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The DNA-based Stable isotope probing (SIP) method has become a powerful tool for identifying functional groups of microorganisms that participate in the metabolic processes of ^{13}C labeled substances. Our study used SIP to explore the PCP-degrading microorganisms in iron-rich paddy soil under anaerobic conditions. Combined with the terminal restriction fragment length polymorphism and 16S rRNA clone libraries methods, *Burkholderiales*-affiliated microorganisms were responsible for PCP degradation in anaerobic paddy soil. These findings provide direct evidence for the microorganisms responsible for PCP degradation and induce a new insight into microorganisms linked with PCP degradation in paddy soil with no need of the prerequisite of cultivation.

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Authors: Hui Tong, Min Hu, Fangbai Li, Manjia Chen, Yahui Lv

Corresponding Author: Fangbai Li, Tel.: +86 20 87024721; fax: +86 20 87024123. E-mail address: cefbli@soil.gd.cn

Corresponding Author's Institution: Guangdong Key Laboratory of Agricultural Environment Pollution Integrated Control, Guangdong Institute of Eco-Environmental and Soil Sciences, Guangzhou, P. R. China

Burkholderiales participating in pentachlorophenol biodegradation in iron-reducing paddy soil as identified by stable isotope probing

Hui Tong [a,b,c,1], Min Hu [b,1], Fangbai Li [*b], Manjia Chen [b], Yahui Lv[b,d]

[a] Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, PR China

[b] Guangdong Key Laboratory of Agricultural Environment Pollution Integrated Control, Guangdong Institute of Eco-Environmental and Soil Sciences, Guangzhou 510650, PR China

[c] Graduate University of Chinese Academy of Sciences, Beijing 100049, PR China

[d] School of Chemical and Environmental Engineering, Shanghai Institute of Technology, Shanghai 201418, PR China

*** Corresponding author**. Tel.: +86 20 37021396; fax: +86 20 87024123.

E-mail address: cefbli@soil.gd.cn (F.B., Li).

[1]These authors contributed equally to this work.

Abstract

As the most prevalent preservative worldwide for many years, pentachlorophenol (PCP) has attracted much interest in the study of biodegradation in soil and aquatic ecosystems. However, the key microorganisms involved in anaerobic degradation are less well understood. Hence, we used DNA-based stable isotope probing (SIP) to identify the PCP-degrading microorganisms in iron-rich paddy soil under anaerobic conditions. ^{12}C- and ^{13}C-labeled PCP were almost completely degraded in 30 days under iron-reducing condition. The results of terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA genes showed that 197 and 217 bp (*Hae*III digests) restriction fragments (T-RFs) were enriched in heavy DNA fractions of ^{13}C-labeled samples, and the information from 16S rRNA gene clone libraries suggested that the microorganisms corresponding with these T-RF fragments, which increased in relative abundance during incubation, belonged to the order of *Burkholderiales*, in which 197 and 217 bp were classified as unclassified *Burkholderiales* and the genus *Achromobacter*, respectively. The results of the present study indicated *Burkholderiales*-affiliated microorganisms were responsible for PCP degradation in anaerobic paddy soil and induced a new light on in situ bioremediation in anaerobic PCP contaminated soil.

***Keywords*:** Pentachlorophenol; Stable isotope probing; Biodegradation; T-RFLP**;** Paddy soil

1. Introduction

Iron is the fourth most abundant element in the Earth's crust, and the redox reactions of iron drive element cycling and pollutant transformation in terrestrial and aquatic ecosystems.[1] Under anaerobic conditions, iron reduction coupled to organic compound degradation is the major energy metabolism for microbes in the iron-rich environments.[2] The iron-reducing microorganisms can use Fe(III) as the electron acceptor and mineralize organic matter completely to carbon dioxide.[3] Among the chlorinated organic compounds, the relationship between iron reduction and degradation of chlorinated ethenes, such as tetrachloroethene or trichloroethene has been well studied.[4, 5] However, the effect of iron reduction on chlorophenol degradation in soils is little understood.

Since the 1980s, pentachlorophenol (PCP) has been widely used as a pesticide in Chinese paddy fields, which has negative influence on aquaculture and soil ecosystems.[6] Because of its persistence in soil,[7] abiotic and biotic transformation of PCP in anaerobic soils have received attention during the past decades. Although most of the previous reports focused on the fate of PCP in the environment, the biological mechanism of PCP degradation remains to be further explored. In our previous study, the degradation of PCP was stimulated by indigenous microbial communities under iron-reducing conditions in paddy soil,[8] but which microorganism in the microbial communities were responsible for PCP degradation was remained unclearly.

A large variety of microorganisms have been linked to PCP degradation in pure or complex cultures, and several PCP degraders have been isolated from soil, sediments and wastewater, including *Flavobacterium*, *Arthrobacter*, *Pseudomonas*, *Mycobacterium* and *Sphingomonas*.[9, 10] Several studies

have also reported that the microbial consortium could completely mineralize PCP under anaerobic conditions.[11] *Desulfitobacterium frappieri* PCP-1 isolated from a methanogenic consortium was able to degrade 5 mg L^{-1} PCP in less than one day.[12] Another microorganism *Desulfitobacterium hafniense* also showed the ability to degrade PCP.[13] However, the organisms that have been isolated and cultivated represent a small percentage of PCP degraders in the nature, so it remains a puzzle to determine which organisms are carrying out activities on PCP degradation in the complex systems. Furthermore, the well-studied PCP degraders were all isolated from methanogenic or sulfate-reducing environments, and less is known about the microbes participating in PCP degradation in paddy soil under anaerobic iron-reducing conditions.

