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# Bioremediation of Nitrobenzene-Polluted Sediments by *Pseudomonas putida*

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**Abstract** Natural attenuation, biostimulation and bioaugmentation of nitrobenzene (NB) contaminated sediments were investigated and compared. The removal rate of NB from contaminated environments with bioaugmentation was much faster than with biostimulation and natural attenuation. Within 10 days, 6 mg/kg and 53 mg/L nitrobenzene in sediment and water, respectively, was degraded with the augmentation of *Pseudomonas putida* isolated from the contaminated sediment. There was no distinct performance difference between natural attenuation and biostimulation, demonstrating that addition of nutrients had no effect on the bioremediation process. The information on the current phase is a crucial step in making policy decisions for the application of bioremediation.

**Keywords** Bioremediation · Nitrobenzene · Sediments · *Pseudomonas putida*

Nitrobenzene (NB) is one of the top 50 industrial chemicals produced in United States and has been on the United States Environmental Protection Agency (EPA) priority pollutant list (Majumder and Gupta 2003). About 19 million pounds of NB is released into the environment annually due to its usage, leakage or industrial accidents (Haigler and Spain 1991). For example, the Songhua River in northeast China was suffered from major water pollution owing to the explosion of a petrochemical corporation on November 13th, 2005. More than 500,000 tons of NB was discharged into the Songhua River, resulting in a highly NB contaminated environment (http://www.chinaview.cn 2005). Due to its recalcitrant and hydrophobic nature, NB accumulates in the sediment of a water body, posing a concern for ecosystem health.

Conventional treatment of NB has largely been through chemical or physical methods. These processes, however, have led to secondary contamination. Biological methods for the bulk removal of these pollutants are therefore generally preferred. Major advantages of bioremediation are the lower capital costs and the ability to perform the task on site. As an emerging alternative technology for restoration of contaminated environments, bioremediation normally includes bioaugmentation (addition of microorganisms) and biostimulation (addition of nutrients) (Dillewijn et al. 2007).

In a previous study, a nitrobenzene-degrading bacteria was isolated from the sediments of Songhua River (Li et al. 2007). The bacteria, identified as *Pseudomonas putida*, can mineralize 20 mg/L nitrobenzene completely from 2.5 to 35°C. The main objective of this study was to assess the bioremediation potential of nitrobenzene contaminated sediment using *P. putida*. The effectiveness of different remediation strategies, such as natural attenuation, biostimulation and bioaugmentation, was investigated and compared.

## Materials and Methods

*Pseudomonas putida* isolated from the river sediment (Li et al. 2007) was inoculated in river water collected from the Songhua River in a plastic container with 20 mg/L nitrobenzene as the sole carbon and energy source. After the

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bacterial grow to their exponential growth phase ($10^7$ cells/mL), they are ready to be used for bioremediation.

Bioremediation experiments were conducted in glass cylinder containers (diameter: 0.4 m, height: 0.6 m) with water and sediment (Table 1) collected from the Songhua River. Water and sediment samples were collected from the Songhua River near the wastewater discharge point of Jilin Petrochemical Corporation. The sediment consisted of light gray, calcareous particles and had a brown color. After mixed adequately with 100 mg/L NB, the sediments were paved (about 0.15 m in height) on the bottom of each container with the same volume of river water on the top. For bioaugmentation, bacterial grown in 20 mg/L nitrobenzene was added into the container with a volume ratio to water inside of 1:100. For biostimulation, nutrients were added into the container to make the same N and P concentrations as those in mineral solution (Li et al. 2007). No measures were taken in the natural attenuation experiment. The experiments were conducted in triple at a temperature of 10°C which is close to the daily average temperature in the Songhuajiang River. Samples were taken at appropriate time for analysis.

Nitrobenzene degrading bacteria was enumerated using a tailored version of the Brown and Braddock (1990) sheen screen MPN method. An initial dilution of ca. 1 g soil in 10 mL basal nitrobenzene medium was used for a 6 tube MPN, with seven 1:10 serial dilutions in a 96 well microtitre plate. After serial dilution, 5 μL of filter-sterilised nitrobenzene was applied to the surface of the medium in each well. The last row only contained medium and nitrobenzene as an un-inoculated control. Microtitre plates were incubated for up to 30 days and examined every 5 days. Tubes were scored as positive if the hydrocarbon sheen was disrupted.

