

New findings in effect of different crude oil concentrations on bacterioplankton communities

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Abstract: [Objective] To investigate the effect of crude oil concentrations on bacterial community structures and diversity, and attempt to elucidate the mechanism of this effect. [Methods] Seawater was sampled near a marine drilling platform and was treated with five different crude oil concentrations (0 to 10 g/L) for a week in the laboratory. Then the bacterial communities were detected using terminal restriction fragment length polymorphism (TRFLP) method. [Results] Some new findings were exhibited, such as the bacterial diversity did not simply decrease with the increase of oil concentrations, but decreased at first, then increased and decreased again. Bacterial communities of treatments with 0.1 g/L (M0.1) and 0.5 g/L (M0.5) crude oil, treatments with 2.5 g/L (M2.5) and 10 g/L (M10) crude oil were similar, respectively, and bacterial communities in the oil added groups (M0.1, M0.5, M2.5, M10) were significantly different from the control (M0). Classification results of the dominant terminal restriction fragments (TRFs) in the oil treatments mainly attached to Proteobacteria, Firmicutes and Bacteroidetes. Based on the relative ratios of TRFs in different treatments, the 52 TRFs could be divided into six types (I to VI): low (I)/middle (II)/high (III)/broad (IV)/narrow (V) concentrations of crude oil adapted bacteria and crude oil sensitive bacteria (VI). Furthermore, “Carbon & Energy sources-Toxicities” hypothesis was proposed to explain effects of oil pollution on bacterioplankton. [Conclusion] Impacts of crude oil pollutions on the marine bacterioplankton are closely related to the concentration of oil and the original bacterial communities in the seawater, these bacteria could be classified upon their adaptability to the crude oil. And the “Carbon & Energy sources-Toxicities” hypothesis could explain effects of oil pollution on bacterioplankton very well.

Keywords: Crude oil pollution, TRFLP, Bacterioplankton, Community structures, Hypothesis

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不同石油浓度对浮游细菌群落影响的新发现

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摘要: 【目的】研究不同浓度石油污染对海水中浮游细菌群落结构及多样性的影响,并尝试阐述造成这种影响的机理。【方法】将采集自海上钻井平台附近的表层海水样品,在实验室条件下用不同浓度的石油(0-10 g/L)处理1周后,用末端限制性片段长度多态性(TRFLP)方法检测其细菌群落结构及多样性变化情况。【结果】得到了一些与以往研究结果不同的新发现,如细菌多样性不是简单地随石油浓度的升高而降低,而是呈现先降低再升高最后又降低的趋势。0.1 g/L石油处理组(M0.1)与0.5 g/L处理组(M0.5)、2.5 g/L石油处理组(M2.5)和10 g/L处理组(M10)的细菌群落结构更相似,且添加石油处理组(M0.1、M0.5、M2.5、M10)与未添加石油的对照组(M0)间细菌群落结构相差较大。与石油降解相关的细菌主要集中于变形菌门(Proteobacteria)、厚壁菌门(Firmicutes)和拟杆菌门(Bacteroidetes)。基于TRFs在不同处理组的相对比例,可将实验得到的52个TRFs划分为6种类型:低浓度(I)、中浓度(II)、高浓度(III)、广浓度(IV)、窄浓度(V)石油适应型和石油敏感型(VI)细菌。另外,首次提出“碳(能)源-毒素”假说来解释石油污染对浮游细菌群落的影响。【结论】石油污染对浮游细菌群落的影响与石油的浓度和海水中原有的历史细菌群落密切相关,可以按照浮游细菌对石油的适应性对其进行分类,并且“碳(能)源-毒素”假说能较好地解释石油污染对浮游细菌群落的影响。

关键词: 石油污染, TRFLP, 浮游细菌, 群落结构, 假说

1 Introduction

Crude oil as a complex mixture of hydrocarbons and other organic compounds is classified into four fractions: aliphatics, aromatics, resins and asphaltenes^[1]. Meanwhile, it includes some heavy metal constituents, most notably mixture of vanadium and nickel^[2-3]. So crude oil released into seawater affects marine organisms in many ways, but it is gradually removed by the action of oil-degrading microorganisms, especially bacteria^[2].