Culture-dependent techniques have been widely used in the studies of environmental microbiology. However, only a small proportion of the microbiota has been successfully isolated and cultivated from natural ecosystem thus far.[14, 15] The advent of culture-independent methods, in particular PCR-DGGE, real-time quantitative PCR (qPCR), microarrays and next-generation high throughput sequencing, have been revolutionary the study of soil microbial ecology.[16] However, linking the identity of bacteria with their function in the environment is still a problem in microbial ecology. The recently developed stable isotope probing (SIP) method is a powerful tool for identifying specific functional groups of microorganisms that participate in the metabolic processes of ^{13}C labeled substances.[17] To date, many microorganisms have been identified by SIP, such as phenol,[18] 2,4-dichlorophenoxyacetic acid,[19] 2,4-dichlorophenol,[20] toluene,[21] and polychlorinated biphenyls (PCBs).[22] It has been shown that iron reduction drives organic contaminants transformation under anaerobic conditions.[3] Our previous research suggested that microbial communities could stimulate anaerobic transformation of

pentachlorophenol in paddy soils.[8] However, the group of PCP degradation microorganism in soil has not yet been explored. In our study, SIP was used to investigate the microorganisms responsible for degradation of PCP under iron reduction in anoxic paddy soil enrichment. SIP usually was applied to detect mineralization processes, and complete mineralization of PCP under anaerobic condition has been observed in continuous-flow system[11] and a fixed-film reactors.[23] Combined with the T-RFLP (terminal restriction fragment length polymorphism) and 16S rRNA clone libraries methods, SIP could provide detailed information on indigenous microbes that play active roles in PCP degradation under anaerobic soil environment, and that may provide natural materials for bioremediation of organic pollutants.

2. Materials and methods

2.1. Chemicals

Pentachlorophenol (PCP, ≥ 98% purity) and 1,4-piperazinediethanesulfonic acid (PIPES, ≥ 98% purity) were purchased from Sigma-Aldrich (St Louis, MO, USA). [^{13}C]-PCP (99% atom ^{13}C6) was obtained from AccStandard (New Haven, Connecticut, USA). All other analytical grade chemicals were obtained from the Guangzhou Chemical Co. (Guangzhou, China). Deionized water (18.2 mΩ) was prepared by an ultrapure water system (EasyPure II RF/UV, ThermoScientific, USA) and used in all experiments.

2.2. PCP-degrading microcosms

Soil samples were collected in a paddy soil in Shuilou village (22°21′N, 112°47′E), Taishan, P. R. China. The method for soil collection was described previously.[24] The physicochemical properties of

the soil were analyzed with the method described previously,[25] and the results are as follows: pH (4.75), cation exchange capacity (CEC) (11.06 cmol Kg^{-1}), organic matter (62.46 g Kg^{-1}), complex-Fe (0.99 g Kg^{-1}), dithionite-citrate-bicarbonate (DCB) (10.33 g Kg^{-1}), amorphous-Fe (7.64 g Kg^{-1}), SiO_2 (52.56 %), Al_2O_3 (21.06 %).

Microcosms containing 5 g soil (wet weight), 10 mM lactate, 30 mM PIPES buffer and 8 mg L^{-1} labeled [^{13}C]-pentachlorophenol (99% atom ^{13}C6) or unlabeled pentachlorophenol were incubated in triplicate at a constant temperature of 30 ± 1 °C and pH 7.0 ± 0.1 in serum bottles (100 ml). Neutral or slightly acidic conditions were the optimum pH for PCP biodegradation in soils, and the pH could influence the chemical forms of PCP in environments.[26, 27] Thus we buffered the incubation at pH 7 to maximum the microbial activity in the process of PCP degradation and minimum the amount of PCP sorption on soil particles.[26, 27] The reactors were purged with O_2-free N_2 for 30 min before they were sealed with butyl rubber stoppers and aluminum crimp seals. The experimental reactors were incubated at constant temperature in a dark anaerobic chamber. Sterile controls were obtained by γ-irradiation at 50 KGy. At given time intervals, the bottles were sampled for reaction solution analyses and DNA was extracted from all microcosms.

2.3. Analyses of PCP and intermediates

PCP concentration in the samples was determined by highperformance liquid chromatography (HPLC). The PCP in the soil suspension with 2 ml was extracted with water/ethanol mixtures (1:1 in volume) by shaking on a horizontal shaker (180 rpm min^{-1}) for 1 h.[28] The filtrate from the 0.45 μm syringe filters was collected for HPLC analysis to quantify PCP, using a Waters Alliance 1527-2487 HPLC

system fitted with a Symmetry C18 column (5 μm, 4.6 × 250 mm, Waters, USA)[24]. The PCP transformation intermediates in the suspension were extracted with hexane and identified by Gas Chromatography/Mass Spectrometry (GC/MS) on a Thermo Trace-DSQ-2000 with electron ionization and an Agilent silicon capillary column (0.25 mm × 30 m)[24].

