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Remediation of diesel contaminated sediments by worms (*Perinereis* sp.) bioturbation

Z Wenbo, Z Xin, L Xianbin* & Y Bo

Tianjin University of Science & Technology, Tianjin Marine Environmental Protection and Restoration Technology Engineering Center, Tianjin – 300457, China

*[E-mail: lxb0688@tust.edu.cn]

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Impacts of bioturbation by worms on remediation of diesel contaminated sediments were studied in a laboratory experiment. The total petroleum hydrocarbons (TPH) were represented by the oil or diesel particles which were detected in seawater as well as in the upper, middle and lower layer of sediments at every three days of experiment. The whole experiment was lasted for 21 days and the TPH in the worms was measured by fluorescence analysis and the components of residual oil in the sediments were detected by gas chromatography-mass spectrometry (GC-MS) at the end of the experiment. The results showed that the TPH were declined to 18.37±0.62 and 20.85±1.74 mg/kg in the surface and sub-surface sediments, respectively, which were the worms' frequently active areas. TPH was found to increase slightly in deeper sediments as well as in the water column because of bioturbation by worms. The feeding behavior of worms led to the accumulation of TPH in the worm's body and was reached from 0.998±0.171 to 23.764±3.878 mg/kg. Many components of the diesel such as alkanes, Polycyclic Aromatic Hydrocarbons (PAHs) etc. were removed through bioturbation by worms. In conclusion, the petroleum hydrocarbons in the area where the worms are located will decrease over time.

[**Keywords**: Bioturbation, Diesel, Remediation, Sediments, TPH]

Introduction

Marine oil spill, which is harmful to marine environment, has always been a hot issue along the world coastline. In general, the oil in the marine oil spill pollution is diesel, which is insoluble in water but can easily associate with particulate matter[1].Petroleum hydrocarbon, a component of oil, is a kind of liposoluble substance, which can be absorbed and accumulated by certain marine organisms, leading to adverse health effects. The offshore areas are the important locations of petroleum exploitation and transportation[2], and thus are the hotspots of diesel pollution. Through a series of physical and chemical changes such as tidal impact, light degradation, and oxidation, the hydrocarbon components of the diesel will associate with suspended particulate matter[3] and then enter the sediments in the intertidal zones, where the intersection of marine and terrestrial environments occur. After being polluted, sediments may release these hydrocarbons back to the water and may act as secondary sources of the pollutants[4,5]. In addition, some of the petroleum hydrocarbons will be assimilated by the benthos, which are used to live in the sediments. Diesel pollutants do great harm to the invertebrates which live in sediments (benthos) and some components such as PAHs, due to high toxicity, will lead to death of such organisms. Therefore, the problem of diesel pollution must be solved in time to ensure the safety of marine environment. One of the efficient and economical methods to tackle such problems is biological method, wherein living organisms are being used to dissociate the pollutants *i.e*. bioremediation.

The biological activities of benthos such as ingestion, excretion, burrowing in the sediments are called bioturbation[6]. The primary structure even the physicochemical properties of sediments can be changed by bioturbation. At the same time, bioturbation changes the ingredients or chemical composition in the sediments such as changing the community structure of microorganisms, influencing the decomposition of the organic matter[7] etc. Significant quantities of contaminants such as petroleum hydrocarbons[8], heavy metals[9] and PAHs[10] will accumulate in the sediments and influence the conducts of the organisms living in the sediments. Therefore, a variety of researches discussed how to use the marine organisms as the biomarkers to monitor and evaluate the changes of the marine environment under these pollutants[9,11,12]. On the other hand, the contaminants can be degraded by marine organisms[13,14] especially by benthos. The activity of bioturbation by benthic organisms such as worms, crabs will lead to the movement of pollutants so that they can be removed from sediments.

Perinereis sp., which belongs to the lugworm, is the model organism of ecotoxicology studies and has an important ecological significance in the scientific researches[15]. It is accustomed to live in the intertidal zones and can be easily affected by human activities[16]. *Perinereis* sp. has a strong ability of bioturbation. The average depth it can reach is 8 cm with the maximum reachability of 15 cm in the sediments[17]. Therefore, in the presence of *Perinereis* sp. in the sediments, the pollutants may be released back to the seawater or even can be transported to deeper layers of sediments by its bioturbation activity.

