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Effects of nitrate concentration in interstitial water on the bioremediation of simulated oil-polluted shorelines

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Abstract

Nutrient addition has been proved to be an effective strategy to enhance oil biodegradation in marine shorelines. To determine the optimal range of nutrient concentrations in the bioremediation of oil-polluted beaches, nitrate was added to the simulated shoreline models in the initial concentration of 1, 5 and 10 mg/L. Whenever the NO_3-N concentration declined to 70% of its original value, additional nutrients were supplemented to maintain a certain range. Results showed adding nutrients increased the oil biodegradation level, the counts of petroleum degrading bacteria (PDB) and heterotrophic bacteria (HB), and the promoted efficiency varied depending on the concentration of nitrate. Oil degradation level in 5 mg/L (NO_3-N) group reached as much as 84.3% accompanied with the consistently highest counts of PDB; while in 1 mg/L group oil removal efficiency was only 35.2%, and the numbers of PDB and HB were relatively low compared to the other groups supplemented with nutrients. Although counts of HB in the 10 mg/L group were remarkable, lower counts of PDB resulted in poorer oil removal efficiency (70.5%) compared to 5 mg/L group. Furthermore, it would need more NO_3-N (0.371 mg) to degrade 1 mg diesel oil in the 10 mg/L group than in the 5 mg/L group (0.197 mg). In conclusion, Nitrate concentration in 5 mg/L is superior to 1 and 10 mg/L in the enhancement of diesel oil biodegradation in simulated shorelines.

Key words: diesel oil 0#; bioremediation; nutrient concentration; interstitial water

Introduction

Many microorganisms presented in seawater possess the enzymatic systems to degrade petroleum hydrocarbons, and they respond quickly to an oil spill (Atlas, 1995). These microorganisms require not only carbon and mineral nutrients for incorporation biomass, but also nitrogen and phosphorus, so the absence of N and P often restricts their growth rate.

Some of the previous results demonstrated the feasibility of nutrient addition in enhancing the degradation of oil (Bragg *et al*., 1994; Swannell *et al*., 1996). Therefore, adding nutrients at a certain concentration is a commonly used strategy in the bioremediation of oil-polluted shorelines. Theoretically, approximately 150 mg nitrogen and 30 mg phosphorus are consumed when converting 1 g hydrocarbon to cell material (Rosenberg and Ron, 1996). If all the petroleum could be biodegraded, it would be convenient to calculate the amount of N and P required for biodegradation. However, the composition of petroleum is so complicated that the optimal nutrient types and concentrations vary widely based upon the oil properties and the environmental conditions.

In the earlier studies simulating environmental conditions of Qingdao shorelines, nutrient type and optimal N/P ratios have been explored (Xia *et al*., 2005; Xia *et al*., 2006). Results showed the optimal N/P ratio was 10:1, and petroleum degrading microorganisms tend to utilize NO_3-N as the nitrogen source. These findings are consistent with those of other researchers (Ramstad and Sebum, 1995).

Oil biodegradation takes place mainly at the interface between oil and water, so the nutrient concentrations in the interstitial water were often measured during the study (Atlas and Bartha, 1992; Bragg *et al*., 1994). Using nitrate as N source, Venosa *et al*. (1994) found 1.5–2.0 mg/L supported nearly maximal biodegradation of heptadecane immobilized onto sand particles. While in continuous flow beach systems, Du (1999) discovered nitrate concentrations below approximately 10 mg/L limited the rate of oil biodegradation. Higher nutrient requirements may attribute to the more complex composition of crude oil.

In this study, to determine the concentration and frequency of nutrient addition in the bioremediation of oil-polluted shorelines, nitrate was added in 1, 5 and 10 mg/L concentrations, and a certain amount of PO_4-P was added to maintain the N/P ratio of 10:1 in the simulated shoreline models.

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1 Materials and methods

1.1 Experimental design

Polyethylene boxes (50 cm × 15 cm × 30 cm) which could move horizontally were used as microcosms to simulate shoreline environments (Fig.1). Each microcosm consisted of a water zone (10 L) and a sand zone (12 L) parted by a stainless sieve (ϕ 1 mm). The interstitial water was taken from the sampling ports located in the middle of each zone.