The HCl-extractable Fe(II) in the reaction suspension was determined with the 1,10-phenanthroline colorimetric method.[29] The soil suspension sampled from each reactor was extracted with 0.5 M HCl for 1.5 h and then filtrated. The filtrate was analyzed with a spectrophotometer at 510 nm.

2.4. Soil genomic DNA extraction and ultracentrifugation

The sample suspension was centrifuged for collection ~ 0.25 g soil, and then the DNA in soil was extracted from ^{13}C-labeled and unlabeled PCP microcosms using a PowerSoil™ DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was quantified by Qubit 2.0 fluorometer DNA (Invitrogen, NY, USA), then ~10 μg DNA was loaded into Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 ml, Beckman Coulter) along with a Tris-EDTA (TE, pH 8.0)-CsCl solution. Before the tubes were sealed, buoyant densities (BD) were measured with a model AR200 digital refractometer (Reichert, Inc., USA). The centrifugation was performed at 178,000 × g (20 ^{o}C) for 48 h in a Stepsaver 70 V6 vertical titanium rotor (eight tubes, 5.1 ml capacity each).[21] Following centrifugation, the tubes were placed onto a fraction recovery system (Beckman), and fractions (150 μl) were collected. The BD of each fraction was measured, and DNA was retrieved from each fraction with the EZNA™ MicroElute DNA Clean Up kit (OMEGA Biotek, USA).

2.5. Experiments for PCR, T-RFLP and 16S rRNA gene sequencing

The ultracentrifugation fractions of DNA from ^{12}C- and ^{13}C-PCP amended microcosms were used as template to recover 16S rRNA gene sequences. T-RFLP fingerprinting of density-resolved DNA fractions was done with primers 27F-FAM (5'-AGAGTTTGATCMTGGCTCAG-3', 5' end-labeled with carboxyfluorescein) and 1492R (5'-GGTTACCTTGTTACGACTT-3'); the purified PCR products were digested with *Hae*III, *Alu*I and *Rsa*I (New England Biolabs) and the data were analyzed with GeneScan software, all as described previously.[21]

To identity the taxonomic information of each T-RF fragment, numbers of clones were randomly selected and sequenced from clone libraries of heavy fraction DNA. Then the predicted sites of restriction endonuclease on each 16S rRNA gene sequences were computed in silicon and the most close fragment length was matched to the corresponding T-RF. Lastly, the represented bacteria for each T-RF were identified through the taxonomic information of the 16S rRNA gene sequences. The purified heavy fraction ^{13}C-labeled and unlabeled PCR products were cloned into vector pGEM-T Easy (Promega, USA) and then transformed to *E. coli* DH5*α* competent cells. Selected clones were grown in 1.5 mL Luria-Bertani medium with 50 μg L^{-1} ampicillin. Clones were screened for inserts with PCR primers M13F(5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-AACAGCTATGACCATG-3') and subsequently sequenced with an ABI 3730*xl* sequencer. The high quality 16S rRNA sequences were subjected to chimera removal and phylogenetic classification using mothur software.[30]

2.6. Quantification of bacterial 16S rRNA genes in SIP gradient

The bacterial 16S rRNA genes were determined by qPCR on a MyiQ™ 2 Optics Module (BIO-RAD, USA) with the primers 338F and 518R and the reaction mixture of the system was based on previously reported methods.[31]. The qPCR calibration curves were generated with serial dilutions of plasmids containing the cloned target sequences. The plasmid DNA concentration was quantified by Qubit 2.0 Fluorometer (Invitrogen, NY, USA), and the corresponding gene copy number was calculated relatively to the plasmid size, insert lengths and Avogadro number.[32]

The nucleotide sequence data were deposited in GenBank under accession numbers KM100457 – KM100567.

3. Results

3.1. PCP degradation in Soil Microcosms

The PCP degradation processes in the soil of the microcosm experiments under different conditions are presented in Fig. 1. The PCP concentration declined rapidly, with approximately 50% PCP removal after 10 days and complete degradation after approximately 30 days, compared with low percentage (~ 5%) of PCP removal in the sterile control, which was likely due to soil sorption. The difference between the sterile and unsterile soil confirmed a biological removal mechanism (Fig. 1). Two degradation mechanisms involving dechlorination and ring-cleavage are expected for PCP degradation. The dechlorination products were analyzed by GC-MS, and during the microbial degradation of PCP, several intermediates were detected, including the major products were 3,4,5-TCP, 4-CP and phenol (Fig. 2), and the mass balance of chlorophenols (PCP and its intermediates) showed that chlorophenols were ring-cleavage after 10 days.

3.2. Microbial community in PCP biodegradation

To investigate the distribution of bacterial 16S rRNA genes in the microcosms of PCP degradation, the qPCR was performed with general bacterial primers 338F and 518R and one time point 28 d was choose in this study. Each fraction collected from the ultracentrifuge tubes was used for qPCR to access comparative DNA distribution in light and heavy fractions (Fig. 3). The results showed that the maximum copies had a significant shift in the heavy fractions between ^{12}C and ^{13}C-PCP samples, which indicated higher label incorporation into the DNA. The peak shift suggested that a portion of bacteria assimilated the ^{13}C during the anaerobic biodegradation of PCP.