Although there are some researches about the removal of contaminants such as organics[13], nutrients[14] and heavy metals[18] from the sediments, few studies have reported the removal of petroleum hydrocarbons, which is commonly found in oil spills, by bioturbation. Bioturbation is a key activity of the benthic worms leading to the removal of pollutants from the sediments[19]. The feeding behavior of benthic organisms i.e. head down results in the exchange of water and sediments, including the contaminants within the sediments. Therefore, to better understand the complete process a laboratory experiment was carried out to study how the diesel particles are being transported in the different layers of sediments, water column and to worm's body, and what ingredients of diesel would stay in the upper, middle and lower sediment layers. In addition, the terminal fate of the diesel pollutants was tested in order to provide a broad idea for the restoration of persistent organic pollution from the sediments.

Materials and Methods

Experimental animal

The worms were obtained from Taizhou, Zhejiang Province, China, and were domesticated in artificial seawater with a salinity of 26 psu in the laboratory. The artificial seawater, which is formulated by sea crystal, was added in the glass cylinder for domestication of worms. In order to ensure that the worms can breathe freely, the depth of the water for domestication was set as 1-1.5 cm. The domestication was lasted for one week, during which a proper amount of clean sediments were added into the water as a source of food for the worms. Artificial seawater was replaced after every 2 days during the experiment and the dead individuals were picked out in time. The worms were used for the experiment if the mortality did not exceed 5 %. Immobility of the worms was considered when no movement was observed after three-time repeated tactile stimulation with a needle and the wait period to confirm the immobility of the worm was 3 seconds between each tactile stimulation[20].

Sediment treatment

The sediments used in the experiment were collected from the intertidal zone of Gaoshaling, 117°37` 48` E, 38°50` 39` N, Tianjin, naturally dried, grinded and sieved to remove impurities such as gravels and shells. Then the contents of the petroleum hydrocarbon were measured. After that, the original hydrocarbon present in the sediments was evaporated through the Muffle furnace (SX3-4-10, Tianjin Zhonghuan Experimental Electric Furnace Co., Ltd. China) at 450 ℃ for 4 h. In order to maintain uniformity in the sediments, the diesel was dissolved in acetone (AR) and then mixed with the sediments. The concentration of the diesel in the sediments was approximately 85 mg/kg, which was slightly higher than the areas where the sediments samples were collected.

Bioturbation experiment

The bioturbation experiment was conducted in a beaker with a height of 19 cm, diameter 13 cm having a volume capacity of 2 L. The total experiment was divided into two groups *viz*. control and disturbance group, and each group was set up in triplicate. 9 cm thick contaminated deposit was added into each beaker and then 1500 ml artificial seawater was slowly added into the beaker. About five strong domesticated worms (10±0.5 g) were selected to carry out bioturbation. Each group was aerated for 12 h everyday in order to ensure adequate oxygen supply. All the beakers were covered with aluminum foil to prevent volatizing of hydrocarbons. The total petroleum hydrocarbon (TPH) in the sediments and seawater were measured after every 3 days of the experimentation and the experiment was ended after 21 days. The total petroleum hydrocarbon in the worms was measured at the end of the experiment.

TPH analysis

TPH in seawater

The method is based on the specifications for marine monitoring[21]. 50 ml of seawater was taken out with the help of beaker, diluted to 500 ml and the TPH content was measured by UV Spectrophotometer (UV765, Shanghai Youke Instrument Co., Ltd. China). In order to ensure precise results, the clean seawater was not added into the system even if there is evaporation.

TPH in sediments

A cylindrical glass tube with a diameter of 0.7 cm was inserted vertically into the deposit for sediment sampling. To ensure veracity, the same operation was repeated 4 times on different locations of sediments. Then the collected sediment samples were divided into three layers *viz*. upper, middle, lower layer. The sediments from the same layer were mixed with each other. The upper, middle and lower layer soil samples were fully pooled separately in order to achieve reliable mean values. Thereafter, the samples were naturally dried and the concentration of total petroleum hydrocarbon was measured by UV Spectrophotometer according to the specification given for marine monitoring[22].

