Front. Environ. Sci. Eng. 2018, 12(1): 11
https://doi.org/10.1007/s11783-018-1016-0

RESEARCH ARTICLE

The greater roles of indigenous microorganisms in removing nitrobenzene from sediment compared with the exogenous *Phragmites australis* and strain JS45

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HIGHLIGHTS

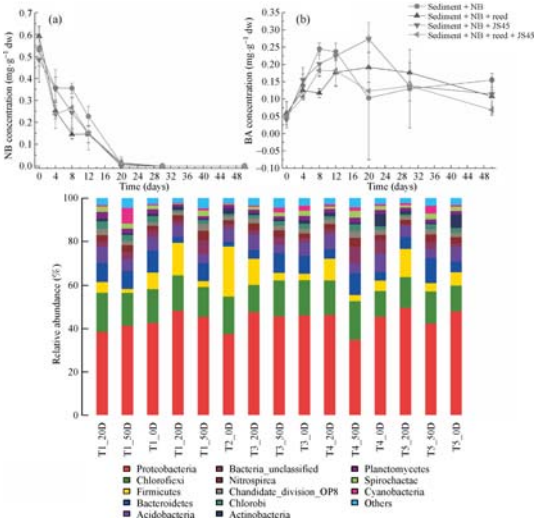
- Nitrobenzene degraded rapidly and was removed completely in native sediments.
- Indigenous microorganisms in native sediments are abundant.
- *Proteobacteria* and *Firmicutes* might play important roles in nitrobenzene removal.
- *P. australis* could provide a more suitable environment for *Thauera*.

ARTICLE INFO

Article history:
Received 19 June 2017
Revised 28 October 2017
Accepted 30 October 2017
Available online 4 December 2017

Keywords:
Community structure
Indigenous microorganisms
Nitrobenzene
Plant-microbe associated remediation
Sediment

GRAPHIC ABSTRACT



ABSTRACT

The feasibility of using *Phragmites australis*-JS45 system in removing nitrobenzene from sediments was conducted. However, it was observed that nitrobenzene degraded rapidly and was removed completely within 20 days in native sediments, raising the possibility that indigenous microorganisms may play important roles in nitrobenzene degradation. Consequently, this study aimed to verify this possibility and investigate the potential nitrobenzene degraders among indigenous microorganisms in sediments. The abundance of inoculated strain JS45 and indigenous bacteria in sediments was quantified using real-time polymerase chain reaction. Furthermore, community structure of the indigenous bacteria was analyzed through high throughput sequencing based on Illumina MiSeq platform. The results showed that indigenous bacteria in native sediments were abundant, approximately 10^{14} CFU/g dry weight, which is about six orders of magnitude higher than that in fertile soils. In addition, the levels of indigenous *Proteobacteria* (*Acinetobacter*, *Comamonadaceae_uncultured*, *Pseudomonas*, and *Thauera*) and *Firmicutes* (*Clostridium*, *Sporacetigenium*, *Fusibacter*, *Youngiibacter*, and *Trichococcus*) increased significantly during nitrobenzene removal. Their quantities sharply decreased after nitrobenzene was removed completely, except for *Pseudomonas* and *Thauera*. Based on the results, it can be concluded that indigenous microorganisms including *Proteobacteria* and *Firmicutes* can have great potential for removing nitrobenzene from sediments. Although *P. australis* - JS45 system was set up in an attempt to eliminate nitrobenzene from sediments, and the system did not meet the expectation. The findings still provide valuable information on enhancing nitrobenzene removal by optimizing the sediment conditions for better growth of indigenous *Proteobacteria* and *Firmicutes*.

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1 Introduction

Nitrobenzene (NB), one of the nitroaromatic compounds, is the primary starting material for the synthesis of aniline and the raw material for manufacturing explosives, pesticides, and drugs. Nitrobenzene degradation is difficult to achieve due to strong electron-withdrawing nature of its nitro group [1]. It can accumulate in human bodies via skin exposure, air inhalation, and oral contact [2]. It can harm major organs and can be teratogenic, mutagenic, and carcinogenic. Nitrobenzene is listed as a priority pollutant by United States Environmental Protection Agency (USEPA) [3]. In China, water bodies with serious nitrobenzene contamination have been reported [4]. In 2005, after a major spill of 10^5 kg of nitrobenzene and its related compounds into the Songhua River of China, the concentration of nitrobenzene in the river increased to 30 times more than the national standard (GB3838—2002, 0.017 mg/L). Nitrobenzene is insoluble and denser than water, is readily adsorbed to suspended particles, and can accumulate in the sediments. Secondary pollution can occur when it is released from sediments toward the overlying water through desorption, dissolution, and decomposition. The elimination of nitrobenzene from sediments is thus necessary to reduce risks to both human health and aquatic environments.

Biological remediation is considered as a promising option to mitigate the pollutions of organic matters, toxic heavy metals and so on [1,5,6]. It has been applied to remove nitrobenzene contamination from soil and aquatic environments. For example, *Phragmites australis,* a large perennial grass, can be utilized for phytoremediation of nitrobenzene contaminated environments [7]. Meanwhile, bioremediation of nitrobenzene contamination with *Pseudomonas spp.* have been reported [1,8,9]. However, both plants and microbes suffer some limitations (soil conditions, availability of nutrients, water, etc.) with respect to their individual competencies to remove pollutants [10]. Methods, such as immobilization, semiconductor quantum dots and plant-microbe associated remediation, can accelerate the removal of contaminants [5,10–12]. Among them, plant-microbe associated remediation, which refers to the removal of pollutants by possible synergy between plant roots and microbes, may benefit and enable both microorganisms and plants to achieve an enhanced remediation of soils contaminated with recalcitrant organic compounds or toxic heavy metals [10,13]. *Pseudomonas pseudoalcaligenes* JS45 was reported to have the ability to utilize nitrobenzene as the sole carbon and nitrogen sources with concomitant release of NH_4^+ [14]. Therefore, *P. australis*- JS45 associated remediation was constructed to study the effectiveness of acceleration of nitrobenzene removal.