Bacterial 16S rRNA gene clone libraries were constructed from heavy gradient fractions (BD up to 1.737 g ml^{-1}) with ^{12}C- and ^{13}C-PCP amended microcosms. The bacterial community composition was shown in (Table 1). Most clones belonged to *Proteobacteria*, and the percentage of α, γ and δ subdivision of *Proteobacteria* were roughly the same in the ^{13}C and ^{12}C libraries. Only the *Burkholderiales*-related sequences constituted 28.6% of total sequences in the ^{13}C library compared with 1.8% in the ^{12}C library. In addition, *Actinobacteria*, *Acidobacteria*, and *Firmicutes*-related sequences were also detected in the ^{13}C and ^{12}C libraries.

Fragments were shown throughout the T-RFLP fingerprinting from all gradient fractions for ^{12}C and ^{13}C-PCP treatment, however only two fragments (197 and 217 bp) were enriched in the heavy ^{13}C fractions, while such enrichment was not observed in the corresponding ^{12}C fractions (Fig. 4). The relative abundance (RA) of two dominant peaks in the T-RFLP profiles were presented in (Fig. 5). This trend indicated that the ^{13}C labeled PCP was incorporated into the biomass of particular

organisms. In our investigation, PCP degradation occurred rapidly after 10 days, and the RAs of 197 and 217 bp at the two later time points in heavy fractions were higher than at the first time point 10 days. During the cultivation process, the maximum RA of T-RF 197 and 217 bp in ^{13}C sample were 36.86% (1.74 g ml^{-1}) and 38.5% (1.74 g ml^{-1}), respectively (Fig. 5). After 49 days, the microorganisms mainly assimilated ^{13}C with the intermediate products of PCP degradation. The microorganisms represented by the two dominant T-RF fragments should be responsible for the PCP and its breakdown products degradation.

To identify the represent active microorganisms of the key T-RF fragments involved in PCP degradation, the 16S rRNA clone library in the PCP degradation microcosms was investigated (Table S1). The 16S rRNA sequences correspond to the two PCP-degrading related T-RF fragments (197 and 217 bp) belonging to order *Burkholderiales*. 197 bp T-RF was affiliated with unclassified *Burkholderiales*, and the other T-RF of 217 bp was assigned to genus *Achromobacter* or *Duganella* (each has an endonuclease recognition site of 217 bp from analysis of the clone sequences). To confirm which microorganisms were truly responsible for the 217 bp in the ^{13}C-DNA heavy fraction, three additional restriction enzymes (*Alu*I, *Hha*I and *Rsa*I) were used for the ^{13}C enriched heavy fractions. These dominant T-RFs obtained from each restriction enzyme were compared to those endonuclease recognition sites in each 16S rRNA gene clone library (Table S2). From the above T-RFLP results, the microorganism enhancing the PCP degradation of 217 bp was the genus *Achromobacter*. The slight difference (two or three bases) between the measured fragment lengths and those predicted using sequence data have also been noted by others.[33] In addition, the clone libraries showed that not only the *Burkholderiales* but also *Enterobacteriales* had been very frequent.

Enterobacteriales had been found in the ^{13}C-labeled and unlabeled heavy fractions with the similar abundance, simultaneously, indicating no shift in the heavy fractions between the ^{12}C and ^{13}C samples. Therefore, the enrichment of *Enterobacteriales*'s DNA in heavy gradient fraction should not account for the ^{13}C assimilation. The distribution of DNA in the different gradient fraction in CsCl is not only controlled by the ^{13}C-labeled nucleic acid, but also the content of G + C.[34] The distribution of *Enterobacteriales*'s DNA in the heavy gradient fraction may be owing to its feature in high GC content.

4. Discussion

In this study, the results confirmed the degradation of PCP by indigenous bacterial community of paddy soil with no chlorinated phenols detected in soil. And the similar research have been presented in grassland soil by Mahmood, in which PCP concentration decreased from initial 200 mg Kg^{-1} to 92 mg Kg^{-1} for 9 weeks at 15 °C.[35] The degradation rate was lower than observed in our study, in which PCP (80 mg Kg^{-1}) was completely degraded after 4 weeks incubation at 30 °C. The higher rate of PCP degradation may arise from high incubation temperature and high-activity of indigenous microorganisms.

The degradation pathways of PCP were similar with polychlorinated biphenyls (PCBs) , including dechlorination and mineralization. Anaerobic and aerobic biodegradation of PCBs have been the subject of a large body of research during the past decades. Then, a sequential anaerobic-aerobic treatment of PCBs has been successfully tested in microcosms with sediments.[36] Recently, several bacteria and genes involved in the PCB degradation process were identified by SIP. The main degraders in a biofilm community on PCB droplets were revealed as Burkholderia species by using