With the development of microbial molecular methods, culture-independent methods, such as TRFLP, DGGE (denaturing gradient gel electrophoresis), 16S rRNA gene clone library and high-throughput sequencing, showed great advantages in monitoring dynamics and diversity of microbial communities. Due to the high throughputs, high reproducibility and robustness, quantitative in species ratios and web-based RDP database^[4-5], TRFLP method has been widely used in recent microbial diversity and dynamics researches^[6-7]. Depending on culture and/or culture-independent methods, a large number of studies have examined the effects of crude

oil or its partial components on microbial communities in different environmental samples. MacNaughton et al. found that the structure and diversity of the dominant bacterial community changed substantially and oil treatment encouraged the growth of gram-negative microorganisms within the Alphaproteobacteria and Bacteroidetes during bioremediation of an experimental oil spill^[8]. Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were demonstrated to be the adapted microbiota during the degradation of Tunisian zarzatine oil, and a clear decrease in bacterial diversity happened according to the incubation time in seawater^[9]. The polycyclic aromatic hydrocarbons (PAHs) as major components of crude oil were found to reduce the microbial diversity both with the exposure time and the PAH concentration in the mangrove sediment^[10].

However, the studies in relationships between crude oil concentration gradients and bacterioplankton communities are very limited. Systematic and general theories or hypotheses to explain the effects of crude oil pollution on bacterial communities are rarely

proposed. In this study, we reported the effects of different crude oil concentrations on the bacterioplankton communities in the Yellow River estuary. Based on the data, "Carbon & Energy sources-Toxicities" hypothesis was proposed.

2 Materials and Methods

2.1 Sampling site and physiochemical parameters

The Yellow River estuary is situated on the confluence among the Yellow River, Bohai Bay and Laizhou Bay of China, and the Shengli Oil Field is located here. Therefore, it is threatened by crude oil pollution, for instance, the ConocoPhillips oil leak in Bohai Bay happened in 2011. Surface seawater was sampled beside an oil field in the Yellow River estuary (119°18'29.0"E, 37°43'49.5"N). When sampled, the in situ water temperature was 14.1 °C, pH was 8.10, salinity was 26.67‰, and the dissolved oxygen was 7.50 mg/L. Total nitrogen of the sample was 4.50 mg/L, total phosphorus was 0.079 mg/L and the chemical oxygen demand (COD) was 53.60 mg/L. The crude oil from Shengli Oil Field was collected in a sterile flask. Sample bottles were stored in ice box and were transported back to laboratory immediately.

2.2 Samples management in laboratory

Sampled seawater was mixed sufficiently and was equally transferred into fifteen 250 mL sterile flasks (100 mL in each flask). Different concentrations of sterilized crude oil were added into the flasks, final oil concentrations in each treatment were 0, 0.1, 0.5, 2.5 and 10 g/L, respectively, and triplicate for each treatment. Shaking (170 r/min) and sealing cultured at 30 °C for a week. Then triplicates seawater of same oil concentration were mixed together, filtered by 0.22 µm millipore filters and stored at -80 °C until the DNA extraction.

2.3 DNA extraction and TRFLP analysis

The DNA extraction followed the manufacturer's protocol of E.N.Z.A.TM Water DNA Kit (Omega, USA). The DNA extracts were examined by electrophoresis in a 1% (W/V) agarose gel. The TRFLP analysis followed previous procedures, 27F-FAM and 1492R primer sets were used, and approximate 1 500 bp's 16S rRNA gene products were obtained for analysis^[7]. The purified PCR products (10 µL) were digested with two different restriction enzymes, *Hae* III and *Msp* I (TaKaRa, Japan),

respectively.