In this study, a *P. australis*- JS45 associated remediation system was constructed to study the effectiveness of nitrobenzene removal from sediments. Meanwhile, the responses of microbial abundance and community structure in different microcosms were analyzed by quantitative real-time PCR and high-throughout sequencing based on the MiSeq platform, respectively.

2 Materials and methods

2.1 Plant, bacterial strain, and sediment samples

Phragmites australis (approximately 40–60 cm tall) with four to five leaves were used in this experiment. *Pseudomonas pseudoalcaligenes* JS45 was obtained from ATCC (Manassas, VA, USA, ATCC 700437) and maintained in lysogeny broth medium containing 100 μg/mL of ampicillin. Sediment samples were collected randomly at 0 to 20 cm depth in the middle of East Lake (Wuhan, Hubei, China) using Peterson grab sampler. Total nitrogen content of the sediment was determined to be 3.845±0.154 g/kg dw (dry weight) according to the previous report [15], and total phosphorus content was 1.602±0.121 g/kg dw (inorganic phosphorous: 0.357±0.061 g/kg dw; organic phosphorous: 1.382±0.152 g/kg dw) as measured previously [15]. The organic matter (OM) was 120.83±3.64 g/kg dw, the pH of the sediment was 7.31±0.03, and the water content was 4.35%±0.63%.

2.2 Remediation microcosms of nitrobenzene-contaminated sediments

Microcosms were set up using Perspex vessels (30 cm × 20 cm × 25 cm) containing 5 kg of sediment and 1 L tap water. Five different treatments were carried out: T1) native sediment; T2) native sediment with nitrobenzene; T3) native sediment with nitrobenzene and *P. australis*; T4) native sediment with nitrobenzene and strain JS45; T5) native sediment with nitrobenzene, *P. australis* and strain JS45. In addition, treatment Ts, which contained sterilized sediment (500 g) with nitrobenzene added post-sterilization (1 mg/g dw). All treatments were run in triplicate. Nitrobenzene and sediment were mixed by stirring the described mixtures for at least 1 h. The initial concentration of nitrobenzene was approximately 1 mg/g dw in each treatment, while *P. australis* density was 60 reeds/m^2. Plants were fixed in holes of polyvinyl chloride-board which was used to cover the vessels to prevent evaporation of the nitrobenzene. *P. australis* plants were acclimated to native sediment for one week before exposure to nitrobenzene. Strain JS45 grown to the late exponential phase was resuspended in sterile saline (0.85% (w/v)) and inoculated into bioaugmentation treatments T4 and T5 at a final concentration of 1.5 × 10^8 colony forming units (CFU)/g dw. The detailed treatment setting is shown in

Table 1 Treatment setup

Treatment No.	Contents	NB[a] concentration (mg/g dw)	*P. australis*[b] density (reeds/m^2)	Strain JS45 abundance (cfu/g dw)
T1	Native sediment	0	0	0
T2	Native sediment + NB	1	0	0
T3	Native sediment + NB + *P. australis*	1	60	0
T4	Native sediment + NB + Strain JS45	1	0	1.5×10^8
T5	Native sediment + NB + *P. australis* + strain JS45	1	60	1.5×10^8
Ts	Sterilized sediment + NB	1	0	0

Notes: a) NB: nitrobenzene; b) *P. australis*: *Phragmites australis*

Table 1. During the experimental operation, tap water was added regularly to maintain a constant depth. The entire experiment lasted 50 days and was conducted in a glasshouse where temperature was maintained in the range of 25°C–35°C.

2.3 Sediment sample collection for analysis

Sediment samples were collected within 2 cm from the rhizosphere in the surface-layer sediment by a scoop. The free water in the scoop was taken back into the corresponding vessel. The samples were collected at 0, 4, 8, 12, 20, 30 and 50 days for subsequent analyses.

2.4 Chemical analysis

Nitrobenzene and aniline (an intermediate of nitrobenzene degradation) were extracted from the sediment as described previously [1,16,17]. In detail, soil samples (0.50 g each) were thoroughly mixed with 1 mL methanol by shaking at 200 r/min for 30 min and then centrifuged at 10000 g for 10 min. The supernatant was filtered for nitrobenzene and aniline analyses using high performance liquid chromatography (HPLC). For HPLC, Agilent series 1200 system (Agilent Technologies, Palo Alto, CA, USA) with a C_{18} reversed-phase column (5 μm, 4.6 × 250 mm; Agilent Technologies, Palo Alto, CA, USA) was set at 30°C. The mobile phases consisted of 70% methanol and 30% water, the flow rate was set at 0.8 mL/min, and UV detection was set at 280 nm. The retention times for nitrobenzene and aniline were 5.7 min and 3.9 min, respectively. The concentrations of NH_4^+ [18], NO_2^- [19] and NO_3^- [20] were colorimetrically determined as described previously [21].

2.5 DNA extraction

DNA was extracted from 0.25 g sediment samples by using PowerSoil® Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). DNA samples were stored at −20°C for the subsequent experiments.