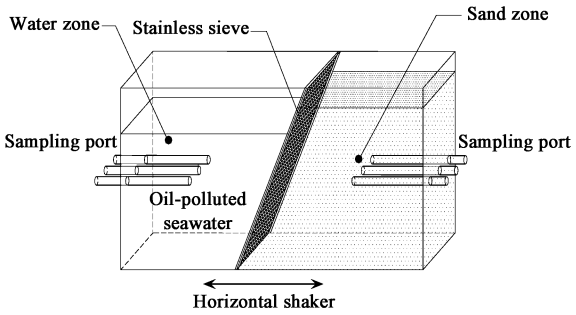

Fig. 1 Simulated shoreline microcosm.

The sands were taken from a natural area in Jin Beach, and seawater was collected from Huiquan Bay in Qingdao, China, and was sterilized before used. Diesel oil 0# obtained from Shengli Oilfield was added to the sterilized seawater with some surfactant then pumped in circulation for 2 h to make oil-polluted seawater. After this treatment, oil concentration in the seawater was 38.6 mg/L. In order to give consistency to the results by alleviating the problem of losses due to volatilization, diesel oil 0# was weathered following standardized method by heating to 521°C under a nitrogen environment (Salvador *et al*., 1997).

The experimental design included nutrient and non-nutrient controls. First, natural clean sands were added into sand zone in each simulated systems, then diesel oil 0# contaminated seawater was poured in and submerged the sands.

Before the experiment, seawater which has been polluted by oil was collected from Dagang Wharf No.6 and incubated on a rotary shaker at 25°C for 6 weeks to obtain a mixed inoculum. To obtain a standard inoculum, the mixed cells were harvested by centrifugation, rinsed three times in sterile saline before being resuspended in sterile liquid basal medium to yield an absorbance reading of 0.5 at 540 nm, and then 3 ml of the inoculum was added to sand zone. With the experiment going on, inorganic nutrients were added to the microcosms to make NO_3-N concentration of 1, 5 and 10 mg/L, respectively, while the concentration of PO_4-P was adjusted to 0.1, 0.5 and 1.0 mg/L to maintain the N/P ratio 10:1. Each microcosm was supplied with nutrients whenever the N concentration decreased to 70% of its original value, and the contaminated sand was tilled.

1.2 Chemical analysis

The samples were analyzed every other day for total petroleum hydrocarbons (TPH) by Oil Analyzer of infrared spectrometer (Jilin BeiGuang Optical Instrument Factory, China), and the concentration of TPH was expressed as C (mg/L) while C_0 represented the original value of TPH in each group. NO_3-N, and PO_4-P were measured using cadmium-copper reduction method and phosphomolybdenum blue method (State Oceanic Administration of China, 1999), respectively.

1.3 Microbiological analysis

The numbers of heterotrophic bacteria (HB) and petroleum degrading bacteria (PDB) were determined following the most-probable-number (MPN) method (Zheng *et al*., 2004), and each dilution was replicated thrice. Bushnell-Hass medium supplemented with 2% (w/w) NaCl was used as the growth medium for PDB and Marine Broth 2216 (Difco) for HB. Plates for enumeration of PDB and HB were incubated at 25°C for 3 weeks and 48 h, respectively.

All the chemicals used in this research are analytical grade and were purchased from certified laboratories and suppliers.

2 Results and Discussion

2.1 Oil biodegradation efficiency

During the experiment, TPH in the sample was measured and the biodegradation efficiency was calculated (Fig.2). The biodegradation level on day X was the difference in TPH values between day 0 and X divided by the value of day 0 and expressed as a percentage.

The results show that the addition of inorganic fertilizers significantly enhanced the efficiency of oil biodegradation in comparison to the control group, while the degree of stimulation varied with the nitrate concentration. In the experiment, a higher biodegradation level (84.3%) was observed in the 5 mg/L group, and the biodegradation level was lower (70.5%) in 10 mg/L group. When NO_3-N concentration was 1 mg/L, oil biodegradation efficiency was 35.2%, which was accelerated to some extent compared to the control group (19.1%). It is apparent that adding NO_3-N 5 mg/L could accelerate oil biodegradation efficiency.