2.4 Statistical analysis

A TRF (terminal restriction fragment) represented an OTU (operational taxonomic unit) and the relative abundance was calculated by the TRFs' relative peak area. Sizes of TRFs between 60 and 700 bp were adopted in further analysis and TRFs with relative abundance <1% were excluded. Based on the effective TRFs, the non-metric multidimensional scaling (NMDS) and diversity indices were conducted using the PAST software^[11], Bray-Curtis coefficient was adopted to visualize the dissimilarity among bacterial community. Diversity indices, Margalef, Shannon, Pielou and Simpson index, denoted the richness, diversity, evenness and dominance, respectively. The PAT+ online program of MiCA 3 was used to confirm the phyla of dominant TRFs^[12].

3 Results

3.1 Change of bacterioplankton diversity with the concentrations of crude oil

Different restriction endonuclease, *Hae* III and *Msp* I, were used to digest the 16S rRNA gene fragments, separately. Interestingly, the bacterial richness did not simply decrease with the increase of crude oil concentrations. Both the two digestion exhibited consistent trends, the bacterial richness decreased at first (from 0 to 0.5 g/L) and then increased (0.5 to 2.5 g/L) and decreased again (2.5 to 10 g/L) with the increase of crude oil concentrations (Figure 1). The bacterial richness in 0.5 g/L crude oil was lower than that in 2.5 g/L, and was comparable with that in 10 g/L treatment (Figure 1). Because of the better polymorphism of *Msp* I digesting results (Figure 1), further analysis in the study mainly based on the data from *Msp* I digestion. The Shannon, Margalef and Simpson indices also showed that the bacterial diversity decreased from M0 to M0.5, and then increased in M2.5, and decreased again in M10 (Table 1).

3.2 Bacterioplankton community structures among different treatments

Through *Msp* I digestion, a total of 52 different effective TRFs were obtained. Control without crude oil (M0) was detected the most TRFs (22 TRFs), followed by M0.1 (21 TRFs), M2.5 (20 TRFs), M0.5 (16 TRFs) and M10 (16 TRFs). The bacterial communities

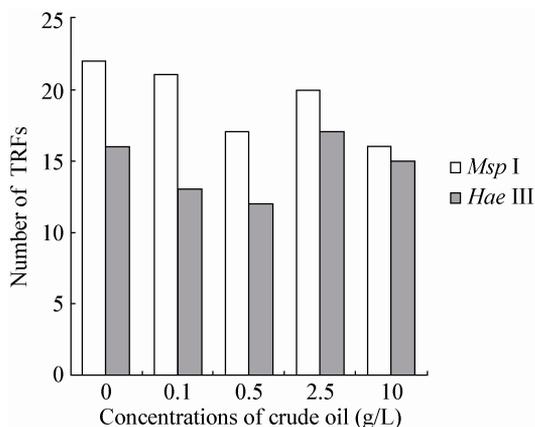


Figure 1 Changing trends of bacterial richness with crude oil concentrations

图 1 细菌丰富度随石油浓度变化趋势

Note: *Msp* I and *Hae* III are different restriction endonuclease.
注: *Msp* I 和 *Hae* III 代表两种不同的限制性核酸内切酶。

Table 1 Diversity indices of different treatments				
表 1 不同浓度石油处理组多样性指数				
Groups	Margalef	Shannon	Pielou	Simpson
M0	4.56	2.70	0.87	0.089
M0.1	4.34	2.49	0.82	0.117
M0.5	3.47	2.38	0.84	0.114
M2.5	4.13	2.65	0.88	0.081
M10	3.26	2.38	0.86	0.123

Note: “M” refers to digestion results of *Msp* I, and the followed number denotes concentrations of crude oil.

