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Degradation of crude oil by indigenous microorganisms supplemented with nutrients

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Abstract: Different kinds of mineral nutrients(NO_3-N, NH_4-N and PO_4-P) were applied in the simulated oil-polluted seawater for enhancing oil biodegradation in the N/P ratio 10:1 and 20:1. Although indigenous microorganisms have the ability to degrade oil, adding nutrients accelerated biodegradation rates significantly. For the group amended with NO_3-N and PO_4-P in the ratio 10:1, the reaction rate coefficient was 4 times higher than the natural biodegradation. Chemical and microbiological analysis showed that the optimal N/P ratio in the system is 10:1, and microorganisms tend to utilize nitrate rather than ammonium as N source.

Keywords: oil spill; biodegradation kinetics; N/P ratio; microbial population

Introduction

Oil spill would bring about tremendous disaster to the ecosystem. Statistics of oil spillage in Qingdao, China during the period of 1979—1989 indicate that incidence of oil spills occurred 208 times, and approximately 5810 tons of oil was released into the sea. For instance, in 1989, 3342 tons of oil was spilled from a Chinese tanker, "Oriental Ambassador", which impacted over 230 km of coastlines(Qu, 2001).

Many microorganisms possess the enzymatic capability to degrade petroleum hydrocarbons, and do appear to respond quite rapidly to the presence of petroleum(Atlas, 1995). Bioremediation is a process whereby the natural biodegradation is enhanced, and it can be carried out by optimization of environment conditions. Compared to physical and chemical methods, bioremediation is the most promising technologe currently in use, with such advantages as cost effectiveness and the potential ability to remediate an environment without causing environmental damage (Pritchard, 1991). Bioremediation technology holds a lot of promise not only for developed countries, but also for developing countries such as China(Xia, 2003).

Since microorganisms require nitrogen, phosphorus and other mineral nutrients for incorporation into biomass, the availability of these nutrients is critical(Walker, 1984). Concentrations of available nitrogen and phosphorus in seawater generally are severely limiting to microbial degradation(Atlas, 1981; Leahy, 1990). In earlier studies on the bioremediation of oil with fertilizers, some of the results in the laboratory and field demonstrated the feasibility of bioremediation in enhancing the degradation of oil(Bragg, 1994; Swannell, 1996). However, some researchers found that fertilization increased the biodegradation rate only slightly (Venosa, 1996; Oudot, 1998).

In this study, different kinds of mineral nutrients(NO_3-Ns, NH_4-N and PO_4-P) were applied to the simulated oil-contaminated seawater in different N : P ratio, and the effectiveness of nutrient application to indigenous, marine microbial populations were assessed.

1 Materials and methods

1.1 Sample collection and preparation

Seawater was collected from natural source(36° 03′ N, 120° 20′ E) and used within 48 h after collection with no microbial inoculums added. The background concentrations of NO_3-N, PO_4-P and NH_4-N are 1.8 μmol/L, 0.32 μmol/L and 8.3 μmol/L respectively.

In the experiment, crude oil from the Shengli Oilfield was used. In order to give consistency to the results by alleviating the problem of losses due to volatilization, the petroleum was weathered prior to experiments following standardized method by heating to 521°F under a nitrogen environment to prevent oxidation(Salvador, 1997).

Fertilizers used in the experiment were mineral nutrients: $NaNO_3$, KH_2PO_4 and NH_4Cl.

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

1.2 Experimental design

The experiment design included nutrient and non-nutrient controls which are summarized in Table 1. Erlenmeyer flasks(250 ml) were used as incubation reactors. The sterile flasks were filled with 100 ml of seawater, 0.2 g crude oil, and with a certain amount of N, P nutrients. To alleviate the influence of volatilization, crude oil was added to the sterilized seawater as blank.

Table 1 The composition of nutrients added in the samples

Group number	Mean value of nutrients added, μmol/L	Approximate ratio of N/P	Description
1	$503NO_3$-N + $49PO_4$-P	10:1	$10NO_3$-P
2	$496NH_4$-N + $48PO_4$-P	10:1	$10NH_4$-P
3	$1017NO_3$-N + $51PO_4$-P	20:1	$20NO_3$-P
4	$973NH_4$-N + $48PO_4$-P	20:1	$20NH_4$-P
5	None	30:1	Control

All samples were incubated on a rotary shaker at 150 r/min at 25℃. A set of flasks was sampled every week for oil analysis. Microbiological analysis was conducted every two week after incubation.

1.3 Chemical analysis

The samples were analyzed every week for total petroleum hydrocarbons (TPH). After the extraction of aqueous phase with carbon tetrachloride(CCl_4), the carbon tetrachloride solutions were dehydrated over anhydrous sodium sulphate, and then the value of TPH was quantified by Oil Analyzer of infrared spectrometer (Jilin Beiguang Optical Instrument Factory, China).

At the end of the experiment, NO_3-N, PO_4-P and NH_4-

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N were measured using cadmium-copper reduction method, phosphomolybdenum blue method and indophenol blue method (State Oceanic Administration of China, 1999) respectively.

