RESEARCH ARTICLE

Effects of nitrobenzene contamination and of bioaugmentation on nitrification and ammonia-oxidizing bacteria in soil

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Received 7 April 2009; revised 1 August 2009; accepted 5 August 2009.
Final version published online 23 September 2009.

DOI:10.1111/j.1574-6941.2009.00773.x

Editor: James I. Prosser

Keywords
ammonia-oxidizing bacteria; bioaugmentation; denaturing gradient gel electrophoresis; multivariate analyses; nitrobenzene; *Pseudomonas putida* ZWL73.

Abstract

Bioaugmentation of nitrobenzene-contaminated soil was performed by inoculation with *Pseudomonas putida* ZWL73, which can grow on nitrobenzene as carbon and nitrogen sources and release free ammonium from the aromatic ring via a partial-reductive pathway. Removal of nitrobenzene was effectively enhanced with concurrent accumulation of ammonium in the bioaugmented soil. Moreover, the negative impact of nitrobenzene contamination on culturable bacterial types and soil nitrification was reduced by strain ZWL73. Changes in the community structure of ammonia-oxidizing bacteria, determined by denaturing gradient gel electrophoresis, were associated with changes in environmental factors in nitrobenzene-contaminated soil, including concentrations of nitrobenzene, ammonium, nitrite and nitrate, but their influence was attenuated in the bioaugmented soil. Overall, *P. putida* ZWL73 shows promising abilities for effective removal of nitrobenzene and for attenuating the negative effects of nitrobenzene contamination on soil functioning.

Introduction

The release of nitroaromatic compounds into the environment either intentionally (pesticides and herbicides) or through accidental spills has caused significant environmental damage (Parales, 2000). Their stability, persistence and toxicity make nitroaromatic compounds hazardous when released into the environment (Nishino *et al*., 2000). As one of the nitroaromatic compounds, nitrobenzene, which is usually used as the primary starting material of aniline production, has great pollution potential in the environment (Nishino & Spain, 1993). In particular, nitrobenzene is extremely recalcitrant to enzymatic attack due to the stability of the benzene ring caused by the electron-withdrawing nature of the nitro group. On November 13, 2005, the Songhua River in the north-east of China suffered serious contamination of > 100 tons of nitrobenzene and related compounds, which were accidentally released due to an explosion at an aniline production factory. The fact that this contamination had serious impacts on the life of millions of habitants along the river has been internationally recognized (World Health Organization Department for Health Action, 2005). Removal of nitrobenzene contamination in an economical and effective way is thus of main concern for human health.

Bioaugmentation has now become an accepted technology for biodegradation of persistent or toxic organic pollutants (Gentry *et al*., 2004; Chen *et al*., 2005). A number of successful bioaugmentations have been reported for the removal of such pollutants, including polycyclic aromatic hydrocarbons (Cunliffe & Kertesz, 2006) and 4-chlorophenol in soil (Elväng *et al*., 2001), 3-chloroaniline in active sludge (Boon *et al*., 2000) and nonylphenol in bioreactors (Chang *et al*., 2005). Although a combined selective adsorption and bioaugmentation approach was reported to treat artificial wastewater containing nitrobenzene and *p*-nitrophenol on a laboratory scale (Hu *et al*., 2008), no bioaugmentation of nitrobenzene-contaminated soil has so far been reported.

Pseudomonas putida ZWL73, isolated from 4-chloronitrobenzene (4CNB)-contaminated soil, can grow on 4CNB or nitrobenzene as both carbon and nitrogen sources and release free ammonium from an aromatic ring via a partial-reductive pathway of 4CNB or nitrobenzene in a culture

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medium (Xiao *et al.*, 2006; Zhen *et al.*, 2006). In a previous study, bioaugmentation by strain ZWL73 against 4CNB proved to be feasible at the laboratory scale (Niu *et al.*, 2009). During the process, some bacterial populations were stimulated and became active, whereas the effects of 4CNB on soil indigenous bacteria were neutral. However, soil diversity and complexity, and other environmental factors that often make the results of bioaugmentation unpredictable, were not investigated.

The current study therefore evaluated the ability of strain ZWL73 to degrade other aromatic pollutants (nitrobenzene) in the soil environment and further analyzed the shifts in ammonia-oxidizing bacteria (AOB) community structure as a function of all the environmental parameters measured. During the process of nitrobenzene bioaugmentation, we aimed to investigate nitrification and the relationships between the AOB community and environmental factors in artificially nitrobenzene-contaminated and in bioaugmented soil, respectively, using multivariate analysis rather than visual analysis and cluster analysis of denaturing gradient gel electrophoresis (DGGE) profiles, as in the previous study.

Materials and methods

Setup of microcosms

The soil used in this study was collected from a noncontaminated area (0–15 cm depth) in Wuhan Botanical Garden, Chinese Academy of Sciences (Wuhan, China). Thereafter, the soil was sieved (2 mm) and stored at 4 °C. The soil type was a sandy loam [total carbon content 1.40%, total nitrogen 1.59 $mg\,g^{-1}$ dry soil (ds) and pH 6.1].

Microcosms were set up with 77.2 g of wet soil (equivalent to 70 g ds) in 250-mL glass bottles with tightened screw caps. There were three different treatments: Tc, natural soil (control); T −, natural soil artificially contaminated with nitrobenzene (contamination); and T+, natural soil contaminated with nitrobenzene and inoculated with strain ZWL73 (bioaugmentation). All the treatments were conducted in triplicate. Microcosms were equilibrated at 30 °C for 72 h before use. Contaminated treatments were prepared by thoroughly mixing nitrobenzene with soil at a final concentration of 0.4 $mg\,g^{-1}$ ds. When grown to the late exponential phase in Luria–Bertani (LB) medium at 30 °C, *P. putida* ZWL73 was collected with sterile saline (0.85% w/v) and inoculated into the bioaugmented T+ at approximately 4×10^8 CFU g^{-1} ds. In parallel, the same volumes of sterile saline were applied to Tc and T −. Finally, the moisture content of all three treatments was adjusted to approximately 20% w/v with distilled water. After vigorous stirring, bottles sealed with screw caps were kept in the dark at 30 °C and sampled for analysis.

