Toxic effect of oil spill on the growth of *Ulva pertusa* by stable isotope analysis

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**Abstract.** The oil spills occur frequently during the offshore oil exploration and transportation, resulting in the destruction of the marine environment. After an oil spill, petroleum can stay in the sea for a long time and pose a toxic effect on marine organism. Under the action of the waves, oil continues to diffuse, flows to the intertidal zone of the coast, where it accumulates. *Ulva pertusa* Kjellman (Ulvales, Chlorophyta) is the marine green algae and is widely distributed and easy to breed in the intertidal zone. The study investigated the growth rates, chlorophyll-\(a\), and carbon and nitrogen stable isotopes of *U. pertusa* under the stress of the water accommodated fraction (WAF) for two kinds of oils (0\# diesel oil and Russian crude oil). The results showed that the growth rate and chlorophyll-\(a\) initially increased and then decreased under the stress of WAF. High concentration of WAF posed the obviously inhibitory effect on algae; and oil spill also causes the distinct fraction of carbon and nitrogen stable isotopes of *U. pertusa*, especially for carbon stable isotope. The change trends of bulk carbon and nitrogen isotopes were similar to those of growth rates of *U. pertusa*. Therefore, the application of stable isotope techniques can quickly evaluate the toxic effects of petroleum on algae and can be used as a new method to evaluate the toxicity of the oil spill in marine environment.

1. Introduction

With the rapid development of the global economy, petroleum energy is becoming more and more important, and offshore oil transportation, as well as exploitation, is also prospering. At the same time, oil spill accidents are aggravated, which seriously threaten the marine ecosystem [1]. After the oil spill, a large amount of oils float on the surface water, impeding photosynthesis of marine plants. Furthermore, it can also accumulate in sand and sediments, leading to long-lasting impact on marine ecology [2]. Some low-chain normal alkanes and polyaromatic hydrocarbon can be dissolved in seawater, and damage to the marine organisms [3].

*Ulva pertusa* is a kind of macroalgae, and is widely distributed in the coastal intertidal zones around the world. Due to its ability to rapidly absorb nutrients such as nitrogen and phosphorus, *Ulva pertusa* is often used to treat aquaculture wastewater to slow down eutrophication [4], and spore to evaluate metal toxicity via susceptibility to metal ions [5]. *U. pertusa* is fast growing species, and its leaves are thin and edible. It is often used as bait to feed marine animal. Therefore, *U. pertusa* has been gained the increasing attention on its environmental effect [6]. As the autotroph, *U. pertusa* can absorb and utilize CO\(_2\) and HCO\(_3^-\) as inorganic carbon sources, and synthesize its own organic substance through photosynthesis. When oil spill occurs, the toxic substances (N-alkanes and PAHs, etc.) in petroleum will dissolve in the seawater, and are absorbed by algae to destroy the physiological...
structure and photosynthetic system of algal cells, thus prevent the normal growth of marine organisms and threaten the ecological balance of the coastal zone [7].

Stable isotope technique has been wildly applied in the field of environmental ecology. Isotope fractionation refers to the phenomenon that isotopes of an element are distributed in different proportions among different substances during physical, chemical and biological reactions, etc. [8]. Isotopic fractionation can be caused by natural processes such as adsorption and biochemical reactions [9]. The algae preferentially absorb lighter $^{12}\text{C}$ and $^{14}\text{N}$ when nutrient elements are sufficient. Under the stress of organic pollution, membrane permeability and enzyme activity are affected by toxic substances, lead to algal metabolism disorder. The absorption rate of C and N elements changes, which can cause dynamic fractionation, and $\delta^{13}\text{C}$ (carbon stable isotope) and $\delta^{15}\text{N}$ (nitrogen stable isotope) also change significantly [10]. When marine algae are ingested by other marine animals, organic pollutants accumulate and change the carbon-nitrogen ratio of marine animals, thus affect the transmission of food chains and biodiversity [11]. Therefore, the stable isotope technique can be used to study algae growth status and the internal metabolism of algae.