2.6 Quantitative real-time polymerase chain reaction (real-time PCR)

The *cnbA* gene encodes the enzyme responsible for the initial degradation of nitrobenzene in strain JS45. The quantitative real-time PCR of *cnbA* was used to quantify the abundance of strain JS45. Real-time PCR was performed on a CFX Connect™ Optics (Bio-Rad Laboratories, Hercules, CA, USA) using a 20 μL reaction mixture containing 10 μL 2 × *TransStart*® Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China), 0.5 μL template DNA, and 0.2 μmol/L of each primer (cnbA-RT-S2/ cnbA-RT-A2). PCR programs were 30 s incubation at 94°C followed by 45 cycles of 5 s at 94°C, 15 s at 60°C, and 10 s at 72°C. A melting curve measuring fluorescence continuously was then analyzed for the specificity of the PCR product after the temperature increased from 65°C to 95°C. The quantification of total bacterial 16S rRNA gene was performed as described previously [16,22,23]. The primers and probe used to quantify the *cnbA* gene and 16S rRNA gene are listed in Table 2.

2.7 Bacterial 16S rRNA gene sequencing and analysis

Days 0, 20 and 50 represent initial, nitrobenzene elimination and later stages of the different microcosms, respectively. DNA samples extracted in triplicate from each microcosm were mixed and used as the template for 16S rRNA gene amplification. The primers 515F (5′-barcode-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) were used to amplify the V4 hypervariable regions, which provided sufficient resolution for microbial sequence classification [24]. The barcode was an eight-base sequence unique to each sample. The bacteria 16S rRNA genes were amplified by PCR (95°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min). PCR reactions were performed in 20 μL volumes containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 μmol/L dNTPs, 0.8 μL of each primer (5 μmol/L), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

Table 2 Primers and probe used in this study

Primers	Target	Sequence (5′-3′)	Reference
cnbA-S	*cnbA*	ACCAGCCCGTTCATTGATG	this study
cnbA-A	*cnbA*	GGACGAAGGTGGTGAACTCT	this study
cnbA-RT-S2	*cnbA*	TTGCTATGGCTGCTTTCTCC	this study
cnbA-RT-A2	*cnbA*	CACTGCCAGGCTATTGTCG	this study
BACT1369F	16S rRNA gene	CGGTGAATACGTTCYCGG	[22]
PROK1541R	16S rRNA gene	AAGGAGGTGATCCRGCCGCA	[22]
TM1389F[a)]	16S rRNA gene	CTTGTACACACCGCCCGTC	[22]
F27	16S rRNA gene	AGAGTTTGATCMTGGCTCAG	[23]
R1492	16S rRNA gene	TACGGYTACCTTGTTACGACTT	[23]

Notes: a) Probe

Each sample was amplified three times. The resulting amplicons from triplicates were mixed and purified with an AxyPrepDNA kit (Axygen Biosciences, Union City, CA, USA). Following purification, the samples were quantified using the QuantiFluor™-ST System (Promega, USA). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2 × 250) on an Illumina MiSeq platform following the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP066935).

The raw data of sequences were analyzed and optimized using Trimmomatic and FLASH [25]. The reads were truncated at any site with an average quality score < 20 over a 50 bp sliding window, and all truncated reads below 50 bp were removed. Subsequently, quality sequences were assigned to samples based on the unique barcodes. Sequences that overlapped more than 10 bp were assembled according to their overlapping regions. Sequences were chimera-checked and clustered into OTUs with Usearch (version 7.1) where phylotypes were defined at the 97% sequence similarity level. The taxonomy of each 16S rRNA gene sequence was studied by RDP Classifier (http://rdp.cme.msu.edu/) against the silva (SSU115)16S rRNA database with confidence threshold level of 70%.

2.8 Statistical analysis

The statistical analysis of sequences was performed using various packages within the R statistical computing environment. Rarefaction curves were produced using Mothur [26]. Heatmap analysis was run using the Bray–Curtis dissimilarity index and the complete linkage method through the functions vegdist and hclust in the vegan statistical package. SPSS (IBM SPSS Statistics for Windows, Version 20.0; IBM Corp, Armonk, NY, USA) was used in the general linear model analysis of variance (ANOVA) followed by Tukey test to check for quantitative differences between different treatments. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Nitrobenzene removal and aniline accumulation

In the native sediment sample, nitrobenzene and aniline (an intermediate of nitrobenzene degradation) were not detected. The concentration variations of nitrobenzene and aniline in nitrobenzene-contaminated treatments are shown in Fig. 1. Added nitrobenzene was completely removed in nitrobenzene-contaminated treatments (T2, T3, T4 and T5) in the first 20 days. No obvious accelerated nitrobenzene removal was observed in T4 treatment (JS45 remediated sediment) compared to the T2 nitrobenzene-contaminated control ($P = 0.14$, $P>0.05$). The *P. australis*-JS45 associated remediation (T5 treatment) could not significantly promote the removal of nitrobenzene compared with T2 treatment ($P = 0.093$, $P>0.05$). Nitrobenzene disappearance rate in T5 was not significantly different from that in T3 treatment ($P = 0.946$, $P>0.05$) (Fig. 1(a)). Together, nitrobenzene disappearance rates were not significantly different in all bioremediation treatments, indicating that *P. australis* alone, JS45 alone, or *P. australis*- JS45 associated system had limited effects on the acceleration of nitrobenzene removal. In the nitrobenzene-contaminated sediment without any bioremediation, nitrobenzene decreased rapidly and was removed completely in the first 20 days. It is most likely that the indigenous microorganisms in sediment play a role in removing nitrobenzene from sediments.

