Feasibility study of in-situ bioremediation for nitrobenzene-contaminated groundwater

Na Liu, Yue Wang, Yonglei An, Feng Ding, Xiaolong Yu and Kang Ye

ABSTRACT

Although many studies have simulated in-situ bioremediation of contaminated groundwater, most of them have not considered hydrochemical conditions and indigenous microorganisms, thus potentially rendering results inapplicable to actual in-situ groundwater bioremediation projects. This study focused on a nitrobenzene-contaminated groundwater site located in Jilin City, China. The actual nitrobenzene-contaminated groundwater was taken from Jilin City to simulate in-situ groundwater bioremediation in the laboratory. The feasibility of in-situ bioremediation for nitrobenzene-contaminated groundwater was studied according to actual site conditions and characteristics of nitrobenzene-degrading microorganisms in groundwater. The results showed that nitrobenzene-degrading bacterium strain NB1 was the dominant species that could effectively and rapidly degrade nitrobenzene by a partial reductive pathway. No negative factors on the growth or degrading function of this strain in groundwater could be detected. During a laboratory simulation experiment, combined in-situ bioremediation technologies, namely air sparging and bioaugmentation, could readily remove approximately 89.56% of nitrobenzene from groundwater without adding nutrients; oxygen was found to be the important growth factor for strain NB1. As the substrate of nitroreductase, encoded by the nitrobenzene nitroreductase (*nbzA*) gene, nitrobenzene was likely to significantly affect the expression of this gene. In conclusion, in-situ bioremediation is a feasible way to solve the problem of nitrobenzene-contaminated groundwater in Jilin City as long as sufficient oxygen and biomass of strain NB1 is ensured.

Key words | gene, groundwater, in-situ bioremediation, nitrobenzene

Na Liu
Yue Wang
Yonglei An (corresponding author)
Feng Ding
Xiaolong Yu
Kang Ye
Key Laboratory of Groundwater Resources and Environment,
Ministry of Education Jilin University, College of Environment and Resources, Jilin University,
Changchun 130021,
China
E-mail: *anyonglei85@163.com*

INTRODUCTION

Groundwater contamination by organic pollutants is a universal and serious problem (An *et al.* 2012a; Dong *et al.* 2015; Yu *et al.* 2015). In-situ bioremediation technology is recognized as the main remediation strategy for this issue as organic pollutants can be biodegraded completely without generating secondary pollution in groundwater (An *et al.* 2012b; Majone *et al.* 2015). Generally, a successful in-situ bioremediation requires the following: (1) the presence of pollutant-degrading microorganisms in contaminated groundwater and (2) the capacity of these microorganisms to degrade pollutants rapidly and effectively (Gianfreda & Nannipieri 2001).

Nitrobenzene is a common organic groundwater pollutant and can be degraded by microorganisms present in the environment (Liu *et al.* 2011, 2013; An *et al.* 2012c; Lee *et al.* 2014). Most of these microorganisms use a partial reductive pathway to degrade nitrobenzene under aerobic conditions (Li *et al.* 2008). Although many reports related to in-situ bioremediation of nitrobenzene-contaminated groundwater exist, feasibility studies considering actual site conditions are less common (An *et al.* 2012a, 2016; Liu *et al.* 2016). Many of these studies focusing on bioremediation were performed in laboratories. Groundwater was not sampled directly from the polluted site, but experimental

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groundwater was manually confected with organic pollutants, mineral salts, and distilled water (Park & Kim 2000; Sun *et al.* 2011). These studies did not consider hydrogeological conditions and chemistry and, furthermore, probably did not consider the feasibility of in-situ bioremediation before conducting laboratory simulation studies. Therefore, these results may be inapplicable to actual in-situ bioremediation of groundwater. Feasibility studies of in-situ groundwater bioremediation methods should be carried out before performing simulated studies under laboratory conditions. If in-situ bioremediation proved feasible, the results of the simulated study would have practical significance and theory value.

This study investigated the feasibility of in-situ bioremediation for nitrobenzene-contaminated groundwater in Jilin City, China. Three aims were formulated: (1) to determine the presence of nitrobenzene-degrading microorganisms in groundwater contaminated with nitrobenzene, (2) to determine whether these microorganisms could adapt to the groundwater conditions, and (3) to determine whether these microorganisms could grow in contaminated groundwater and effectively degrade nitrobenzene.

