Bioturbation enhances the removal of petroleum hydrocarbons in sediments by bacteria

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Abstract. A laboratory experiment was designed to study the removal of total petroleum hydrocarbons (TPH) by bacteria-bioturbation in the sediments. TPH were detected on 0, 5, 10, 14, 17, 20, 24 d in different phases and the removal rate in different layers of TPH in sediments was measured. The results showed that there were three results about the fate of TPH in the sediments: transferring to seawater by bioturbation; accumulating in the worms by feeding and degrading by bacteria. The removal rate of TPH in joint repairing group (JRG) was obviously higher than other three groups and reached to 86.01%, 83.58% and 75.56% in upper, middle and lower layers, respectively. The concentration of TPH increased 2.9264 mg/L in seawater and 12.7514 mg/kg in worms, imply that feeding by worms is an effective way to remove TPH in the sediments. The result of orthogonal experiment in the laboratory demonstrated that the best conditions to remove TPH by bacteria-bioturbation is: A. density of worms, 200 ind/m²; B. volume of bacterial fluid vaccination, 25 mL; C. salinity of seawater, 23; D. pH of seawater, 8.5; E. cultivation time, 12 d.

1. Introduction
With the development of the maritime transportation, the marine environment, especially sediments [1] in the intertidal zone, has been seriously polluted by the oil spills [2]. Petroleum hydrocarbons, the main components of the oil, are so strong hydrophobicity but easily combined with particles [3] that do harm to sediments. Furthermore, some hydrocarbons will release to the water from sediments, which caused the water polluted again [4]. On the other hand, oil pollution may arise and create a threat to organics however the oil is produced, transported, stored or used in the ocean [5]. Therefore, it is urgent to solve the problems caused by oil spills in order to ensure the safety of the organisms.

In many kinds of methods, bioremediation methods can meet the needs of efficiency without causing harm to sediments. Biodegradation by microorganisms is the most popular method to eliminate the organic pollutants, including diesel [6]. The bacterial consortium from Cerqueira [7] reduced 90.7% of the aliphatic fraction and 51.8% of the aromatic fraction, which demonstrated an excellent oily sludge degradation capacity. In addition, as a kind of biological methods, bioturbation was similarly got widespread attention in the field of repairing the contaminated sediments because of its economy and convenience. Bioturbation is defined as the behavior of feeding, digging, excretion and building by benthics [8], which causes the diversification of the primary structure even the physicochemical property of sediments. At the same time, the pollutants will remove from sediments so that the polluted sediments can be repaired [9].
Microbial degradation of oils is relatively a kind of mature method and widely used in the laboratory [10] or practice [11]. But the ability of removing hydrocarbons by microorganisms is vulnerable to environmental factors especially oxygen content. In order to improve the efficiency of the method, some other methods are usually used to combine with microorganisms, in which bioturbance is a common way. The bioturbation behavior of the worms can also change the community structure of microorganisms so that indirectly affecting the environmental behavior of petroleum hydrocarbons [12]. A laboratory experiment was carried out to learn how the total petroleum hydrocarbons (TPH) were migrated during the sea water and sediments in the presence of both bacteria and worms. The goal of the experiment is to find a more efficient way to repair oil pollution. The joint remediation of microorganisms and benthics is a relatively novel approach, which would not cause secondary pollution to environment. What is more, orthogonal experiment was designed to explore the best conditions of oil degradation, which provide examples for practical applications.

2. Materials and methods
The whole experiment was conducted in the beaker with a height of 19 cm, a volume of 2 L. About 1.5 kg sediments and 1.5 L artificial seawater were added in every beaker. All domesticated worms were similar in length and every condition was set up in three parallels. The overlying water was poured out for 12 h and return to the original beakers for 12 h everyday to simulate the effect of tide and ensure normal breathing of worms.

2.1. Sediments repairing experiment
Four conditions were setted in the beakers (Table 1). The sediments and water were sampling on day 0, day 5, day 10, day 14, day 17, day 20, day 24 and TPH would be measured in them. TPH in worms would be measured on day 24.

### Table 1. Four different conditions for petroleum hydrocarbon degradation.

| Serial number | Groups                     | Conditions                                                                 |
|---------------|----------------------------|-----------------------------------------------------------------------------|
| 1             | Control Group (CG)         | 20 mL enriched medium (without bacteria)                                   |
| 2             | Microbe Group (MG)         | 20 mL enriched medium (with bacteria)                                      |
| 3             | Bioturbation Group (BG)    | 20 mL enriched medium (without bacteria) and                              |
|               |                            | four strong worms                                                          |
| 4             | Joint Repairing Group (JRG)| 20 mL enriched medium (with bacteria) and                                  |
|               |                            | four strong worms                                                          |

2.2. Joint repair experiment optimization design
Five-factor and four-level orthogonal experiment was designed (Table 2, 3). After the orthogonal experiment, the degradation rate of TPH in the sediments was calculated.