注: M0–M10: 限制性核酸内切酶 *Msp* I 酶切后不同浓度梯度的实验组。

changed largely both in the TRF’s sizes and their relative ratios among different experimental groups (Figure 2). The 435 bp TRF (22.82%) was the most dominated one in group M0, followed by 295 bp (12.85%), 485 bp (8.96%), 489 bp (7.23%), 487 bp (6.38%) and 545 bp (5.02%), however, most of the dominant bacteria were replaced by some low abundance bacteria in M0 when crude oil was added (Figure 2). In the oil added groups (M0.1 to M10), there were 11 different dominant TRFs which covered approximately 70% in each group. The most predominant TRF became 425 bp both in group M0.1 and M0.5 (24.22% in M0.1 and 20.57% in M0.5), followed by 151 bp (20.22% in M0.1 and 17.82% in M0.5). Nevertheless, a 435 bp TRF turned into the

most dominated TRF in higher concentrations of crude oil groups (16.95% in M2.5 and 29.21% in M10), the second dominant TRF was 485 bp (13.69%) in M2.5, while 433 bp (9.83%) in M10. These TRFs, 425 bp, 435 bp and 485 bp, existed in all oil-contained groups (M0.1 to M10) and were abundant (Figure 2). Through the MiCA 3 online program, the dominant TRFs (relative ratio > 5%) in the oil treated groups mainly were Proteobacteria, Firmicutes, Bacteroidetes and maybe in phyla of Verrucomicrobia, Actinobacteria and Cyanobacteria.

To clarify the dissimilarity of the bacterial communities among different groups, NMDS plot was conducted (Figure 3). In the plot, the community structure of group M2.5 and M10 were closer, and group M0.1 and M0.5 was more similar, while the bacterial communities of group M0 was very different from them (Figure 3). These results indicated that the bacterial communities changed largely after crude oil was added, and some similar bacterial species existed in different degrees of oil polluted environments.

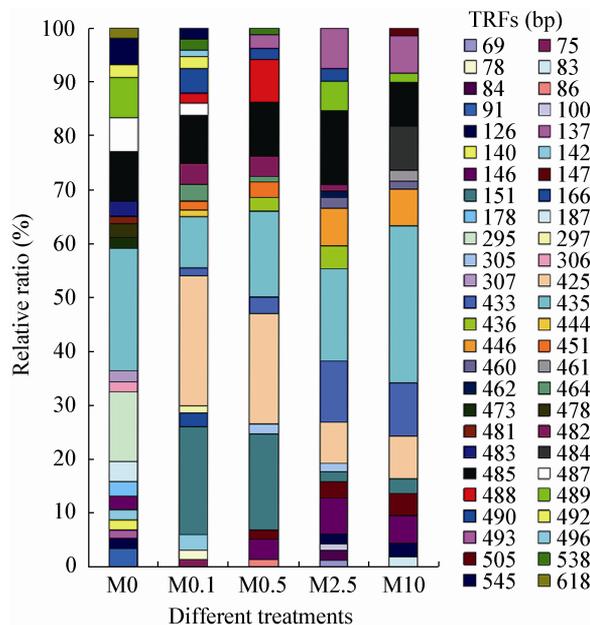


Figure 2 The distributions of bacterioplankton TRFs in different experimental groups

图 2 不同实验组中浮游细菌 TRFs 的分布状况

Note: Each color is on behalf of a specific TRF. M0–M10 represents different treatments.

注: 不同的颜色代表不同片段长度的 TRFs; M0–M10 代表不同的实验处理组。

3.3 Six types: the selective effects of crude oil on the bacterioplankton

In this study, selective effects of crude oil concentrations on bacterioplankton were discovered. Based on the relative ratio of a certain TRF among different groups, the 52 different TRFs can be divided into six types (I to VI, Figure 4). And the characteristics of the six types were as follows (Figure 4):

Type I: low concentrations of crude oil adapted bacteria. After the oil pollution happened, relative ratios of bacteria were higher in low concentrations of oil than those in other concentrations, that is, type I was more adaptive in low concentrations crude oil seawater.