MATERIALS AND METHODS

Nitrobenzene-contaminated groundwater

The experimental groundwater was sampled from a nitrobenzene-contaminated site located in Jilin City, and the characteristics of the contaminated groundwater were described as follows: nitrobenzene, 40.40 mg/L; pH, 7.95; total organic carbon, 65.58 mg/L; total nitrogen, 16.88 mg/L; ammonia nitrogen, 11.91 mg/L; fluorinion, 0.32 mg/L; chloridion, 200.75 mg/L; dissolved oxygen, 0.2–0.5 mg/L; nitrite, 12.85 mg/L; nitrate, 1.56 mg/L; and sulfate, 565.84 mg/L.

Isolation and identification of nitrobenzene-degrading microorganisms

Nitrobenzene-degrading microorganisms were sourced from nitrobenzene-contaminated groundwater with liquid selective culture medium (nitrobenzene, 100 mg/L; KH_2PO_4, 1,000 mg/L; $Na_2HPO_4{\cdot}12H_2O$, 3,800 mg/L; $MgSO_4{\cdot}7H_2O$, 200 mg/L). The microorganisms were isolated via streak plate cultivation and identified by gram staining. Sequences of 16S rDNA and key nitrobenzene-degrading genes, as well as metabolites of nitrobenzene-N were also analyzed.

Inoculation of nitrobenzene-degrading microorganisms to actual groundwater

The isolated nitrobenzene-degrading microorganisms were inoculated into the sampled groundwater in three triangular flasks without additional nutrition and cultured at 10 °C (the average annual groundwater temperature in this contaminated site in northeast China). Variations in nitrobenzene concentrations in each triangular flask were monitored, and the relative expressions of key degrading genes were also quantified.

Simulation of in-situ bioremediation

The in-situ bioremediation experiments were performed in four similar simulated groundwater systems (Figure 1). To simulate actual nitrobenzene-contaminated groundwater conditions, medium sand was chosen as the experimental groundwater medium, and the permeability coefficient was approximately 50.5 m/d. The four simulated groundwater systems were labeled as S1, S2, S3, and S4. The velocity of groundwater flow was controlled at 0.1 m/d (similar to that of the actual nitrobenzene-contaminated groundwater). S1 was set as the control, i.e., with no treatment; S2 was treated with air sparging, and the aeration flux was 2 L/min; S3 was treated with bioaugmentation, with

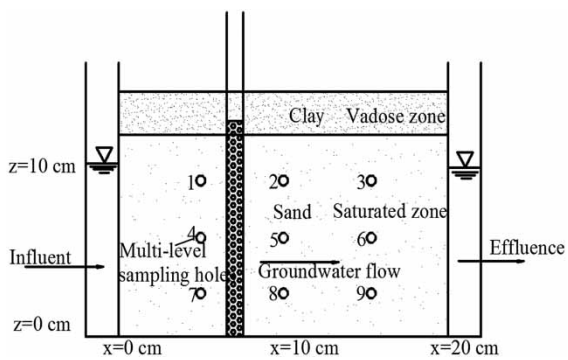

Figure 1 | Simulated groundwater system.

100 mL of isolated nitrobenzene-degrading microorganism suspension (1.32×10^{12} cfu/mL); and S4 was treated with a combination of air sparging and bioaugmentation. In other words, four kinds of technology were used to simulate the in-situ remediation of actual nitrobenzene-contaminated groundwater, involved natural attenuation of S1, air sparging of S2, bioaugmentation of S3, and combined air sparging and bioaugmentation of S4. During the experimental process, groundwater samples were collected from multi-level sampling holes and nitrobenzene concentrations were analyzed. At the conclusion of the experiment, the mixtures of groundwater and aquifer media in the center of each simulated groundwater system were collected to examine biological characteristics for three times, including the density of nitrobenzene-degrading microorganisms, dehydrogenase activity, biological diversity, and the relative abundance and expression of key nitrobenzene-degrading genes.

