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Effect of nitrate addition on reductive transformation of pentachlorophenol in paddy soil in relation to iron(III) reduction

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ABSTRACT

Reductive dechlorination is a crucial pathway for anaerobic biodegradation of highly chlorinated organic contaminants. Under an anoxic environment, reductive dechlorination of organic contaminants can be affected by many redox processes such as nitrate reduction and iron reduction. In the present study, batch incubation experiments were conducted to investigate the effect of nitrate addition on reductive dechlorination of PCP in paddy soil with consideration of iron transformation. Study results demonstrate that low concentrations (0, 0.5 and 1 mM) of nitrate addition can enhance the reductive dechlorination of PCP and Fe(III) reduction, while high concentrations (5, 10, 20 and 30 mM) of nitrate addition caused the contrary. Significant positive correlations between PCP degradation rates and the formation rates of dissolved Fe(II) (pearson correlation coefficients $r = 0.965$) and HCl-extractable Fe(II) ($r = 0.921$) suggested that Fe(III) reduction may enhance PCP dechlorination. Furthermore, consistent variation trends of PCP degradation and the abundances of the genus *Comamonas*, capable of Fe(III) reduction coupled to reductive dechlorination, and of the genus *Dehalobacter* indicated the occurrence of microbial community variation induced by nitrate addition as a response to PCP dechlorination.

1. Introduction

Pentachlorophenol (PCP), extensively used as a herbicide, insecticide, fungicide, wood preservative, resin, lubricant and dye intermediate, has been commonly found in ground waters, sediment and surface soils (Field and Sierra-Alvarez, 2008). It can be absorbed into the body through inhalation, diet, or skin contact (Eisler, 1989) and has caused numerous occupational illnesses and deaths (Wood et al., 1983). Its acute toxicity results from its ability to interfere with the production of high-energy phosphate compounds essential for cell respiration (Eisler, 1989; Liu et al., 2008). PCP can be degraded in the environment by chemical, microbiological and photochemical processes (Choudhury et al., 1986). In soil, microbial decomposition appears to be an important and potentially dominant removal mechanism (Choudhury et al., 1986). In addition, anaerobic reductive dechlorination achieved mainly by anaerobic microbes is a crucial pathway for the degradation of highly chlorinated organic compounds such as PCP (Adrian and Gorisch, 2002).

Iron, the most abundant element on earth and the most frequently utilized transition metal in the biosphere, plays a particularly important role in environmental biogeochemistry (Kappler and Straub, 2005). Previous studies have indicated that iron is involved in the transformation of many elements and contaminants, such as nitrogen and organic pollutants (Borch et al., 2010; Lovley et al., 2004). In particular, iron is closely linked to the transformation of nitrogen (Borch et al., 2010). For example, pyrite oxidation is able to couple denitrification in fertilizer-impact aquifers (Postma et al., 1991). In addition, nitrate-dependent Fe(II)-oxidizing bacteria mediate Fe(II) oxidation in neutrophilic, anoxic environments, where nitrate can serve as an electron acceptor (Kappler and Straub, 2005). Moreover, under anaerobic conditions, the dissimilatory reduction of Fe(III) can be coupled with ammonium oxidation (Clement et al., 2005). Furthermore, dissolved and adsorbed Fe(II) can effectively enhance the reductive dechlorination of organic pollutants (Amonette et al., 2000; Li et al., 2008). On the other hand, nitrogen, as a necessary element for microorganisms, can influence the growth and abundance of many

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microorganisms (Vitousek and Howarth, 1991). It has been reported that N addition can accelerate the degradation of organic contaminants by stimulating microbial activity (Braddock et al., 1997), while N addition can also inhibit enzymatic systems responsible for the degradation of organic pollutants, resulting in an inhibition of the degradation of organic pollutants (Abdelhafid et al., 2000). In addition, the application of nitrate, which serves as an electron acceptor, can compete for electrons with the reductive dechlorination of chlorinated organic compounds (Yoshida et al., 2007). As mentioned above, iron and nitrogen can each affect the degradation of organic pollutants separately, but the transformations of iron and nitrogen can also affect one another. Therefore, iron and nitrogen together can also affect the degradation of organic pollutants. However, few studies have considered the effects on the degradation of contaminants with respect to this interaction.