Type II: middle concentrations of crude oil adapted bacteria. Bacterial relative proportions were higher in moderate concentrations of oil than those in other concentrations.

Type III: high concentrations of crude oil adapted bacteria. Bacteria relative ratios were higher in high concentrations of crude oil than those in other

concentrations.

Type IV: broad concentrations of crude oil adapted bacteria. Their relative ratios were stable in broad concentrations ranges of crude oil. Type IV could exist in various degrees of oil pollutions with a relative stable ratio.

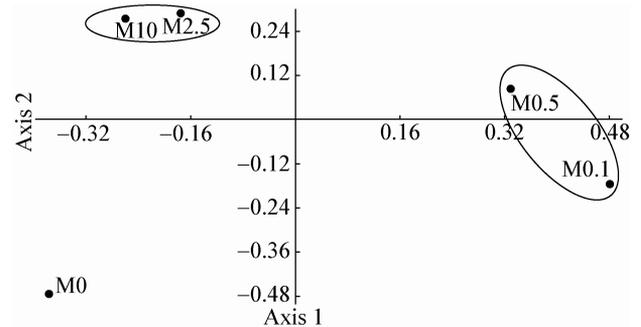


Figure 3 NMDS plot of different experimental groups

图3 不同实验组的非参数多维尺度分析(NMDS)

Note: M0–M10: Experimental groups treated with different concentrations of crude oil.

注: M0–M10: 不同石油浓度处理的实验组。

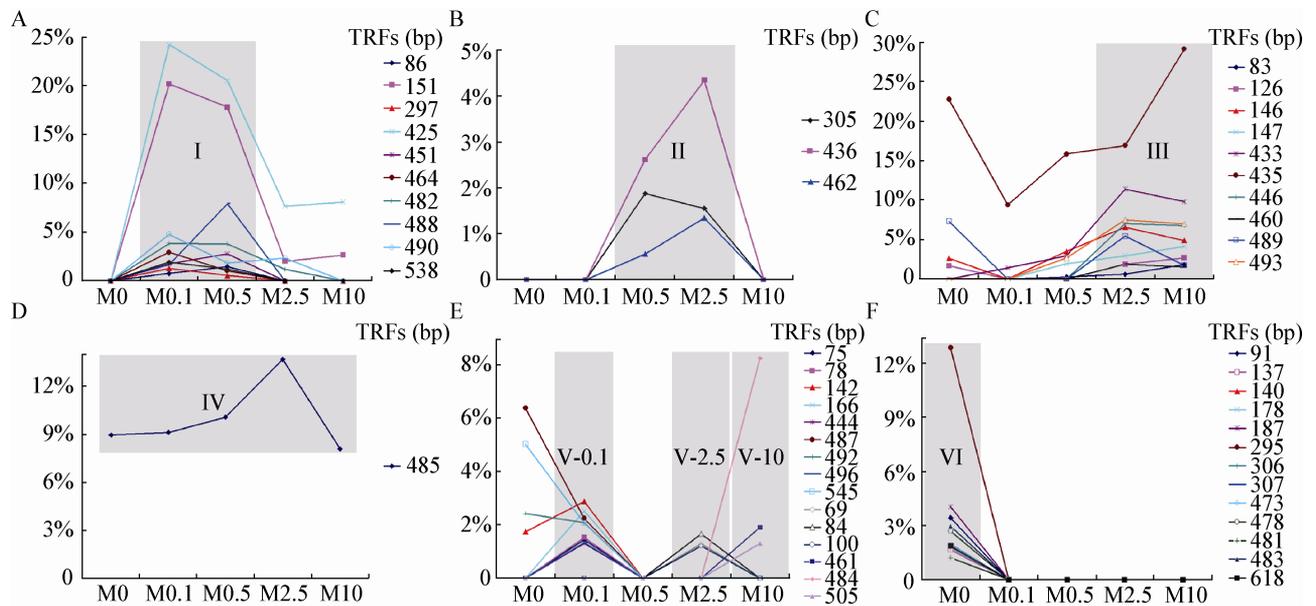


Figure 4 The characteristics of six types (I to VI) in different concentrations of crude oil

