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Toxicity of Fuel Oil Water Accommodated Fractions on Two Marine Microalgae, *Skeletonema costatum* and *Chlorela spp*

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Abstract In this paper, the acute toxicity of four fuel oils including F120, F180, F380 and No.-20 was evaluated by exposing the marine microalgae *Chlorela spp.* (Chlorophyta) and *Skeletonema costatum* (Bacillariophyta) in the fuel oil water accommodated fractions (WAF). The bioassay showed that F180 WAF was the most toxic to both microalgae. The 96 h EC_{50} value of F180 WAF for *Skeletonema costatum* and *Chlorela spp.* was 9.41 and 13.63 mg/L expressed in concentration of total petroleum hydrocarbons, respectively. WAFs of F120, F180 and F380 were more toxic to *Skeletonema costatum* than to *Chlorela spp.* In contrast, No.-20 WAF did not show significant toxicity for both *Skeletonema costatum* and *Chlorela spp.*

Keywords Water accommodated fractions · *Skeletonema costatum* · *Chlorela spp.* · Fuel oil

The previous research reported that there had around five million tons of crude oil and fuel oil to enter the marine environment by a variety of sources each year (Kennedy and Farrell 2005). Among which, oil spill is a major source. The oil spill incidents had been a crucial disaster endangering all the trophic level organisms of marine ecosystem (MacFarlane et al. 2004; Solé et al. 2008).

With the rapid economic development and energy demand these years, petroleum import amount is growing dramatically in China. Marine petroleum transportation and harbor throughput increase year after year, causing the frequency oil spill accident. Total 46 times oil spill accidents larger than 50 t each time had been recorded from 1978 to 2000 and more than 1.7941×10^4 tones of crude oil or marine fuel oil were spilled into the marine environment (Chen et al. 2007). In the light of the protection of marine environment and fishery resources, it is necessary and crucial to study the impact of oil pollution to marine organisms for the emergency management of oil spill.

In 2008, a program focusing on oil spill emergency management system of China was funded by National Key Technology R&D Program of China (2006BAC11B03). In this program, the toxicity of crude oils and fuel oils that are at the risk of spill into coastal waters of China were assessed by bioassay test. Before the bioassay, oils were treated into water accommodated fractions (WAFs). WAFs had been recommended as a standard method of the preparation of oil test media by Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) (Singer et al. 2000). The WAFs can be considered to contain the highest possible concentration of dissolved petroleum hydrocarbons expected from a spill (Faksness et al. 2008).

The objective of this paper is to evaluate the fuel oil toxicity on microalgae with WAFs. Marine microalage, as the most important primary producer, its dynamics and accumulation of oil contaminants (Binark et al. 2000) under the stress of spilled oil can transfer the effects to higher trophic level consumers including zooplankton, benthos, fish larvae and juvenile fish (Duffy and Stachowicz 2006). Two dominant species in coastal waters of China were selected as test species, *Skeletonema costatum* (Bacillariophyta) and *Chlorela spp.* (Chlorophyta). The two species are easy to be cultured and very sensitive to environment toxicants, and therefore can be good indicator species in the evaluation of oil toxicity. The

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calculated toxicity data can be implemented into the oil toxicity database of China.

Materials and Methods

Sample of four fuel oils including F120, F180, F380 and No.-20 and the oil characteristic data were provided by Waterborne Transportation Institute, Ministry of Transport of the People's Republic of China. Oil characteristic included the viscosity, straight chain alkanes content, and aromatic hydrocarbons (AH) percentage. Viscosity of oil was measured according to GB/T 2676-88. The chemical composition was determined by GC/MS (Agilent 6890N/592). To avoid losing the most volatile components, samples were kept in refrigerated containers for up to 2 weeks.

WAF of each fuel oil was prepared according to Tsvetnenko and Evans (2002) with some modification. Briefly, 40 g fuel oil was added into 1,500 mL sterilized seawater. The content of closed flask was magnetically stirred with a 34 mm-length bar at 190 rpm rotation speed in room temperature (20 ± 2°C) for 24 h. The speed can also be changed a little to acquire maximum oil column in the flask. The aqueous phase was siphoned into a separating funnel and remained for 1 h. Then aqueous phase was set into brown bottle and stored at 4°C. Total petroleum hydrocarbons (TPH) for each fuel oil WAF was quantified by UV-spectroscopy according to the specification for China marine monitoring GB/T 17378-2007.