In this study, the stable isotope technique was applied to study the effects of different water-soluble components of two kinds of oils on the stable isotope composition of carbon and nitrogen in the coastal dominant species $U.\ pertusa$, and the toxic effects of WAF on macroalgae. It provides an effective means for intertidal environmental ecological risk assessment and monitoring of oil spill pollution.

2. Materials and methods

2.1. Materials

The $U.\ pertusa$ and seawater were gathered in the Heishijiao of Dalian (China). The seawater was sterilized and filtered prior to use to remove impurities and microorganisms. First four-layer 500 mesh nylon filter cloth was used, followed by 0.2 μm microporous membrane (Whatman, Buckinghamshier, UK). $U.\ pertusa$ was repeatedly washed with the sterilized seawater to remove impurities, pre-incubated for one week under aerated conditions and replaced with seawater every two days.

2.2. Preparation and concentration determination of WAF

Preparation of the water accommodated fraction, petroleum and sterilized seawater (1:9 v/v) were mixed in a beaker, and stirred at room temperature for 24 hours with a magnetic stirrer. After the mixture was keep the whole night, the lower crude oil dispersion was collected into brown bottle by siphon method, and stored at 4℃ for further analysis. The WAF concentration was measured by ultraviolet spectrophotometer (SP-756P, Shanghai Spectrographic Instrument Co., Ltd, China).

Two kinds of oils were purchased from China Marine Bunker Co., Ltd (Petro China). Specific parameters are as shown in table 1:

| Sample | Oil species      | Density (20°C) kg/m³ | API gravity (°) | Viscosity (50°C) mm²/s | WAF (mg/L) |
|--------|------------------|----------------------|----------------|------------------------|-------------|
| 0#     | light diesel oil | 840                  | 38.2           | 4.1                    | 2.79        |
| RUS    | light crude      | 851.2                | 34.7           | 7                      | 8.91        |

2.3. Culture conditions

The experimental components were shown in table 2. The modified Conway nutrient and vitamins were added and cultured in a constant temperature light incubator. The temperature was maintained at 18 ± 0.1°C; the light-dark period of 12 h: 12 h and intensity was 3000 lx; and the culture solutions were shaken once a day. Each 24 hours, one piece of $U.\ pertusa$ was taken out from different concentration gradient cultures with tweezers and used ultrapure water to wash, and then placed in a
freeze dryer for drying. The experiment lasted for 96 hours and each treatment was carried out in triplicate.

Table 2. The experimental components.

| Component     | Control | 10% | 20% | 30% | 40% | 50% |
|---------------|---------|-----|-----|-----|-----|-----|
| U. pertusa/g  | 0.50    | 0.50| 0.50| 0.50| 0.50| 0.50|
| Seawater/L    | 1.0     | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 |
| WAF/L         | 0.0     | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |

2.4. Determination of growth rate and chlorophyll a
At the end of the 96 h experimental period, sterilized filter paper was used to absorb water from the algal surface and weigh each sample. The specific growth rate was calculated using the following formula:

\[
\text{Specific growth rate} = \left[ \frac{W_t}{W_0} \right]^{1/t} - 1 \times 100\%
\]

where \(W_0\) and \(W_1\) (m/g) represent the wet weight of algae at the beginning and end of the experiment, respectively; and \(t\) (d) is the experimental time.

The dried U. pertusa of different groups were taken to be grinded into powder with a mortar. The 0.20 g of sample was put it in a 10 mL plug colorimetric tube, and added 10 mL of 90% acetone at 55°C. The tubes were placed under the condition of 4°C for 4 h before centrifuged at 3850 g for 15 min [12]. The absorbance of supernatant was measured by ultraviolet spectrophotometer, and the calculated the content of chlorophyll a according to the Jeffrey and Humphrey [13].