The biodegradation rates of all the groups were estimat-

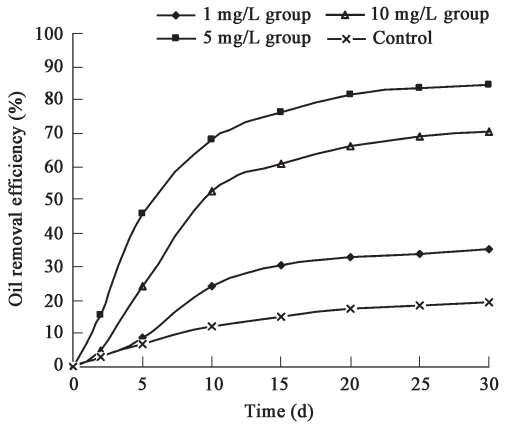

Fig. 2 Oil removal efficiency under different nitrate concentrations.

ed in the study. Suggesting oil biodegradation process fits to a first order model ($r = KC^n$, where $n = 1$), we plot ln (C/C_0) versus time, the reaction rate coefficient K could be obtained from the slope of the regression line (Venosa *et al*., 1996; Stewart *et al*., 1993). From the biodegradation kinetics equation, the half time $t_{1/2}$ for the samples treated with nutrients and control group can be calculated as $t_{1/2}$= 0.693/K, and the $t_{1/2}$ values of all groups are also shown in Table 1.

Table 1 indicates the noticeable effect of NO_3-N addition in 5 mg/L group: it can accelerate biodegradation rates up to 8 times compared to the control group. This means if no nutrients were added in the systems, the biodegradation rates were extremely slow although petroleum degradation microorganisms were applied.

The biodegradation rates of diesel oil 0# in other samples treated with NO_3-N in 10 and 1 mg/L group to some extent were lower than the 5 mg/L NO_3-N. This indicated that the stimulating effects of the degradation rates varied with the nutrient concentration. In this simulated shoreline microcosms, 5 mg/L NO_3-N supported nearly complete biodegradation of diesel oil 0#. This would be useful in selecting the nutrient concentration on bioremediation of oil-polluted beach, for the application of nutrients under an optimal range may result in higher biodegradation efficiency and lower adverse effect. However, the main cause of this result needs to be further explored.

2.2 Counts of microorganisms

The bioremediation process of simulated oil-polluted shoreline was investigated for 30 d under laboratory conditions, and variations in the numbers of HB and PDB of the nutrients amendment samples and control group are shown in Figs.3 and 4.

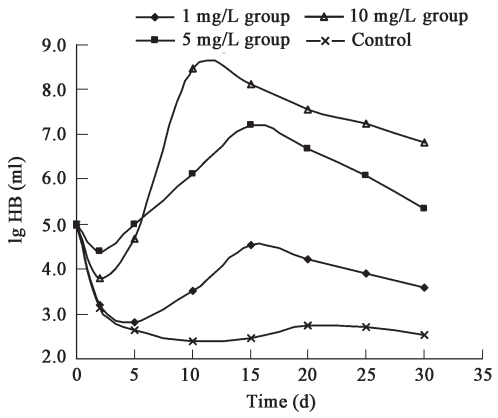

Fig. 3 Counts of HB under different nitrate concentrations.

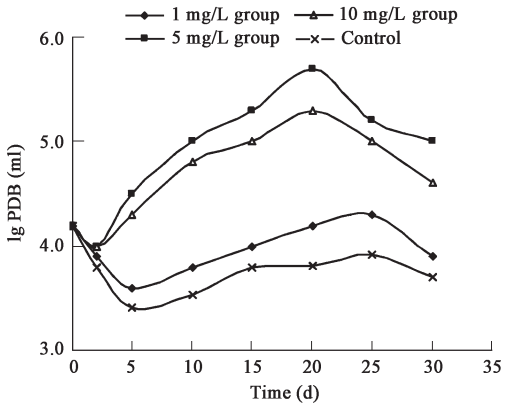

Fig. 4 Counts of PDB under different nitrate concentrations.

