Environmental Pollution 172 (2013) 33–41

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

# Bioaugmentation with a consortium of bacterial nitrophenol-degraders for remediation of soil contaminated with three nitrophenol isomers

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ARTICLE INFO

*Article history:*
Received 2 March 2012
Received in revised form
25 May 2012
Accepted 4 August 2012

*Keywords:*
Bioaugmentation
Denaturing gradient gel electrophoresis
Nitrophenol isomers
Pareto–Lorenz curve
Real-time PCR

ABSTRACT

A consortium consisting of *para*-nitrophenol utilizer *Pseudomonas* sp. strain WBC-3, *meta*-nitrophenol utilizer *Cupriavidus necator* JMP134 and *ortho*-nitrophenol utilizer *Alcaligenes* sp. strain NyZ215 was inoculated into soil contaminated with three nitrophenol isomers for bioaugmentation. Accelerated removal of all nitrophenols was achieved in inoculated soils compared to un-inoculated soils, with complete removal of nitrophenols in inoculated soils occurring between 2 and 16 days. Real-time polymerase chain reaction (PCR) targeting nitrophenol-degradation functional genes indicated that the three strains survived and were stable over the course of the incubation period. The abundance of total indigenous bacteria (measured by 16S rRNA gene real-time PCR) was slightly negatively impacted by the nitrophenol contamination. Denaturing gradient gel electrophoresis profiles of total and group-specific indigenous community suggested a dynamic change in species richness occurred during the bioaugmentation process. Furthermore, Pareto–Lorenz curves and Community organization parameters indicated that the bioaugmentation process had little impact on species evenness within the microbial community.



## 1. Introduction

There are three isomeric mononitrophenols, namely, *ortho*-nitrophenol (ONP), *meta*-nitrophenol (MNP) and *para*-nitrophenol (PNP). As a result of equal molecular weight and similar boiling points among them, it is difficult to distinguish them from each another, and these properties have provoked researchers to test various methods to distinguish among them in manufacture (Guo et al., 2004). On the other hand, their wide application in the agricultural, pharmaceutical and chemical industries has led to accumulation of nitrophenols as contaminants in soil. All nitrophenol isomers have high water solubility and potential negative impact if released into the environment, as they can be carcinogenic, teratogenic and mutagenic to plants, fish and many other organisms (Guo et al., 2004). Among the nitrophenol isomers, ONP and PNP were listed as "priority pollutants" by United States Environmental Protection Agency (USEPA) (Keith and Telliard, 1979). Bioaugmentation is considered as a promising approach to clean up contamination in soil (Dechesne et al., 2005) and has been employed in the remediation of soils contaminated by various organic pollutants, such as polycyclic aromatic hydrocarbons (Cunliffe and Kertesz, 2006) and nitroaromatic compounds (Niu et al., 2009; Zhao et al., 2009). A successful bioaugmentation has also been reported for the single nitrophenol isomer (PNP)-contaminated soil using *Arthrobacter protophormiae* RKJ100 (Labana et al., 2005). Moreover, bioaugmentation has also been implemented to clean up soils contaminated with mixtures of PNP and other compounds, for instances, PNP and pentachlorophenol were simultaneously degraded by *Sphingomonas* sp. UG30 in soil perfusion bioreactors (Alber et al., 2000) and PNP, 2-chloro-4-nitrophenol and 2,4-dinitrophenol were removed at the same time by *Rhodococcus imtechensis* strain RKJ300 (Ghosh et al., 2010). However, no study on bioaugmentation for the simultaneous removal of three nitrophenol isomers in a co-contaminated soil has been documented, with either single microorganisms or a consortium.

It is generally accepted that successful bioaugmentation depends upon the efficient and reliable use of microbes with the capability to degrade the target pollutants. Successful bioremediation also requires information on survival and metabolic activity of the inoculated strains (Gomes et al., 2005). Sufficient data on introduced population dynamics in bioremediation can provide insights towards improved methods of inoculation, maintenance and management of contaminated sites (Rodrigues et al., 2002).

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http://dx.doi.org/10.1016/j.envpol.2012.08.002

Plate counting is the most widely used method for quantifying survival of inoculants during bioaugmentation; however, this traditional method is time-consuming and labor-intensive, and may also result in an underestimate of population numbers (Chen et al., 2010). Alternatively, real-time PCR quantification of specific gene copy numbers is becoming an accepted technique for assessment of gene stability and fate of specific strains in soil samples, particularly when a consortium of multiple strains are employed in bioremediation. The use of real-time PCR methods for detecting specific functional genes relies on a comprehensive understanding of microbial catabolic pathways of the target pollutants at both genetic and biochemical level.