Chemical analysis

To extract nitrobenzene, 0.5 g soil sample was mixed with 1 mL methanol by shaking at 200 r.p.m. for 30 min and centrifuged for 10 min at 10 000 ***g***. The resulting supernatant was analyzed using HPLC (Gilson, Villiers le Bel, France), which was performed at the column temperature of 30 °C with a Gilson 715 system equipped with a Gilson 119 UV/VIS detector and a C18 reversed-phase column (25 cm × 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA). The mobile phase was 70% methanol at a flow rate of 0.8 $mL\,min^{-1}$. Nitrobenzene was quantified at 280 nm. Under these conditions, authentic nitrobenzene had a retention time of 5.9 min. The concentrations of NH_4^+-N and NO_2^--N were determined colorimetrically according to published methods (Nkonge & Ballance, 1982; Kandeler, 2002, respectively). The NO_3^--N concentration was measured spectrophotometrically using dual-wavelength UV as described previously (Norman *et al.*, 1985).

Enumeration of *P. putida* ZWL73 and cultivable bacteria in soil

Suspensions of 0.2 g of soil sample in 1.8 mL sterile extracting solution ($Na_4P_2O_7$, 2 $g\,L^{-1}$) (Cavalca *et al.*, 2002), which was used to dissolve soil particles and to maintain ion concentration balance and cell viability until plating, were serially 10-fold diluted in sterile saline and 0.1 mL from appropriate dilutions was subsequently plated. All CFU numbers of culturable heterotrophic bacteria from the different treatments were counted on nutrient agar (NA) plates amended with the fungicide nystatin (25 $g\,mL^{-1}$). Colony types were distinguished by their shape, size and color. CFU abundance of the naturally antibiotic-resistant *P. putida* ZWL73 in T+ was determined on LB agar plates supplemented with nystatin (25 $g\,mL^{-1}$), ampicillin (100 $g\,mL^{-1}$) and chloramphenicol (34 $g\,mL^{-1}$).

DNA extraction and PCR amplification

Microcosms were sampled at 0, 6 and 20 days for DNA extraction. Total DNA from soil was extracted using the UltraClean Soil DNA kit (Mo Bio Labs, Solana Beach, CA) according to the manufacturer's instruction and thereafter 10-fold diluted as a template for PCR.

PCR amplification of the 16S rRNA gene fragments of AOB was performed as described previously (Mahmood *et al.*, 2006). The first round of PCR amplification was performed with universal primers F27 and R1492 (Lane, 1991). Then, the products of the first round of PCR were used as a template for secondary PCR amplification with primers CTO189AB–CTO189C and CTO654R specific for AOB (Kowalchuk *et al.*, 1997). The final amplification

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products, obtained from further amplification with universal 357f-GC-518r primers (Muyzer *et al.*, 1993), were analyzed by DGGE. Thermocycling conditions were as described (Mahmood *et al.*, 2006).

DGGE analyses

DGGE analyses were performed according to a published method (Muyzer *et al.*, 1993) using the Dcode System (Bio-Rad Labs, Hercules, CA). The gel consisted of an 8% polyacrylamide with a gradient of 45–65% of denaturant. Electrophoresis was carried out with $1\times$ TAE buffer at 60 °C at a constant voltage of 65 V for 16 h. Gels were stained with silver nitrate based on a published method (McCaig *et al.*, 2001).

Multivariate analyses

DGGE images were converted to form a 'species' table (i.e. relative abundance of DGGE bands in the different samples) in the QUANTITY ONE software version 4.6 (Bio-Rad Labs). Redundancy analysis (RDA) was used to estimate the extent of the correlation between the AOB community (i.e. DGGE bands) and the environmental factors measured using the software program CANOCO (version 4.15). The total variance of the species table was partitioned into the respective contribution of each set of environmental variables and into their covariations using both standard and partially constrained ordinations using the variation partitioning approach (Ramette, 2007). The environmental variables were divided into two sets: (1) time variables (time as a quantitative variable) and (2) treatment variables (all the other variables, including nitrobenzene, NH_4^+, NO_2^-, NO_3^- and strain ZWL73, as quantitative variables; Tc, T− and T+ as nominal variables). The total variance of species composition was investigated by standard RDA as a function of the two sets of environmental variables taken as explanatory variables. Thereafter, the variance explained by each variable set (i.e. time or treatment variables) was assessed by partial RDA (pRDA) by taking the other set of environmental variables as covariables. Monte Carlo permutation tests were used to test for the significance of the models with 499 unrestricted permutations at the 5% level. Both species and environmental data were log-transformed for these analyses.

Recovery and sequencing of DNA from DGGE bands

Selected bands on the DGGE gel were excised as described previously (Schwieger & Tebbe, 1998). The presence of products from recovered DNA using primers 357f-GC-518r was confirmed by agarose gel electrophoresis and products were then ligated into pGEM-T vector (Promega, Madison, WI) before being transformed into *Escherichia coli* DH5. Positive colonies were selected randomly and their inserts were amplified and screened to determine their migration positions by DGGE. Clones with inserts migrating to the same positions as the original DGGE bands were sequenced (Invitrogen, Shanghai, China).