### Table 2. The factors and levels of experiment.

| Levels | A. Density of worms(ind/m²) | B. Volume of bacteria(mL) | C. Salinity of seawater(‰) | D. pH of seawater | E. Cultivation time (d) |
|--------|-----------------------------|---------------------------|---------------------------|------------------|-------------------------|
| 1      | 100                         | 10                        | 20                        | 5.5              | 9                       |
| 2      | 150                         | 15                        | 23                        | 6.5              | 12                      |
| 3      | 200                         | 20                        | 26                        | 7.5              | 15                      |
| 4      | 250                         | 25                        | 29                        | 8.5              | 18                      |

The method to measure TPH in seawater and sediments was totally according to the Specification for Marine Monitoring by using UV spectrophotometer. And the method to measure TPH in worms was according to the Specification for Marine Monitoring and the method of Cai, Xu and Wu [13] by using fluorescence spectrophotometer.
2.3. Data analysis

The data was showed by “average value ± standard deviation” and the variety of TPH with time was analyzed by bivariate correlation analysis in SPSS. Variance analysis was used in the orthogonal experiment. All the figures related to data were drew by Origin 8.5.

3. Results and discussion

3.1. The variety of TPH in different phases

3.1.1. TPH in Water. The variety of TPH in seawater was slightly different (Figure 1). The change of TPH in BG and JRG were all reducing until the tenth day, but in CG (P>0.05) and MG (P>0.05) were keeping rising until the fourteenth day, which indicated that bioturbance by worms promoted volatilization of TPH in the water. Organic Pollutants, especially hydrocarbons, will release to the air by bioturbation when they accumulate to a certain extent in the water [3]. After that TPH released to water again in BG (P>0.05) and JRG (P>0.05) during 10-14 days, which is due to bioturbation by worms in the sediments. Qin [12] proposed that bioturbation promotes the release of petroleum hydrocarbons from sediments into seawater and the results confirmed this point. Lastly, TPH were slightly rising in CG and MG, but kept reducing in BG and JRG.

3.1.2. TPH in Sediments. The trend of TPH in different layers of sediments was roughly the same downward, which certificated that TPH was removed from sediments. However, the amount of TPH variety in different groups and layers was obviously distinguishing (Figure 2).

Microbial remediation method, as a kind of traditional and popular method, has good repair effects [14] on various pollutants such as petroleum hydrocarbons, PAHs [15] and chemical products [16]. Microbial removal of petroleum hydrocarbons has achieved good results.

Table 3. Orthogonal experiment to determine the optimal conditions for different factors.

| Serial number | A. Density of worms(ind/m²) | B. Volume of bacterial fluid vaccination(mL) | C. Salinity of seawater(‰) | D. pH of seawater | E. Cultivation time (d) |
|---------------|-----------------------------|---------------------------------------------|---------------------------|------------------|------------------------|
| 1             | 1(100)                      | 1(10)                                      | 1(20)                     | 1(5.5)           | 1(9)                   |
| 2             | 1                           | 2(15)                                      | 2(23)                     | 2(6.5)           | 2(12)                  |
| 3             | 1                           | 3(20)                                      | 3(26)                     | 3(7.5)           | 3(15)                  |
| 4             | 1                           | 4(25)                                      | 4(29)                     | 4(8.5)           | 4(18)                  |
| 5             | 2(150)                      | 1                                           | 2                          | 3                | 4                      |
| 6             | 2                           | 2                                           | 1                          | 4                | 3                      |
| 7             | 2                           | 3                                           | 4                          | 1                | 2                      |
| 8             | 2                           | 4                                           | 3                          | 2                | 1                      |
| 9             | 3(200)                      | 1                                           | 3                          | 4                | 2                      |
| 10            | 3                           | 2                                           | 4                          | 3                | 1                      |
| 11            | 3                           | 3                                           | 1                          | 2                | 4                      |
| 12            | 3                           | 4                                           | 2                          | 1                | 3                      |
| 13            | 4(250)                      | 1                                           | 4                          | 2                | 3                      |
| 14            | 4                           | 2                                           | 3                          | 1                | 4                      |
| 15            | 4                           | 3                                           | 2                          | 4                | 1                      |
| 16            | 4                           | 4                                           | 1                          | 3                | 2                      |
**Figure 1.** The variety with time of TPH in seawater.