Cell concentration was determined by measuring the absorbance at a wavelength of 550 nm using a Shimadzu UV-Visible spectrophotometer UV-1200 V. Cell density was obtained from the formula: DCW (mg $L^{-1}$) = 314.5* $OD_{550}$. For analysis of NB concentrations, the sample was centrifuged and extracted with methyl-*t*-butyl ether. A 1 μL extract was then analyzed for NB using a capillary gas chromatograph (Anglient, Model 6890N) with an ECD detector. The GC temperature program was as follows: the initial temperature was 80°C (2 min), and it increased at 10°C/min to 160°C (5 min). The detector and injector temperatures were 300°C and 270°C, respectively. The measured limit of detection for nitrobenzene in water is 0.1 mg/L.

**Table 1** Chemical parameters of water and sediment from Songhua River

| Parameter | Water | Sediment |
|---|---|---|
| Temperature (°) | 10 | N.A. |
| pH | 7.2 | N.A. |
| Dissolved oxygen (mg/L) | 3.5 | N.A. |
| Organic carbon (%) | N.A | 1.26 |
| COD (mg/L) | 25 | N.A. |
| TN | 1.2 | 2.52 |
| TP | 0.23 | 1.02 |

## Results and Discussion

Bacteria growth in river water and sterilized culture media was investigated and compared to evaluate the potential of bacteria inoculation in field conditions. Temporal profiles of nitrobenzene concentration and bacterial growth are shown in Fig. 1.

As can be seen from Fig. 1, NB degraded quickly both in river water and culture media. It took about 14 h and 20 h, respectively for the 20 mg/L NB to completely degrade in the two systems. The final cell density in both systems was all about 23 mg/L. Bacterial growth in river water followed the batch growth curve of a short lag phase, followed by an exponential biodegradation phase and ended in stationary phase. Only about 3% of the initial NB was removed in the control experiment and no increase in cell density was observed.

The results showed that there was no difference of bacterial growth in river water compared with that in culture media. On one hand, this means the nutrient concentration in river water was not the growth-limiting factor for cell growth. On the other hand, the cells can also be inoculated