Sequence and phylogenetic analyses

The sequences of the DGGE bands determined in this study were investigated for homology and closest relatives in GenBank using the BLASTN search tool (http://www.ncbi.nlm.nih.gov). All sequences have been deposited in the GenBank database under accession numbers FJ875113–FJ875116. To construct a cladogram, the sequences determined in this study were aligned with published 16S rRNA gene sequences (Purkhold *et al.*, 2000) with CLUSTALX (version 2.0.11.). A neighbor-joining tree was created using Kimura two-parameter, and cluster stability was assessed by bootstrap analyses based on 1000 replicates with MEGA [version 4.1 (Beta 2)].

Results

Nitrobenzene removal

Nitrobenzene removal from bioaugmented and nonbioaugmented soils as determined by HPLC measurements of nitrobenzene concentrations was clearly different (Fig. 1a). A large variation occurred in the initial nitrobenzene concentration because it was difficult to mix small volumes of liquid nitrobenzene thoroughly and uniformly, an issue common to many bioaugmentation studies in soil (Wang *et al.*, 2004; Gomes *et al.*, 2005). Therefore, in the first 2 days, the difference between T− and T+ was not very evident, but the nitrobenzene concentration of T+ gradually decreased from 0.4 to $6.8\pm6.03\times10^{-4}$ mg g^{-1} from day 2 on. In contrast, the nitrobenzene concentration in T− was still 0.24 ± 0.034 mg g^{-1} ds on day 20 in the absence of strain ZWL73.

As ammonium was released via the partial-reductive pathway of nitrobenzene degradation by strain ZWL73, the NH_4^+-N concentration was also determined during the 20-day period (Fig. 1b). The NH_4^+-N concentration in bioaugmented T+ increased to 183 ± 17.4 μg g^{-1} ds in the first 2 days, to 211 ± 8.8 μg g^{-1} ds on day 6 and subsequently showed no significant change. In contaminated T−, the NH_4^+-N concentration gradually increased and finally reached 95.5 ± 3.9 μg g^{-1} ds on day 20. In control Tc, the amount of NH_4^+-N appeared to be constant and low without apparent changes in 20 days.

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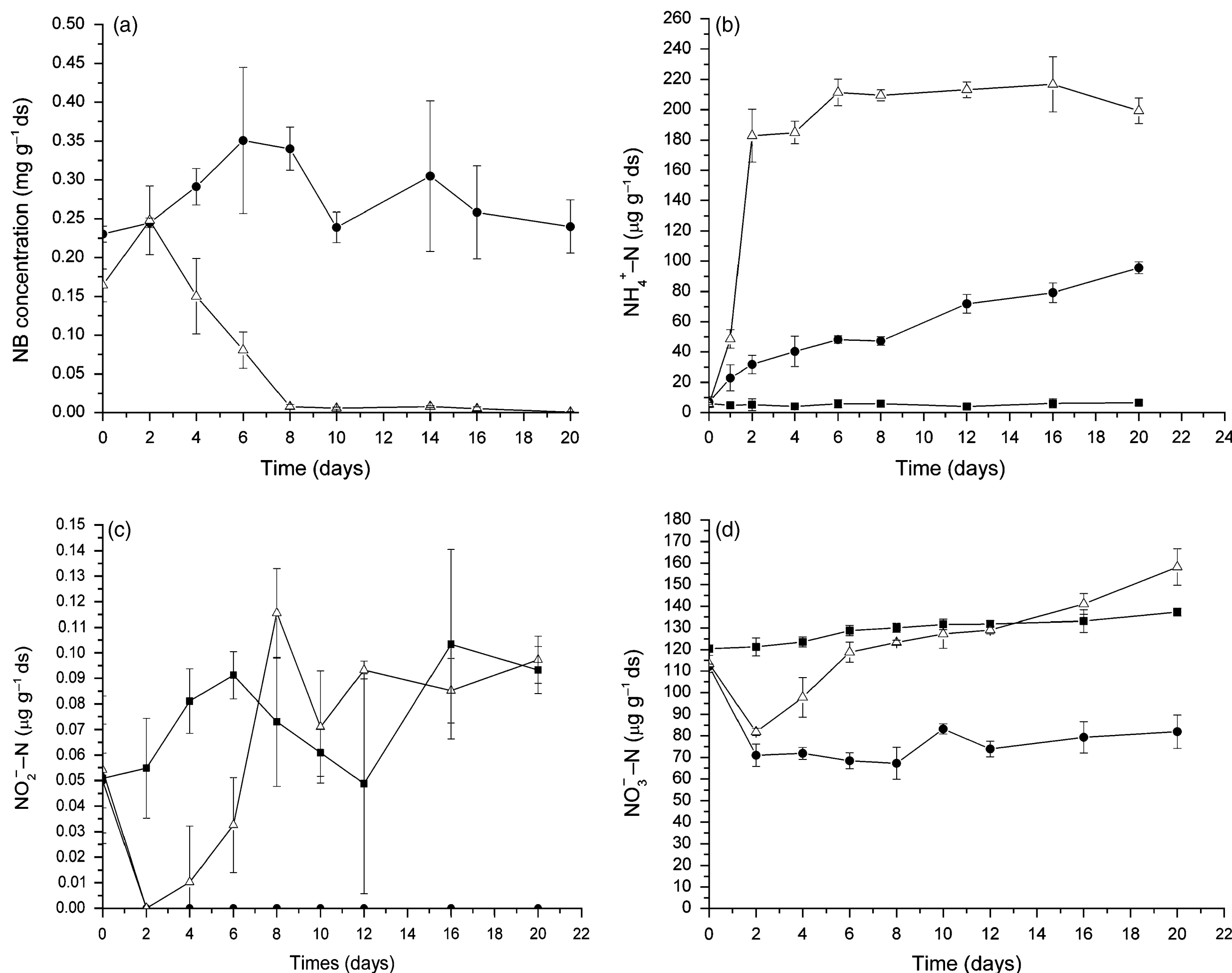

Fig. 1. Results of chemical analysis. (a) Removal of nitrobenzene in contaminated T− (•) and bioaugmented T+ (△) soils. Variation in the concentrations of NH_4^+-N (b), NO_2^--N (c) and NO_3^--N (d) in control Tc (■), contaminated T− (•) and bioaugmented T+ (△). Means of triplicates are shown with SDs (error bars).