Test methods

The concentration of nitrobenzene in groundwater was quantified by high performance liquid chromatography (Liu *et al.* 2009). The 16S rDNA was amplified with universal primers F27/R1492 (Yang *et al.* 2010). The key degrading gene was amplified with primers of the nitrobenzene nitroreductase (*nbzA*) gene, which encodes nitroreductase (Kadiyala *et al.* 2003; Lee *et al.* 2014). The polymerase chain reaction (PCR) products of 16S rDNA and *nbzA* were sequenced by Sangon Biotech (Shanghai, China) and sequencing reactions were run on an ABI 3730 apparatus. The relative abundance and expression of key degrading genes were quantified by real-time fluorescence-based quantitative PCR (Lee *et al.* 2014). The metabolites of nitrobenzene-N were analyzed by Nessler's colorimetric method, and the density of nitrobenzene-degrading microorganisms was monitored by the plate counting method with selective solid culture medium. Microbial dehydrogenase activity was determined by the 2,3,5-triphenyltetrazolium chloride (TTC) method (Dun *et al.* 2008). Biological diversity was determined by PCR denaturing gradient gel electrophoresis (PCR-DGGE) technology and diversity indexes, including the Shannon–Weaver, Pielou, and Simpson indexes (Susumu & Makoto 2008; Ying *et al.* 2008; Moura *et al.* 2009).

RESULTS AND DISCUSSION

Isolation and identification of nitrobenzene-degrading microorganisms

One strain of nitrobenzene-degrading bacterium, NB1, was isolated successfully from the nitrobenzene-contaminated groundwater. Gram stain results showed that strain NB1 was Gram negative and rod shaped. Comparison of strain NB1's 16S rDNA sequence with that found in GenBank identified strain NB1 as *Pseudomonas putida* with a similarity of 99%. Most aerobic bacteria use a partial reductive pathway to mineralize nitrobenzene (Li *et al.* 2008), and the *nbzA* gene encoding nitroreductase was the key functional gene for the biodegradation of nitrobenzene. In the partial reductive pathway, nitrobenzene is catalyzed by nitroreductase, which is encoded by the *nbzA* gene, and generates phenylhydroxylamine (Li *et al.* 2008).

Therefore, strain NB1 was found to use a partial reductive pathway when degrading nitrobenzene, since the *nbzA* gene was detected successfully in this strain (Figure 2). The sequence of the *nbzA* gene was compared with that found in GenBank and the distance tree was constructed (Figure 3). It was clear that the *nbzA* gene amplified from strain NB1 encoded *nbzA* and the degree of similarity with *Pseudomonas* plasmid (pNB1) *nbzA* was 99% (Figure 3), confirming that strain NB1 used the partial reductive pathway.

In addition, two kinds of degradation pathways are generally selected by microorganisms by which to degrade nitrobenzene under aerobic conditions – one is the

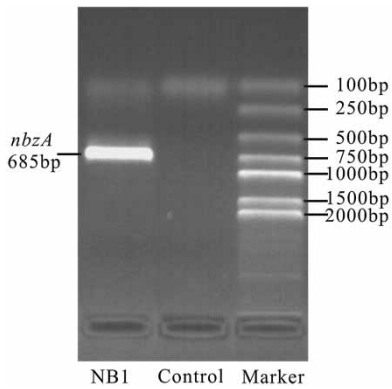

Figure 2 | Amplified results of the *nbzA* gene.

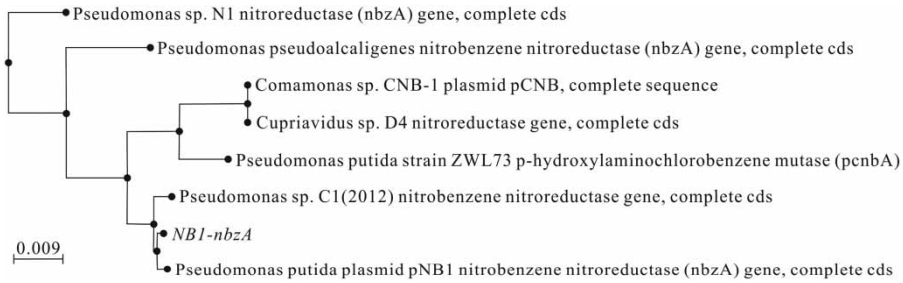

Figure 3 | Distance tree of the *nbzA* gene.

dioxygenase metabolic pathway with nitrite-N as the metabolite of nitrobenzene-N and the other is the partial reductive pathway with ammonia-N as the metabolite of nitrobenzene-N (Li *et al.* 2008). Consequently, the variation of ammonia in the nitrobenzene biodegrading system was monitored (Figure 4).