2.5. Determination of carbon and nitrogen stable isotope
The powder of U. pertusa for 0.45 mg was wrapped into a tin cup. The samples were measured by elemental analyzer (Flash EA 1112, Thermo Fisher Scientific, USA) and stable isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, USA). The \(\delta^{13}\)C and \(\delta^{15}\)N values of U. pertusa were obtained. The natural abundance of stable N and C isotopes are expressed as

\[
\delta X = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 10^3
\]

where \(X\) represents \(^{15}\)N or \(^{13}\)C; \(R\) represents for \(^{15}\)N/\(^{14}\)N or \(^{13}\)C/\(^{12}\)C. The \(\delta^{15}\)N value is relative abundance of N\(_2\) in the air, \(\delta^{13}\)C value is relative to the natural abundance of PDB standard. \(\delta^{15}\)N and \(\delta^{13}\)C analysis were accuracy of ± 0.30‰ and ± 0.20‰, respectively.

2.6. Statistical analysis
The data were analyzed by SPSS20 software. When experimental data follow normality and homoscedasticity, one-way ANOVA method was used for the difference analysis, and then multiple comparisons (Dunca) was used to determine significant differences between them. For all statistical tests, \(p < 0.05\) was considered to be significantly standard.

3. Results and discussion

3.1. Specific growth rate and chlorophyll a
The WAF addition contents were 0, 10, 20, 30, 40, and 50%. These percentages corresponded to WAF concentrations of 0# diesel oil of 0.00, 0.28, 0.55, 0.83, 1.10, and 1.38 mg L\(^{-1}\) and Russian crude oil of 0.00, 0.90, 1.78, 2.67, 3.56, and 4.46 mg L\(^{-1}\).

Figure 1 shows the U. pertusa specific growth rate and chlorophyll a content after 96 h under 0# diesel stress. The growth rate and chlorophyll a initially increased and then decreased with the increase of WAF concentration. However, the growth rate reached a maximum of 6.75% at 10%, while the chlorophyll a reached a maximum at 3.71 mg L\(^{-1}\) at 30%. Due to the relatively short duration of acute
toxicity experiments, the chlorophyll content not decreased in every cell although the biomass of *U. pertusa* has changed. As a result, when WAF was 30%, the chlorophyll a content increased even if the growth was inhibited [14]. Figure 2 shows the *U. pertusa* specific growth rate and chlorophyll a content after 96 h under Russian crude oil stress. The change trend of the *U. pertusa* under Russian crude oil stress was the similar to that of 0# diesel, under the stress of Russian crude oil, when the WAF was 30%, the maximum growth rate was 7.01%, while chlorophyll a maximum value was 2.47 mg L$^{-1}$ when the WAF was 20%. The change trend of chlorophyll a was similar to that of growth rate.

![Figure 1](https://example.com/figure1.png)  
*Figure 1.* Specific growth rate (bar chart) and chlorophyll a content (line chart) of *U. pertusa* exposed of different WAF concentrations of 0# diesel oil for 96 h.

![Figure 2](https://example.com/figure2.png)  
*Figure 2.* Specific growth rate (bar chart) and chlorophyll a content (line chart) of *U. pertusa* exposed of different WAF concentrations of Russian crude oil for 96 h.

At the low WAF concentrations promoted the growth of *U. pertusa* and increased the chlorophyll a content, which conform to the "hormesis" theory. The "hormesis" theory refers to the low dose stimulation and high dose inhibition effect when the organism was subjected to toxic stress [15]. When the concentration of WAF was low, the repair mechanism may be activated, leading to the normal growth. Moreover, algae can also degrade the components of polycyclic aromatic hydrocarbons contained in WAF and generate other metabolites to reduce toxicity [16]. Under the stress of high WAF concentration, the growth rate and chlorophyll a of *U. pertusa* were significantly slower than those of the control group ($p < 0.05$). When the WAF was 50%, chlorophyll a content and specific growth rate reached the minimum in both oils. The results show that high concentration of WAF inhibits the growth and reduced chlorophyll a. *U. pertusa* as a primary producer, belong to autotrophic organisms and needs photosynthesis. The hydrocarbons contained in WAF were absorbed by algae and accumulated in the cells, inhibited the photosynthesis by reduce electron transport capacity, initial photochemical yield, etc. Due to the inability to perform photosynthesis, chlorophyll a content gradually decreases and growth rate slows down [17]. Toxic substances can also cause imbalance of oxidative balance in organisms, in which a large amount of free radicals were generated to destroy cells and induce algae lipid peroxidation. The activity of antioxidant enzymes was inhibited, and the excess free radicals cannot be removed in time, thus affecting the mitosis and reproduction of cells, destroying the structure and function of chloroplasts, and impeding cell growth [16,18].

