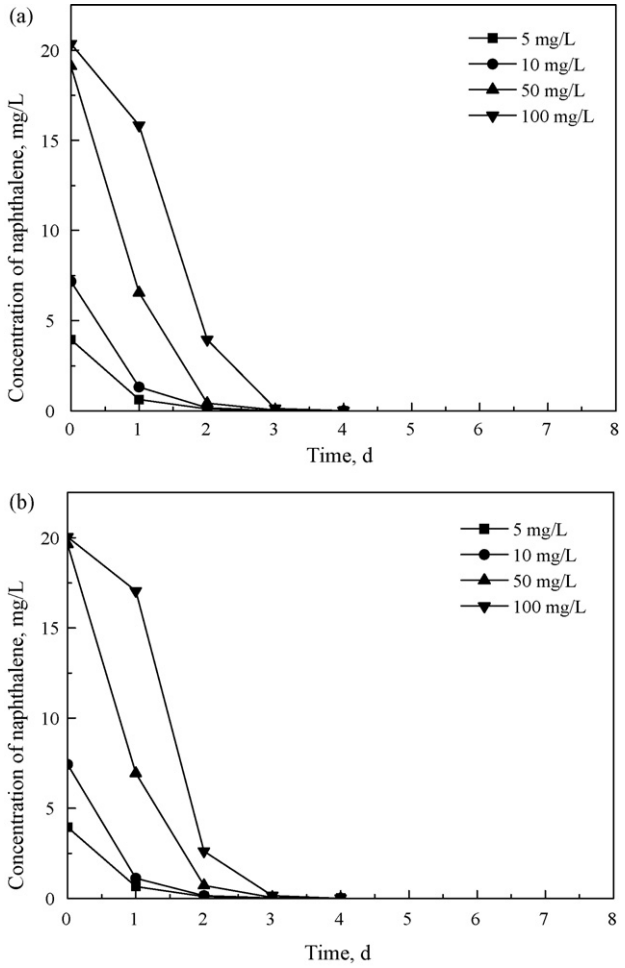

Fig. 4. The changes of concentration of naphthalene in the culture medium with time under N,P-enriched condition (a) and N,P-starved condition (b) with initial concentrations of naphthalene at 5, 10, 50 and 100 mg/L, respectively.

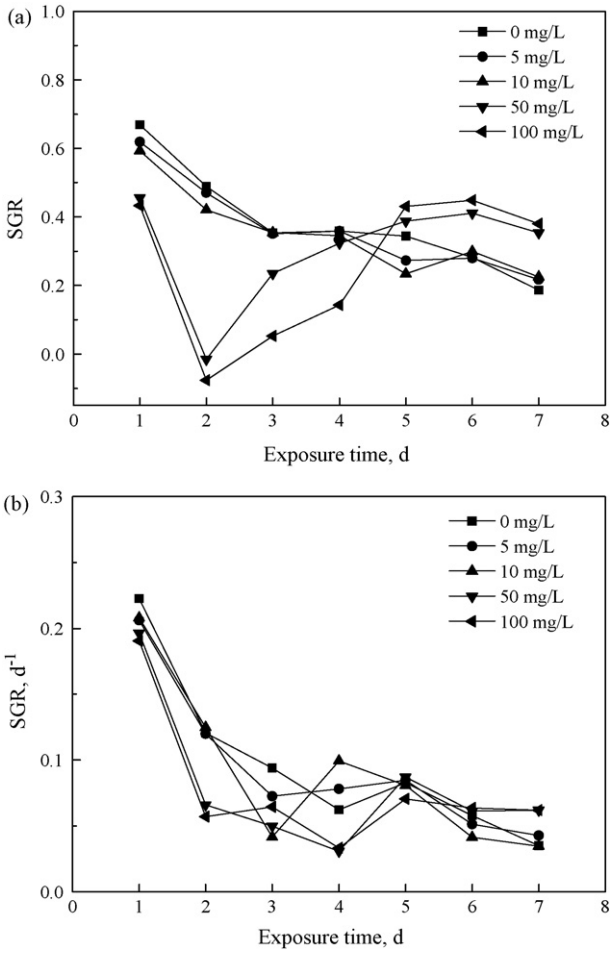

Fig. 5. The SGR of *C. vulgaris* N,P-enriched condition (a) and N,P-starved condition (b) with initial concentrations of naphthalene at 0, 5, 10, 50 and 100 mg/L, respectively.

increase of MDA. So algal cells under N,P-enriched condition were more sensitive to naphthalene.