1.4 Microbiological analysis

The numbers of heterotrophic and oil-degrading bacteria were determined by most-probable-number (MPN) method, and each dilution was in replicates of three. Bushnell-Hass medium supplemented with 2% (w/w) NaCl was used as the growth medium for petroleum degrading microorganisms (PDM) and Marine Broth 2216 (Difco) for heterotrophic microorganisms (HM). Plates for enumeration of PDM and HM were incubated at 25℃ for 3 weeks and 48 h respectively.

2 Results and Discussion

2.1 Oil biodegradation with and without nutrient

The average value of oil concentration during the biodegradation process in different groups is showed in Fig. 1. Although no inoculum was added, the oil was biodegraded to some extent after 8 weeks of incubation, this indicates that the natural petroleum-degrading bacteria in the seawater possess potentials for oil biodegradation.

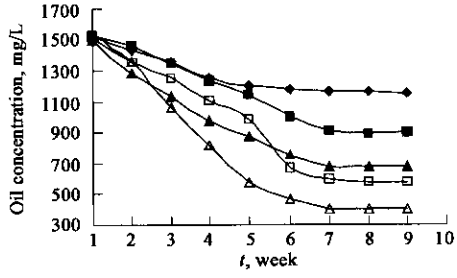

Fig.1 Variation of oil concentration during the 8 weeks experiment
◆control group ■20NH_4-P ▲20NO_3-P □10NH_4-P △10NO_3-P

The biodegradation capabilities of the indigenous microorganisms were enhanced by the addition of nutrients. It demonstrated that although the carbon source provided by the spilled oil is in excess, other elements like phosphorus and nitrogen are limiting in the environment. Supplementing flasks with these elements encouraged proliferation of microorganisms, thus enhancing the usage and breakdown of the oil.

The efficacy of nutrients addition differed according to the type and relative concentration of the N and P source. In the experiment, significant higher biodegradation level was observed when NO_3-N and PO_4-P were added in the ratio of 10:1; on the contrary, when NH_4-N and PO_4-P were added in the ratio of 20:1, the biodegradation level was much lower.

When NO_3-N and PO_4-P were added in the ratio of 20:1, or NH_4-N and PO_4-P were added in the ratio of 10:1, oil biodegradation was accelerated to some extent but not as much as 10NO_3-P group done.

In this study, the biodegradation rates for all of the groups were estimated. The time and concentration data fitted to a first order model ($r = KC^n$, where $n = 1$) as suggested by Stewar and Venosa (Stewar, 1993; Venosa, 1996). Plotting $\ln(C/C_0)$ versus time, the slope of the regression line was the value of the reaction rate coefficient K. From the biodegradation kinetics equation, the half-time for the samples and control was estimated as shown in Table 2.

Table 2 Biodegradation kinetics equation and the half-time of oil pollutants

Group description	Biodegradation kinetics equation	K, d^{-1}	R^2	Half-time, d
10NO_3-P	$\ln C = 7.26-0.0261t$	0.0261	0.9263	23
10NH_4-P	$\ln C = 7.35-0.0191t$	0.0191	0.9456	38
20NO_3-P	$\ln C = 7.25-0.0149t$	0.0149	0.9418	43
20NH_4-P	$\ln C = 7.32-0.0102t$	0.0102	0.9501	68
Control	$\ln C = 7.29-0.0051t$	0.0051	0.8818	128

The effect of nutrient amendment with 10NO_3-P was most noticeable: it can accelerate biodegradation rates up to 5 times compared to natural biodegradation. That is to say it will take natural degradation without nutrient enhancement about 128 d to achieve the result obtained in 23 d with the 10NO_3-P application. The results are comparable to those of other authors (Song, 1990), and would be useful in predicting effects of different nutrients on oil biodegradation in marine environment.

2.2 Microbial populations with and without nutrient amendment

Bacteria counts obtained from the samples are showed in Fig.2(a, b).

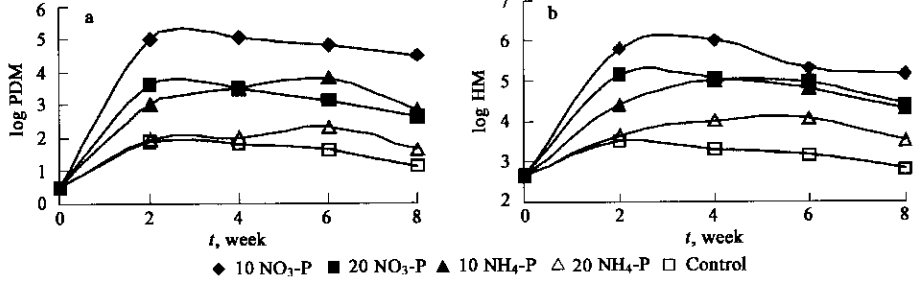

Fig.2 Average counts of petroleum-degrading microorganisms (PDM) (a) and heterotrophic microorganisms (HM) (b)

The response of the petroleum-degrading microorganisms (PDM) to the bioremediation treatment was positive, and it varied according to the type of nutrients. Groups treated with nitrate reached their maximum population density after 2 weeks of incubation, whereas the maximum density in ammonium-treated group occurred in week 6. The number of PDM in the 10NO_3-P group remained consistently higher than the control and other groups. It is thought that during the experiment, the microbial population in 10NO_3-P group was growing at the expense of an optimal source of N and P. As for other groups, the relative lower PDM might result from either the unfavorable form of nutrients, or the unsuitable value of N/P ratio.