Under the stress of 0# diesel and Russian crude oil, the specific growth rate and chlorophyll a of *U. pertusa* were different. Because of the different oils, the WAF toxicity varies with the proportion of the main components dissolved in seawater [19]. Although the two oils were light petroleum in this study, even similar hydrocarbon compositions showed different effects on macroalgae [20].

### 3.2. Nitrogen stable isotope
The nitrogen stable isotope values of \textit{U. pertusa} under two oils stress were shown in figure 3. The $\delta^{15}$N increased gradually over time in the control group and the low WAF concentration, and no significant change at the high concentration. At the low WAF concentrations, \textit{U. pertusa} accelerated growth and need to absorb a large amount of nutrients. When nitrogen content was sufficient in water, algae will preferentially absorb lighter $^{14}$N. As time goes on, the $^{14}$N gradually consumed, and the algae absorbed $^{15}$N to continue to supply growth, so $\delta^{15}$N was gradually positive. Under the stress of exposure to 0# diesel oil and Russian crude oil for 96 h, the overall trend in $\delta^{15}$N values was first increase and then decrease. The range of variation was 8.47‰ ~ 8.79‰ and 7.72‰ ~ 9.66‰, respectively. However, the change trend of $\delta^{15}$N was not obvious under 0# diesel stress. The WAF concentration was also different due to types of oil. The WAF concentration of Russian crude oil was 8.91 mg L$^{-1}$, which has significant effect on the fractionation of nitrogen. The 0# diesel has maximum WAF concentration was 2.8 mg L$^{-1}$, resulting in insignificant nitrogen isotope fractionation.

\textbf{Figure 3.} Nitrogen stable isotope of \textit{U. pertusa} over time when exposed to different concentrations of WAF for 96 h: (a) 0# diesel oil and (b) Russian crude oil.

\textit{U. pertusa} is a kind of large algae with large leaf area, so it has a strong ability to absorb nutrients and photosynthesis. Therefore isotope fractionation occurs when nitrogen element was sufficient [21]. When the concentration of WAF was low, the metabolic rate of algae will be accelerated, so the algae would preferentially absorb $^{14}$N in the water. When $^{14}$N was exhausted, the algae would absorb $^{15}$N and the $\delta^{15}$N value was positive. With the gradual deepening of pollution, most WAFs contain strong lipophilicity, and toxins may accumulate inside the cells and affect the growth and reproduction of marine organisms, leading to the depletion in $\delta^{15}$N [22]. In this study, nitrate was the main nitrogen source and contains little ammonium salt. Algae give priority to NH$_4$-N. Nitrate needs to be used under the action of some enzymes by phytoplankton, and converted to ammonia nitrogen by reduction reaction [23,24]. The nitrate reductase plays an important role in the metabolism of phytoplankton, reducing nitrate to nitrite [25]. Karsh [26] found that when nitrate reductase was lowly expressed, it affected the conversion of nitrate. Therefore, nitrate reductase activity may be inhibited under high concentration of WAF, and the nitrogen absorption ratio decrease leaded to the negative $\delta^{15}$N.

3.3. \textit{Carbon stable isotope}

The carbon stable isotope values of \textit{U. pertusa} under two oils stress were shown in figure 4. In the control group, $\delta^{13}$C of \textit{U. pertusa} was gradually negative in the first 48 h, and stabilized in the last 48 h; it was different form $\delta^{13}$N. Because of the carbon source mostly converted from carbon dioxide in the air, and this experiment was carried out in an open system and CO$_2$ supply sufficient. When algae undergo photosynthesis, it will preferentially utilize $^{12}$CO$_2$, and $\delta^{13}$C gradually negative and then stabilize [27,28].
Figure 4. Carbon stable isotope of *U. pertusa* over time when exposed to different concentrations of WAF for 96 h: (a) 0# diesel oil and (b) Russian crude oil.