Currently, China consumes approximately 30% of global nitrogen fertilizer. Between 1961 and 1999, the global application of nitrogen fertilizer increased from 11.6×10^6 t to 85.5×10^6 t (approximately 6.4 times), whereas, during the same period, the application amount of nitrogen fertilizer in China increased 43.8 times (Peng et al., 2002). Guangdong province, located in southeast China, is one of China's most economically prosperous regions and has been well developed agriculturally. Results from a study investigating the nitrate and nitrite pollution of vegetables in typical areas of Guangdong province showed that 36.5% of samples contained nitrate at higher levels than national standards (Yang et al., 2007). Therefore, the excessive application of nitrogen fertilizer has resulted in high residual levels of nitrate in the agricultural environment of Guangdong province. In addition, PCP has also been used extensively as a wood preservative, molluscicide and clean-pond reagent in Guangdong province (Hong et al., 2005). Relatively high levels of PCP are found in pond sediment of this area, especially in Zhongshan, containing comparable levels of PCP to those found in a historic severe schistosomiasis plague area in China (Peng et al., 2002). In addition, the soil in Guangdong province is characteristic of red soil, which is rich in iron oxides (Li et al., 2006).

The objective of the present study was to examine the effect of nitrate addition on reductive dechlorination of PCP in paddy soil with consideration of the involvement of the process of iron transformation. It is expected that the results derived from the interactions among nitrate, iron and PCP in the microcosm will offer theoretical guides for further research on the interaction mechanisms among the nitrogen cycle, iron cycle and reductive dechlorination of organic pollutants under natural conditions.

2. Materials and methods

2.1. Soil sampling

Paddy soil sample used in the present study was collected from the town of Shahu in the city of Enping (22°23.11′N, 112°26.85′E), located in southwest Guangdong province in south China during September and December 2008. Upon return to the laboratory, the soil was sealed in polytetrafluoroethylene (PTFE) bag and stored in glass bottles at 4 °C prior to use.

2.2. Chemicals

PCP (≥98% purity) and 1,4-piperazinediethanesulfonic acid (PIPES) (≥98% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA). $NaNO_3$ was purchased from Sinopharm Chemical Reagent Co., Ltd, China. All other analytical-grade chemicals were obtained from Guangzhou Chemical Co., China. Deaerated deionized water was prepared by deoxygenating ultrapure water (18 MΩ-cm, Easy Pure II RF/UV, USA).

2.3. PCP transformation experiments in soil

Experiments were conducted in triplicate at a constant pH of 7.0 ± 0.2 with 30 mM PIPES as a buffer solution. The batch experiment procedures were as follows: the soil samples (0.5 g dry weight) were transferred into 20 mL serum bottles with silicone-lined septa and aluminum sealing caps, and 10 mL PIPES buffer solution was then added. Seven batch experiments, including control, were conducted in this study: (1) control (0 mM $NaNO_3$); (2) 0.5 mM $NaNO_3$; (3) 1 mM $NaNO_3$; (4) 5 mM $NaNO_3$; (5) 10 mM $NaNO_3$; (6) 20 mM $NaNO_3$; and (7) 30 mM $NaNO_3$. Subsequently, PCP and lactic acid at final concentrations of 0.0188 mM and 10 mM, respectively, were added to each vial. The mixture was then purged with O_2-free N_2 for 30 min and sealed with teflon-coated butyl rubber stoppers and crimp seals. The closed bottles were mixed on a rotary shaker and incubated at 30 ± 1 °C in an anaerobic chamber. Finally, at predetermined sampling intervals, the bottles were removed for analysis.

2.4. Chemical analysis methods

The PCP in the soil suspension was extracted using water/ethanol mixtures (50:50 by volume) on a horizontal shaker (180 rpm) for 1 h. The suspension was then filtered through a 0.45 μm syringe filter and collected for high performance liquid chromatography analysis. The detailed analysis procedures for PCP have been described in a previous study (Lan et al., 2008). HCl-extractable Fe(II) and dissolved Fe(II) were measured using the 1,10-phenanthroline colorimetric method at 510 nm on a UV–Vis spectrophotometer (TU-1810PC, Beijing Purkinje General Instruments, China) (Viollier et al., 2000). Concentrations of NO_3^-/NO_2^- were determined by ion chromatography (Dionex ICS-90) with an ion column (IonPac AS14A4 × 250 mm) and detection limits for both NO_3^- and NO_2^- were 0.003 mM. The mixed solution of Na_2CO_3 (8.0 mM) and $NaHCO_3$ (1.0 mM) was used as mobile phase at a flow rate of 1.0 mL min^{-1}.