TPH in worms

TPH content was also measured in domesticated and tested worms. After homogenization, the dead worms were weighed (3.00-5.00 g), uniformly mixed with 20 ml sodium hydroxide solution (6 mol/l) and 20 ml absolute ethanol. Then the samples were subjected to Saponification for 18 h in room temperature by adding 15 ml saturated sodium chloride solution. Then 25 ml cyclohexane (HPLC) was used to extract TPH. The extracts were combined and subjected to centrifugal separation. The final volume was made up to 25 ml with cyclohexane, then was analyzed by Fluorescence analysis (F-7100FL, China), to which the test parameters of the instrument were: excitation wavelength (310 nm), emission wavelength (360 nm), and the excitation and emission wavelength slit were 5 nm and the detection limit was 1.0×10^{-6} wet weight[23]. In addition, the standard curve line was drawn according to the above method combined with the specification for marine monitoring and made appropriate changes[24]. The TPH contents in the worms were calculated by the formula as follows:

$$w_{oil} = (m \cdot V)/(F \cdot M)$$

Where, w_{oil} is the concentration of TPH in the worms (quality score, 10^{-6}), m is the concentration of TPH on the standard curve line (μg/ml), V is the volume of extracting agent (cyclohexane, ml), F is the rate of dry/wet weight of samples, and M is the weight of samples.

Analysis of residual oils in the sediments

The components of diesel were measured by GC-MS before the experiment. Diesel was passed through an activated alumina column and was eluted with 100 ml mixture composed of 20 % hexane and 80 % dichloromethane. 10 μl of eluent was diluted with 1 ml acetone and then measured[25]. After the bioturbation experiment, the residual oil was extracted by hexane (HPLC) from the sediments and the hydrocarbons were measured by GC-MS in SIM mode. The column temperature was started at 50 ℃ (2 min) and was then increased with 15 ℃ min^{-1} to 100 ℃ and then with 10 ℃ min^{-1} to 170 ℃, and lastly with 3 ℃ min^{-1} to 300 ℃.

Data analysis

Data in the results was shown by "average value ± standard deviation", and the variation in TPH with time was analyzed by SPSS. $P < 0.05$ suggests there exists a significant difference, in contrast, $P > 0.05$ suggests no significant difference. Figures were drawn by using Origin 8.5.

Results

During the experiment, the worms tend to move towards upper and middle layers of sediments. There were some small holes and so many fecal mounds on the upper sediment layer in the disturbance groups, which were caused by bioturbation and excretion of worms. Moreover, the quality of water in the disturbance groups is relatively turbid and viscous compared to control groups. The mortality rate of worms was 0, and the worms were still active at the end of experiment which indicated the strong ability of worms to withstand with diesel pollution.

Sediment contamination

The background value of TPH concentration in the collected sediment samples was (42.72±2.97 mg/kg) which was lowered to somewhat negligible concentration (6.93±1.64 mg/kg) by subjecting the sediments to high temperature in the muffle furnace. Most of the impurities had been removed and the diesel was added to the sediments. At this time, the contents of TPH can be used to represent the contents of diesel. After multiple sampling and estimation, the concentration of TPH was recorded as 88.26±3.49 mg/kg after contaminating, which was similar to the expected value. This indicated the uniform distribution of diesel particles in the sediments.

The change of TPH in three phases

The contents of TPH in water, sediments and worms were strongly influenced by bioturbation. Numerous portion of the TPH was transferred from the sediments to the water and worms by bioturbation. In addition, TPH from different layers of the sediments was removed in different degrees.

TPH in seawater

In general, the TPH concentration in the water was increased initially till day 6 and then decreased gradually till the end of the experiment (Fig. 1, $P < 0.05$, control; $P < 0.05$, bioturbation, significant difference). The concentration of TPH in the water reached the highest point of 22.03±2.20 mg/kg on day 6 in the bioturbation group. After comparison with the control group, TPH released from the sediments to water by bioturbation was 5.15 mg/l. The concentration of TPH in the water was 12.50±0.03 and 8.28±0.71 mg/l in the bioturbation and control groups, respectively at the end of the experiment, which means the concentration of TPH remained in the seawater was 4.22 mg/l when compared to control group.