In all nitrobenzene-contaminated treatments (T2–T5), the concentrations of aniline increased progressively in the process of nitrobenzene elimination (0–20 days) while nitrobenzene decreased gradually over the 20 days and retained a constant amount thereafter (Fig. 1(b)). The maximum molar ratio of nitrobenzene disappearance to the accumulation of aniline was approximately 1:0.5. In sterile treatment Ts (sterilized sediment with nitrobenzene), the reduction of nitrobenzene concentration was not accompanied by any accumulation of aniline (data not shown), suggesting that the decrease of nitrobenzene in all non-sterilized treatments (T2–T5) was not a result of

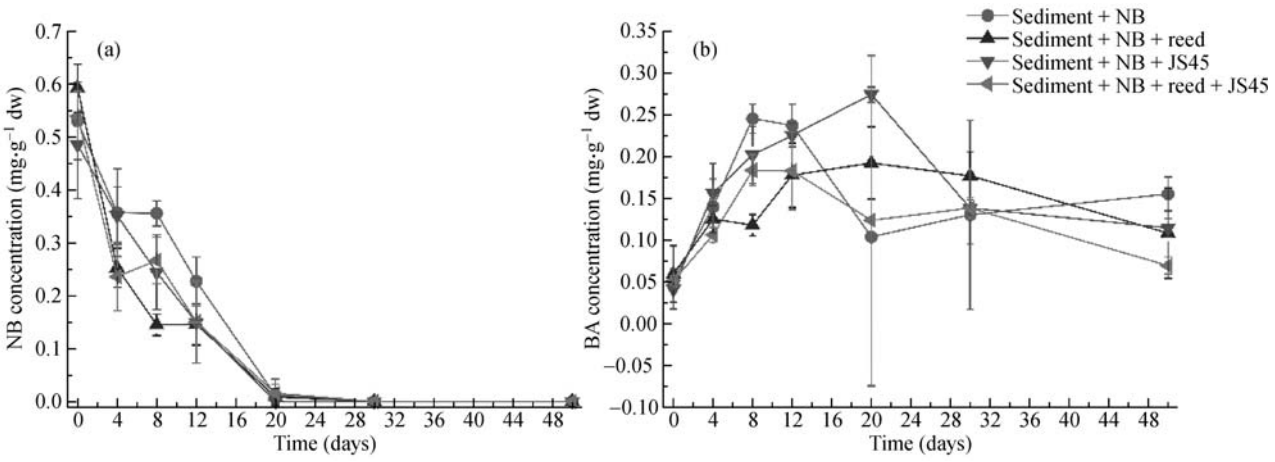

Fig. 1 Nitrobenzene removal and aniline accumulation among different microcosms. (a) Removal of nitrobenzene; (b) the variations of aniline concentrations. Values are means±SD (error bars) (n = 3) (NB: nitrobenzene; BA: aniline)

volatilization. The concentrations of aniline among different remediation treatments did not show remarkable variations during the full experimental period (P = 0.153, P>0.05), further confirming that nitrobenzene removal rates showed no significant difference among different microcosms.

NH_4^+ is released via nitrobenzene reduction by strain JS45 [14]. Consequent changes of NO_2^- and NO_3^- concentration may also occur. Therefore, concentrations of NH_4^+, NO_2^- and NO_3^- were monitored in treatment T4 (strain JS45 remediation) and other treatment groups (Supplementary Fig. S1). The results showed that there were no significant changes in NH_4^+ concentration among different treatments during the reduction process of nitrobenzene.

3.2 Inoculated strain JS45 abundance variation in bioaugmentation treatments

To examine whether JS45 system could be applied for remediation of nitrobenzene contaminated sediment, the abundance of inoculated strain JS45 was monitored by quantitative real-time PCR targeting the *cnbA* gene, which encodes the enzyme responsible for the initial degradation of nitrobenzene in this strain. Quantitative real-time PCR with *cnbA* gene primer showed that *cnbA* gene was not detected from the native sediment, indicating that *cnbA* gene is only in inoculated strain JS45 in the treatments. JS45 abundance was not significantly different between the treatments T4 (sediment + nitrobenzene + JS45) and T5 (sediment + nitrobenzene + *P. australis* + JS45) (P = 0.737, P>0.05) (Fig. 2). In the course of nitrobenzene disappearance (the first 20 days), the copy numbers of *cnbA* gene in both treatments T4 and T5 maintained at the same order of magnitude (approximately 10^7 CFU/g dw) as the initial level. After 20 days, when nitrobenzene was removed completely, the amount of *cnbA* gene in both treatments T4 and T5 declined sharply and then vanished on day 30 (Fig. 2). The results show that strain JS45 could not adapt to the complex sediment environment.