*Pseudomonas* sp. strain WBC-3 (Zhang et al., 2009), *Cupriavidus necator* JMP134 (formerly *Ralstonia eutropha* JMP134) (Schenzle et al., 1997; Yin et al., 2010) and *Alcaligenes* sp. strain NyZ215 (Xiao et al., 2007) are able to utilize PNP, MNP and ONP, respectively, as the sole source of carbon, nitrogen and energy. The underlying degradation mechanisms at both genetic and biochemical levels have also been elucidated for each of these three strains. It has been shown that *pnpA*-encoded PNP 4-monooxygenase, *mnpA*-encoded MNP nitroreductase and *onpA*-encoded ONP 2-monooxygenase catalyze the initial reactions of PNP, MNP and ONP catabolism, respectively, with concomitant release of nitrite, ammonium and nitrite, respectively (Xiao et al., 2007; Yin et al., 2010; Zhang et al., 2009). In this study, we report the successful bioaugmentation of an inoculated consortium consisting of the above three nitrophenol degraders in soil artificially contaminated with all three nitrophenol isomers. Simultaneously, the changes in bacterial abundance and community structure of the dominant indigenous microbial community in the presence or absence of the inoculated consortium were also analyzed by denaturing gradient gel electrophoresis (DGGE).

## 2. Materials and methods

### 2.1. Soil characteristics and preparation

The soil samples were collected from an un-contaminated area on the campus of the Chinese Academy of Sciences, Wuhan, China. The top layer (0–15 cm) of the soil was sampled, sieved ($\leq$2 mm) and stored at 4 °C. Total organic carbon content of the soil sample was 0.70% and total nitrogen content was 0.06%. The pH of soil was 7.6 and moisture content was 14.5%.

### 2.2. Preliminary experiment

We have set 6 groups of different concentrations (5, 10, 20, 50, 70, 100 μg $g^{-1}$ ds of each isomer with equal concentration) of nitrophenol in contamination and bioaugmentation treatments. All three nitrophenols were degraded completely when the concentration of each isomer did not exceed 50 μg $g^{-1}$ ds. Therefore, each isomer with a final concentration of 40 μg $g^{-1}$ ds was added into soils in all subsequent experiments. In addition, we also found each isomer at the concentrations of no more than 50 μg $g^{-1}$ ds could be degraded completely in bioaugmentation of single nitrophenol isomer-contaminated soil.

### 2.3. Soil microcosm experiments

Microcosms were prepared in 250 mL glass bottles with tightened screw cap tops with 114.5 g wet weight of native or sterile soil (sterilized by autoclaving for 20 min at 121 °C three times, every other day during treatment), corresponding to 100 g soil dry weight (dw). Five different treatments were applied: (T1) native soil; (T2) native soil with three nitrophenol isomers added; (T3) native soil with three nitrophenol isomers added and with the microbial consortium inoculated; (T4) sterilized soil with three nitrophenol isomers added; (T5) sterilized soil with three nitrophenol isomers added and with the microbial consortium inoculated. All treatments were prepared in triplicate. Microcosms were equilibrated at 30 °C for 48 h prior to commencing the experiment. Contaminated treatments were prepared by thoroughly mixing the three nitrophenols with soil to give a final concentration of 0.04 mg nitrophenol $g^{-1}$ dw (dry weight) each. Strains NyZ215, JMP134 and WBC-3 were grown to the late exponential phase in lysogeny broth (LB) medium at 30 °C, and then resuspended in sterile saline (0.85% (w/v)). Cells were inoculated into bioaugmentation treatments T3 and T5 to give a final concentration of approximately $1 \times 10^8$ colony forming units (CFU) $g^{-1}$ dw for each strain. In parallel, equivalent volumes of sterile saline were added to non-inoculated soils (T1, T2 and T4) to adjust the final moisture content (approx. 20% (w/w)) in each microcosm. After vigorous stirring, microcosms were maintained in the dark at 30 °C and sampled for analysis.