2.5. DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis

Total soil DNA was extracted using the PowerSoil™ DNA isolation kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. The bacterial 16S rRNA gene was amplified using PCR primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′; 5′ end labeled with 6-FAM) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The PCR mixture contained 1.2 μL of each primer (20 μM), 15 μL of the 2× PCR mix containing Taq polymerase, dNTP and buffer solution, 1 μL of template DNA and 11.6 μL of ultrapure water. PCR was performed using an initial denaturation step of 94 °C for 4 min followed by a program of 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. Triplicate PCRs per sample were performed, and the labeled PCR products were purified with a commercial PCR purification kit (OMEGA biotek, USA). Aliquots of these products were digested with restriction enzyme AluI (TaKaRa Biotechnology, China) at 37 °C for 6 h. The digested PCR products were resolved by electrophoresis using an ABI 3730xl sequencer (Applied Biosystems, USA), with GS-500 Rox as an internal size standard in each lane. The fragment sizes and peak fluorescence intensities were analyzed using GENESCAN software. The relative abundance of individual terminal restriction fragment (T-RF) was calculated as the percentage of its peak area

accounting for the total peak areas in the T-RFLP profile. Only those T-RFs with a relative abundance > 1% were included in the following analysis.

2.6. Clone library construction

For clone library construction, the bacterial 16S rRNA gene was amplified with the same bacterial primers (27F and 1492R) as for T-RFLP analysis except that the 27F primer was not labeled with 6-FAM on its 5′ end. Each PCR mixture (final volume, 30 μL) contained 0.6 μL of each primer (20 μM), 3 μL of the 10× PCR buffer, 2.4 μL of dNTP (2.5 mM), 0.15 μL of Taq polymerase (5 U μL^{-1}), 1 μL of template DNA and 22.25 μL of ultrapure water. Amplification was performed under the same PCR condition as for T-RFLP analysis. The purified PCR products were ligated into the vector PCR2.1 TOPO (Invitrogen, USA) and transformed into *E. coli* DH5α competent cells (TaKaRa Biotechnology, China). Selected clones were grown in 1.5 mL of Luria Bertani (LB) medium amended with 50 μg mL^{-1} of ampicillin. The cloned inserts were reamplified using the PCR primers M13F and M13R (30 cycles of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 1.5 min) and subsequently sequenced with an ABI 3730xl sequencer. Quality filtering and chimera removal was performed using the mothur software. The taxonomy of the 16S rRNA gene sequence was then classified using RDP pipelines.

2.7. Quantitative real-time PCR

Quantification of total bacterial, *Shewanella* spp., *Geobacter* spp., and *Dehalobacter* spp. was performed using a MyiQ™ 2 Optics Module (BIO-RAD, USA) with the primers listed in Supplemental Materials, Table S1. The reaction mixture (total volume, 25 μL) contained 12.5 μL 2× SYBR Premix *Ex Taq* Mix (TaKaRa Biotechnology, China), 0.3 μL of each primer (10 μM), 0.3 μL template DNA and 11.6 μL of ultrapure water. All samples were run in triplicate, and the PCR conditions are listed in Supplemental Materials, Table S1. Finally, a melting curve ranging from 60 °C to 95 °C was performed with steps of 0.5 °C and a hold of 30 s. The quantitative real-time PCR calibration curves were generated using serial dilutions of plasmids containing the cloned target sequences. The plasmid DNA concentration was quantified using a Qubit 2.0 Fluorometer (Invitrogen, NY, USA), and the corresponding gene copy number was calculated according to a previous study (Whelan et al., 2003).

2.8. Accession number of nucleotide sequences

Bacterial 16S rRNA gene sequences have been deposited in the GenBank database under accession numbers KF460281 to KF460396.

3. Results and discussion

3.1. Reductive transformation of PCP

The time course of the degradation of PCP could be fitted to a logistic degradation curve, which has been reported for the degradation of several other organic compounds (Gunasekara et al., 2005; Yang et al., 2011). Based on the logistic model, the maximum transformation rate (μ_m) and kinetic rate constants (k) of PCP can be derived (Supplemental Materials, Table S2). The maximum transformation rate of PCP increased gradually as the concentration of $NaNO_3$ varied between 0 mM and 1 mM (Fig. 1). In contrast, the maximum transformation rate of PCP showed a decreasing trend as the concentration of $NaNO_3$ increased from 1 mM to 30 mM (Fig. 1). These results indicate that low concentrations of $NaNO_3$ addition

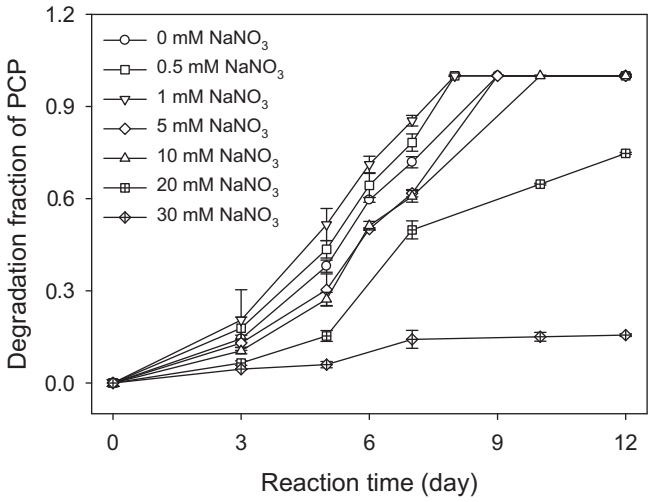

Fig. 1. The variations of the degradation fraction (the percentage of amount of PCP degraded accounting for total amount of PCP added) of pentachlorophenol (PCP) under different concentrations of $NaNO_3$ addition with increasing incubation time.