A 96 h algae bioassay was carried out to test their toxicity of the above four fuel oils. Microalgae *Chlorela spp.* and *Skeletonema costatum* for bioassay were cultured with Guillard's f/2 medium (Guillard and Ryther 1962) at 25°C, salinity 25‰, 300 μmol/m^2 s in a light controlled incubators, set Light/dark ratio at 14:10. WAFs was added into f/2 medium with 5 mL exponential growth stage microalgae sample at 1%, 10%, 25%, 50%, 75% dilution rate in different group. Total volume was 100 mL of each flask. Cells of microalgae were counted with 7230/G Spectrophotometer (Shanghai analyzer company, China) at 680 nm every 24 h.

Algae growth rate μ were calculated with formula

$$\mu = \frac{\ln N_t - \ln N_0}{t} \times \ln 2 \quad (1)$$

Parameter N_t was the algae biomass at 72 h and N_0 is the initial algae biomass.

Growth rate inhibition percentage (y) of each group was calculated with formula

$$y = \frac{1-\mu}{\mu_0} \times 100 \quad (2)$$

μ_0 is the algae growth rate of control group.

Growth rate inhibition percentage (y) versus TPH concentration of WAF (x) were fit using graphing software to the equation

$$y = 50 + \left[\frac{100}{\pi} \times arctg\left(\frac{x-T}{W}\right)\right] \quad (3)$$

Parameters T and W were calculated by automatic iterations. This equation is only valid if operated in radians mode. T is the EC_{50} value, because

$$x = T + W \times tg\frac{y-50}{100} \quad (4)$$

and the second term of is 0 if $y = 50$. W is a parameter related to the shape of the curve (Hampel et al. 2001).

Results and Discussion

Four fuel oils had different physical characteristics which can be informed from the viscosity value. No.-20 was light fuel oil, whereas the other three were heavy fuel oils (ASTM 1992). Chemical analysis data of four fuel oils (Table 1) revealed that AH content percentage of F180 was the highest (44.53%) and that of No.-20 was the lowest (10.29%). Low concentration of AH in No.-20 was due to the lower concentrations of 2–3 and 4–6 ring PAHs in light fuel oil. Normally, heavy fuel oil had higher concentration of 2–3 and 4–6 ring PAHs than light fuel oil (Faksness et al. 2008).

The concentration of TPH in four WAFs was listed in Table 1. F120 WAF had the highest concentration of TPH (142.6 mg/L), and No.-20 had the lowest concentration of TPH (36 mg/L). WAFs are an effective test medium that can help to acquire the maximal toxicity of water extracts of fuel oil (Faksness et al. 2008). The highest TPH concentration in a fuel oil can be gotten by mixing for 24 h at the agitator velocity of 190 rpm (Tsvetnenko and Evans 2002). However TPH concentration and chemical components in WAF depend on oil characteristic. The TPH in light fuel oil WAF was lower than heavy fuel oil because more light components dissolved into WAF than heavy

Table 1 Oil characterization data

Fuel oil	Viscosity (mm^2/s)	Straight chain alkanes content (%)	AH content (%)	TPH concentration of fuel oil WAFs (mg/L)
No.-20	2.5–8.0 (20°C)	55.05	10.29	36.0
F120	15 (100°C)	36.38	32.91	142.6
F180	25 (100°C)	37.23	44.53	120.6
F380	20.5 (50°C)	36.97	35.67	105.4

fuel oil. For the heavy fuel oils, the chemical component dissolved in the WAF was affected by the viscosity. F120 had the lowest viscosity, therefore the concentration of TPH in F120 WAF was highest among the three heavy fuel oils.

Three heavy fuel oil WAF caused toxicity to *Chlorela spp*. Figure 1a–c shows a good fit curve between *Chlorela spp.* growth inhibition and TPH concentration of WAF of the three fuel oil WAF ($p < 0.01$). No.-20 WAF did not show a good dose–response relation ($p > 0.05$) which indicated that No.-20 WAF had no significant toxicity to *Chlorela spp.* (Fig. 1d). A thin film of oil occurred in the No.-20 WAF medium during bioassay. The 96 h EC_{50} expressed as TPH concentration for F120, F180 and F380 was 17.97, 13.63 and 73.15 mg/L, respectively (Table 2). F180 WAF was the most toxic to *Chlorela spp*.

In the bioassay of *Skeletonema costatum*, good fit curves between *Skeletonema costatum* growth inhibition and TPH of concentration of F120, F180 and F380 WAF were also observed (Fig. 2a–c). No.-20 WAF also had no significant effect on the growth rate of *Skeletonema costatum* (Fig. 2d). A thin film of oil also occurred in the No.-20 WAF medium during bioassay. The 96 h EC_{50} value of WAFs of F120, F180 and F380 for *Skeletonema costatum* were 12.69, 9.41 and 16.13 mg/L, respectively. F180 WAF was the most toxic to *Skeletonema costatum*.