Survival of strain ZWL73 in nitrobenzene-contaminated soil and of cultivable bacteria in all treatments

During bioaugmentation in T+, survival of strain ZWL73 was detected by plate counting on an LB agar plate supplemented with ampicillin and chloramphenicol. During nitrobenzene removal, the CFU abundance of strain ZWL73 decreased from $6.59 \pm 2.78 \times 10^6$ CFU g^{-1} ds on day 0 to $2.26 \pm 0.49 \times 10^6$ CFU g^{-1} ds on day 8. CFU abundance then decreased by one order of magnitude to $3.49 \pm 1.76 \times 10^5$ CFU g^{-1} ds on day 12 and changed little during the rest of the treatment period.

The abundance of cultivable heterotrophic bacteria in Tc and T− varied little (in the range of 1.24 ± 0.65–$2.31 \pm 0.34 \times 10^6$ CFU g^{-1} ds) during the experiments. In bioaugmented T+, the bacterial abundance was $1.42 \pm 0.56 \times 10^7$ CFU g^{-1} ds on day 0, due to the inoculation of strain ZWL73, but was reduced to $2.37 \pm 0.27 \times 10^6$ CFU g^{-1} ds on day 4, $1.17 \pm 0.59 \times 10^7$ CFU g^{-1} ds on day 12 and decreased to $2.52 \pm 0.37 \times 10^6$ CFU g^{-1} ds on day 20. In all three treatments, nine colony types of cultivable heterotrophic bacteria were observed on NA plates on day 0 and were detected in control Tc at all sampling times. In contaminated T−, however, only five colony types were observed from day 2 to the end of the experiment. In bioaugmented T+, the number of colony types was 5 on day 2, but 9 on day 4, with no subsequent change until the end of the experiment.

Impact of nitrobenzene contamination and bioaugmentation on nitrification and the AOB community

In order to investigate soil nitrification, the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were determined. In the first 2 days, NH_4^+-N accumulated (Fig. 1b) and NO_2^--N and NO_3^--N concentrations simultaneously decreased in T−

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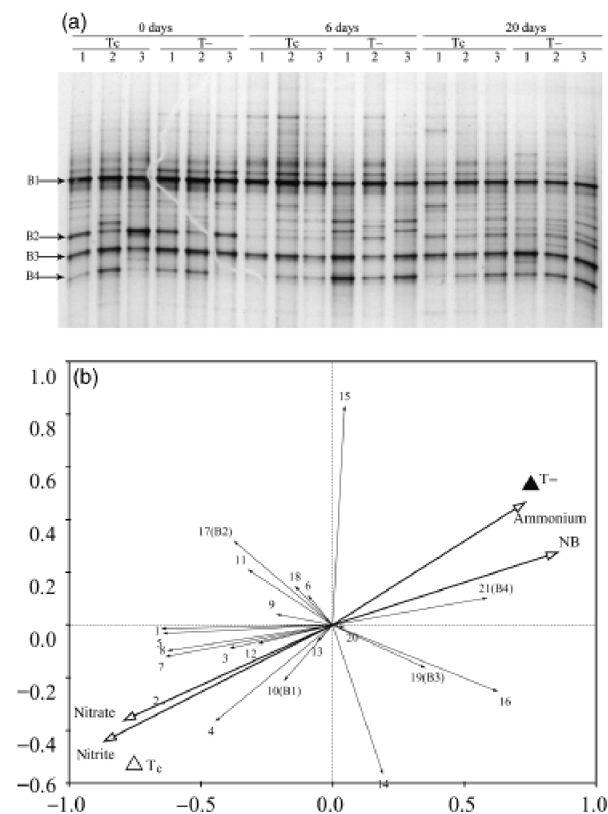

Fig. 2. (a) PCR-DGGE analysis of AOB communities in control Tc and contaminated T− (0, 6 and 20 days) soils. Bands marked with arrows were excised and sequenced. (b) Ordination biplots generated by pRDA of DGGE profiles. Solid arrow, species; empty arrow, environmental variables; △, Tc and ▲, T−, as qualitative variables. Qualitative variables were plotted based on their centroid position in the diagram. The correlation between environmental factors and species composition was evaluated by the cosine of the angle between the corresponding arrows. The arrow length implied its contribution in the model. Bands that were excised and sequenced are indicated in brackets.