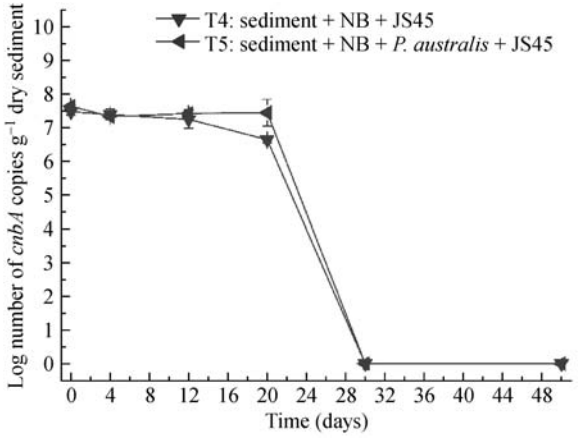

Fig. 2 *cnbA* gene abundance variation in inoculated microcosms. Values are means±SD (error bars) (n = 3) (NB: nitrobenzene)

3.3 Variation of total indigenous bacterial amount in each treatment

The total bacterial abundance in each treatment using real-time PCR targeting bacterial 16S rRNA gene was determined. The initial amount was approximately 10^{14} CFU/g dw in native sediment. During the experimental period, the changes to 16S rRNA gene copy numbers in each treatment did not exceed one order of magnitude (Supplementary Fig. S2), indicating that the contamination and bioremediation of nitrobenzene-contaminated sediment did not significantly influence the abundance of indigenous bacteria.

3.4 Reasonable quantities and depths of the 16S rRNA gene sequencing

Days 0, 20, and 50 represent the initial, nitrobenzene elimination and later stages of the different microcosms, respectively. The samples in those phases from all the treatments were sequenced. In each sample, there were more than ten thousands of reads with the average length of 396 bp. As indicated in Fig. 3, all rarefaction curves of phylotypes (defined at the 97% sequence similarity level) reached its plateau, indicating that both quantities and depths of these sequences were reasonable. The Shannon index of microbial diversity (Fig. 4) indicates that the most microbial diversity in each sample could be analyzed when all the curves had reached the plateau.

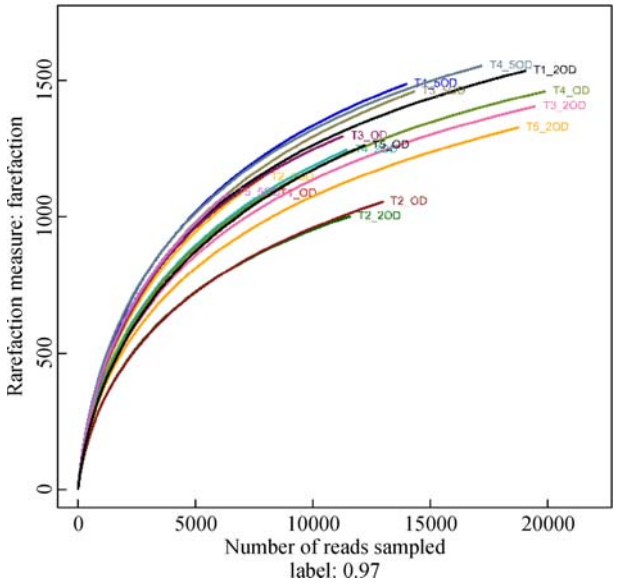

Fig. 3 Phylotype rarefaction curve of each sample (97% level)

Taxonomically annotated sequences of each sample were evaluated and Fig. 5 shows a barplot of the microbial community (phylum classification). Five categories of microorganisms (*Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Bacteroidetes*, and *Acidobacteria*) accounted for 80% of total microorganisms in all treatments, among which *Proteobacteria* had the highest abundance and occupied about 40% of the total. The abundance of *Chloroflexi* kept stable in the range of 12%–18% in each sample of different treatments, indicating that the abundance of *Chloroflexi* was not significantly influenced by both nitrobenzene contamination and time variations. On the contrary, the abundance of *Firmicutes* remarkably increased concomitantly with the elimination of nitrobenzene in the first 20 days and decreased once nitrobenzene was degraded in the nitrobenzene-contaminated treatments (T2–T5), indicating that nitrobenzene greatly affected the abundance of indigenous *Firmicutes* in sediments.

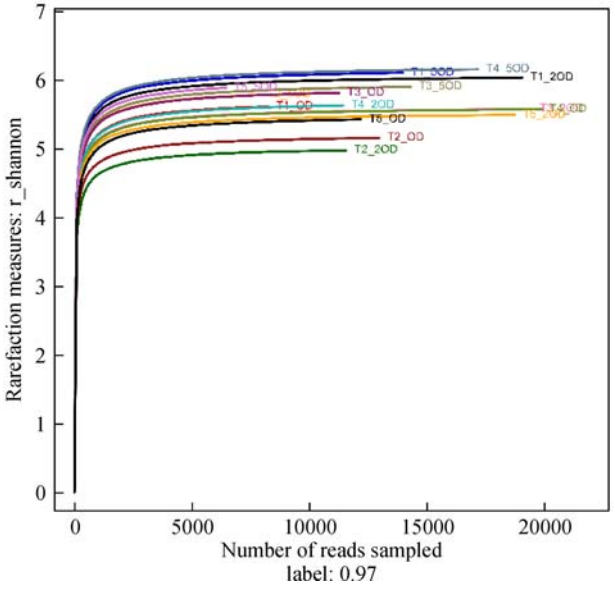

Fig. 4 Shannon index of microbial diversity in each sample

3.5 Similar microbial community structures in different treatments at the end of the experimental period

The principal component analysis of microbial community structure (Fig. 6) showed that the samples on day 50 in all treatments were closer to each other than those samples in other phases, indicating that the samples on day 50 in all treatments were more similar in microbial community structure. Conversely, no predictable patterns were observed in the distribution of these samples in day 0 and day 20. The microbial community structure and diversity of all samples tended to be similar in the absence of nitrobenzene and JS45, suggesting that the microbial communities were influenced by the involvement of nitrobenzene contamination or presence of JS45. Most of the *P. australis* plants remained alive during the experimental period, and are therefore unlikely to be the source of differences between microbial community structures.