can enhance the degradation of PCP, while high concentrations of $NaNO_3$ addition may inhibit the degradation of PCP. In the present study, the concentration of NO_3^- addition seemed to be an important factor affecting the degradation of PCP, and 1 mM was a turning point for the variation in the degradation of PCP.

The effect of nitrate addition on the degradation of organic pollutants has been controversial (Braddock et al., 1997; Chen et al., 2002). Previous study has suggested that the degradation of organic pollutants can be accelerated by nitrate addition (Braddock et al., 1997), while other studies have reported that nitrate addition inhibits the degradation of organic pollutants (Chang et al., 2003; Chen et al., 2002). It should be noted that nitrate was added at concentration levels lower than 0.5 mM in Braddock et al.'s study and higher than 3 mM in Chen and Chang et al.'s studies, respectively. From the results of our study in combination with previous reports (Braddock et al., 1997; Chen et al., 2002), we speculate that the concentration of nitrate addition can affect the degradation of organic pollutants. In particular, low concentrations of nitrate addition as a nitrogen source could enhance the reductive dechlorination of organic pollutants by stimulating microbial activity, while high concentrations of nitrate addition may inhibit the degradation of organic pollutants attributable to electron competition. This speculation can be corroborated further with the following microbial community structure analysis.

3.2. Iron and nitrate reduction

The concentrations of dissolved and HCl-extractable Fe(II) in all treatments followed similar variation trends to those of PCP degradation (Fig. 2), increasing markedly during the first 3 days, and significant correlations were also found between the formation rates of dissolved and HCl-extractable Fe(II) and PCP transformation rates (Supplemental materials, Table S2). In other words, the concentrations of dissolved and HCl-extractable Fe(II) increased successively in the treatments with 0.5 mM and 1 mM $NaNO_3$ addition compared with those in the control; in the treatments with 1 mM–30 mM $NaNO_3$ addition, these concentrations decreased successively. These results suggest that low concentrations of $NaNO_3$ addition can enhance the formation of dissolved and HCl-extractable Fe(II), whereas high concentrations of $NaNO_3$ addition may inhibit the formation of dissolved and HCl-extractable Fe(II). However, denitrification processes in all

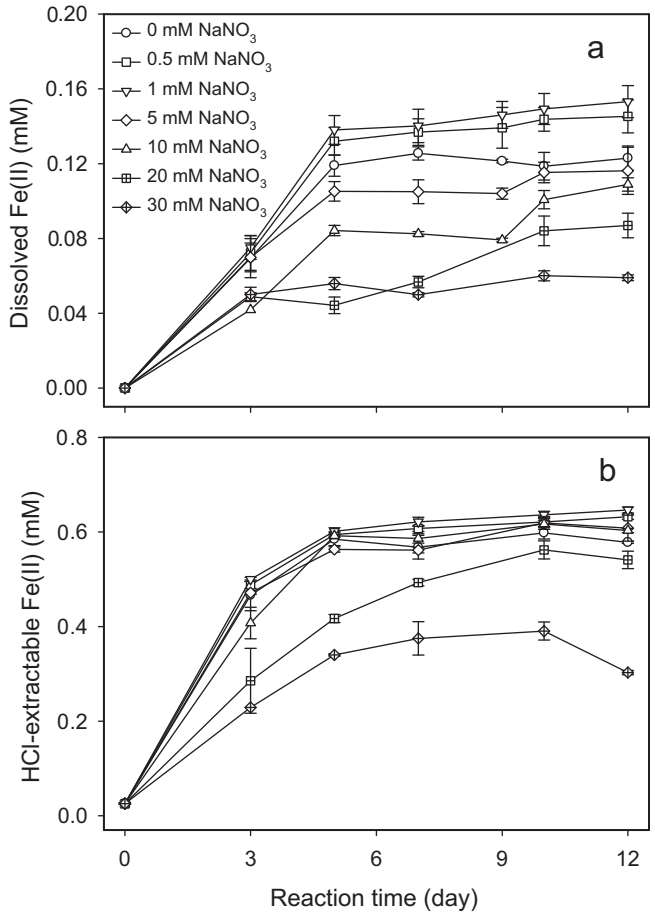

Fig. 2. The concentration variations of dissolved and HCl-extractable Fe(II) in all treatments under different concentrations of $NaNO_3$ addition with increasing incubation time.