DNA-SIP.[37] In another DNA-SIP study, the genera Achromobacter and Pseudomonas that acquired carbon from ^{13}C-biphenyl were found in the PCB-contaminated river sediment.[22] In addition, the functional genes were explored using the Geochip and PCR amplified sequences in ^{13}C-DNA heavy fraction from PCB-contaminated soil.[38] The aerobic transformation of chlorinated aromatic compounds involves oxygenase enzymes, molecular oxygen, and a source of reducing equivalents.[39] But, under anaerobic condition, the oxygen is replaced by nitrate, Fe(III) and sulfate as electron acceptors, and the biodegradation of chlorinated aromatic compounds are promoted by nitrate, Fe(III) and sulfate reduction.[40] Anaerobic PCP degradation has been studied under nitrate-reducing, sulfate-reducing, iron-reducing and methanogenic condition.[8, 41-43] Being the fourth most abundant element on earth and the most frequently utilized transition metal in the biosphere, iron naturally undergoes active reactions between ferrous and ferric states in circumneutral pH or acidic environments.[44] It is worthwhile to note that in anaerobic environments microbial Fe(III) reduction is an important pathway of anaerobic mineralization of organic matter.[2]

In our previous study, it was suggested that an electron donor (lactate) and electron shuttle (anthraquinone-2,6-disulfonate)could accelerate PCP transformation in iron-rich paddy soils,[8] and that the microbial community structure changed after biostimulation by the additions of lactate and/or AQDS during PCP degradation processes where *Clostridium* sp. increased its abundance during incubation. However, direct evidence is lacking to support *Clostridium* sp. as the PCP-degrading bacteria in iron-reducing paddy soil. The previous research combined DNA- and RNA-SIP with DGGE (denaturing gradient gel electrophoresis) methods to explore the bacteria involved in degradation of PCP in pristine grassland soil under oxic condition.[35] However, large differences

existed between the geochemical properties of aerobic oxidizing grassland soil and anaerobic reducing paddy soil contained large amounts of iron oxides. The pathways and mechanisms of PCP degradation were complete different under aerobic and anaerobic conditions, involving different microorganisms and functional genes.[9, 45] In the current study, SIP was applied to identify the key microorganisms responsible for PCP degradation in anaerobic reducing paddy soil. During the PCP degradation in microcosms, the generation of 0.5 HCl-extractable Fe(II) increased steadily, which indicated a dominant Fe-reducing process (data not shown). It has been shown that microbial Fe(III) reduction can promote the dechlorination of chloroalkane,[4] but the effect of Fe(III) reduction on chlorophenol biodegradation is less understood, especially the natural microbiota involved in the dechlorination or mineralization processes.

In present study, during the PCP degradation, the percentage of the same heavy fragment increased greatly and enriched highly (~ 40%). The PCP degradation process included dechlorination and ring-cleavage stages, thus the microorganisms may also catalyze the ^{13}C intermediate products. Therefore, it is difficult to distinguish whether the ^{13}C-DNA originated from microorganisms directly utilizing the ^{13}C-PCP substrate or the intermediate products, and cross-feeding might occur. The cross feeding may result in dispersal of label among microorganisms not directly involved in PCP degradation. At the early time point (10 d), the two T-RF fragments (197 and 291 bp) were enriched in the ^{13}C-DNA heavy fraction and had not been detected in the control ^{12}C-DNA heavy fractions (Fig. 4). And the maximum relative abundance of T-RFs in the ^{13}C heavy fractions (BD > 1.74 g mL^{-1}) fitted well to the pseudo-first order kinetic model (Fig. S1). Therefore, the cross feeding was not a major limitation in our research and these results suggested that the organisms represented by these two T-RF

fragments which initially attacked PCP were the most important candidates involved in PCP degradation.

The 16S rRNA sequences corresponding to T-RF fragments 197 bp and 219 bp (*Hae*III digestion) belonged to the *Burkholderiales* of *β-Proteobacteria*， which carried out the biodegrading potential for aromatic compounds.[45] Previous reports had linked *Burkholderiales* to the degradation of organic compounds, such as pentachlorophenol,[11, 35] 2,4-dichlorophenoxyacetate,[19] phenol,[18] toluene.[21] Organic contaminant degraders include members belonging to the *Burkholderiales* order such as *Burkholderiaceae*, *Comamonadaceae* and *Alcaligenaceae*. *Burkholderia* was reported as one of the most relative cultivated microorganisms in PCP degradation in grassland soil,[35] and the DNA-SIP revealed *Burkholderia* species were the active polychlorinated biphenyls degraders in a biofilm community.[37] *Comamonadaceae*-related bacteria have been isolated from forest sediment and have the capability to degrade 2,4-dichlorophenoxyacetic acid under iron-reducing conditions.[46] *Comamonadaceae* has also been identified by the SIP method as the major benzene degrader in different soil types.[21, 47] *Alcaligenaceae* has been reported as the dominant microorganism in PCP degradation.[48]