3.6 Responses of indigenous microbial community in different remediation microcosms

To find indigenous microorganisms that are most likely responsible for removing nitrobenzene from sediments, a genus level heatmap analysis for the microbial community was performed (Fig. 7). The abundance of several genera, such as *Acinetobacter*, *Youngiibacter*, *Clostridium_sensu_stricto*_12, *Fusibacter*, *Sporacetigenium*, *Trichococcus*, and *Comamonadaceae_uncultured* varied greatly among different phases. In the initial phase (day 0), microorganisms from all genera were at very low levels. When nitrobenzene was added, quantity of microorganisms increased significantly, particularly for the genera

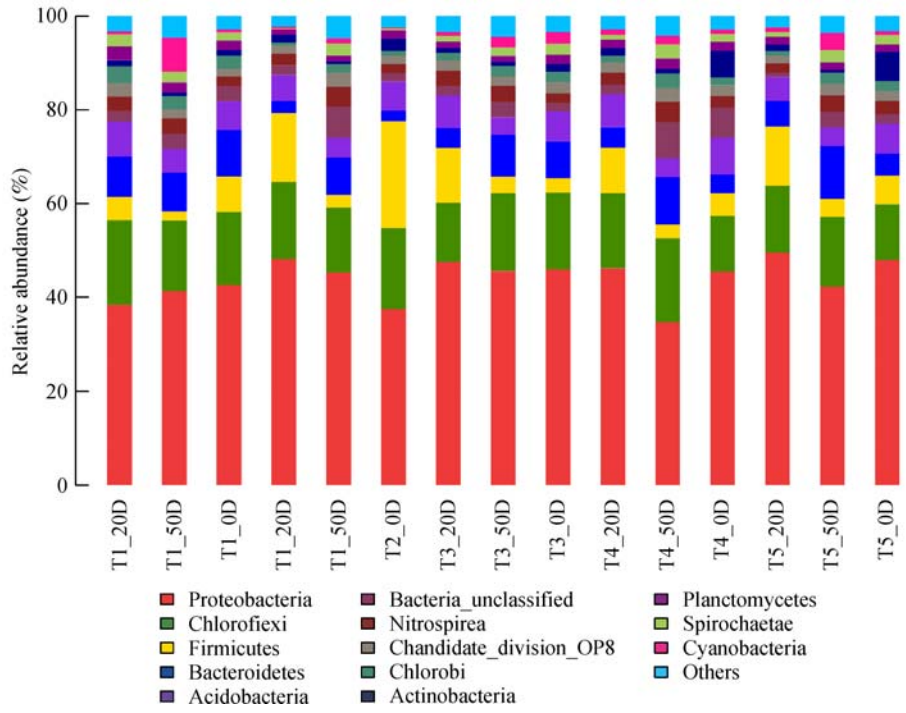

Fig. 5 Barplot of indigenous microbial community structure of samples at different times and in different microcosms

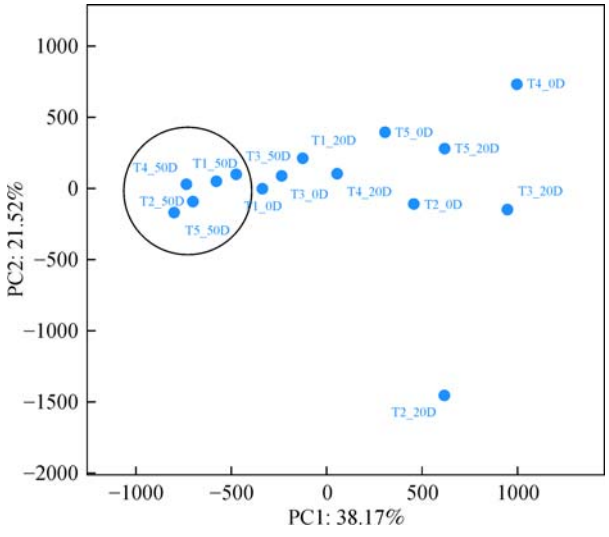

Fig. 6 PCA analysis of microbial community structures in each sample

of *Acinetobacter*, *Youngiibacter* and *Fusibacter*. After nitrobenzene was removed completely, the population of these genera decreased to the initial low levels (Fig. 7).

As shown in Fig. 7, although the genera of *Pseudomonas*, *Cloacibacterium*, and *Thauera* were undetectable in the initial stage, they accumulated rapidly with the addition of nitrobenzene. Subsequently, *Cloacibacterium* was no longer detected when nitrobenzene was removed completely on day 50. On the other hand, while the *Pseudomonas* population did decrease by day 50, its level on day 50 remained significantly higher than that of the initial stage. *Thauera* continued to grow even after the complete removal of nitrobenzene. These results indicate that *Pseudomonas* and *Thauera* can continue grow by utilizing local resources even after nitrobenzene were removed completely. Furthermore, on day 50, *Thauera* abundance in both the *P. australis* -JS45 associated remediation (T5) and *P. australis* remediation (T3) treatments were much more than those in the JS45 remediation treatment alone (T4). It is likely that *P. australis* can provide a more suitable environment for *Thauera* growth.