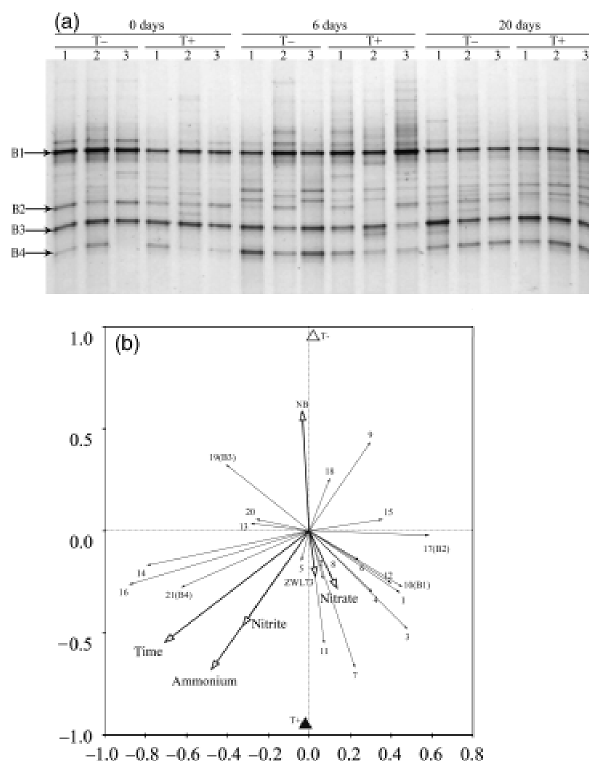

Fig. 3. (a) PCR-DGGE analysis of AOB community in contaminated T− and bioaugmented T+ (0, 6 and 20 days) soils. Bands marked with arrows were excised and sequenced. (b) Ordination biplots generated by RDA of DGGE profiles. Solid arrow, species; empty arrow, environmental variables; △, T− and ▲, T+, as qualitative variables. The correlation between environmental factors and species composition was evaluated by the cosine of the angle between the corresponding arrows. The arrow length implied its contribution in the model. Bands that were excised and sequenced are indicated in brackets.

and T+ (Fig. 1c and d), indicating that nitrification was inhibited. Subsequently, nitrification in bioaugmented T+ began to recover gradually after day 4, along with increasing NO_2^--N and NO_3^--N concentrations. As the NH_4^+-N concentration increased (Fig. 1b) in contaminated T−, however, the NO_2^--N concentration was still below the detection limit and the NO_3^--N concentration remained at a low level for 20 days (Fig. 1c and d).

Changes in the AOB community structure were examined by DGGE (Figs 2a and 3a) with samples on 0, 6 and 20 days, representing the initial, processing and later stages of nitrobenzene contamination in T− and bioaugmentation in T+, respectively. In order to analyze the relationships between the AOB community and environmental factors, multivariate analysis (i.e. RDA and pRDA) were used. To reduce subjectivity in analyses, datasets with or without faint bands were analyzed, but the results were virtually the same (data not shown), and subsequent analysis was carried out using all DGGE bands. For the control Tc and contaminated T− treatments, standard RDA showed that 51.2% of the total species variance was explained by environmental variables (both time and treatment) (Fig. 2a). Monte Carlo permutation tests showed that this model significantly explained AOB community variation ($P < 0.05$). However, the effects of time only (i.e. when treatment was considered as a covariable) were found to be nonsignificant, as determined by a permutation test, and explained only 7.5% of the total community variance. In contrast, treatment explained 43.8% of the total biological variance and was significant ($P < 0.05$) when time was considered as a covariable in pRDA models.

The first axis of the pRDA accounted for 26.5%, while the second axis accounted for 10.7% of the total species variance (Fig. 2b). Meanwhile, 72.6% of the cumulative variance of the species–environment relation was explained by the first two axes. Ammonium clearly showed a negative correlation with nitrite and nitrate (Fig. 2b), as nitrobenzene resulted in

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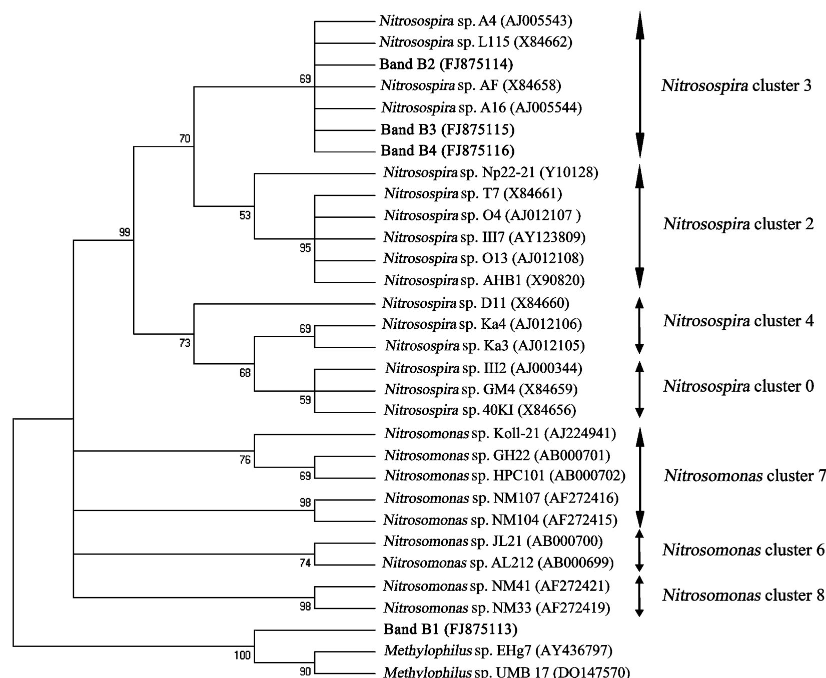

Fig. 4. 16S rRNA gene-based cladogram of the betaproteobacterial AOB. Cluster designations were performed according to Purkhold *et al*. (2000). Bootstrap values are shown at nodes when they exceed 50% of replicates. Species sequences determined in this study are depicted in bold.

nitrification inhibition. The presence of many species arrows pointing in the opposite direction to nitrobenzene arrows suggested that nitrobenzene may have a negative influence on the AOB community structure. In contrast, the nitrobenzene concentration had a strong positive correlation with the ammonium concentration.

The AOB communities in contaminated T− and bioaugmented T+ were analyzed by DGGE (Fig. 3a) and variation partitioning was also applied to investigate the effect of environmental factors on the AOB community. Standard RDA showed that 52.5% of the total species variance was significantly explained by both explanatory variables time and treatment ($P < 0.05$). However, pRDA with time as an explanatory variable and treatment as a covariable showed that time explained 6.5% of the total species variance and was statistically nonsignificant ($P > 0.05$). When treatment was considered as an explanatory variable and time as a covariable, 36.7% of the total species variance could be explained, but this was not statistically significant ($P > 0.05$).