Nutrient deficiency caused an unhealthy photosynthetic activity [38], and enhanced the accumulation of MDA. The decreased activities of some ROS elimination enzyme like CAT and SOD resulted in the accumulation of the MDA produced in the normal metabolism after 7 days incubation. Under N,P-enriched condition algae produced more ROS and ROS elimination enzyme like CAT and SOD, so no more ROS would be accumulated. Finally algae cultured in N,P-enriched medium had a lower MDA content.

3.3. The relationship of SGR and the concentrations of naphthalene

The specific growth rates were calculated for the SGR of a culture should reflect the growth supporting ability of the medium [39].

The concentrations of naphthalene were monitored for 72 h till all the concentrations were below 0.1 mg/L. 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 no significant difference between the two nutrient conditions for 5 mg/L ($p = 1.000$), 10 mg/L ($p = 0.996$), 50 mg/L ($p = 0.963$) and 100 mg/L ($p = 0.995$) groups (Fig. 4).

The concentration of naphthalene in the medium decreased to almost zero after 48 h for the 5, 10 and 50 mg/L group, 72 h for 100 mg/L group. In Fig. 5(a), under N,P-enriched condition, SGR of the control decreased from 0.669 to 0.186 d^{-1} during the 7 days exposure. During the first 48–72 h when the concentration of naphthalene was above 0.1 mg/L, the SGRs of all treated groups decreased with time and there was a negative ralationship between SGR and naphthalene concentration. For 50 and 100 mg/L naphthalene treated groups, SGRs decreased sharply and reached the lowest point, −0.0152 and −0.0762 d^{-1} respectively after 48 h exposure, and then they increased gradually, which indicated that some algal cells died from the damage of naphthalene. When the concentrations of naphthalene were below 0.1 mg/L, algal cells began to recover from the damage of naphthalene.

In Fig. 5(b), under N,P-starved condition, SGR decreased sharply in 48 h and it was above 0 for all treated groups, which showed that algae were not killed by naphthalene. Under N,P-enriched condition naphthalene concentration was limiting factor for the growth supporting ability of the medium. However, nutrition elements became limiting factor under N,P-starved condition, resulting in less change of SGR with naphthalene concentration.

Soto et al. [40] reported that high percentages of *Chlamydomonas angulosa,* a green microalgal species, inoculated into a medium saturated with naphthalene, were killed but the remaining live cells could restore their growth when naphthalene gradually reduced its concentration through evaporation, suggesting that microalgae have some resistance to PAHs toxicity and were able to recover after PAHs exposure.

4. Conclusions

Naphthalene showed less toxicity to *C. vulgaris* under N,P-starved condition than that under N,P-enriched condition. The inhibitory rates were less than 15.3% during 7 days exposure when the initial concentrations of naphthalene were 5, 10, 50, 100 mg/L, respectively under N,P-starved condition. While they were 7.5–72.3% under N,P-enriched condition. The MDA content suggested that naphthalene induced oxidative damage to *C. vulgaris* when initial concentration was above 50 mg/L under N,P-starved condition in 7 days exposure, while it was above 5 mg/L under N,P-enriched condition. SGR and the naphthalene concentration in the medium had a negative relationship. Moreover, SGR of the naphthalene treated group decreased sharply during the first 2–3 days when the dissolved concentration of naphthalene was above 0.1 mg/L, and then increased gradually with the evaporation of naphthalene.

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