In the current study, the two identified PCP degrading microorganisms were classified as the unclassified *Burkholderiales* and the genus *Achromobacter* of the class *β-Proteobacteria*. The closest relatives of three 16S rRNA gene sequences of the unclassified *Burkholderiales* were obtained from the surface water of Kalahari Shield (DQ223206, 98%), polluted soil (GQ487960, 98%), and Songhuajiang River sediments (DQ444086.1, 97%). The three similar sequences were not associated

with PCP degradation, however, others had been identified as able to assimilate similar labeled organics, such as 2,4-dichlorophenoxyacetate and PCBs.[19, 37] Another identified PCP degrading microorganism *Achromobacter* was a well-known organic pollutant degrader. *Achromobacter* sp. was carried on the 2,4-D degrading in an airlift inner-loop bioreactor.[49] In the polychlorinated biphenyl degradation, *Achromobacter* was revealed as the dominant organism by SIP.[22] Compared to the above aerobic condition, the organic compounds anaerobic degradation by *Achromobacter* has been rarely described. Under nitrate-reducing conditions, *Achromobacter* sp. strain PC-07 was able to degrade *p*-cresol.[50] Under similar conditions, the isolated 1,2-dichloroethane degrading microorganisms were closest to *Achromobacter xylosoxidans*.[51] Furthermore, *Achromobacter* related bacteria were isolated from a PCP-contaminated soil,[52] which suggested its important role in PCP-biodegradation. The results of the present study indicated that *Achromobacter* targeted by ^{13}C was a prominent anaerobic PCP degrader within the family of *Burkholderiales*. However, further research is necessary to understand the fundamental mechanisms of biodegradation PCP by *Achromobacter*.

5. Conclusion

Previous research has successfully applied SIP to identify organisms capable of degrading PCP in grassland soil,[35] but as far as we know, this is the first application of the SIP technique to an anaerobic soil system involving PCP biodegradation. Our study demonstrates that a DNA-SIP combined molecular biology method, such as T-RFLP, is a useful tool to link phylogeny of microorganisms to their capacity to degrade and assimilate particular organic pollutants. The data also suggests that *Burkholderiales* is responsible for PCP degradation in the anaerobic iron-reducing environment and may help to understand the biological mechanism of chlorophenol degradation under anaerobic

conditions.

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Figure Captions

Fig. 1. Concentration of pentachlorophenol (PCP) over time in sterile control and samples amended with ^{13}C-PCP or ^{12}C-PCP. The error bars represent standard deviations.

Fig. 2. The PCP (8 mg L^{-1}) transformation products concentration across reaction time.

Fig. 3. Quantitative of bacterial 16S rRNA gene distribution in DNA gradients from soil samples amended with ^{12}C- or ^{13}C-PCP.

Fig. 4. Compare of heavy fraction TRFLP profiles from ^{12}C and ^{13}C-PCP amended soils to illustrate the dominance of fragments 197 bp and 217 bp in labeled heavy fractions.

Fig. 5. Relative abundance of fragments (digested by *Hae*III) assigned to unclassified *Burkholderiales* (197 bp) and *Achromobacter* (217 bp). Symbols: A, PCP, ~ 50% degraded, 10 days; B,PCP, ~ 100% degraded, 28 days; C, the reaction after 49 days.