**Figure 2.** The variety of TPH contents with time and removal rate of TPH in Upper (A, B), middle (C, D) and lower (E, F) sediments.
In the upper sediments, the concentration of TPH was intensely reducing in MG (P<0.05) during the first ten days, which proved bacteria were the most energetic in this period and TPH removal rate reached 76.51%. Petroleum hydrocarbons were not accumulate in the sediments despite their continuous introduction, mainly due to the specific bacteria ingest hydrocarbons as carbon and energy sources [17]. After that the variety of TPH was slight and the finally removal rate reached 79.93%. TPH variety were decreasing in first two weeks in BG (P<0.05) because of bioturbation. Then hydrocarbons were released from the water, which was the reason that the concentration in upper sediments was rising a little [12]. Finally, TPH continued to reduce and the removal rate reached 82.01%. However, a single repair method, because of some disadvantages, sometimes does not meet the needs of production and life. The limitation of microbial degradation of TPH was insufficient oxygen content and it was made up by bioturbation [18]. The trend of TPH in JRG (P<0.05) was basically the same as in BG but the inflection point appeared later, which indicated there were stronger effect in JRG and removal rate reached 86.01% (Figure 2 A and B). Obviously, bioturbation promotes the degradation of petroleum hydrocarbons by microorganisms.

TPH trend of CG (P<0.05) and MG (P<0.05) in middle (Figure 2 C) and lower sediments (Figure 2 E) were the same as in upper sediments. In MG, the removal rate of TPH was obviously lower than in upper sediments. This is because the oxygen content decreases with increasing depth and adequate oxygen content enhances bacterial activity [19]. TPH trend of BG (P<0.05) and JRG (P<0.05) in middle and lower sediments were similar. Removal rate in middle and lower sediments reached 75.74%, 69.58% in BG and 83.57%, 75.56% in JRG, which means the deeper the level, the smaller the role that microorganism can play. The different of TPH between BG and JRG was only that TPH was rising and then reducing in BG but it was opposite in JRG in first ten days. The removal rate of TPH in middle layers in JRG was higher than in MG, which proved that microbial removal of TPH was improved by bioturbation.

Table 4. The results of orthogonal experiment.

| Serial number | A. Density of worms (ind/m²) | B. Volume of bacterial (mL) | C. Salinity of seawater (%) | D. pH of seawater | E. Cultivation time (d) | Results (average value ± standard deviation) |
|---------------|----------------------------|-----------------------------|----------------------------|-------------------|------------------------|---------------------------------------------|
| 1             | 1(100)                     | 1(10)                       | 1(20)                      | 1(5.5)            | 1(9)                   | (29.45±3.17)%                              |
| 2             | 1                          | 2(15)                       | 2(23)                      | 2(6.5)            | 2(12)                  | (37.00±2.20)%                              |
| 3             | 1                          | 3(20)                       | 3(26)                      | 3(7.5)            | 3(15)                  | (31.91±3.71)%                              |
| 4             | 1                          | 4(25)                       | 4(29)                      | 4(8.5)            | 4(18)                  | (27.25±4.61)%                              |
| 5             | 2(150)                     | 1                           | 2                          | 3                 | 4                      | (31.54±5.17)%                              |
| 6             | 2                          | 2                           | 1                          | 4                 | 3                      | (31.18±3.14)%                              |
| 7             | 2                          | 3                           | 4                          | 1                 | 2                      | (26.60±3.61)%                              |
| 8             | 2                          | 4                           | 3                          | 2                 | 1                      | (31.03±3.99)%                              |
| 9             | 3(200)                     | 1                           | 3                          | 4                 | 2                      | (73.02±8.51)%                              |
| 10            | 3                          | 2                           | 4                          | 3                 | 1                      | (33.13±4.97)%                              |
| 11            | 3                          | 3                           | 1                          | 2                 | 4                      | (32.43±6.97)%                              |
| 12            | 3                          | 4                           | 2                          | 1                 | 3                      | (54.59±1.40)%                              |
| 13            | 4(250)                     | 1                           | 4                          | 2                 | 3                      | (22.69±9.29)%                              |
| 14            | 4                          | 2                           | 3                          | 1                 | 4                      | (38.38±9.83)%                              |
| 15            | 4                          | 3                           | 2                          | 4                 | 1                      | (60.68±4.94)%                              |
| 16            | 4                          | 4                           | 1                          | 3                 | 2                      | (52.97±5.12)%                              |

In general, correlation analysis shows that the change of petroleum hydrocarbon concentration over time is significantly correlated. The removal rate of TPH in upper and middle layers in joint repair group (JRG) was higher than there were only microorganisms (MG), the result of which is roughly the
same with Zhang [20]. The reason is bioturbation increases oxidation reduction potential (ORP) of sediments [21] thus improves oxygen content thereby enhances the activities of bacteria. However, the result was opposite in the lower layers, which was removal rate of TPH in joint repair group (JRG) was lower than microbial group (MG).