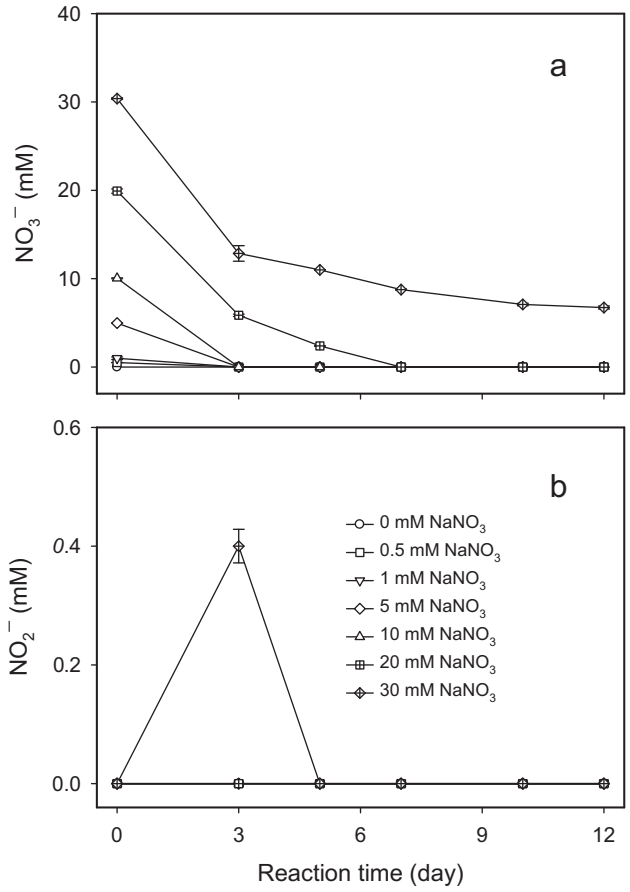

Fig. 3. Concentrations of NO_3^- and NO_2^- in all treatments under different concentrations of $NaNO_3$ addition with increasing incubation time.

treatments progressed rapidly in the first 3 days, and in the treatments with 0.5 mM, 1 mM, 5 mM and 10 mM $NaNO_3$ addition, NO_3^- was reduced completely within 3 days (Fig. 3a). By contrast, NO_3^- needed 7 days to be reduced completely in the treatment with 20 mM $NaNO_3$ addition, and 6.7 mM NO_3^- residue remained in the treatment with 30 mM $NaNO_3$ addition. Furthermore, NO_2^- was not detected in most of the treatments except for 30 mM $NaNO_3$ addition, in which trace amounts of NO_2^- was detected but then disappeared quickly (Fig. 3b), indicating a high transformation rate of NO_2^- in these treatments. Altogether, these results indicate that NO_3^- was transformed to NO_2^- first and then quickly to other forms of nitrogen, such as N_2O and N_2. In addition, no NH_4^+ was detected in any treatment.

Based on the results of the present study, the concentration of NO_3^- addition appeared to be an important factor affecting Fe(III) reduction. However, previous studies (Cooper et al., 2003; Zhang et al., 2012) suggested that Fe(III) reduction in the presence of nitrate can be inhibited, possibly due to the competition of nitrate reduction and Fe(III) reduction for electrons. Nevertheless, the lowest concentration of nitrate addition in these studies was 1 mM, which was a turning point for the results in the present study. Thus, the effect of nitrate addition at concentrations lower than 1 mM on Fe(III) reduction under the same conditions as these studies remains unclear. In addition, it should be noted that similar variation trends were found for concentrations of dissolved and HCl-extractable Fe(II) and the PCP degradation fraction (the percentage of amount of PCP degraded accounting for total amount of PCP added) in the present study. The nitrate reduction rates in the present study seem to be affected mainly by the concentration of nitrate addition, showing an increasing trend with the increase in the concentration of nitrate addition (Fig. 3a). Currently, there are two opinions about the effect of iron oxide on nitrate reduction. First, nitrate reduction can be inhibited by Fe(III) reduction as result of Fe(III) (hydr)oxide coatings formed by the reaction of Fe^{2+} with NO_2^- (Coby and Picardal, 2005). Second, nitrate reduction can be enhanced by iron oxide in the presence of fermentative bacteria capable of Fe(III) reduction under anaerobic conditions, attributable to iron oxide as a conduction band accelerating electron transfer from the fermentative bacteria to nitrate (Zhang et al., 2012). The uncorrelated variation trends of Fe(II) and nitrate reduction and the fast reduction of NO_3^- in the present study most likely indicate the simultaneous existence of both cases mentioned above. If so, fermentative bacteria should be detected in the microcosm, which can be shown by the following microbial community structure analysis.