453 **Figure 1**

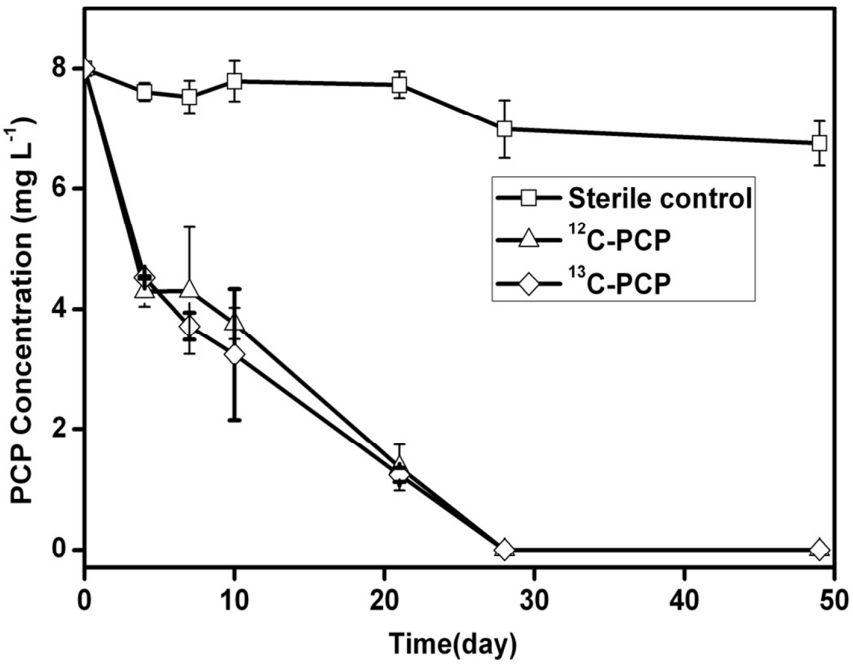

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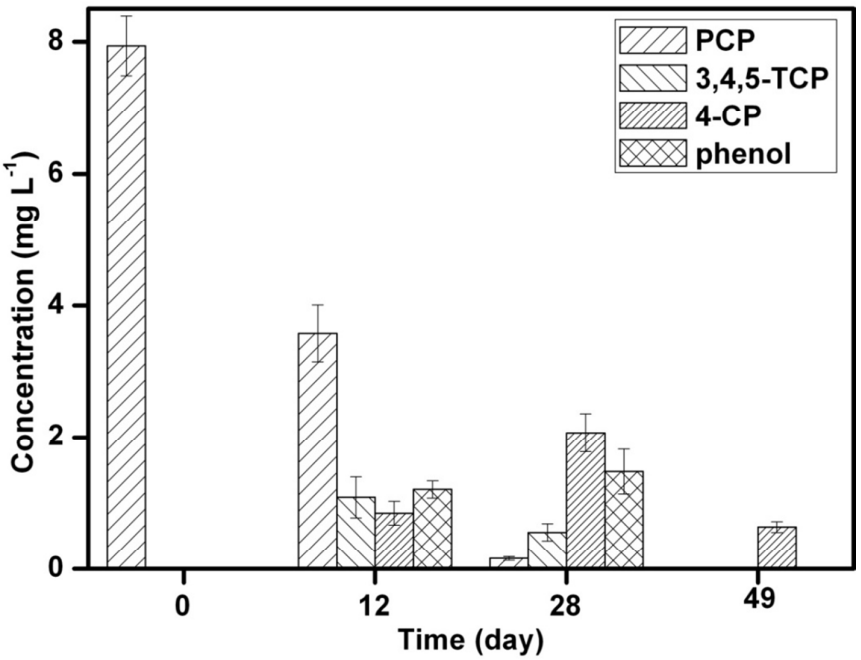
Figure 2