4 Discussion

This study showed that bioremediation treatments (*P. australis*-remediation, JS45-remediation and *P. australis*-JS45 associated remediation) have limited effects on the removal of nitrobenzene in sediments. However, it was previously reported that bioaugmentation of nitrobenzene-contaminated soil inoculated with exogenous strains can significantly decrease nitrobenzene [1,27]. The reason behind such differences is likely because microbes are far more abundant in sediments than in soil, as indicated by the fact that the 16S rRNA gene copy numbers in sediment is six orders of magnitude higher than that in soil (10^8 CFU/g dw) (Fig. 3).

Differences in microbial abundance can also partly explain why the JS45 strain did not have much effect on

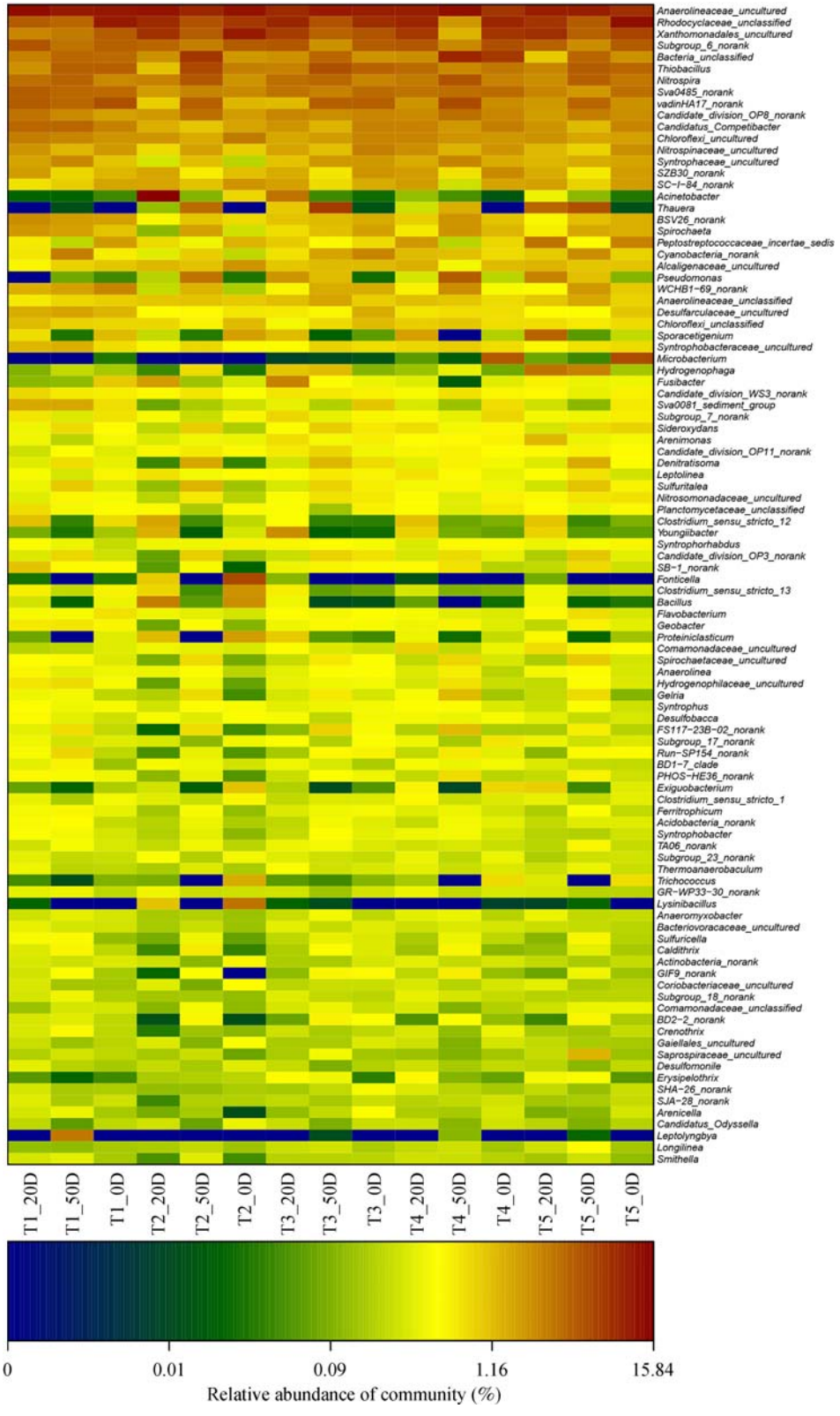

Fig. 7 Heatmap analysis of indigenous microbial community in each sample

nitrobenzene removal from contaminated sediments, as the level of inoculated JS45 was six orders of magnitude lower than that of indigenous microorganisms. Another possible reason is that the strain JS45 could not adapt to the complex microbial interactions in sediment, although it was able to degrade nitrobenzene in an anaerobic environment as described by the previous report [14]. Previous bioaugmentation of soils showed that exogenous microorganisms could survive by utilizing local resources after their preferred substrates had been degraded completely [1,27]. In the experimental process of this study, the disappearance JS45 strain, as measured by quantitative real-time PCR, indicated that the strain could not compete with the native microbial communities after nitrobenzene removal, and the integration and establishment of strain JS45 in the sediment microbial community could not be maintained.

All nitrobenzene in T2 (nitrobenzene-contaminated treatment) disappeared completely in the first 20 days. Furthermore, the accumulation of aniline (an intermediate of nitrobenzene degradation) was not observed in Ts (sterilized sediment with nitrobenzene) while its accumulation was detected in T2–T5 treatments (native sediments), indicating that the reduction of nitrobenzene in T2–T5 was not just a result of natural volatilization. This result, in addition to the limited effect of JS45 and *P. australis*, indicates that indigenous microorganisms in native sediment are most likely responsible for nitrobenzene disappearance.