TPH in sediments

The contents of TPH were different among the three layers (Fig. 2) of sediments *i.e.* in upper (surface), middle (sub-surface) and the lower layer. Overall, the trend of TPH in the surface ($P < 0.01$, control; $P < 0.01$, bioturbation) and sub-surface ($P < 0.01$, control; $P < 0.01$, bioturbation) layers was observed to be declining (Figs. 2A & B), which indicated that these are the main regions in which the worms tended to migrate leading to the removal of TPH from these layers. The value of TPH in sediments showed ups and downs throughout the experimentation period and eventually stabilized at

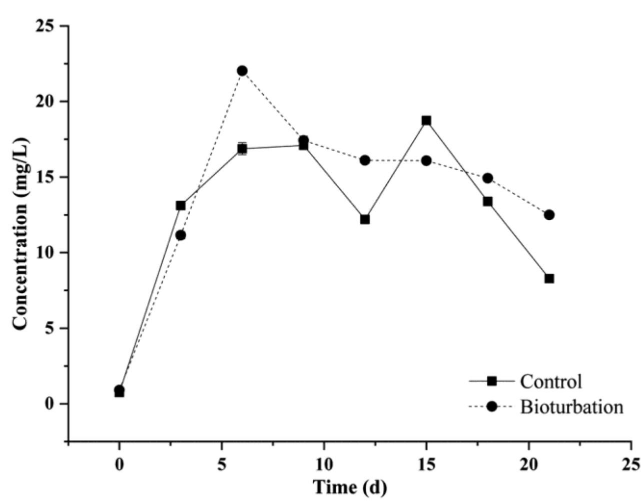

Fig.1 — The variation in TPH with time in the seawater with (●) and without (■) worms

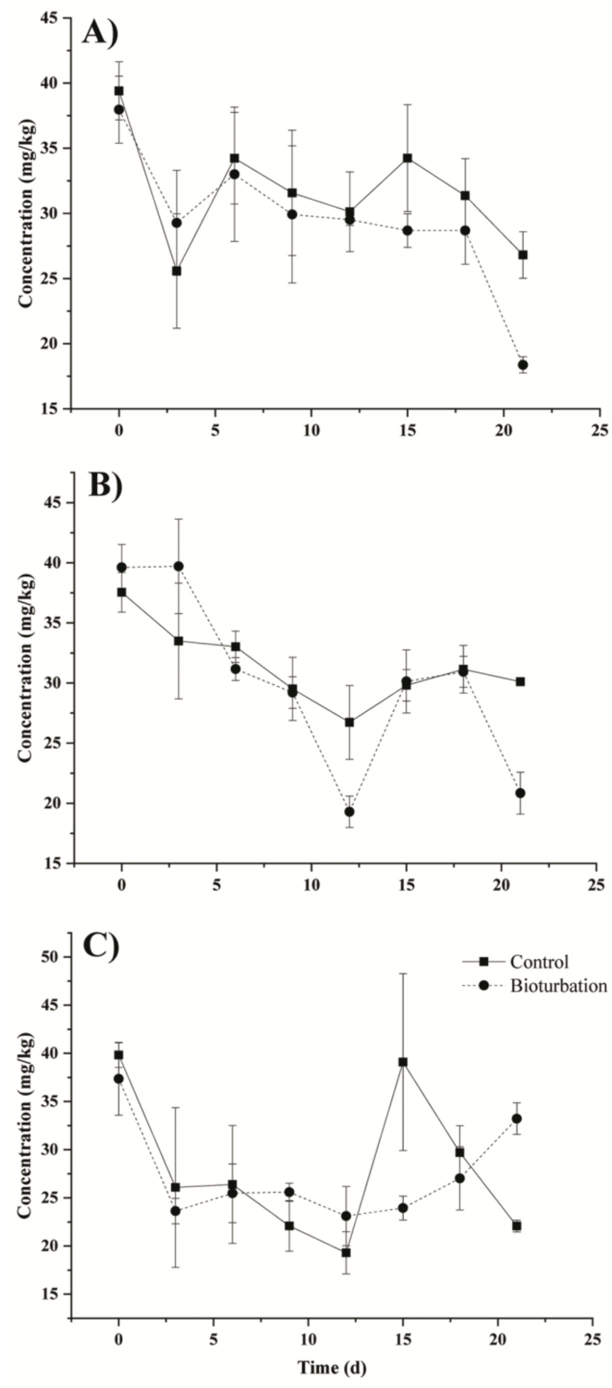

Fig. 2 — The variation in TPH with time in upper (A), middle (B), and lower (C) layer of sediments with (●) and without (■) worms