图4 不同石油浓度实验组中“6种类型”(I–VI)的分布特征

Note: M0–M10: Experimental groups treated with different concentrations of crude oil; Vertical coordinate: The relative ratios of TRFs; Different lines: Different bacterial TRFs; Gray shades: The dominant concentration region of a certain type; I to VI in the shadow mean six different types.

注: M0–M10: 不同处理组; 纵坐标: 细菌 TRFs 的相对比例; 不同颜色的线条: 不同的细菌 TRFs; 灰色阴影: 某种类型的优势浓度区间; I–VI: 6 种不同类型。

Type V: narrow range concentrations of crude oil (light, midrange or heavy) adapted bacteria. They thrived in certain narrow range concentrations of crude oil, and this type included some sub-types.

Type VI: crude oil sensitive bacteria. They dominated in the environment without crude oil pollution, and these bacteria disappeared or existed in a state of very low abundance after oil pollution happened.

In the present study, the type I had higher proportions in the 0.1 g/L and 0.5 g/L crude oil concentrations than those in other concentrations (Figure 4A). Relative ratios of the type II were higher in 0.5 g/L and 2.5 g/L crude oil concentrations than those in other groups (Figure 4B). The type III was more dominant in higher concentrations crude oil (2.5 g/L and 10 g/L) (Figure 4C). The type IV were relative abundant and stable in all concentrations (Figure 4D). The type V were those narrow range crude oil concentration adapted bacteria which could be detected only in a narrow range of crude oil concentration, so this group included 0.1 g/L crude oil adapted bacteria (V-0.1), 2.5 g/L adapted bacteria (V-2.5) and 10 g/L adapted bacteria (V-10) (Figure 4E). And the type VI were found only in original environment and disappeared after crude oil pollution happened (Figure 4F). The type VI owned the most TRFs (13 TRFs), and the fact indicated that a large number of bacteria were not related to crude oil degradation in the sampling site. In the bacteria related to oil degradation, the type I and type III had the most TRFs (10 TRFs, respectively), followed by the sub-type V-0.1 (8 TRFs), V-10 (4 TRFs), sub-type V-2.5 (3 TRFs), type II (3 TRFs), and type IV (1 TRF). These results showed that bacteria adapted the low concentrations (<0.5 g/L) and high concentrations (>2.5 g/L) of crude oil exhibited the highest diversity, the broad crude oil concentrations adapted bacteria were very rare. In addition, some bacteria could be only found in a narrow range of concentrations crude oil, especially in low concentrations.

4 Discussion

4.1 The bacterioplankton related to crude oil degradation

Hydrocarbon-degrading microorganisms usually exist in very low abundance in marine environments^[13].

Pollution by petroleum hydrocarbons, however, may stimulate the growth of such organisms and cause changes in the structure of microbial communities in the contaminated area^[14]. In our study, a consistent trend was found that most bacterioplankton related to crude oil degradation existed in a state of low abundance in the original seawater (M0) and became thriving after oil was added. Meanwhile, the dominant TRFs (relative ratio >5%) related to crude oil degradation were mainly affiliated to Proteobacteria, Firmicutes and Bacteroidetes in our study and this result was in accordance with the previous result^[9].