The 96 h EC_{50} values of F120, F180 and F380 WAFs for *Skeletonema costatum* were all smaller than that for *Chlorela spp.* (Table 2), which indicated that *Skeletonema costatum* was more sensitive to WAFs of heavy fuel oils than *Chlorela spp*. Field investigation after oil spill accident of "TASMAN SEA" tanker showed diatoms were more sensitive than dinoflagellates. Species number of diatom decreased from 40 to 18, and contrarily the species number of dinoflagellates did not change (Gao et al. 2007). Many studies had also confirmed that phytoplankton community happened abnormal succession under the stress of oil pollution. The dominant degree of less tolerant species gradually decreased, even vanished, while more tolerant species gradually became the dominant ones (Paixăo et al. 2007; Sargian et al. 2007). The abundance of *Skeletonema costatum* will be primarily affected by oil WAFs once oil spill occurring in coastal waters of China. The change of phytoplankton community structure can influence the biomass of zooplankton, benthic invertebrates, or other filter feeders by bottom-up effects.

The bioassay revealed that F180 WAF was the most toxic to both *Chlorela spp.* and *Skeletonema costatum* among four fuel oil WAFs. However, the TPH concentration in F180 WAF was lower than in F120 WAF, which illustrated that more toxic substances existing in F180 WAF than that in F120 WAF. According to the research of Saco-Alvarez et al. (2008), the difference in toxicity

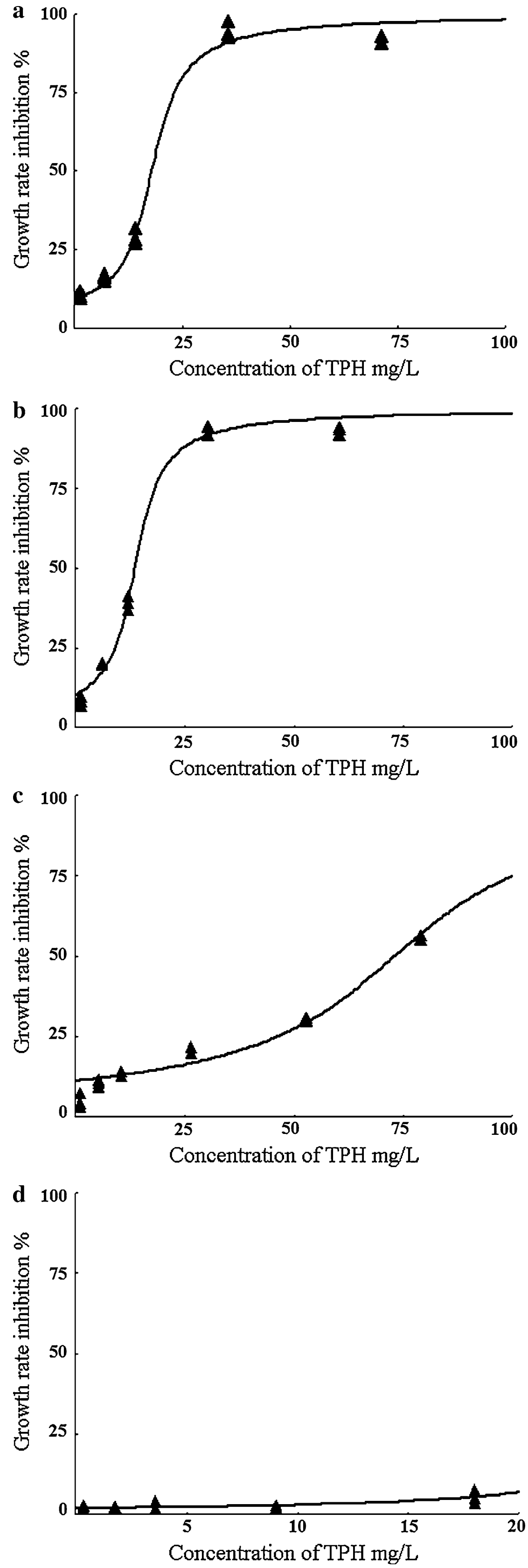

Fig. 1 WAFs toxicity curves for *Chlorela spp.* **a** F120 (a), **b** F180, **c** F380, **d** No.-20