Overall, these results suggest that the AOB community was significantly influenced by the covariation of both treatments (strain ZWL73, nitrobenzene, ammonium, nitrite, nitrate, etc.) and time. Figure 3b shows the relationship between AOB species composition and environmental factors. The first and second axes accounted for 23.4% and 11.7% of the total species variance, respectively. Moreover, 66.9% of the cumulative variance of species–environment relationships was explained by the first two axes. More specifically, strain ZWL73 showed a strong negative correlation with nitrobenzene, but positive relationships with other variables (i.e. ammonium, nitrite, nitrate and time; Fig. 3b). Most species vectors seemed to be almost perpendicular to the nitrobenzene vector, showing less correlation with nitrobenzene than observed in Fig. 2b.

Four dominant bands on AOB DGGE profiles were excised and four clones per band were sequenced. Phylogenetic analysis indicated that three of the four bands (B2, B3 and B4) belonged to *Nitrosospira* cluster 3, except B1, which was related to *Methylophilus* (Fig. 4). Analyses with and without the band B1, as a product of nonspecific amplification, proved that this had no effects on the above results of multivariate analyses.

Discussion

Catabolic capability and survival of *P. putida* ZWL73 in soil

Successful bioaugmentation depends on the catabolic capability of degrader strains. Strain ZWL73 in this study showed a strong ability to degrade nitrobenzene in soil. Nitrobenzene was effectively removed in bioaugmented T+ compared with noninoculated T− (Fig. 1a). Nitrobenzene could be metabolized via a partial-reductive pathway, leading to the release of considerable amounts of ammonium (Xiao *et al*., 2006; Zhen *et al*., 2006) into soil from aromatic rings in T+ (Fig. 1b), indicating that strain ZWL73 enhanced nitrobenzene degradation in soil. We have reported that strain ZWL73 could degrade 4CNB in soil,

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resulting in the simultaneous accumulation of ammonium and chloride (Niu *et al*., 2009).

Bioaugmentation *in situ*, however, depends not only on the catabolic capability of the inoculum but also on its survival in contaminated soils (Singer *et al*., 2005; Cunliffe & Kertesz, 2006). For instance, poor survival of the inoculated degraders may result in bioaugmentation failure (Bouchez *et al*., 2000). In principle, the persistence and growth of inoculated strains in any soil will depend on their ability to utilize local resources but also on the presence of special contaminants they can degrade (Cunliffe & Kertesz, 2006). Numerous bioaugmentation reports have revealed that inoculated bacteria may increase in the presence of their substrates and then decline when the substrates are removed (Elväng *et al*., 2001; Gomes *et al*., 2005; Cunliffe & Kertesz, 2006; Niu *et al*., 2009). Native communities have been demonstrated to resist ecological invasion in grasses (Kennedy *et al*., 2002) and this theory has generally been applied to microbial diversity (Cunliffe & Kertesz, 2006). In our study, however, in the presence of contaminants in soil, the inoculated contaminant utilizer could establish within the local microbial communities. Furthermore, when the contaminants were finally removed, the new inoculated member was still maintained in the community, indicating that it successfully adapted to the complex microbial interactions in the soil environment. In our previous study on bioaugmentation of 4CNB-contaminated soil, ZWL73 grew better in sterile soil than in natural soil where both 4CNB and ZWL73 were spiked, indicating that the native microbial communities resisted the establishment of ZWL73. Although sterile soil spiked with nitrobenzene and ZWL73 was not performed in this study, a similar assumption can be made here. In this study, strain ZWL73 seemed to decrease slowly when nitrobenzene was present rather than increase, as mentioned in the previous studies, possibly because the low nitrobenzene concentration was unable to support its growth or the low toxicity of nitrobenzene was insufficient to impact on the native microbial communities. Finally, ZWL73 decreased by one order of magnitude and remained stable after complete degradation of nitrobenzene, indicating that it was able to survive and to utilize local resources in soil when nitrobenzene was removed and a new community was established.

Reduced effects of nitrobenzene contamination on nitrification and the AOB community structure with *P. putida* ZWL73 inocula

The important process of chemolithotrophic nitrification is a two-step process involving bacterial and archaeal ammonia-oxidizers, oxidizing NH_3 to NO_2^-, and nitrite-oxidizing bacteria, oxidizing NO_2^- to NO_3^- (Harms *et al*., 2003). Ammonia oxidation is the first and often the rate-limiting step of nitrification and, therefore, is critical for the global nitrogen cycle (Chu *et al*., 2007).

Multivariate analyses are useful to assess the relationship between biotic communities and/or their environmental conditions (Ramette, 2007). In both T− and T+ of the current study, nitrification appeared to be completely inhibited as soon as nitrobenzene was added to soil (Fig. 1b, c and d), because the ammonium concentration increased while the nitrite and nitrate concentrations decreased simultaneously. Correspondingly, in Fig. 2b, the ammonium vector shows a nearly perfect negative correlation with nitrite and nitrate vectors, indicating that complete inhibition of ammonium conversion to nitrite and nitrate occurred in the nitrobenzene-contaminated soil. It has been shown that nitrobenzene could inhibit nitrification as a suicide substrate for ammonium monooxygenase (Keener & Arp, 1994; McCarty, 1999). Generally, soil nitrification was affected by polycyclic aromatic compounds (Sverdrup *et al*., 2002).