the end. The concentration of TPH in the upper layer of bioturbation group reached 18.37±0.62 mg/kg in the end, which was 51.59 % lower than day 0 and 31.48 % lower than control group in the end. This indicated that the removal rate by bioturbation in the upper layer reached 31.48 %. In the middle layer, the concentration of TPH was decreased by 47.38 % (20.85±1.74 mg/kg) compared with day 0, which was 39.70±3.92 mg/kg. In comparison to the control group, TPH was removed 30.77 % in the bioturbation group of middle layer. But the concentration of TPH in the lower layer was increased compared to control group, although 11.07 % lower than day 0 (Fig. 2C, $P < 0.01$, control; $P < 0.01$, bioturbation). The results demonstrated that the worms have removed a part of TPH by bioturbation in their active areas and hence renewed the areas contaminated with diesel to a certain extent.

TPH in worms

The standard line measured by fluorescence spectrophotometer for TPH content in worms were 310 nm (Ex), 360 nm (Em), and 5 nm (Em Slit) and the curve is depicted in Figure 3. The formula of the curve is as follows:

$$Y = 5961.5x+642.06\ (R^2 = 0.998)$$

Where, Y is the fluorescence value of oil, and x is the concentration of oil (μg/ml).To ensure accuracy of the experiment, TPH accumulated in the worms were measured only at initial phase and at the end of the experiment. Initially, at the start of the experiment (after domestication) the TPH content in the worms was 0.998±0.171 mg/kg whereas, at the end of experiment it was recorded as 23.764±3.878 mg/kg, which indicated that the feeding behavior of the worms caused a large accumulation of TPH content in the worms body. Feeding is a kind of crucial behavior in the process of bioturbation by worms.

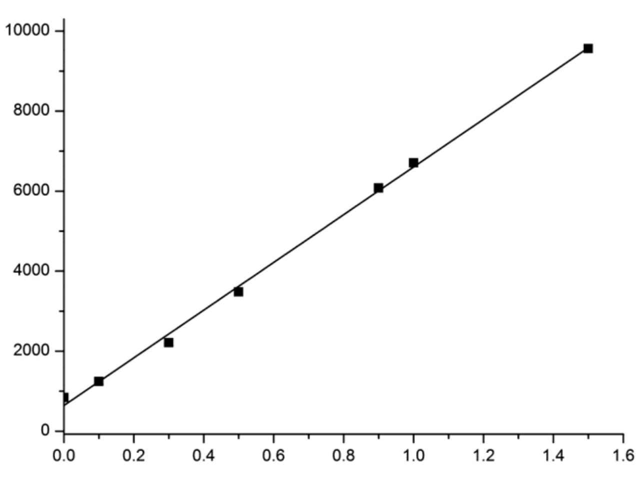

Fig. 3 — The variation in fluorescence value along with the concentration of TPH

Distribution of TPH in three phases

There were some variability in the contents of TPH in the systems with and without worms, and the change was more obvious in the bioturbation group (Fig. 4). At the initial stage of the experiment, the contents of TPH were negligible in both water column and worms body compared to the sediments. As time went on, a large amount of TPH content was transferred from sediments (particularly from surface and sub-surface) to the water column in the bioturbation group. Additionally some part of TPH was entered into the deeper sediments, leading to increase in concentration of TPH in lower layers of sediment. At the end of the experiment, increased TPH content was also detected in the body of worms, indicating continuous accumulation of TPH in worms throughout the experimentation time. Moreover, some part of TPH content was lost due to volatilization and thereby recorded a decline in the gross TPH content.

