Effect of petroleum hydrocarbons on the carbon and nitrogen stable isotope composition of *Nitzschia closterium*

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**Abstract.** The microalgae is an important test organism of Petroleum hydrocarbon pollution. The present study was selected the *N. closterium* under laboratory conditions, the acute toxicity test of 180# fuel oil CE-WAF was investigated. We found the main content of the PAHs of CE-WAF were C₀-N, C₂-N, C₁-N and C₉-P by GC-MS analysis, indicating the toxicity of PAHs could affect the growth and development of microalgae. The EA-IRMS was used to analyze the carbon and nitrogen stable isotope composition of *N. closterium* under the CE-WAF stress. More importantly, the value of δ¹³C increased with the culture time at the control group, but there was no significant difference in δ¹³C value with increased culture time at the high (5%, 7%, and 10%) CE-WAF concentrations. In addition, the δ¹⁵N value of microalgae was increased in the culture time at the same CE-WAF concentration. However, there was no significant difference in δ¹⁵N value with increasing CE-WAF concentration at the same culture time. This study illustrated that the toxic effects of PAHs could be detected by the δ¹³C value to reveal the impact on marine ecosystems, and provided basic data and related theoretical support for marine pollution detection systems.

1. Introduction
The rapid expansion of transportation and development of offshore oil has added to the economic prosperity of China, but led to the generation of a large volume of hazardous marine oil spills that warrants immediate attention[1]. As a developed city in northeast China, Dalian is believed to be suffering from the pollution of oil spill due to the oil pipeline explosion on July 16, 2010. The prime technological solution to the accident was to collect oil in the core area and spray GM-2 type dispersant on the periphery. When the dispersant was added to disperse the crude oil into oil in water emulsified particles, this method could only reduce the floating hazard on the sea surface[2]. However, the use of dispersant will inevitably bring secondary pollution to the marine ecological environment. Thus, the studies on insight into the comprehensive roles of crude oil and dispersant on the marine of oil spill are urgently needed.

The primary producer of microalgae is ubiquitous in the ocean supplying energy for the basic marine food chain[3]. Furthermore, microalgae are often used for objective evaluation of environmental effects and toxicity due to their advantages of small size, rapid reproduction, and sensitivity to poisons in the study of aquatic toxicology. Inevitable oil spills could negatively affect attached microalgae that form water-soluble petroleum hydrocarbons. Previous studies reported that
the oil spill could destroy the structure and membrane system of microalgae cells, resulting in reduced photosynthesis ability of microalgae[3]. Moreover, the petroleum hydrocarbons also inhibited the synthesis of nucleic acids and proteins[4], interfered with the normal operation of the microalgae antioxidant defence system[5]. Therefore, this study selected microalgae as a test organism for evaluating the toxic effects of petroleum hydrocarbons.

The stable isotope analysis, which will be the topic of the following review, are the most effectively revealed the circulation path of organic matter and widely used in marine ecosystem research[6]. Disruption of photosynthesis or energy metabolism is one of the major physiological processes affected by petroleum hydrocarbon. So the microalgae was stimulated by external pollutants, the carbon stable isotopes ($\delta^{13}$C) enrichment in the photosynthetic system of microalgae will be affected[7]. In addition, the nitrogen cycle in marine ecosystems is also completed by microbial metabolism, including nitrogen assimilation and nitrogen nitrification, which directly affected the composition of nitrogen stable isotopes ($\delta^{15}$N) in marine ecology[8]. Experimental research is therefore needed to determine the oil spill of a direct impact of microalgae growth and mechanisms by the carbon and nitrogen stable isotopes. The aim of the present study is to evaluate the toxicity mechanism of petroleum hydrocarbons of microalgae, which provided a valuable reference for the protection of marine ecosystems.

In the present study, it was simulated the 96 h acute toxicity effects of 180# fuel oil chemical enhanced water-accommodated fractions (CE-WAF) on *Nitzschia closterium* (*N. closterium*) under the conditions of laboratory[9]. The inhibitory effects of the following CE-WAF on the growth of the microalgae was evaluated. Furthermore, the objectives were to use stable isotope analysis technology to explore the changes of $\delta^{13}$C and $\delta^{15}$N in microalgae under CE-WAF stress, thus revealing the C and N source fixation effects of oil spill on microalgae. More importantly, toxicity tests play an important role in detecting the marine environment and marine environmental assessment, as well as in providing a basis for the optimal management and use of dispersants and the marine ecological safety assessment.

2. Materials and method

2.1. Materials

The *N. closterium* was provided by the National Marine Environmental Monitoring Center (Dalian, China). Microalgae was cultured in sterilized seawater (pH = 8, salinity is 35 ‰) from Heishijiao (38°52'N, 121°33'E), an oceanic bay southwest of Dalian, China. 180# fuel oil was from China Marine Bunker (Petro China) Co., Ltd. And GM-2 dispersant was purchased from Qingdao Guangming Environmental Protection Technology Co., Ltd.