RNA- and DNA-based molecular approaches have been applied for characterization of microbial communities mainly targeting metabolically active and total bacteria, respectively (Duineveld *et al*., 2001). In this study, DNA-based DGGE analysis was selected for investigation of betaproteobacterial AOB. Variation partitioning revealed that the AOB community was significantly affected by nitrobenzene contamination. Although the incubation period was rather short, in bioaugmentation microcosms, strain ZWL73 was shown to play a key role in protecting the AOB community from nitrobenzene contamination. Standard RDA showed that the AOB community was significantly affected by both time and treatment variables. pRDA showed that neither time nor treatment alone had a significant influence on the AOB community, indicating that the effects of nitrobenzene on the AOB community were weakened by strain ZWL73 inoculated into soil. Most species were not correlated with the presence of nitrobenzene (Fig. 3b), suggesting that the impact of nitrobenzene on the species composition may be reduced in the bioaugmented microcosms. Strain ZWL73 showed an ability to alleviate the influence of nitrobenzene on nitrification and on the AOB community. Boon *et al*. (2003) reported that, in semi-continuous activated-sludge reactors, complete inhibition of nitrification was observed immediately after a 3-chloroaniline (3-CA) shock load. Meanwhile, in 3-CA-contaminated treatments, the AOB community clearly changed and the percentage of AOB in the whole bacterial community decreased dramatically, but these negative effects of 3-CA on AOB and nitrification were removed by inoculation with the 3-CA-degrading strain *Comamonas testosteroni* I2 *gfp* (Boon *et al*., 2003). Likewise, the AOB community structure has been reported to be negatively affected by addition of toxic metals in soil and yet protected by inoculating metal-resistant bacteria into soil (Stephen *et al*., 1999).

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Dominance of *Nitrosospira* in soil

All known terrestrial AOB belong to a monophyletic group within the *Betaproteobacteria*, and the currently accepted classification recognizes only two genera within this group: *Nitrosospira* and *Nitrosomonas* (Stephen *et al*., 1998; Chu *et al*., 2007). The dominance of *Nitrosospira* in soil has been reported in previous studies (Stephen *et al*., 1998; Kurola *et al*., 2005; Chu *et al*., 2007) and was found in this study. Four bands with a high relative intensity were recovered from DGGE gels and sequenced. B2, B3 and B4 fell in *Nitrosospira* cluster 3 (Fig. 4), which has been found in soil, freshwater and sand dune (Purkhold *et al*., 2000). However, the sequence obtained from band B1 was closely related to *Methylophilus* (with 98% identity). Cébron *et al*. (2004) also found a band with 94% similarity to *Methylophilus leisingeri* during DGGE analysis of AOB in lower Seine River by either direct PCR with CTO or nested PCR with CTO and 357f-GC-518r primers. This nonspecific amplification arose mainly due to defects of CTO primers. None of the published primers targeting 16S rRNA genes of AOB show complete specificity under all conditions (Purkhold *et al*., 2000; Cébron *et al*., 2004), but the PCR strategy used in this study has been found to provide the best method targeting 16S rRNA genes to analyze AOB communities in soil and marine sediments (Mahmood *et al*., 2006). Indeed, universal bacterial 27f-1492r primers that are considered to be unbiased against AOB may be used for the first amplification of environmental templates, followed by CTO primers (Mahmood *et al*., 2006). The 357f-GC-518r primer set then provides a high resolution for DGGE analysis of the final products (Freitag & Prosser, 2003; Mahmood *et al*., 2006).

Acknowledgements

This work was supported by the National High Technology Research and Development Program of China (grant 2006AA10Z403) and Knowledge Innovation Program of the Chinese Academy of Sciences (grant KSCX2-YW-G-009).

References

Boon N, Goris J, De Vos P, Verstraete W & Top EM (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. *Appl Environ Microb* **66**: 2906–2913.

Boon N, Top EM, Verstraete W & Siciliano SD (2003) Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. *Appl Environ Microb* **69**: 1511–1520.

Bouchez T, Patureau D, Dabert P, Juretschko S, Dore J, Delgenes P, Moletta R & Wagner M (2000) Ecological study of a bioaugmentation failure. *Environ Microbiol* **2**: 179–190.

Cavalca L, Colombo M, Larcher S, Gigliotti C, Collina E & Andreoni V (2002) Survival and naphthalene-degrading activity of *Rhodococcus* sp. strain 1BN in soil microcosms. *J Appl Microbiol* **92**: 1058–1065.

Cébron A, Coci M, Garnier J & Laanbroek HJ (2004) Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine River: impact of Paris wastewater effluents. *Appl Environ Microb* **70**: 6726–6737.

Chang BV, Chiang F & Yuan SY (2005) Biodegradation of nonylphenol in sewage sludge. *Chemosphere* **60**: 1652–1659.

Chen SC, Chen SL & Fang HY (2005) Study on EDTA-degrading bacterium *Burkholderia cepacia* YL-6 for bioaugmentation. *Bioresource Technol* **96**: 1782–1787.

Chu H, Fujii T, Morimoto S, Lin X, Yagi K, Hu J & Zhang J (2007) Community structure of ammonia-oxidizing bacteria under long-term application of mineral fertilizer and organic manure in a sandy loam soil. *Appl Environ Microb* **73**: 485–491.

Cunliffe M & Kertesz MA (2006) Effect of *Sphingobium yanoikuyae* B1 inoculation on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. *Environ Pollut* **144**: 228–237.

Duineveld BM, Kowalchuk GA, Keijzer A, van Elsas JD & van Veen JA (2001) Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Appl Environ Microb* **67**: 172–178.

Elväng AM, Westerberg K, Jernberg C & Jansson JK (2001) Use of green fluorescent protein and luciferase biomarkers to monitor survival and activity of *Arthrobacter chlorophenolicus* A6 cells during degradation of 4-chlorophenol in soil. *Environ Microbiol* **3**: 32–42.

Freitag TE & Prosser JI (2003) Community structure of ammonia-oxidizing bacteria within anoxic marine sediments. *Appl Environ Microb* **69**: 1359–1371.

Gentry TJ, Rensing C & Pepper IL (2004) New approaches for bioaugmentation as a remediation technology. *Crit Rev Env Sci Tec* **34**: 447–494.