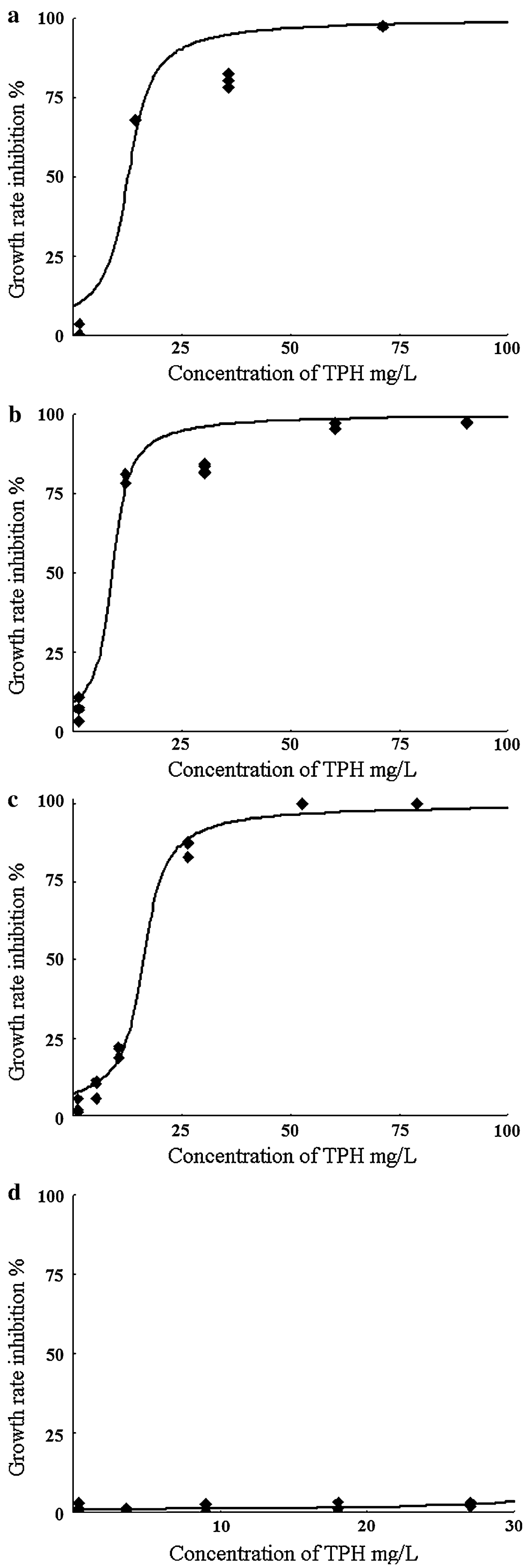

Fig. 2 WAFs toxicity curves for *Skeletonema costatum*. **a** F120 (a), **b** F180, **c** F380, **d** No.-20

Table 2 Microalgae *Chlorela spp.* and *Skeletonema costatum* growth inhibition 96 h EC_{50} values expressed in TPH concentration of WAF for fuel oil F120, F180, F380, and No.-20

Fuel oil WAF	96 h EC_{50} (CI 95%) *Chlorela spp.*, mg/L TPH	96 h EC_{50} (CI 95%) *Skeletonema costatum*, mg/L TPH
F120	17.97 (16.40, 19.54)	12.69 (9.27, 16.11)
F180	13.63 (12.78, 14.35)	9.41 (6.39, 12.42)
F380	73.15 (68.98, 77.26)	16.13 (14.23, 18.02)
No.-20	n.c.	n.c.

Confidence intervals are expressed in parenthesis (CI 95%)

n.c. indicated that an EC50 could not be calculated because the WAF was not toxic and growth was inhibited by less 10% at all tested WAF concentrations

between standard marine fuel oil and prestige fuel oil could not be explained on the basis of total aromatic hydrocarbon content. He thought there must be other unmeasured toxic compounds also responsible for toxicity in prestige WAF except the aromatic hydrocarbons. These compounds probably were unresolved complex mixture and polar compounds (Neff et al. 2000), or some hydroxylated derivatives of PAHs (Saco-Alvarez et al. 2008).

Short (2003) thought that the light compounds including BTEX, naphthalene and its homologues were water soluble and easily volatile so cannot stay long in the water. In our study, No.-20 WAF did not have significant toxicity to both *Chlorela spp.* and *Skeletonema costatum*. The low toxicity of No.-20 WAF was mainly caused by the low TPH concentration in WAF on one hand, and the light compounds volatilized during bioassay on the other hand.

In conclusion, F180 is the most toxic to microalgae, F120 and F380 are also toxic, especially for marine diatom. WAF toxicity tests on microalgae in the laboratory have been proved near to the real oil spill environment. Then the result of our study can afford basic toxicity data of fuel oil for the construction of an oil spill emergency management system in China.

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