2.2.1 Numbers of HB

Numbers of HB in all groups decreased at first in response to the oil-polluted seawater, which may due to the toxicity of diesel oil 0#. After 5 d of adaptation, the numbers of HB in 5 and 10 mg/L groups began to increase and reached approximately the original value. However, the numbers of HB in 1 mg/L and control group were about 150–230 times lower than the original ones.

During the course of the experiment, the numbers of HB in each group began to increase. In 10 mg/L group, the counts of HB reached the maximal value by three orders of magnitude compared to the original one on day 10, and they remained consistently declining very slowly during the entire experimental process. In 5 mg/L group, the maximum HB occurred on day 15 and was approximately 150 times higher than the original value. On the contrary, samples in 1 mg/L group showed relatively little change in the number of HB compared to the control group during the first 5 d, and on day 10 it began to increase, reaching the maximum on day 15, but it failed to reach the original value. In the control group, counts of HB did not increase until day 20, and they were much lower than other groups during the experiment, indicating a lack of nutrients. After reaching their maximum values, counts of HB in all groups began to decline. However, the values are quite higher in the groups with amended nutrition compared to the control group.

2.2.2 Numbers of PDB

The variations of the counts of PDB in the systems were similar to HB, and they also decreased on day 2. In 5 and 10 mg/L groups, the numbers of PDB in the experiment began to increase on day 5, but in 1 mg/L and in the control groups, they did not increase until day 10, and the counts of PDB in the latter were much lower than those in the former.

Table 1 Biodegradation kinetics equation* and the half-time of diesel oil 0#

Group (mg/L)	1	5	10	Control
Regression equation	$y = 0.0156x + 0.0414$	$y = 0.0642x + 0.2301$	$y = 0.0439x + 0.0989$	$y = 0.0071x + 0.0294$
	$R^2 = 0.8887$	$R^2 = 0.9086$	$R^2 = 0.9126$	$R^2 = 0.9137$
Half-time (d)	44.4	10.8	15.8	97.6

*y stands for ln(C/C_0), and x stands for time (d), R^2 is interrelated coefficient.

Groups in 5 and 10 mg/L reached their maximum population density on day 20, whereas the maximum density in 1 mg/L and control groups occurred on day 25. It seems nitrate in 5 and 10 mg/L concentrations are apt to be utilized by oil degraders. During the experiment, the numbers of PDB in the 5 and 10 mg/L groups remained consistently higher than the control and 1 mg/L groups. Compared to 10 mg/L group, the effect of adding nitrate in 5 mg/L quantities is extraordinary: the counts of PDB increased 30 times on day 20 compared to the original counts, and the counts of bacteria declined very slowly afterwards. It is thought that during the experiment, the microbial population in the 5 mg/L group was growing at the expense of an optimal N concentration. As for other groups, the relatively lower PDB might result from either the scarcity or the excess of the nutrients.

It is well-known that oil contamination causes significant changes in microbial populations. In uncontaminated areas, the oil degraders fluctuated only very slightly (Wright *et al*., 1997). However, as soon as the oil pollution began, a selective enrichment of degrading bacteria occurred, which produced changes in the composition of the microbial community. In our study, oil degrading microorganisms in 5 mg/L group were much higher than other groups, and the level of the oil biodegradation in this group was also the highest one.

In spite of the large numbers of HB in 10 mg/L group, these findings are not surprising that oil degradation efficiency in it was only the second due to the relatively low numbers of PDB. Considering this, the concentration of NO_3-N in 5 mg/L is more appropriate to the enhancement of oil degradation than 10 and 1 mg/L.

2.3 Variations of nutrients concentration

At the beginning of the experiment, NO_3-N was added in three concentrations in each microcosm, and PO_4-P was added according to N/P ratio 10:1 at the same time. In order to maintain the nutrients concentration in interstitial water, periodical addition of nutrients was done whenever the concentration decreased to approximately 70% of its original value. Take NO_3-N as an example, the variation of its value in each group was showed in Fig.5.