3.3. *Effect of $NaNO_3$ addition on microbial community structure in the microcosm*

From the discussion in the preceding sections, microorganisms should be of great importance for the reductive dechlorination of PCP, nitrate reduction and iron reduction in the microcosm. Therefore, samples incubated for 0 (control only), 5, 7 and 12 days in all treatments were chosen for examining the variation of microbial community structure by T-RFLP analysis targeting the

bacterial 16S rRNA genes. T-RFs of 69, 154, 175, 229, 235 and 435 bp were detected in all treatments, and the 235 bp T-RF was the most dominant fragment in most of the treatments (Supplemental materials, Fig. S1). The abundances of the 69 and 435 bp T-RFs in all treatments showed no significant variation trends. Moreover, the abundances of the 235 bp T-RF in the treatments with $NaNO_3$ addition incubated for 5 and 7 days were lower than in the control, indicating growth inhibition of this microorganism by $NaNO_3$. However, the abundances of the 154 and 175 bp T-RFs in the treatments with $NaNO_3$ addition were higher than in the control, indicating an enhancement of the growth of these microorganisms due to $NaNO_3$ addition.

The phylogenetic associations of the dominant T-RFs were evaluated by constructing clone libraries from bacterial 16S rRNA genes. A total of 12 dominant T-RFs and their corresponding putative phylogenetic associations are presented in Table 1. The most dominant clones identified in all of the enrichments showed sequence similarity to the *Comamonas* genus (Fig. 4). It has been reported that the *Comamonas* genus is capable of Fe(III) and humic substance reduction coupled to the reductive dechlorination of chlorinated organic compounds under anaerobic conditions (Wang et al., 2009) and the strain in Wang's study has sequence similarity of 98% to our study. The abundances of the *Comamonas* genus in the treatments with a higher degradation rate of PCP and concentration of HCl-extractable Fe(II) (control and 1 mM $NaNO_3$ addition) increased during the first 7 days and then decreased, consistent with the variations in the PCP degradation fraction (Fig. 1) and in the concentration of HCl-extractable Fe(II) (Fig. 2b). The degradation rate of PCP and the concentration of HCl-extractable Fe(II) both showed an increasing trend during the first 7 days and then became steady, indicating that the *Comamonas* genus may be involved in the processes of Fe(III) reduction coupled to the reductive dechlorination of PCP during the first 7 days. After 7 days, the concentrations of Fe(II) nearly reached a stable level (Fig. 2). Therefore, *Comamonas* reduced PCP directly during this period of time. Nevertheless, the reductive dechlorination ability of the microorganism is relatively weak (Wang et al., 2009), so the transformation rates of PCP in the other NH_4Cl treatments were still lower. Reductive dechlorination of chlorinated organic compounds is less thermodynamically favorable than nitrate reduction in terms of electron competition, as indicated by a previous study (Wei and Finneran, 2011). As shown in Fig. 1, the degradation fraction of PCP in the first 3 days was lower than in the next 3 days, which may be attributable to the competition between nitrate reduction and reductive dechlorination during the first 3 days. In addition, the genus *Sedimentibacter*, detected in most treatments (Fig. 4), can assist the genus *Dehalobacter* in completing reductive dechlorination under anaerobic conditions (Maphosa et al., 2012) and the strain in Maphosa's study has sequence similarity of 96% to our study. Finally, the genus *Bacillus*, fermentative bacteria capable of Fe(III) reduction under anaerobic conditions (Boone et al., 1995) with sequence similarity of 96% to our study, was also detected in the microcosm, corroborating the likelihood of enhanced nitrate reduction by iron oxide.

Table 1
Predicted genus associations for dominant terminal restriction fragment (T-RF) lengths based on bacterial 16S rRNA gene sequences.