2.2. Experimental methods

The experiment to culture microalgae was carried out after the period was 12 L: 12 D, the light intensity was 3000 Lux, and the temperature was (20 ± 1) °C. In addition, the flask was shaken four times a day to prevent microalgae cells from adhering or sinking into the vessel.

This study was prepared on the 180# fuel oil of CE-WAF, the ratio of 180# fuel oil to seawater to GM-2 dispersant was (1:10:90/v:v:v), after magnetic stirring for 24 h and settling for 4 h. The lower layer of liquid was collected by siphoning and stored at 4℃. The total petroleum hydrocarbon (TPH) concentrations were determined by ultraviolet spectrophotometry. In addition, the gas chromatography mass spectrometry (GC-MS) was used to analyse and identify polycyclic aromatic hydrocarbons (PAHs) in CE-WAF.

The experimental settings were the control group and five groups of CE-WAF concentrations gradients, which was 1%, 3%, 5%, 7%, and 10%, respectively. Moreover, each group was set to three sets of parallel. The acute toxicity test of 96 h was performed and sampled every 24 h.
Table. 1 TPHs concentration of each group of CE-WAF.

| CE-WAF(mg/L) | Ctrl   | 1%   | 3%   | 5%   | 7%   | 10%  |
|-------------|--------|------|------|------|------|------|
|             | 0.001±0.01 | 1.12±0.03 | 3.37±0.03 | 5.62±0.01 | 7.87±0.03 | 11.24±0.15 |

2.3. The determination of $\delta^{13}$C and $\delta^{15}$N value

The GF/F glass fiber membrane (Whatman of Buckinghamshire, UK) was fired in advance at 450°C for 4 h. The experimental microalgal solution was filtered through a GF/F glass fiber membrane dried at 60°C for 24 h. Carbon and nitrogen stable isotope values ($\delta^{13}$C, $\delta^{15}$N) were determined on a Flash EA 1112-ConFlo IV-IRMS (Delta V Advantage, Thermo Fisher Scientific). Insert a standard sample for every 10 samples measured to maintain the accuracy of the experimental results and the stability of the instrument. The $\delta^{13}$X value is calculated according to Eq. (1):

\[
\delta^{13} X = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 10^3
\]

Where X is $^{13}$C or $^{15}$N; $R_{\text{sample}}$ is the $^{13}$C/$^{12}$C or $^{15}$N/$^{14}$N ratio of the sample; $R_{\text{standard}}$ is the $^{13}$C/$^{12}$C or $^{15}$N/$^{14}$N ratio of the international isotopic standard, Vienna Pee Dee Belemnite standard (VPDB). The standard deviation of these analyses was $\delta^{13}$C $< 0.10\%, \delta^{15}$N $< 0.20\%$.

2.4. Statistical analysis

Statistical analysis data was calculated by the one-way analysis of variance (ANOVA) in SPSS Statistics 19.0. Differences were considered significant at P $< 0.05$ and highly significant at P $< 0.01$.

3. Results and discussion

3.1. Analysis of the composition of polycyclic aromatic hydrocarbons in CE-WAF

Figure 1. The relative abundance of PAHs in CE-WAF.

The CE-WAF was a complex mixture of alkanes, cycloalkanes, and PAHs, main of the PAHs effected the growth and development of microalgae. We found the main content of the PAHs of CE-WAF were naphthalene (C$_2$-N), 2-methylnaphthalene (C$_2$-N), 1-methylnaphthalene (C$_1$-N) and phenanthrene (C$_0$-P) (26.3%, 25.8%, 17.8% and 7.1%, respectively), indicating the addition of GM-2 dispersant could effectively increase the solubility of TPHs (Figure 1). When the microalgae was actually exposed to the CE-WAF, which was present in the aqueous phase in a dispersed or soluble form, the main of PAHs may be affected the growth and development of microalgae[10].
3.2. The influence of CE-WAF on the growth of N. closterium

Figure 2. Cell density of N. closterium in each of CE-WAF concentrations at different times.

Summary of the 96 h acute toxicity test of N. closterium under different CE-WAF concentrations was presented in Figure 2. The control group density of the N. closterium increased exponentially, but the inhibition rate at 96 h with the CE-WAF concentration groups was 64.13%, 72.31%, 76.88%, 89.98% and 88.55%, respectively. In addition, the low concentration group (1% and 3%) grew slowly, while the high CE-WAF concentration group (5%, 7% and 10%) was significantly inhibited. It can be indicated that the higher the concentration of CE-WAF, the more obvious the inhibition of microalgae biomass[11]. This can be attributed to addition of dispersant promoted dissolution of crude oil, which increased their toxicity to microalgae.