Figure 4 clearly illustrates that the concentration of ammonia in the culture medium increased gradually from 2.97 mg/L to 23.09 mg/L over time, which demonstrated that the nitrogen in nitrobenzene was transformed into ammonia-N in the nitrobenzene biodegrading system. Therefore, it could further be confirmed that strain NB1 used the partial reductive pathway and the metabolic rate of nitrogen obeyed the first-order kinetic mode (Figure 4).

The adaptability of strain NB1 in actual nitrobenzene-contaminated groundwater

Strain NB1 was inoculated into actual nitrobenzene-contaminated groundwater in a triangular bottle and simultaneously inoculated into the culture medium as the control. Variation of nitrobenzene concentration and relative expression of the *nbzA* gene were monitored to investigate the adaptability of strain NB1. The results showed that strain NB1 could effectively degrade nitrobenzene in groundwater from 300.00 mg/L to 0.27 mg/L in 144 h. The degradation rate constant (value of m1) of nitrobenzene in groundwater was significantly larger than that in the culture medium, and the degradation speed of nitrobenzene in groundwater was therefore faster than that in the culture medium (Figure 5). In addition, the result of real-time fluorescence-based quantitative PCR concurred with the nitrobenzene degradation speed in that the relative expression of the *nbzA* gene in groundwater was 9.46 times higher than that in the culture medium (Figure 6). These results convincingly demonstrated that strain NB1 could not only adapt to the groundwater environment, but also that some hydrochemical factors may have benefited strain NB1.

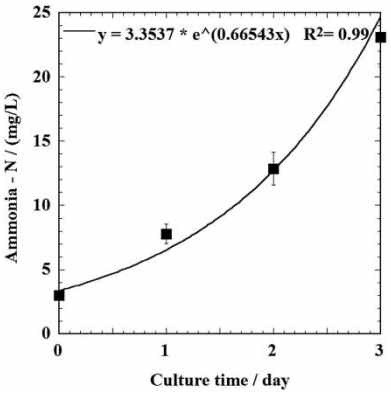

Figure 4 | Variation of ammonia in the nitrobenzene biodegrading system.

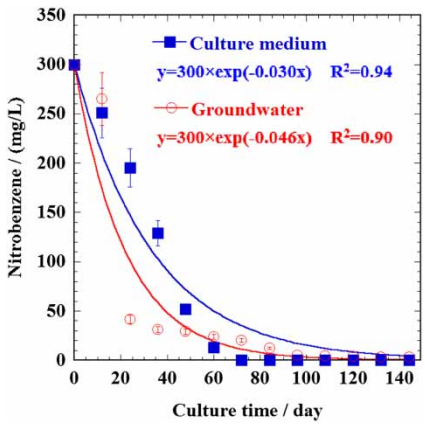

Figure 5 | Removal effects of nitrobenzene degraded by strain NB1 in culture medium and groundwater.

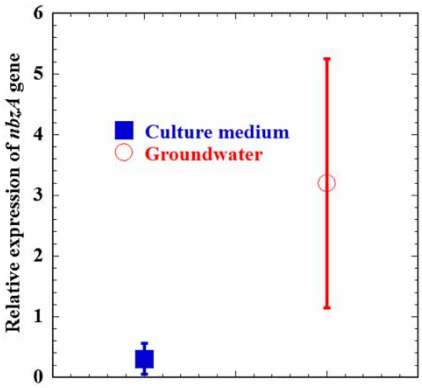

Figure 6 | Relative expression of the *nbzA* gene in culture medium and groundwater.