Previous studies showed that indigenous microbes in natural soil environments have the potential to mitigate the harmful effects of xenobiotic substances [28]. For example, indigenous microbes mineralized 20% *para*-nitrophenol in everglades soils [28]. In various sediment-water samples, microbial communities could also degrade radiolabelled *para*-nitrophenol and the mineralization ratio ranged from 20% to 60% [29]. However, it should be noted that nitrobenzene elimination caused by indigenous microorganisms may not be a complete mineralization processes. Previous reports showed that in an anaerobic environment, nitrobenzene can be reduced to aniline, which may then be oxidized to catechol and then further degraded [30,31]. In this study, although the indigenous microorganisms in native sediment could eliminate nitrobenzene contamination, the concomitant generation of aniline indicates that the indigenous microorganisms can not mineralize nitrobenzene. Aniline is also an organic chemical compound. Aniline and its derivatives are suspected carcinogens and are highly toxic to aquatic life. In most circumstances, a secondary pollution may result from biostimulation with indigenous microorganisms alone. So it is still necessary to utilize bioaugmentation with inoculated microbes capable of mineralization for targeting contaminants.

The community structure and biodiversity data showed that *Firmicutes* changed most significantly with the addition and removal of nitrobenzene. It is possible that indigenous *Firmicutes* plays a critical role in degrading nitrobenzene in sediment. In addition, the genera showing great variations of abundance in different treatments (*Clostridium*, *Sporacetigenium*, *Fusibacter*, *Youngiibacter*, and *Trichococcus*) were all categorized within the phylum of *Firmicutes*. The abundance of these genera increased significantly during the process of nitrobenzene disappearance and decreased after nitrobenzene was eliminated. Therefore, it is most likely that the genera of *Clostridium*, *Sporacetigenium*, *Fusibacter*, *Youngiibacter*, and *Trichococcus* significantly accelerated the removal of nitrobenzene in sediments. Previous studies also showed that *Comamonadaceae* and *Firmicutes* had a great potential for degrading nitrobenzene, based on the changes of bacterial communities in sediments of Songhua River in China after a nitrobenzene contamination event [32].

In addition to *Firmicutes*, it is possible that *Proteobacteria* with the highest abundance in native sediment played an important role in degrading nitrobenzene. The genera of *Acinetobacter*, *Comamonadaceae_uncultured*, *Pseudomonas*, and *Thauera* are all categorized in the *Proteobacteria* phylum, and their levels increased with nitrobenzene disappearance. After nitrobenzene was removed completely, *Acinetobacter* and *Comamonadaceae* abundances decreased to initial low levels while *Pseudomonas* and *Thauera* continued to grow with the consumption of local resources. In fact, all four kinds of microbes had been reported to have a great potential of degrading nitrobenzene. An *Acinetobacter*, which was screened from sewer sediment and activated sludge of waste water treatment system in a chemical factory, was regarded as an effective nitrobenzene degrading strain [33]. *Comamonas* sp. strain CNB-1 (belonging to *Comamonadaceae*) was applied for remediation of nitroaromatic pollutants [34]. *Pseudomonas putida* ZWL73 was proven to have the ability to grow using nitrobenzene as the sole carbon, nitrogen, and energy sources [35]. Furthermore, successful bioaugmentations with inoculated strain ZWL73 had been achieved to eliminate nitrobenzene contamination in soils [1,27]. In *Thauera* microbes, MetaCyc database indicated that they may possess metabolic pathway to degrade nitrobenzene. Since *Pseudomonas* and *Thauera* have the potential to degrade nitrobenzene and are able to survive using local resources, bioremediation with *Pseudomonas* or *Thauera* may be a good choice for degrading the corresponding contaminant. Moreover, after the complete removal of nitrobenzene, *Thauera* level was remarkably higher in treatments containing *P. australis* (T3 and T5) than those treatments without *P. australis* (T2 and T4). The existence of *P. australis* likely provided a more suitable environment for the survival of *Thauera*. The relationship between *P. australis* and *Thauera* growth deserves further exploration, and *P. australis*-*Thauera* associated remediation should be considered to eliminate recalcitrant pollutants in contaminated environments.

5 Conclusions

There was no evidence to indicate the use of *P. australis* and strain JS45 in the elimination of NB was effective, raising the possibility that indigenous microorganisms play important roles in degrading nitrobenzene in sediments. The results of this study support this possibility because of the findings of the abundant types of potential nitrobenzene degraders among indigenous microorganisms in sediments. Biostimulation of sediment may be a better choice to achieve an enhanced remediation of recalcitrant nitroaromatic compounds. However, since this study was focus on at a particular site under particular conditions, and further studies in other sites should be conducted. In addition, quantitative PCR conduction or other quantitative analyses may be further needed to identify the important groups as the key players in NB degradation.

Acknowledgements This work is supported by the National Natural Science Foundation of China (Grant Nos. 51408593 and 51578538); The National Key Research and Development Program of China (2016YFC0503601); the China Postdoctoral Science Foundation (2013M531769), and Major projects of science and Technology Department of Zhejiang Province (2015C32011). We thank Professor Ningyi Zhou for his help in the nitrobenzene detection by HPLC. We thank Mr. James Wang from United States Environmental Protection Agency for his great help in polishing the English writing of this manuscript.

Conflict of Interest Statement The authors declare that they have no conflict of interest and financial disclosures.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s11783-018-1016-0 and is accessible for authorized users.

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