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457 **Figure 3**

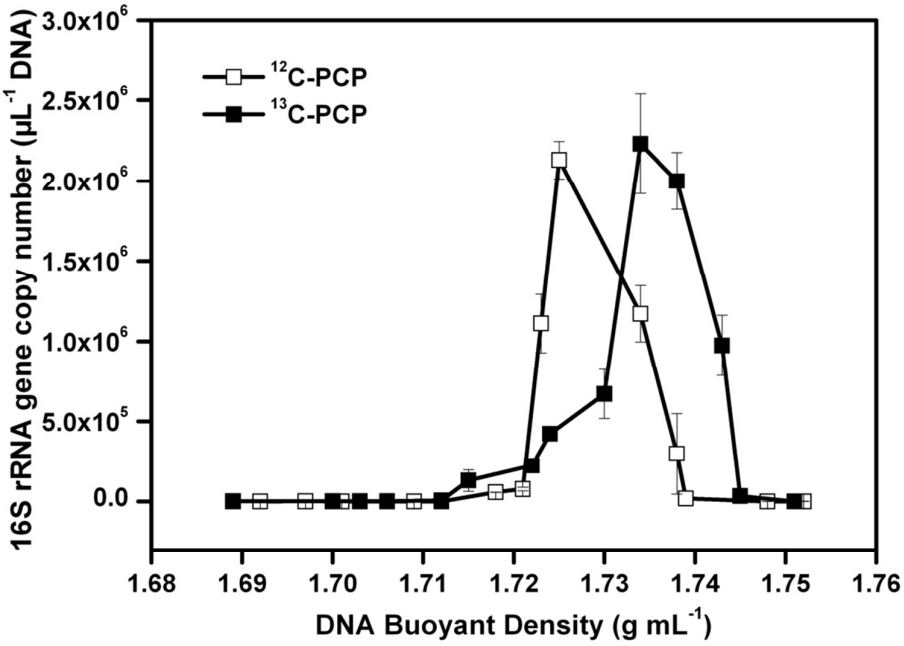

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Figure 4

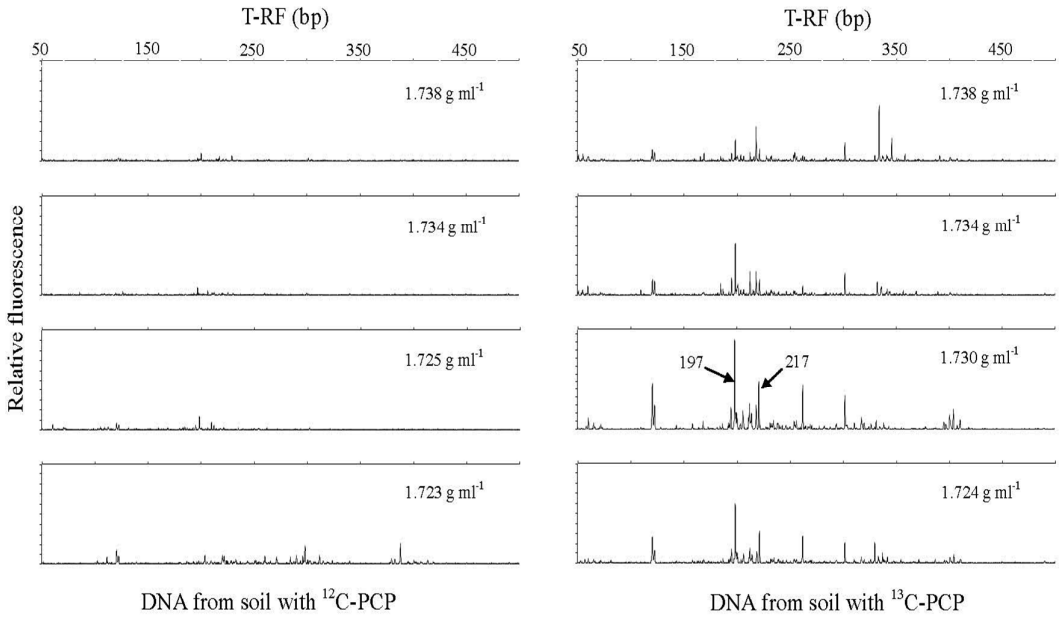

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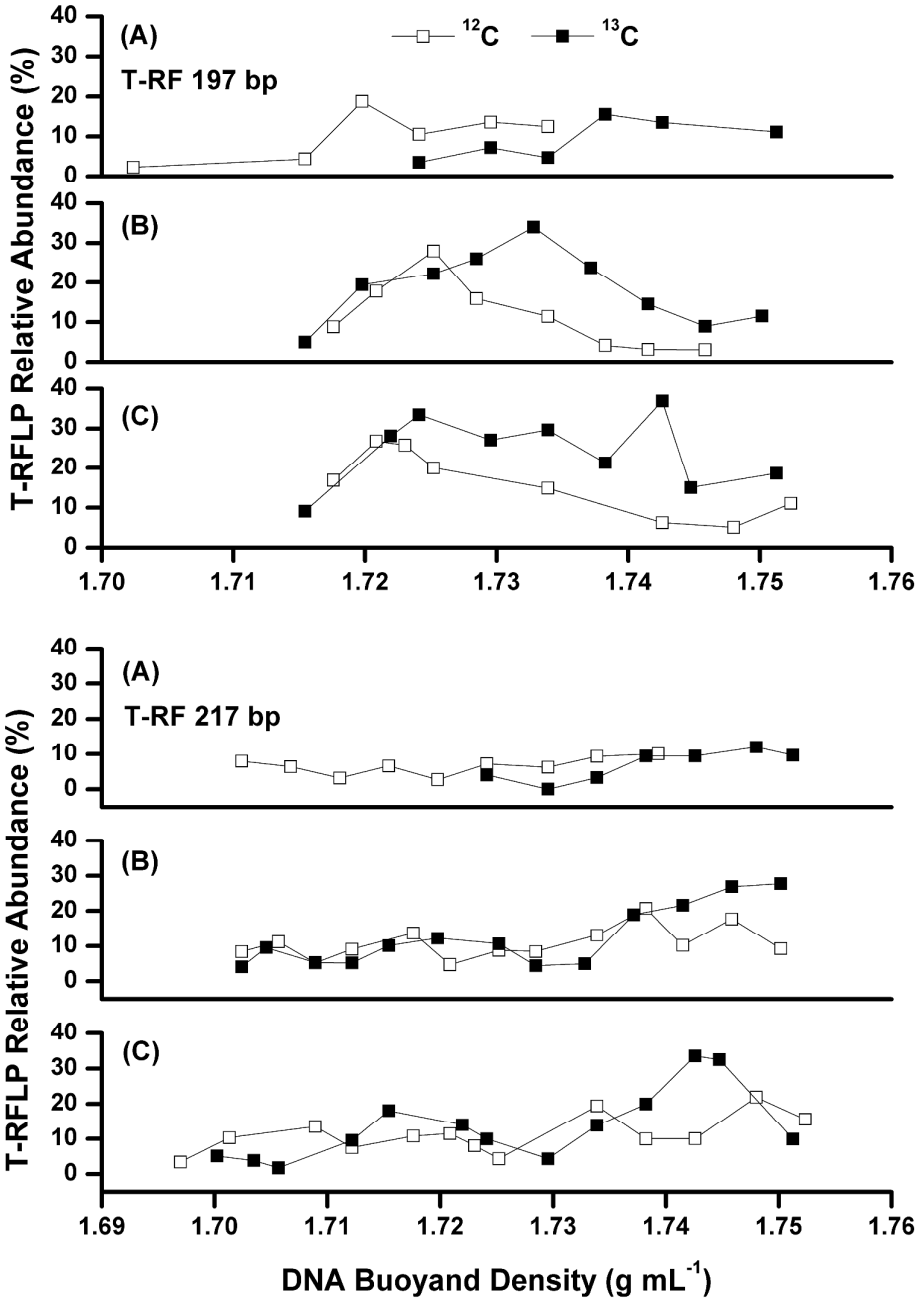
Figure 5



462

Table 1. Phylogenetic affiliations and numbers of 16S rRNA clone sequences retrieved from heavy fractions of microcosm incubated with ^{13}C-PCP and ^{12}C-PCP (control treatment).

Phylogenetic Group	Heavy fraction Clones (n)	Control treatment Clones (n)
β-proteobacteria		
Burkholderiales	16 (28.6%)	1 (1.8%)
Hydrogenophilales	1	
Unclassified	5	7
γ-proteobacteria		
Xanthomonadales	5	3
Enterobacteriales	12	12
Pseudomonadales	4	
Unclassified	1	1
δ-proteobacteria		
Syntrophobacterales	2	2
Desulfuromonadales	2	2
Nannocystineae		2
α-proteobacteria		
Rhodospirillales		2
Rhizobiales		1
Unclassified	1	
Actinobacteria		
Actinobacteria	1	1
Planctomycetacia		
Planctomycetales		6
Chloroplast		
Chloroplast	1	
Clostridia		
Clostridiales	3	1
Acidobacteria	1	6
Unidentified affiliation	1	8
Total	56	55