Residual oil in the sediments

The results of GC-MS analysis of diesel indicated that diesel consists of many kinds of hydrocarbons (Fig. 5A) including alkanes, olefins, aromatic hydrocarbons and so on, among which the content of alkanes is the most (Fig. 5B). The results of GC-MS analysis of sediments extracted with n-hexane (HPLC) after the experiment indicated that some of

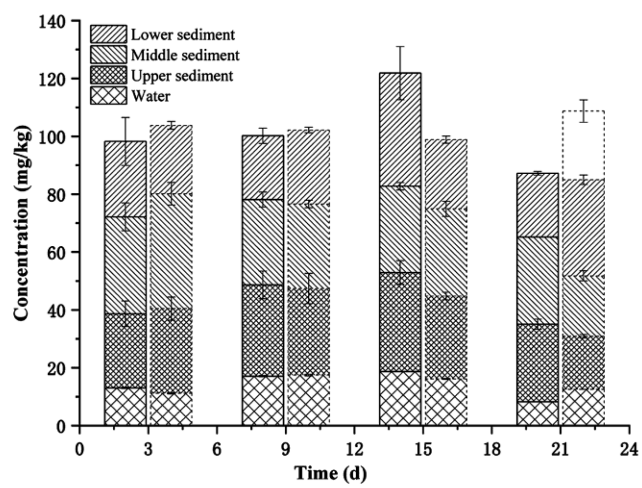

Fig. 4 — The comparison of TPH in different phases with (□) and without (□) worms on day 3, day 9, day 15, day 21, from bottom to top sediment layers and in water column

the components of diesel were removed from the sediments, particularly alkanes and PAHs (Figs. 6A & B) demonstrating the fact that the volatile hydrocarbons could be removed easily than others by bioturbation.

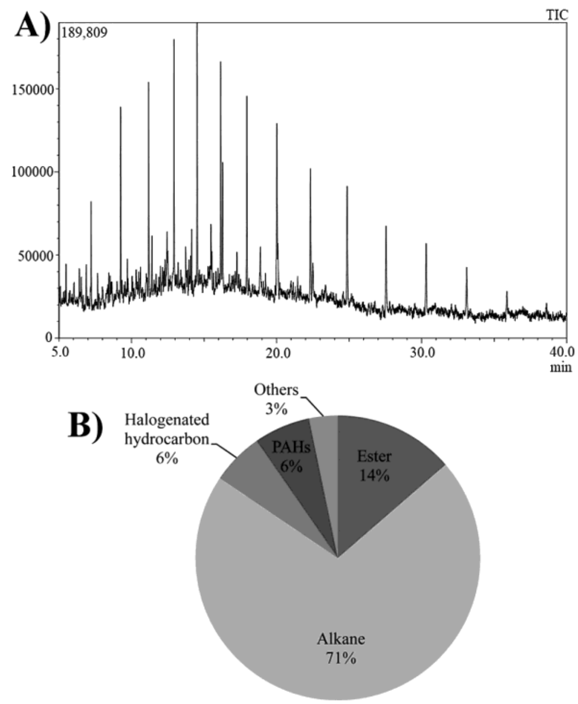

Fig. 5 — (A) GC-MS analysis of untreated diesel; and (B) Components of diesel

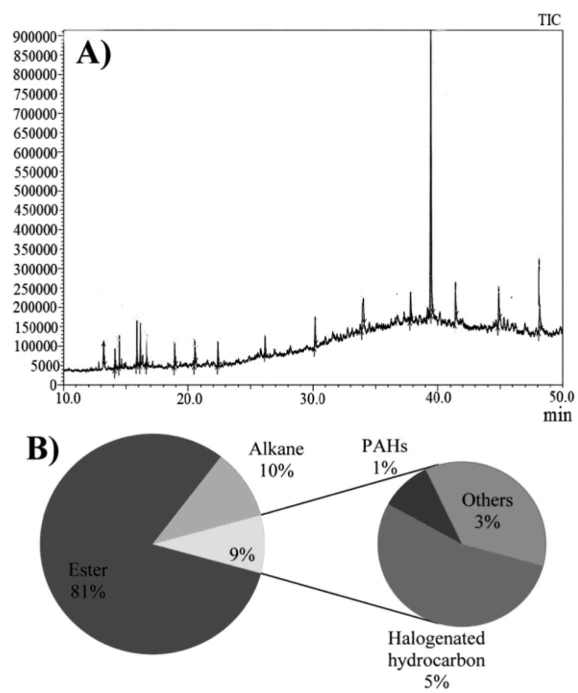

Fig. 6 — (A) GC-MS analysis of residual oil in the sediments after bioturbation; and (B) Components of residual oil