### Table 5. The variance analysis of orthogonal experiment.

| Item               | A       | B       | C       | D       | E       | Deviation |
|--------------------|---------|---------|---------|---------|---------|-----------|
| Upper sediments    |         |         |         |         |         | 0.03      |
| Sum of squared deviation | 0.176   | 0.025   | 0.067   | 0.096   | 0.096   |
| F ratio            | 7.040   | 1.000   | 2.680   | 3.840   | 3.840   |
| Significance       | *       |         |         |         |         |           |
| Factor influence   | A>E>D>C>B|
| Middle sediments   |         |         |         |         |         | 0.03      |
| Sum of squared deviation | 0.135   | 0.073   | 0.033   | 0.064   | 0.045   |
| F ratio            | 6.840   | 2.640   | 1.000   | 2.560   | 2.120   |
| Significance       | *       |         |         |         |         |           |
| Factor influence   | A>B>D>E>C|
| Lower sediments    |         |         |         |         |         | 0.02      |
| Sum of squared deviation | 0.086   | 0.031   | 0.122   | 0.018   | 0.033   |
| F ratio            | 4.778   | 1.722   | 6.778   | 1.000   | 1.833   |
| Significance       | *       |         |         |         |         |           |
| Factor influence   | C>A>E>B>D|
| Degree of freedom  | 3       |         |         |         |         |           |
| F-threshold        | 5.390 (P=0.1) |

*The factor is significant

3.1.3. **TPH in worms.** Ingestion is the main way in which pollutants accumulate in benthics [22]. Feeding led worms to accumulate TPH in their bodies. The value of TPH before the formal experiment was (1.22±0.25) mg/kg, whereas rising to (13.69±2.99) mg/kg in BG and (13.97±3.55) mg/kg in JRG. The measured values were almost the same in BG and JRG, which illustrated the presence of bacteria did not affect the life activities of the worms. But bioturbation significantly enhanced the efficiency to remove petroleum hydrocarbons by microorganisms. In addition, feeding was the mainly action of removing TPH by worms.

All in all, there are three reasons for the change in petroleum hydrocarbons in sediments: physical effect, which are gravity and tides; microbial degradation [23] and bioturbation by worms [24].

### 3.2 Optimization Experiment

TPH in sediments of JRG was removed apparently compared to the other groups. In order to determine the optimal conditions in JRG and provide a basis for pilot experiments, the orthogonal experiment was setted and the results of removal rates showed that the ninth experiment was the best no matter how upper, middle or lower layers. The result of orthogonal experiment in the laboratory (Table 4) demonstrated that the best conditions to remove TPH by bacteria-bioturbation is: A. density of worms, 200 ind/m²; B. volume of bacterial fluid vaccination, 25 mL; C. salinity of seawater, 23; D. pH of seawater, 8.5; E. cultivation time, 12 d. In upper and middle layers, the removal of TPH and the density of worms were significantly correlated. In addition, there was significant deference in salinity of seawater in lower layers (Table 5). The influence of physical factors on the experiment, such as pH and salinity of seawater, which leads to changes in biological activities, is actually impacts on the organisms. Biological activities are susceptible to external factors and then affect the migration conversion of pollutants [25], so it is important to adjust external conditions to improve the efficiency of biological removal of petroleum hydrocarbons. In addition, the difference in optimal degradation conditions is determined by the oxygen content at different levels of the sediments [20]. Bioturbation can also affect the oxygen levels at different layers in the sediments and improve the activities of bacteria.
4. Conclusions
The removal of TPH by bacteria and bioturbation in the the uniformly diesel contaminated sediments was measured and the conditions were optimized in a laboratory experiment. There are three kinds of fates about TPH in the sediments: releasing to the water, accumulated in the worms and degrading by bacteria. By adding worms to the microbial system, the removal rate of petroleum hydrocarbons in sediments increased by 7.6%. However, the experimental site in the laboratory is limited, and it will have better results in practical applications. For worms, feeding is the mainly way to remove TPH in sediments and bioturbation is the way to help microorganisms remove TPH. What is more, gravity and tide also have effects on the fate of TPH in sediments, which have been ruled out in the control groups. Under the conditions of the coexistence of microorganisms and worms, the most influential effect on the removal of petroleum hydrocarbons is the density of worms. In general, the best effect is achieved by keeping the density of the worms at about 200 ind / m².

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