T-RF length (bp)	Clone number	Predicted genus association	Accession number	Similarity (%)
69	4–56	*Sedimentibacter*	EU703420	98%
154	4–40	*unclassified_Lachnospiraceae*	JX222919	95%
160	2–59	*Ralstonia*	DQ227340	99%
175	2–53	*Ralstonia*	DQ227340	99%
217	2–23	*unclassified_Veillonellaceae*	FJ269098	98%
219	2–38	*unclassified_Veillonellaceae*	FJ269098	98%
229	4–14	*Clostridium sensu stricto*	AJ229197	99%
235	5–53	*Comamonas*	EU817492	99%
243	5–5	*Clostridium sensu stricto*	JX133664	96%
248	4–51	*unclassified_Veillonellaceae*	HQ660792	99%
253	5–38	*Clostridium sensu stricto*	FN667108	96%
443	1–40	*Bacillus*	DQ448748	98%

3.4. Quantity variation of total bacteria, iron-reducing bacteria and dechlorinating bacteria in different treatments

To further examine the effect of $NaNO_3$ addition on the main organisms involved in the dechlorination of PCP or in iron reduction, total bacteria, dechlorinating bacteria in the genus *Dehalobacter* and iron-reducing bacteria in the genera *Shewanella* and *Geobacter* were quantified using quantitative real-time PCR (Fig. 5). The *Geobacter* genus was not detected in all treatments. The copy number of total bacteria was clearly higher in the treatments with $NaNO_3$ addition compared with the control, indicating that the enhanced microorganism growth was derived from $NaNO_3$ addition as a nitrogen source. The genus *Shewanella* is by far the most intensively and extensively investigated of all the identified strains capable of iron reduction (Zachara et al., 1998). The abundance of the *Shewanella* genus in the control first increased, then decreased and finally increased again. Moreover, the copy number of the genus *Shewanella* was higher in the control than in the treatments with $NaNO_3$ addition, indicating that $NaNO_3$ can inhibit the growth of the genus *Shewanella*. Furthermore, there was no consistency between the abundance variation of the *Shewanella* genus and the degradation fraction of PCP, suggesting that iron reduction by the *Shewanella* genus may not be very important during the process of PCP dechlorination. It has been reported that the genus *Dehalobacter* has the ability of dehalorespiration (Sun et al., 2002), and their copy number in the present study were orders of magnitude higher than that of the *Shewanella* genus (Fig. 5). In the control, the abundance of the *Dehalobacter* genus slowly increased initially and then increased markedly after 7 days. Comparably, in the treatments with 1 and 10 mM $NaNO_3$ addition, the copy number of the *Dehalobacter* genus slowly decreased initially and then rapidly increased after 5 days, finally decreasing again. The copy number of the *Dehalobacter* genus reached its highest level at day 8, which was consistent with almost complete dechlorination of PCP at day 8 (Fig. 1). In the treatment with 20 mM $NaNO_3$ addition, the copy number of the *Dehalobacter* genus decreased by several orders of magnitude compared with the control, showing an increasing trend at first and then becoming steady. In contrast, in the treatment with 30 mM $NaNO_3$ addition, the copy number of the *Dehalobacter* genus, lower than in the treatment with 20 mM $NaNO_3$ addition, rapidly decreased at first, then increased and finally decreased again. These results indicate that high concentrations of $NaNO_3$ addition may inhibit the growth of the *Dehalobacter* genus, in agreement with the results that the degradation fractions of PCP in the treatments with 20 and 30 mM $NaNO_3$ addition were lower than in the other treatments and the speculation that high concentrations of nitrate addition may inhibit the growth of certain degraders due to electron competition. Thus, the genus *Dehalobacter* should play a role during PCP dechlorination.

3.5. Environmental implications

In the anoxic natural environment, organic pollutants can coexist with many substrates (or electron acceptors), e.g., nitrate and iron oxides. Therefore, many redox processes, such as nitrate reduction and iron reduction, can affect reductive dechlorination of chlorinated organic pollutants. In particular, in terms of electron