Under the stress of exposure to 0# diesel oil and Russian crude oil for 96 h, the overall trend in $\delta^{13}C$ values was first increase and then decrease. The range of variation was $-24.77\%$ ~ $-20.64\%$ and $-18.51\%$ ~ $-13.82\%$, respectively. When the WAF was 0.55 and 1.78 mg L$^{-1}$, $\delta^{13}C$ was positive compared with the control group (2.14% and 4.03%, respectively). At the low concentrations, WAF as a foreign substance stimulates the rapid growth of *U. pertusa*. Inorganic carbon source that algae absorb during photosynthesis was only CO$_2$ and HCO$_3^{-}$ [29]. When the carbon source was sufficient, CO$_2$ can be directly utilized by algae in the absence of CO$_2$, HCO$_3^{-}$ was converted into CO$_2$ by carbonic anhydrase and absorbed by algae, and stable isotope fractionation (about 10‰) occurs during the transport [10,30]. The $\delta^{13}C$ value of *U. pertusa* was higher than the control group. With the increasing concentration of WAF, the toxic effect of petroleum hydrocarbons on *U. pertusa* enhanced, and algae cannot maintain normal growth. On the one hand, WAF can block the absorption of nutrients and CO$_2$, and affects the synthesis of biomolecules such as DNA and protein [31,32]. On the other hand, WAF contain a lot of hydrocarbons (alkanes and aromatics), especially benzenes and naphthalenes, which are water solubility and lipophilicity and tend to accumulate in algae cells, interacting with phospholipid bilayers to change membrane structure and function. Toxic substances also inactivate the biological enzymes involved in biochemical processes in algae cells [33], resulting in the inability of algae to perform normal life activities, so $\delta^{13}C$ was gradually negative.

Table 3. Correlation analysis between growth rate and chlorophyll a, $\delta^{15}N$ and $\delta^{13}C$ at 96 h.

| Growth rate | Chl-a | $\delta^{15}N$ | $\delta^{13}C$ |
|-------------|-------|---------------|---------------|
|             | r     | p             | r             | p             | r             | p             |
| 0#          | 0.040 | 0.87          | 0.43          | 0.06          | 0.686*        | 0.00          |
|             | 3     | 9             | 9             |               |               | 2             |
| RUS         | 0.650* | 0.00          | 0.45          | 0.06          | 0.958*        | 0.00          |
|             | 1     | 1             | 0             |               |               | 0             |

$p < 0.05$ for significant difference; $r \geq 0.8$ for height correlation, $0.5 \leq r < 0.8$ for moderate correlation, $r < 0.5$ for low correlation.

Table 3 shows the correlation between the growth rate of *U. pertusa* and chlorophyll a, $\delta^{13}C$ and $\delta^{15}N$ under the stress of 0# diesel and Russian crude oil after 96 h. In 0# diesel, the growth rate of *U. pertusa* was correlated with $\delta^{13}C$, but no correlated with chlorophyll a and $\delta^{15}N$. In Russian crude oil, the growth rate of *U. pertusa* was correlated with chlorophyll a and $\delta^{13}C$, especially to $\delta^{13}C$. It is indicated that the carbon stable isotope can approximately judge the growth state of algae compared to
chlorophyll a. When algae were subjected to toxic stress, the trend of chlorophyll content was not clear. So that, there was a certain error to judge algae growth status by chlorophyll a [14].

4. Conclusion
Under the stress of different WAF concentrations of two kinds of oils (0# diesel and Russian crude oil), the low concentration of WAF promoted the growth of macroalgae, while the high concentration of WAF significantly inhibited the macroalgae growth. This study was the first time to combine stable isotope technique with toxic stress of oil on the U. pertusa. It was found that toxicity affects the absorption of carbon and nitrogen by U. pertusa, which leads to the stable isotope fractionation of carbon and nitrogen, especially carbon stable isotope basically consistent with the growth rate. Therefore, the application of stable isotope techniques can quickly evaluate the effects of petroleum on algae, and provide a new method for coastal environmental monitor and assessment.

Acknowledgments
This work was supported by the National Science & Technology Pillar Program (grant numbers 2015BAD17B05); and the Fundamental Research Funds for the Central Universities (grant number 3132016332).