Simulation study of in-situ bioremediation for nitrobenzene-contaminated groundwater

The removal effects of nitrobenzene with different in-situ remediation technologies

The concentration of nitrobenzene in raw groundwater was 40.40 mg/L, and the concentration of nitrobenzene in effluent was 31.51 mg/L with natural attenuation treatment, 8.03 mg/L with air sparging treatment, 11.22 mg/L with bioaugmentation treatment, and 4.22 mg/L with a combination of air sparging and bioaugmentation treatments (Figure 7). These results initially demonstrated that the combination of air sparging and bioaugmentation technologies with strain NB1 was an effective and feasible in-situ bioremediation method for nitrobenzene-contaminated groundwater.

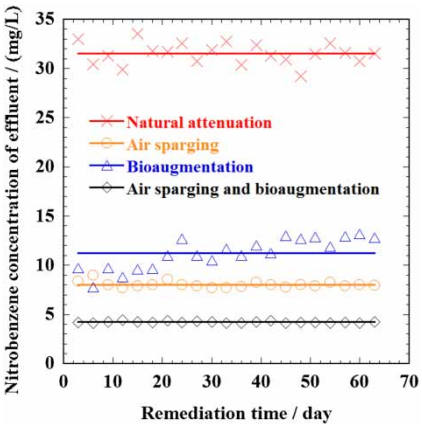

Figure 7 | Concentrations of nitrobenzene in simulated groundwater systems' effluent.

Density of strain NB1 in simulated groundwater systems

Density of functional microorganisms in groundwater is an important factor for successful in-situ bioremediation (Lee *et al.* 2014). The density of strain NB1 in raw nitrobenzene-contaminated groundwater was 2.9×10^5 cfu/mL (Figure 8). The application of air sparging or bioaugmentation increased the density of strain NB1 to a certain extent. However, the combined in-situ remediation technologies of air sparging and bioaugmentation increased the density of strain NB1 significantly from 2.9×10^5 cfu/mL to 4.2×10^5 cfu/mL. These results demonstrated that oxygen was a key factor for the growth of strain NB1 and that this nitrobenzene-degrading bacterium species could overgrow in contaminated groundwater.

Microbial dehydrogenase activities in simulated groundwater systems

Microbial activity was another important factor for in-situ bioremediation and most redox reactions in microorganisms are catalyzed by microbial dehydrogenases (Lee *et al.* 2014). The results showed that both air sparging and bioaugmentation enhanced the activity of microbial dehydrogenase. The combined technologies of air sparging and bioaugmentation improved this activity from 0.32 mcg/g/min to 0.81 mcg/g/min (Figure 9). Although microbial dehydrogenase activity was not solely caused by strain NB1, these results indirectly revealed the activity of functional bacteria.

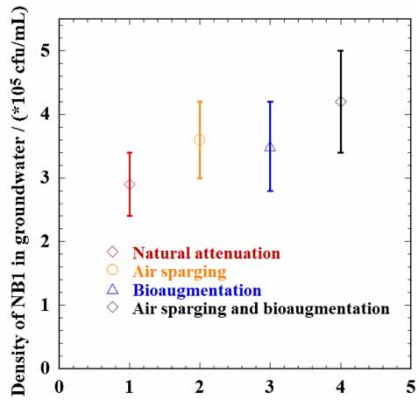

Figure 8 | Density of strain NB1 in simulated groundwater systems.

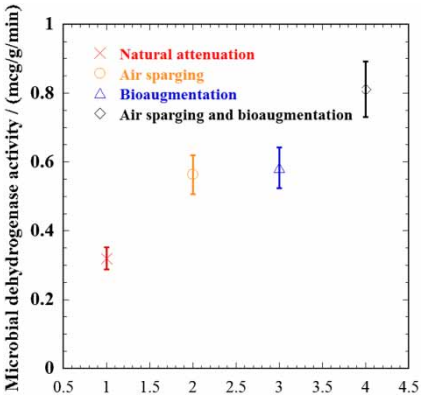

Figure 9 | Microbial dehydrogenase activity in simulated groundwater systems mcg/g/min = microgram TTC/gram groundwater medium/minute.

Changes in microbial community structure

PCR-DGGE was used to investigate changes in microbial community structure in groundwater (Figure 10). Strain NB1 was the dominant species in the raw groundwater. The band brightness of strain NB1 in S1 was relatively dark compared to that in S2, S3 and S4, which demonstrated that air sparging and bioaugmentation made strain NB1 more dominant in groundwater and benefited the in-situ bioremediation.