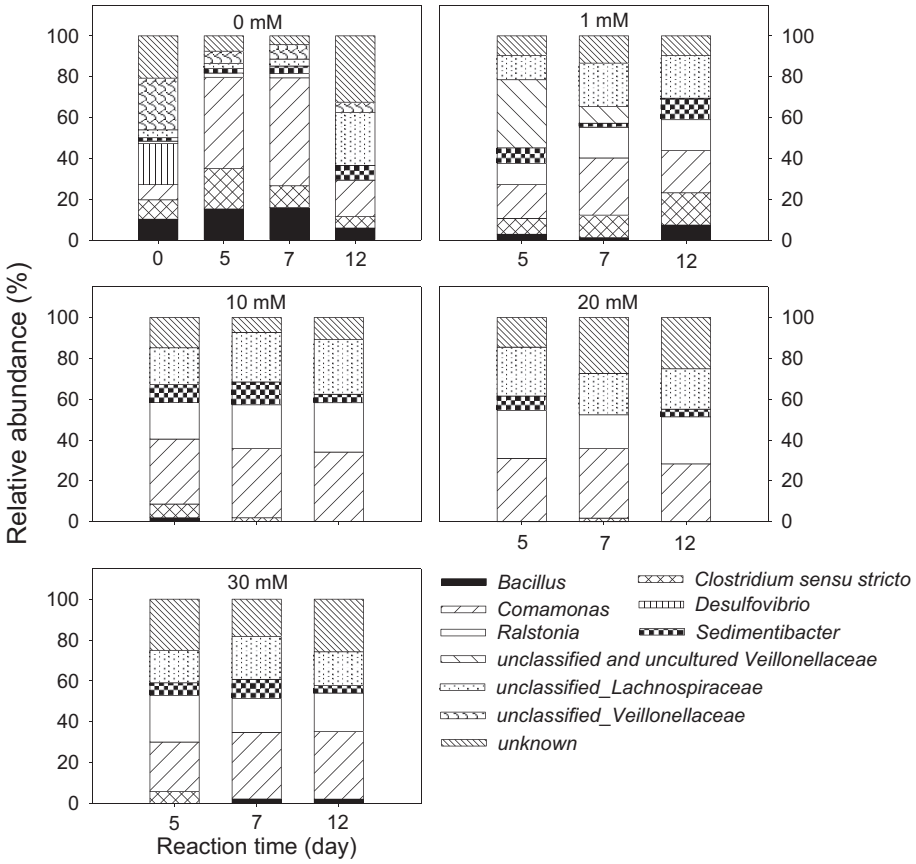

Fig. 4. Microbial community variation in all treatments under different concentrations of $NaNO_3$ addition with increasing incubation time based on terminal restriction fragment length polymorphism (T-RFLP) analysis and clone library construction.

competition, nitrate reduction can inhibit reductive dechlorination and Fe(III) reduction; in terms of electron transfer, Fe(III) reduction can enhance reductive dechlorination. Because of the formation of Fe(III) (hydr)oxide coatings, Fe(III) reduction can inhibit nitrate reduction (Coby and Picardal, 2005). On the other hand, nitrate reduction can be enhanced by iron oxide, as a conduction band accelerating electron transfer in the presence of fermentative bacteria (Zhang et al., 2012). Overall, the results of the present study

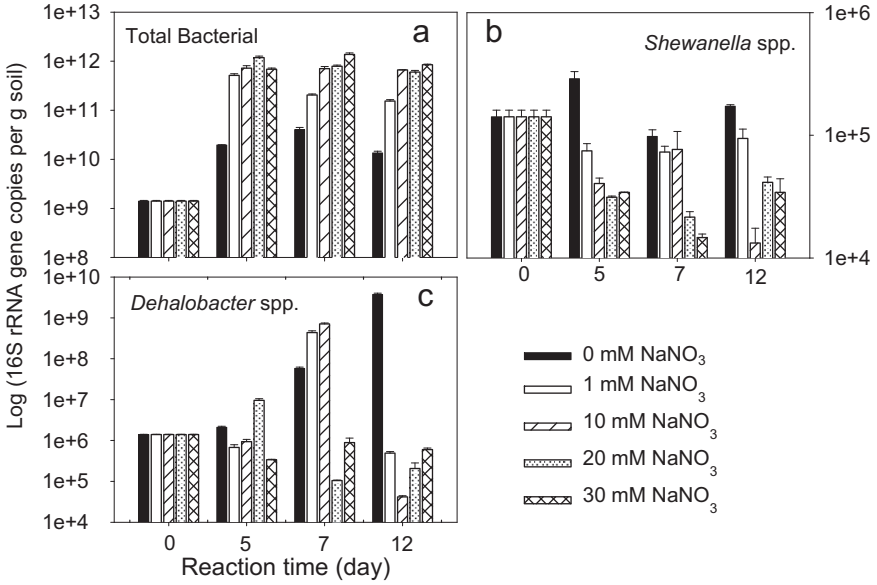

Fig. 5. DNA copy numbers of the 16S rRNA genes of total bacteria (a), *Shewanella* spp. (b) and *Dehalobacter* spp. (c) under different concentrations of $NaNO_3$ addition with increasing incubation time.

provide evidence for interactions between the reductive dechlorination of organic pollutants and the transformation of nitrate and iron species, indicating that microorganisms are very important during these processes. Under natural conditions, these interactions may be more complex than previously thought and thus require further investigation.

4. Conclusions

Concentration of nitrate addition appeared to be an important factor affecting the degradation of PCP, Fe(III) reduction and nitrate reduction. In particular, low concentrations of nitrate as a nitrogen source can enhance reductive dechlorination and Fe(III) reduction, while high concentrations of nitrate can inhibit reductive dechlorination and Fe(III) reduction; nitrate reduction rates increased with the increase in the concentration of nitrate. In addition, iron-reducing bacteria and dechlorinating bacteria may play an important role in the processes of iron reduction and PCP dechlorination.

Acknowledgments

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Appendix A. Supplementary data

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