Gomes NC, Kosheleva IA, Abraham WR & Smalla K (2005) Effects of the inoculant strain *Pseudomonas putida* KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. *FEMS Microbiol Ecol* **54**: 21–33.

Harms G, Layton AC, Dionisi HM, Gregory IR, Garrett VM, Hawkins SA, Robinson KG & Sayler GS (2003) Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ Sci Technol* **37**: 343–351.

Hu X, Li A, Fan J, Deng C & Zhang Q (2008) Biotreatment of *p*-nitrophenol and nitrobenzene in mixed wastewater through selective bioaugmentation. *Bioresource Technol* **99**: 4529–4533.

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Kandeler E (2002) Potential nitrification test. *Methods in Soil Biology* (Schinner F, Öhlinger R, Kandeler E & Margesin R, eds), pp. 146–148. Springer, Berlin.

Keener WK & Arp DJ (1994) Transformations of aromatic compounds by *Nitrosomonas europaea*. *Appl Environ Microb* **60**: 1914–1920.

Kennedy TA, Naeem S, Howe KM, Knops JMH, Tilman D & Reich P (2002) Biodiversity as a barrier to ecological invasion. *Nature* **417**: 636–638.

Kowalchuk GA, Stephen JR, De Boer W, Prosser JI, Embley TM & Woldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microb* **63**: 1489–1497.

Kurola J, Salkinoja-Salonen M, Aarnio T, Hultman J & Romantschuk M (2005) Activity, diversity and population size of ammonia-oxidising bacteria in oil-contaminated landfarming soil. *FEMS Microbiol Lett* **250**: 33–38.

Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 115–175. Wiley, Chichester.

Mahmood S, Freitag TE & Prosser JI (2006) Comparison of PCR primer-based strategies for characterization of ammonia oxidizer communities in environmental samples. *FEMS Microbiol Ecol* **56**: 482–493.

McCaig AE, Glover LA & Prosser JI (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Appl Environ Microb* **67**: 4554–4559.

McCarty GW (1999) Modes of action of nitrification inhibitors. *Biol Fert Soils* **29**: 1–9.

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microb* **59**: 695–700.

Nishino SF & Spain JC (1993) Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Appl Environ Microb* **59**: 2520–2525.

Nishino SF, Spain JC & He ZQ (2000) Strategies for aerobic degradation of nitroaromatic compounds by bacteria: process discovery to field application. *Biodegradation of Nitroaromatic Compounds and Explosives* (Spain JC, Hughes JB & Knackmuss HJ, eds), pp. 7–61. Lewis Publishers, Boca Raton, FL.

Niu GL, Zhang JJ, Zhao S, Liu H, Boon N & Zhou NY (2009) Bioaugmentation of a 4-chloronitrobenzene contaminated soil with *Pseudomonas putida* ZWL73. *Environ Pollut* **157**: 763–771.

Nkonge C & Ballance GM (1982) A sensitive colorimetric procedure for nitrogen determination in micro-Kjeldahl digests. *J Agr Food Chem* **30**: 416–420.

Norman RJ, Edberg JC & Stucki JW (1985) Determination of nitrate in soil extracts by dual-wavelength ultraviolet spectrophotometry. *Soil Sci Soc Am J* **49**: 1182–1185.

Parales RE (2000) Molecular biology of nitroarene degradation. *Biodegradation of Nitroaromatic Compounds and Explosives* (Spain JC, Hughes JB & Knackmuss HJ, eds), pp. 63–89. Lewis Publishers, Boca Raton, FL.

Purkhold U, Pommerening-Röser A, Juretschko S, Schmid MC, Koops HP & Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. *Appl Environ Microb* **66**: 5368–5382.

Ramette A (2007) Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol* **62**: 142–160.

Schwieger F & Tebbe CC (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microb* **64**: 4870–4876.

Singer AC, van der Gast CJ & Thompson IP (2005) Perspectives and vision for strain selection in bioaugmentation. *Trends Biotechnol* **23**: 74–77.

Stephen JR, Kowalchuk GA, Bruns MAV, McCaig AE, Phillips CJ, Embley TM & Prosser JI (1998) Analysis of beta-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. *Appl Environ Microb* **64**: 2958–2965.

Stephen JR, Chang YJ, Macnaughton SJ, Kowalchuk GA, Leung KT, Flemming CA & White DC (1999) Effect of toxic metals on indigenous soil beta-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. *Appl Environ Microb* **65**: 95–101.

Sverdrup LE, Ekelund F, Krogh PH, Nielsen T & Johnsen K (2002) Soil microbial toxicity of eight polycyclic aromatic compounds: effects on nitrification, the genetic diversity of bacteria, and the total number of protozoans. *Environ Toxicol Chem* **21**: 1644–1650.

Wang G, Gentry TJ, Grass G, Josephson K, Rensing C & Pepper IL (2004) Real-time PCR quantification of a green fluorescent protein-labeled, genetically engineered *Pseudomonas putida* strain during 2-chlorobenzoate degradation in soil. *FEMS Microbiol Lett* **233**: 307–314.

World Health Organization Department for Health Action (2005) Health Action in Crises Highlights No. 84, November 21 to December 4.

Xiao Y, Wu JF, Liu H, Wang SJ, Liu SJ & Zhou NY (2006) Characterization of genes involved in the initial reactions of 4-chloronitrobenzene degradation in *Pseudomonas putida* ZWL73. *Appl Microbiol Biot* **73**: 166–171.

Zhen D, Liu H, Wang SJ, Zhang JJ, Zhao F & Zhou NY (2006) Plasmid-mediated degradation of 4-chloronitrobenzene by newly isolated *Pseudomonas putida* strain ZWL73. *Appl Microbiol Biot* **72**: 797–803.

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