References
[1] Beyer J, Trannum H C, Bakke T, Hodson P V and Collier T K 2016 Mar. Pollut. Bull. 110 28-51
[2] Faksness L G, Altin D, Nordtug T, Daling P S and Hansen B H 2015 Mar. Pollut. Bull. 91 229-9
[3] Peterson C H, Rice S D, Short J W, Esler D, Bodkin J L and Ballachey B E 2003 Science 302 2082-6
[4] Largo D B, Diola A G and Marababol M S 2016 Aquacult. Rep. 3 67-76
[5] Han Y S, Kumar A S and Han T 2009 Toxicol. Environ. Health. Sci. 1 24-31
[6] Sónia Costa, Crespo D, Henriques B M G, Pereira E, Duarte A C and Pardal M A 2011 Water Air Soil Poll. 217 689-99
[7] Leliaert F, Zhang X W, Ye N H and Malta E J 2009 Phycol. Res. 57 147-51
[8] Marion H O and Leary 1981 Royal Society of New Zealand Wellington New Zealand 20 553-67
[9] Macko S A, Fogel M L, Hare P E and Hoering T C 1987 Chem. Geol. 65 79-92
[10] Wu Y Y, Xu Y, Li H T and Xing D K 2012 Chinese Science Bulletin 57 786-9
[11] Elliott J E 2005 Arch. Environ. Con. Tox. 49 89-96
[12] Liu Y X, Liu Y, Li N, Lou Y D and Zhao X D 2019 Sci. Total Environ. 649 1443-51
[13] Jeffrey S W and Humphrey G F 1975 Bioche. Physiol. Pflanzen. 167 191-4
[14] Gilde K and Pinckney J L 2012 Estuar. Coast. 35 853-61
[15] Stebbing A R D 1982 Sci. Total Environ. 22 213-34
[16] Liu Y, Luan T G, Lu N N and Lan C Y 2006 J. Integ. Plant Biol. 48 169-80
[17] Wang X, Zhang J, Shi X, Zhu C, An Y and Jun S 2002 Arch. Environ. Contam. Toxicol. 42 272-9
[18] Sargian P, Sébastien Mas, émilien Pelletier and Demers S 2007 Polar Biol. 30 829-41
[19] Djomo J E, Dauta A, Ferrier V, Narbonne J F, Monkiedje A and Njine T 2004 Water Res. 38 1817-21
[20] Paixão J F, Nascimento I A, Pereira S A, Leite M B L, Carvalho G C and Silveira J S C 2007 Environ. Res. 103 365-74
[21] Wada E and Hattori A 1978 Geomicrobiol. J. 1 85-101
[22] Özhan K, Miles S M, Gao H and Bargu S 2014 Environ. Monit. Assess. 186 3941-56
[23] Joseph L and Villareal T A 1998 J. Exp. Mar. Biol. Ecol. 229 159-76
[24] Gu X Y, Li K Q, Pang K, Ma Y and Wang X 2017 Mar. Pollut. Bull. 124 946-52
[25] Berges J A and Hageman P Y 1995 Limnol. Oceanogr. 40 82-93
[26] Karsh K L, Trull T W, Sigman D M, Thompson P A and Granger J 2014 *Geochim. Cosmochim. Ac.* **132** 391-412

[27] Farquhar G D, And J R E and Hubick K T 1989 *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **40** 503-37

[28] Guy R D, Fogel M L and Berry J A 1993 *Plant Physiol.* **101** 37-47

[29] Colman B, Huertas I E, Bhatti S and Dason J S 2002 *Funct. Plant Biol.* **29** 261-70

[30] Marlier J F and O‘Leary M H 1984 *J. Am. Chem. Soc.* **106** 5054-7

[31] Bopp S K and Lettieri T 2007 *Gene (Amsterdam)* **396** 293-302

[32] Huang Y J, Jiang Z B, Zeng J N, Chen Q Z, Zhao Y Q and Liao Y B 2011 *Environ. Monit. Assess.* **176** 517-30

[33] Jiang Z, Huang Y, Chen Q, Zeng J and Xu X 2012 *Mar. Environ. Res.* **81** 12-7