The heterotrophic microorganisms (HM) increased in response to the nutrient addition. For all of the groups, it began to increase quickly, then reached a maximum value and declined. The most significant change was observed in 10NO_3-P group. After 4 weeks of incubation, the HM

reached a value of 9.5×10^5 MPN ml^{-1}, and approximately 380 times higher than the control. On the contrary, samples treated with $20NH_4$-P showed little change in the number of HM compared to the control during the first 2 weeks. The differences of HM between groups treated with $20NO_3$-P and $10NH_4$-P were not significant at 8 week, but the counts of HM were higher than the control and the $20NH_4$-P group.

It is well known that oil contamination causes significant changes in microbial populations. The result showed in Fig.2 that the number of PDM and HM increased with the addition of nutrients. Changes in the number of microorganisms may be an indication of a stimulated biodegradation process, but do not represent an accurate measurement of the actual biodegradation (Zheng, 2004). For instance, if the relationship between oil biodegradation level and microorganisms was analyzed as linear equation, then the interrelated coefficient was lower than 0.2 for both PDM and HM in $10NO_3$-P treated group. However, if we estimated the biodegradation level with the ratio of PDM and HM (PDM/HM), then the interrelated coefficient was 0.7. It is suggested that the ratio of PDM/HM can be used as a good indicator for oil biodegradation.

2.3 Nutrients utilization

The concentrations of mineral nutrients were determined before and after the experiment. The nutrients utilization efficiency is showed in Fig.3.

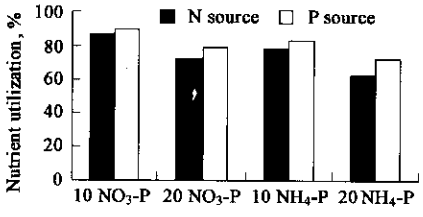

Fig.3 Nutrient utilization efficiency in the samples during the experiment

It is indicated that the utilization of PO_4-P in each group was much higher than that of NO_3-N or NH_4-N, which represented the relative scarcity of P source. In the groups with higher N/P ratio, much more P source was utilized compared to N source. It meant that there was P limitation in the environment.

With the addition of N source, the type of the nitrogen had significant influence on the biodegradation extent. If the nutrients were added in the same ratio of N/P, then the biodegradation efficiency was significantly higher in nitrate-treated groups than that in ammonium-treated groups. This can be seen not only from oil removal efficiency, but also from microbiological analysis that in nitrate-treated groups, there were more HM and/or PDM, which demonstrated that microorganisms tend to utilize nitrate rather than ammonium as some researchers had found (Ramstad, 1995).

The optimal C : N : P ratio generally used in bioremediation studies have been known to be 100 : 10 : 3 (Atlas, 1991). If all the petroleum could be biodegraded, it is convenient to calculate the required amount of N and P to biodegradation. Crude oil is composed of a wide range of hydrocarbons, and microbial utilization of these compounds as sole carbon source is highly dependent on the chemical nature of the compounds within the petroleum mixture and on the environmental determinants (Atlas, 1981), thus the demand of nutrients for oil biodegradation is site-specific.

In our study, the background ratio of N/P is about 30:1. We use nitrogen and ammonium as N source, phosphorus as P source, and add the nutrients with the N/P ratio of about 10 : 1 and 20 : 1 respectively. Results showed that when the nutrients in the N/P ratio were 10 : 1, the oil biodegradation extent was dramatically higher than the 20 : 1 groups in case the same kind of N source was employed, and in the former groups the utilization efficiencies of nutrients (both N and P source) were much higher than the latter. Considering that much more P was utilized, it is suggested that the optimal N/P ratio might be less than 10 : 1.

3 Conclusions

Results of this study suggested that the intrinsic bacteria along Qingdao shorelines have the ability to degrade petroleum. Nutrient amendment in the natural environment can accelerate oil degradation and this may shorten the treatment period to clean up the contaminated environments. Biodegradation dynamics study showed the biodegradation of spilled oil in the seawater follows the first-order model, and the half-time of oil is 23 and 128 d respectively for $10NO_3$-P treated group and the control.

The number of PDM and HM increased with the addition of nutrients. Although the counts of microorganisms alone do not represent an accurate measurement of the actual biodegradation, it is suggested that PDM/HM can be used as a good indicator for oil biodegradation.

Under test conditions, microorganisms tend to utilize nitrate as N source, and the optimal N/P ratio is 10 : 1 rather than 20 : 1.

Because the set-up of this experiment did not emulate a natural environment, it is thought that the application of the nutrients in the sea should be tested before any decision is made. It is believed that this test provides a database for further evaluations.

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