The results of ecological biodiversity indexes showed that a single air sparging treatment could enhance the microbial diversity significantly, but neither single bioaugmentation nor the combination of air sparging and bioaugmentation could significantly enhance microbial diversity (Table 1). One possible reason for this phenomenon could have been that effective removal of nitrobenzene with air sparging and bioaugmentation led to a lack of microbial growth substrate.

Figure 10 | PCR-DGGE profile of microorganisms in simulated groundwater systems. S1: natural attenuation; S2: air sparging; S3: bioaugmentation; S4: air sparging and bioaugmentation; NB1: strain NB1.

Relative abundance of the *nbzA* gene in simulated groundwater systems

Using natural attenuation as the control, the relative abundance of the *nbzA* gene in groundwater with air sparging and bioaugmentation was increased significantly (Figure 11). This demonstrated that the relative abundance of functional nitrobenzene-degrading strain NB1 was increased and that oxygen was a limiting factor for strain NB1.

Relative expression of the *nbzA* gene in simulated groundwater systems

Using natural attenuation conditions as the control, the relative expression of the *nbzA* gene in groundwater was most

Table 1 | Ecological indexes of microorganisms in nitrobenzene-contaminated groundwater

Sample	Band number	Shannon–Weiner index (H)	Pielou index (E)	Simpson index (D)
S1	15	2.651	0.979	0.075
S2	25	3.170	0.985	0.044
S3	15	2.651	0.979	0.076
S4	18	2.675	0.926	0.088

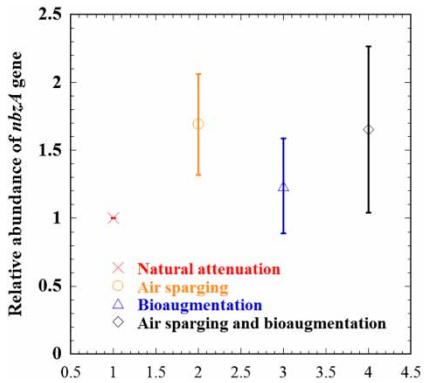

Figure 11 | Relative abundance of the *nbzA* gene in simulated groundwater systems.

significantly increased with air sparging, followed by bioaugmentation, and the combination of air sparging and bioaugmentation (Figure 12). The expression of the functional gene was mainly affected by growth conditions surrounding the cells. Nitrobenzene was the catalytic substrate of nitroreductase encoded by the *nbzA* gene. Therefore, the most probable reason for the low relative expression of the *nbzA* gene with the combination of air sparging and bioaugmentation treatments was that low concentrations of nitrobenzene inhibited the expression of the *nbzA* gene.

CONCLUSIONS

Nitrobenzene-degrading bacterium strain NB1 existed as the dominant indigenous microorganism species in nitrobenzene-contaminated groundwater in Jilin City. This strain could ingest nitrobenzene as the sole nutrition source of carbon and nitrogen under aerobic conditions. Strain NB1 could adapt to the hydrochemical conditions and low temperature (10 °C) of groundwater in this area. Combined in-situ bioremediation technologies of air sparging and bioaugmentation could remove approximately 89.56% of nitrobenzene from groundwater, with a small change in biodiversity. Oxygen was a key factor for the growth of strain NB1 and the induction of nitrobenzene determined the expression of the *nbzA* gene. Considering actual site conditions, in-situ bioremediation was found to be a feasible treatment for nitrobenzene-contaminated groundwater in the study area and the combined technology of air sparging and bioaugmentation was preferable.

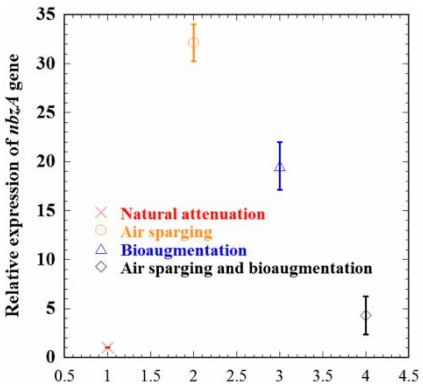

Figure 12 | Relative expression of the *nbzA* gene in simulated groundwater systems.

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