3.3. Effect of the CE-WAF on $\delta^{13}\text{C}$ value of N. closterium

Table 2. Effect of CE-WAF on $\delta^{13}\text{C}$ value of N. closterium.

| Time/h | Ctrl | 1% | 3% | 5% | 7% | 10% |
|--------|------|----|----|----|----|-----|
| 24     | -20.84 ± 0.59 | -21.56 ± 0.24 | -21.87 ± 0.27* | -23.09 ± 0.24** | -23.68 ± 0.31** | -24.20 ± 0.07** |
| 48     | -20.47 ± 0.06 | -22.58 ± 0.18** | -22.06 ± 0.41** | -22.85 ± 0.14** | -23.97 ± 0.06** | -24.10 ± 0.10** |
| 72     | -13.75 ± 0.06 | -16.19 ± 0.11** | -19.45 ± 0.12** | -21.76 ± 0.09** | -23.84 ± 0.10** | -24.55 ± 0.06** |
| 96     | -10.59 ± 0.13 | -13.33 ± 0.11** | -15.82 ± 0.15** | -19.77 ± 0.07** | -22.72 ± 0.051** | -23.74 ± 0.21** |

* p<0.05.  ** p<0.01

The change of $\delta^{13}\text{C}$ value in N. closterium from 24 h to 72 h in control group and 1% CE-WAF concentration respectively was -20.84‰~13.75‰ and -21.56‰~16.19‰, indicating that the $\delta^{13}\text{C}$ value had a significant increased trend at 72 h (Table 2). At the initial phase of microalgae growth, the density of microalgae was in a stable rising phase, which can maintain the stable absorption of $^{12}\text{C}$ and no limited. However, the constant consumption of CO$_2$ lead to decrease of $^{12}\text{C}$ after entering the exponential growth period. Microalgae had to choose the absorption of $^{13}\text{C}$, which eventually led to an increase in the value of $\delta^{13}\text{C}$. At the concentration of 3% CE-WAF, the significant rise point appeared 96 h (-21.87‰~15.82‰). However, there was no significant increased trend at high (5%, 7% and 10%) CE-WAF concentrations. The reason might be due to the inhibition rate of microalgae cell growth and even death under the high concentrations of CE-WAF. In addition, the $\delta^{13}\text{C}$ value decreased in the CE-WAF concentrations increased at the same culture time. In the present study, the
microalgae was exposed in the high concentration of CE-WAF, caused the cell gene mutations[12-13]. The PAHs in oil spills was induced the microalgae to produce a large amount of active oxygen free radicals, resulting in a significant decreasing the cell synthesis rate.

3.4. Effect of the CE-WAF on $\delta^{15}N$ value of N. closterium

| Time/h | Ctrl  | 1%    | 3%   | 5%    | 7%    | 10%   |
|--------|-------|-------|------|-------|-------|-------|
| 24     | 13.28 ± 0.56 | 14.65 ± 0.60 | 14.25 ± 0.15 | 13.64 ± 0.52 | 12.91 ± 0.91 | 12.61 ± 0.43 |
| 48     | 15.37 ± 0.14 | 14.67 ± 0.20 | 14.863± 0.56 | 14.71 ± 0.40 | 13.87 ± 0.16*| 14.16 ± 0.44* |
| 72     | 14.72 ± 0.03 | 14.99 ± 0.30 | 15.12 ± 0.45 | 15.07 ± 0.27 | 14.53 ± 0.47 | 14.23 ± 0.46 |
| 96     | 14.290± 0.41 | 15.37 ± 0.33 | 14.77 ± 0.63 | 15.39 ± 0.31 | 14.49 ± 0.08 | 14.61 ± 0.61 |

* p<0.05, ** p < 0.01

The value of $\delta^{15}N$ in N. closterium increased with the culture time at the same concentration, except the control group (Table 3). However, there was no significant change in $\delta^{15}N$ value under the different CE-WAF concentration at the same culture time. Especially, the concentrations of 7% and 10% CE-WAF were significantly different from the control group (p < 0.05), and there was no significant difference in other groups. The pollutants in CE-WAF provided mostly hydrocarbons, whereas it was no change the nitrogen source in the culture solution. Thus, the change in $\delta^{13}C$ value was no significantly different with the concentrations of CE-WAF.

4. Conclusion

In this study, the acute toxicity test of N. closterium was exposed to different concentrations of CE-WAF at 96 h. The $\delta^{13}C$ value increased with the culture time at the same CE-WAF concentrations, and the value of $\delta^{13}C$ in the control group and CE-WAF concentration group was significantly different. The $\delta^{13}C$ value showed a significant upward trend at 72 h in the control group and 1% CE-WAF concentration, while there was no obvious upward trend at the high (5%, 7% and 10%) CE-WAF concentrations. And then, the $\delta^{15}N$ value in microalgae increased with the culture time, but there was no significant change in $\delta^{15}N$ value under the different CE-WAF concentration. In summary, the $\delta^{13}C$ value in microalgae can be reflected to the extent of PAHs pollution, providing a new means for marine monitoring.

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