Discussion

Oil pollution, especially diesel pollution, which is one of the important sources of pollution in the marine environment, seriously affects the sedimentary environment and also influences the whole ecosystem. Benthos, living in sediments, plays an important role in biomonitoring[26,27] and to evaluate the marine environmental pollution and bioturbance[28,29] to restore polluted sediments. Mussels are often used to monitor the pollution situation[30], whereas worms, mud snails, crabs are usually used as bioturbators[31] in the laboratory experiment. The disturbance intensity of these marine organisms are obviously different[32]. In the process of bioturbation, some part of the pollutants will get accumulated in the bioturbators body through feeding behavior and some gets transferred to other layers of sediments by excretion and burrowing. The present study was committed to analyze how the worms or the bioturbators remove TPH from the sediments. In addition, the fate of TPH was also studied when it left from the sediments by bioturbation in the present study. However, the actual measured values of TPH were lower than the initial estimated values in sediments, because of part of TPH was volatilized during air-dried process.

The fate of TPH

Bioturbation by the worms had a crucial effect on TPH in the surface and sub-surface sediments. In the bioturbance groups, the measured profiles of TPH showed reduction in TPH concentration over time in surface and sub-surface sediment layers but its concentration rose in seawater and deeper sediments (Figs. 1 & 2), which indicated that most of the TPH from surface and sub-surface sediments was transferred to other phases (Fig. 4). Previous experiments also reported the similar results[33,34]. The results of the present study proved that the disturbance intensity by worms was largest in the upper and middle layers of sediments. In addition, the reported decline in TPH content of water is may be due to volatilizing into the atmosphere, transferring to the sediments again and by absorbing by the worms (Fig. 1). Further, part of TPH remained in the water column would gets transferred to the sediments due to gravity, leading to rise in value of TPH in the sediments (Fig. 2A). In control groups, TPH in the upper and middle layers declined slightly (Figs. 2A & B), which proved that part of it has released to the water[35] or transferred to the deeper layers due to volatilation and gravity, respectively. In the lower sediment layer, the concentration of TPH was increased irrespective of presence or absence of worms, which indicated that the part of TPH from the middle and upper layers have transferred to the bottom layer either by bioturbation or gravity action. But the more amount of TPH was accumulated in the worms due to the strong lipophilicity of TPH which is conducive to the absorption of TPH by worms. Therefore, the removal of TPH from the sediments was not only caused by transferring through bioturbation, but also through absorption by the organisms. The longer the time, the more amount of TPH are transferred to other layers. Hence, by the infaunal activities, lot of pollutants from the sediments can be removed[36], and can be restored back.

The fate of residual oils

The actual composition of diesel is very complex. The non-hydrocarbon components of diesel are sulfur, nitrogen, nitrogen compounds[37]and even metals. Hydrocarbon components of diesel are alkanes, olefins, polycyclic aromatic hydrocarbons, alcohol, ester and so on (Fig. 5). Some of them (mainly hydrocarbons) can be removed by bioturbation so that the sediments polluted by diesel can be restored back. The effect of *Nereis diversicolor* bioturbation on the remobilization and bioavailability of polycyclic aromatic hydrocarbons from estuarine sediments were determined in a laboratory experiment and revealed that *N. diversicolor* bioturbation significantly increased PAHs release from sediments to water[38]. The results of GC-MS analysis showed that the removed component of hydrocarbons by bioturbation were mainly alkanes (Figs. 5B & 6B).

Conclusion

The removal of some hydrocarbons (mainly TPH) from uniformly diesel polluted sediments was studied in a laboratory experiment in the absence and presence of worms. The results clearly demonstrated the fate of TPH: when the worms were present, it would transfer to the overlying water column, deeper sediment layers and to the bodies of worms mainly from surface and sub-surface sediment layers. In the absence of worms a bit of TPH content were migrated to the water column or dropped to the deeper layers of sediments because of volatizing and gravity action. The other components of diesel such as alkanes, aromatic hydrocarbons in the sediments were removed to a certain degree by bioturbation, but where these got transferred was not detected. The removal of diesel from upper and middle layers of sediments was strongly influenced by bioturbation process which is the layers where the worms were located in the sediments

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Conflict of Interest

Authors declare no competing or conflict of interest.

Author Contributions

LX & YB: Resources, investigation, and supervision; and ZW & ZX: contributed in writing - original draft; review & editing.

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