4.2 “Carbon & Energy sources-Toxicities” hypothesis (“CE-T” hypothesis)

Previously, Röling and colleagues found that bacterial diversity following addition of oil can be dramatically reduced, and the result was owing to strong selection for hydrocarbon-degrading species^[15]. In our study, we found that bacterial diversity did not simply reduce, but related to the concentrations of crude oil. And the bacterioplankton could be divided into six types following the crude oil concentrations. About the comprehensive influences of crude oil on bacterioplankton, although many studies have examined the effect of crude oil pollution on bacterial communities^[9,16-17], theories or hypotheses to explain the effects are still absent. In order to explain comprehensive influences of crude oil on bacterioplankton, based on our data and previous studies, “Carbon & Energy sources-Toxicities” hypothesis is proposed. The core ideas of the hypothesis are as follows: crude oil is a two-sided mixture due to the “increasing effect” on bacterial abundance from “Carbon & Energy sources” (hydrocarbons and other nutrients) and “decreasing effect” from “Toxicities” (such as PAHs, heavy metals etc.). Different concentrations of crude oil had different selective effects on bacteria that led to six types, and due to the two-sided selective effects of crude oil, different types owned different high efficient “carbon & energy sources” utilization concentrations regions and different “toxicities” tolerance concentrations regions.

4.3 “CE-T” hypothesis explains crude oil pollution related microorganisms’ phenomena

Because of the “Carbon & Energy

sources-Toxicities” effects, six types maybe have different ranges of efficient “carbon & energy sources” utilization concentration regions and different regions of “toxicities” tolerance concentrations. The intersection of two regions determine whether a bacterium existing or dominant in a oil polluted environment. In this research, the intersection of type I concentrates in low concentrations (0.1 g/L to 0.5 g/L crude oil), and the “Toxicities” tolerance region might be the limiting factor. The intersection of type II is mainly in range of middle concentrations (0.5 g/L to 2.5 g/L). “Toxicities” tolerance regions of type III and the type IV were very broad (0.1 g/L to 10 g/L), but the high efficient “carbon & energy sources” utilization concentrations regions were different, the type III distributed mainly in high concentrations (2.5 g/L to 10 g/L), while the type IV had broad utilization region range from 0.1 g/L to 10 g/L. The intersection for the type V concentrated in a narrow range, so they could only thrive in some specific concentration. The type VI could not metabolize crude oil and be sensitive to toxicities, thus they disappeared (under the detection line) when crude oil pollution happened in seawater. After clarifying the characteristics of the six types, it is easy to understand the changing of bacterial diversity in our study. Through the Figure 4, the richness of six types was as follows: VI (13 TRFs)>I=III (10 TRFs)>V-0.1 (8 TRFs)>V-10 (4 TRFs)>II=V-2.5 (3 TRFs)>IV (1 TRFs). In the original seawater, some bacterioplankton related to crude oil degradation existed in a state of very low abundance. Without pollution and relatively stable environment led to high bacterial diversity be detected in M0 (mainly VI). When crude oil was added, “increasing effect” from “carbon & energy sources” and “decreasing effect” from “toxicities” began to work. In the concentration of 0.1 g/L, “toxicities” decreased some “crude oil sensitive bacteria” (VI) and insufficient “carbon & energy sources” could not support “middle and high crude oil concentrations adapted bacteria” (II and III); “increasing effect” mostly acted on the type I and V-0.1, but due to the richness of six types in the original seawater, “decreasing effect” was slightly stronger and “comprehensive effect” was decreasing of bacterial diversity (Table 2). Similarly, when the concentration was up to 0.5 g/L, more “toxicities” continually decreased the V-0.1, although “carbon &

energy sources” increased the II, due to the ratio of the II was lower than the V-0.1, and “comprehensive effect” was further decreasing (Table 2). In the 2.5 g/L crude oil group, “carbon & energy sources” were relatively sufficient, and “toxicities” were in the tolerant range of bacteria (mainly II, III and V-2.5), thus the bacterial diversity increased again. With the concentrations reaching to 10 g/L, “Carbon & Energy sources” were more sufficient, but the toxicities were increasing continually which would make the diversity or richness of the II and V-2.5 decrease, and bacterial diversity decreased in 10 g/L group again (Table 2).