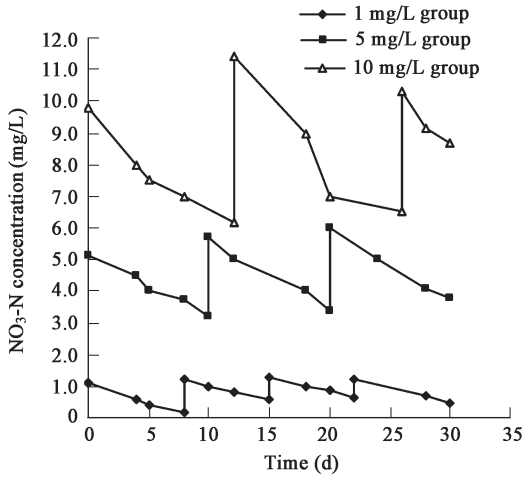

Fig. 5 Concentration of NO_3-N with the variation of time.

In 1 mg/L group, NO_3-N was depleted very quickly, and the extra N was supplied on the day 8, 15, and 22, respectively. In 10 and 5 mg/L groups, NO_3-N was replenished two times in each group, with the addition time on day 10, 20 in the former and day on 12, 26 in the latter. Residual concentration of NO_3-N in each groups are measured at the end of the experiment, and the value was 0.48, 3.8 and 8.7 mg/L in 1, 5 and 10 mg/L groups respectively.

Calculating the amount of oil removed by biodegradation and the consumption of NO_3-N, the nutrient demand of NO_3-N in the biodegradation of diesel oil 0# could be measured, and the results were showed in Table 2.

Table 2 Nutrient consumption in the biodegradation of diesel oil 0#

Group (mg/L)	1	5	10
Biodegradation of oil (mg)	135.9	325.4	272.1
NO_3-N consumption (mg)	18.6	64.0	101.0
Nutrient/oil ratio (mg/mg)	0.137	0.197	0.371

Table 2 shows the amount of NO_3-N depleted in each group varied greatly. In 10 mg/L group, the nutrients consumption was almost 3 times of that in 1 mg/L group. In 5 mg/L group, the consumption of NO_3-N was mediate among the three groups. It can be seen from oil biodegradation efficiency and the counts of HB (PDB) that adding nitrate in 1 mg/L concentration is inadequate to maintain a high level of bioremediation. Due to the scarcity of nutrients, oil degrading microorganisms may live in uncomfortable conditions and result in relatively low degradation efficiency. On the contrary, the addition of nutrients in 10 mg/L may be in excess. In this microcosm, too many HB were stimulated to grow up while the counts of PDB did not increase as much, and as a result, the oil biodegradation efficiency was lower than the 5 mg/L group.

Among all the 3 groups amended with nutrients, adding nitrate in 5 mg/L amounts may be an optimal choice. This would not only result in higher counts of PDB and degradation efficiency, but also the relatively lower residual nutrient concentration. Furthermore, it only needs 0.197 mg NO_3-N to degrade 1 mg diesel oil 0# which would cost less.

Recently, the potential application of resource-ratio theory in hydrocarbon biodegradation was discussed (Head and Swannell, 1999; Smith *et al*., 1998). This theory suggests that manipulating the N/P ratio may result in the enrichment of different microbial populations, and the optimal N/P ratio can be different for degradation of different compounds. Results from this experiment confirmed this hypothesis to some extent from the measurement of PDB and HB, further studies needs to be developed to identify the exact strains of the microorganisms in each microcosm.

3 Conclusions

Three levels of NO_3-N were used to investigate the effects of nutrients on biodegradation of diesel oil 0#

in simulated shoreline microcosms. Oil biodegradation efficiency in the 5 mg/L group was the highest (84.3%) accompanied by the biggest counts of petroleum degrading microorganisms. On the contrary, lower degradation levels in 1 mg/L group or relatively higher counts of HB and residual concentration of NO_3-N in 10 mg/L group indicated the scarcity or excess of nutrients in the systems.

Although laboratory experiments have shown that the addition of growth-limiting nutrients enhanced the rate of oil biodegradation, it is impossible to maintain a certain nutrient ratio because of the dynamic washout of nutrients resulting from the action of tides and waves (Bragg *et al*., 1994; Venosa *et al*., 1996). So it is more practical to maintain the nutrient concentrations within the interstitial water at an optimal range, and adding NO_3-N in the concentration of 5 mg/L may be a good choice.

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