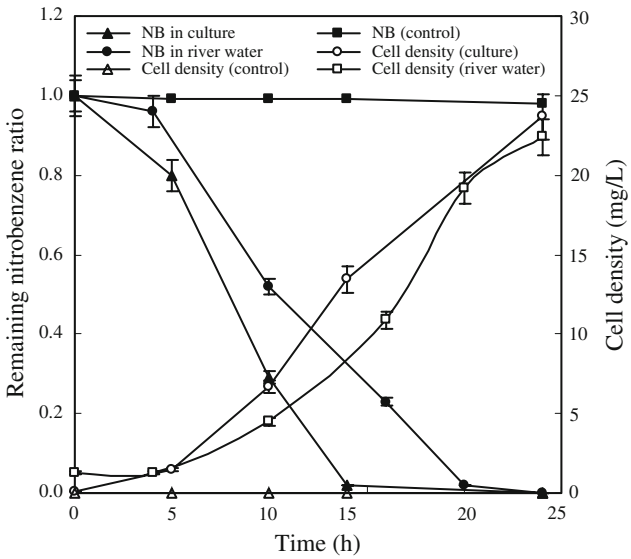


**Fig. 1** Temporal profiles of bacterial growth and NB degradation in river water and culture media

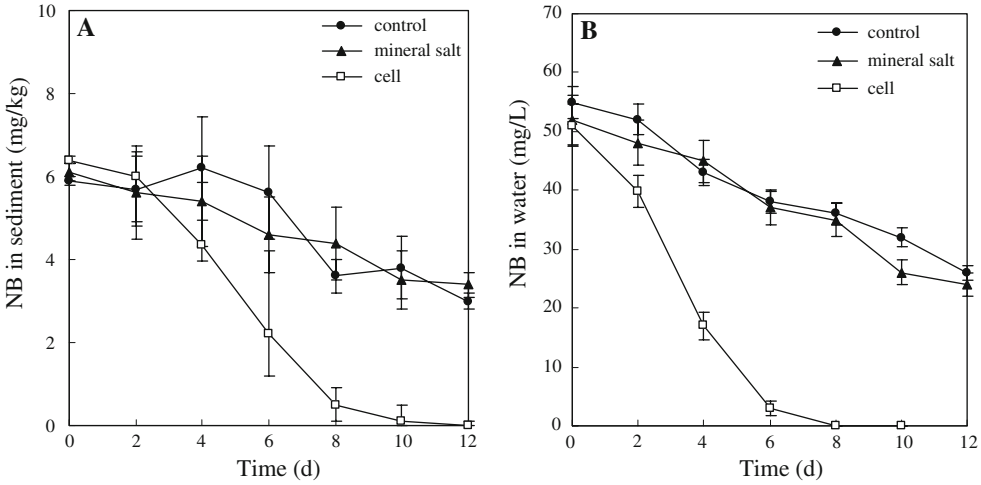


**Fig. 2** Temporal profiles of NB in the sediment **a** and solution **b**

directly in river water for large-scale remediation of NB-contaminated river water or sediment. This is very useful in practical remediation work where disinfection of contaminated environment is impossible.

Figure 2 shows NB concentration profiles in sediment and covered water under different remediation strategies. The initial concentrations of NB in the sediment and water were 6 mg/kg and 53 mg/L. It took about 10 and 8 days for the NB to degrade in sediment and water with bacterial augmentation. NB degradation with bioaugmentation was much faster than that in the other two systems. There was no distinct performance difference between natural attenuation and biostimulation. Until the end of the experiment (12 days), about 50% and 40% NB remained in the sediment and water without bacterial augmentation.

The results affirmed that the added bacterial can grow quickly in the field condition, even at a low temperature of 10°C. As the external bacterial was initially isolated from the sediment and later inoculated in river water, the competition between external and indigenous cell could be alleviated to a minimum level, resulting in a quick remediation process. The persistence and effectiveness of the bacterial during bioremediation is anticipated. It can also be concluded that nutrients were not the limiting factors in the system since the sediment and water were taken from the polluted Songhua River. However, it is necessary to investigate the additional limiting factor with further study.

The bacterial abundance as estimated by MPN was highly variable, resulting in high standard deviations in each microcosm and at each time of sample collection (Fig. 3). The initial culturable NB degrading microbial population were low (between $3 \times 10^3$ g dry soil$^{-1}$ and $9 \times 10^3$ g dry soil$^{-1}$). With the bacterial augmentation, there was an exponential increase in bacterial number for the first 4 days of bioremediation. The culturable bacterial population then remained relatively stable at $5 \times 10^8$ g dry soil$^{-1}$, with no statistical significant difference in the

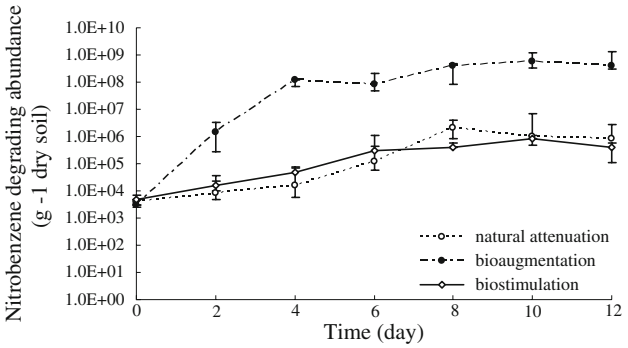


**Fig. 3** Nitrobenzene degrading bacterial abundance during bioremediation

microbial numbers. Without bacterial addition, there was also a slow increase in bacterial numbers for the 12 days of remediation. We attribute the increases largely to the growth of indigenous bacterial. Actually there were about seven different bacteria existing in the polluted sediment (Li et al. 2007). Although the completion of NB degradation could finally be anticipated even without bacterial addition, bioaugmentation is still necessary for a rapid bioremediation procedure.

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