“CE-T” hypothesis not only can explain results in this research, but also provide a way to understand some phenomena related to crude oil pollution in previous papers. Nayar et al. demonstrated that the density of bacteria increased upon the addition of crude oil at a concentration of about 0.001 g/L^[18]. Based on “CE-T” hypothesis, at the concentration of 0.001 g/L, the “toxicities” might not be adequate to decrease the diversity of bacteria in the original environment, but the “carbon & energy sources” could increase part of low abundance bacteria (mainly type I) related to hydrocarbons metabolizing. Thus, the comprehensive effect was increase of bacterial density. Furthermore, some researchers have referred that DMC (defined mixed culture) could enhanced the capabilities of crude oil degradation^[19-20]. But systematic theories or hypothesis were rarely proposed

Table 2 Effects of “CE-T” hypothesis in different treatments

Concentrations 石油浓度(g/L)	Increase 增加效应	Decrease 减少效应	Comprehensive 综合效应
0.1	(I&V-0.1)↑	(VI&II&III)↓	↓
0.5	(II)↑	(V-0.1)↓	↓
2.5	(II&III&V-2.5)↑	(I)↓	↑
10.0	(III)↑	(II&V-2.5)↓	↓

Note: ↑: “Increasing effect” of crude oil makes some types being detected; ↓: “Decreasing effect” of crude oil makes some types becoming under the detection line; I-VI: The six types (I to VI).

注: ↑: 石油对某些类型细菌的“促进效应”, 使其在生境中相对比例增加; ↓: 石油对某些类型细菌的“抑制效应”, 使其在生境中相对比例降低; I-VI: 所划分的6种不同类型细菌.

to explain the fact. Through the “CE-T” hypothesis, different types of bacteria with different “carbon & energy sources” utilization abilities and “toxicities” tolerance abilities should be contained in the DMC. It would allow different types of bacteria to play critical roles in different degrading stages. Therefore, DMC could enhance biodegrading capabilities of oil. In addition, some phenomena in other related studies could also be given good explanations through the “CE-T” hypothesis^[8,21-22].

4.4 Comparison of the “CE-T” hypothesis and the “intermediate disturbance hypothesis”

The conception of “intermediate disturbance hypothesis” (IDH) was presented by Connell^[23], and the IDH demonstrated that diversity of competing species is, or should be expected to be, maximized at intermediate frequencies and/or intensities of disturbance or environmental change^[24]. The IDH was a very universal hypothesis in ecology in the past years, and ecologists had tested the hypothesis for a variety of taxa in a number of different environments^[25-27]. However, in a recent review paper, Fox argued that the IDH has been refuted on both empirical and theoretical grounds, and so should be abandoned^[24]. The “CE-T” hypothesis is different from the IDH mainly in two aspects. On one hand, the concerned periods and aims are different. The IDH aims to predict the diversity-disturbance relationship in a long-time community succession, while the “CE-T” hypothesis focuses on the short-time effects of oil pollutions on the microorganism community. We aim to explain not to predict the behaviors of bacteria after oil spills accidents happening. On the other hand, the disturbance factor is special in our study. Crude oil not only has disturbance effect on bacteria from PAHs etc., but also has promoting effect on bacteria’s growth due to carbon sources etc. The “CE-T” hypothesis gives a good perspective on explaining the effects of crude oil accidents on microorganism community. Although it is difficult to predict the oil pollution and bacteria diversity relationship, it points out the possibility and universality of the six types in all water columns, and the prediction will become available after deducing the proportions of six types through our experiments. Finally, does the bacterial communities in other environments (soil, freshwater etc.) responding to crude oil pollution exhibit similar

changing trend with our results? Are the six types really common in a large scale of environments? And as a hypothesis, different environments and larger scales samples need to be studied to confirm the “CE-T” hypothesis in the future.

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