### 2.4. Chemical analysis

For high performance liquid chromatography (HPLC) analysis, soil samples (0.5 g each) were thoroughly mixed with 1 mL methanol. Soil/methanol mixtures were shaken at 200 rpm for 30 min and then centrifuged at 10,000 × *g* for 10 min. Mononitrophenols in the soil microcosms were determined by HPLC which was performed on an Agilent series 1200 system (Agilent Technologies, Palo Alto, CA) equipped with a $C_{18}$ reversed-phase column (5 μm, 4.6 × 250 mm; Agilent Technologies) maintained at 30 °C. The mobile phase consisted of methanol (40%) and 0.1% acetic acid (60%) at a flow rate of 1 mL $min^{-1}$. All mononitrophenols were monitored at 280 nm with an Agilent ultra-violet detector. The following retention times were observed for each nitrophenol: ONP, 17.0 min; MNP, 12.5 min; PNP, 11.2 min.

The concentrations of ammonium (Fawcett and Scott, 1960) and nitrite (Lessner et al., 2002) were determined colorimetrically. Soil samples (0.5 g each) were first extracted with 2 M KCl and shaken at 200 rpm for 30 min. The samples were then centrifuged at 10,000 × *g* for 5 min.

### 2.5. DNA extraction

Nucleic acids were extracted from 0.5 g soil using MoBio UltraClean™ soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). DNA was eluted with 50 μL of solution S5 (MoBio Laboratories) and stored at −20 °C.

### 2.6. Quantitative real-time PCR

The primers and probe used in this study are listed in Table 1. Real-time PCR was performed on a RT-Cycler™ 136 (CapitalBio, Beijing, China). The reaction solution for the quantification of genes (*pnpA*, *mnpA* and *onpA*) encoding the initial degradations of PNP, MNP and ONP contained 10 μL 2 × TransStart™ Eco Green qPCR SuperMix (TransGen Biotech, Beijing, China), 1 μL template DNA, 0.4 μL Passive Reference Dye and 0.2 mM of each pair of primers (pnpA1/pnpA2 for *pnpA*; mnpA1/mnpA2 for *mnpA*; onpA1/onpA2 for *onpA*) in 20 μL reaction mixture. PCRs were run using the following steps: 5 min incubation at 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. PCR product specificity was then confirmed by a melting curve analysis which measures fluorescence continuously along with the temperature increasing from 60 °C to 94 °C. TaqMan probes were labeled with the reporter dye FAM (6-carboxy-fluorescein) at the 5′ end and with the quencher dye TAMRA (6-carboxy-tetramenthylrhodamine) at the 3′ end (Sangon Biotech, Shanghai, China). Primers, probe and thermal profiles appropriate for quantification of total bacterial 16S rRNA gene in soil were as previously described (Shen et al., 2008). The fragments of almost complete genes *pnpA*, *mnpA*, *onpA* and 16S rRNA gene amplified from extracted DNA from soil with corresponding primers (pnpA96/pnpA1204 for *pnpA*; mnpA2/mnpA680 for *mnpA*; onpA4/onpA1640 for *onpA*; 27F/1492R for 16S rRNA gene) were individually cloned into pGEM®-T Vectors (Promega, Madison, USA). Plasmids were extracted from the recombinant clones and used as standards for quantitative analysis. Plasmid DNA concentrations were determined on an Eppendorf BioPhotometer (Hamburg, Germany), through which the copy numbers of target genes were directly calculated. Ten-fold serial dilutions of known concentrations of the plasmid DNA were used in the real-time PCR assay in triplicate to generate an external standard curve.

### 2.7. PCR amplification for denaturing gradient gel electrophoresis (DGGE) analysis

DNA extracted in triplicate from every microcosm was mixed for use as template for PCR-DGGE. Universal primers GC-341F and 518R were used for amplifying the total bacterial 16S rRNA gene fragments (V3 variable regions of the 16S rRNA gene), according to a published method (Muyzer et al., 1993). For the amplification of Alphaproteobacterial, Actinobacterial and Acidobacterial 16S rRNA gene fragments, a nested PCR approach was performed. In the first round PCR, the group-specific primer was used as the forward primer and R1492 was used as the reverse primer (Niu et al., 2009). The products of the first round PCR were then diluted and used as a template for secondary PCR amplification with primers GC-341F and 518R (Muyzer et al., 1993).

### 2.8. DGGE analyses

DGGE was performed as described (Muyzer et al., 1993) using the Dcode System (Bio-Rad Labs, Hercules, CA). The gels were prepared with 8% polyacrylamide containing a denaturant gradient between 45 and 65%. Gels were run in 1 × TAE buffer at 60 °C at a constant voltage of 80 V for 10 h. After completion, gels were stained with silver nitrate as described previously (McCaig et al., 2001). The DGGE patterns were analyzed using Quantity One 4.62 (Bio-Rad).