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The key microorganisms for anaerobic degradation of pentachlorophenol in paddy soil as revealed by stable isotope probing

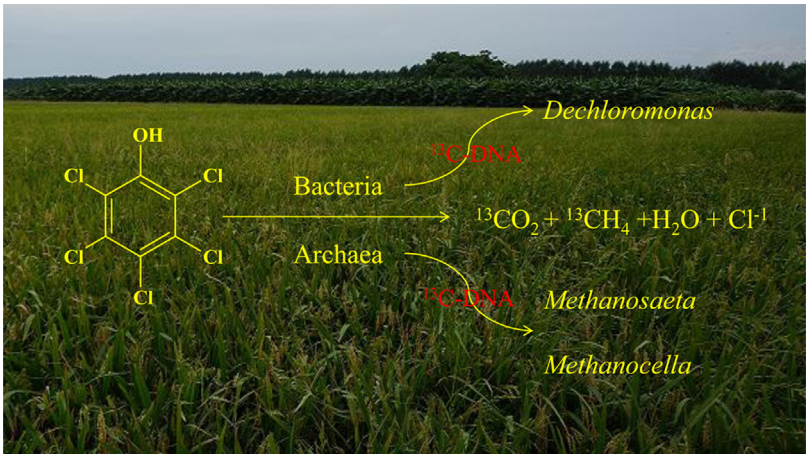
Hui Tong[a,b,c], Chengshuai Liu[d], Fangbai Li[a,*], Chunling Luo[b], Manjia Chen[a], Min Hu[a]

[a] Guangdong Key Laboratory of Agricultural Environment Pollution Integrated Control, Guangdong Institute of Eco-Environmental and Soil Sciences, Guangzhou 510650, PR China
[b] Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, PR China
[c] University of Chinese Academy of Sciences, Beijing 100049, PR China
[d] State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550009, PR China

HIGHLIGHTS

- SIP suggested that *Dechloromonas* can mineralize PCP in soil.
- *Methanosaeta* and *Methanocella* acquired PCP-derived carbon.
- Lactate enhanced microbial degradation of PCP in soil.

GRAPHICAL ABSTRACT



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ABSTRACT

Pentachlorophenol (PCP) is a common residual persistent pesticide in paddy soil and has resulted in harmful effect on soil ecosystem. The anaerobic microbial transformation of PCP, therefore, has been received much attentions, especially the functional microbial communities for the reductive transformation. However, the key functional microorganisms for PCP mineralization in the paddy soil still remain unknown. In this work, DNA-based stable isotope probing (SIP) was applied to explore the key microorganisms responsible for PCP mineralization in paddy soil. The SIP results indicated that the dominant bacteria responsible for PCP biodegradation belonged to the genus *Dechloromonas* of the class β-*Proteobacteria*. In addition, the increased production of $^{13}CH_4$ and $^{13}CO_2$ indicated that the addition of lactate enhanced the rate of biodegradation and mineralization of PCP. Two archaea classified as the genera of *Methanosaeta* and *Methanocella* of class *Methanobacteria* were enriched in the heavy fraction when with lactate, whereas no archaea was detected in the absence of lactate. These findings provide direct evidence for the species of bacteria and archaea responsible for anaerobic PCP or its breakdown products mineralization and reveal a new insight into the microorganisms linked with PCP degradation in paddy soil.

* Corresponding author. Tel.: +86 20 37021396; fax: +86 20 87024123.
E-mail address: cefbli@soil.gd.cn (F. Li).

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1. Introduction

Chlorophenols (CPs) are commonly used as pesticides, herbicides, and wood preservatives [1]. Among the CPs, the pentachlorophenol (PCP) characteristics of high toxicity and persistence have results in their classification as a priority pollutant by the US Environment Protection Agency [2]. Since the 1980s, PCP has been used widely as a pesticide in Chinese paddy fields, which has negatively influenced the aquaculture and soil ecosystems [3]. Due to PCP's relative persistence, slow attenuation and long field half-life in the environment [4], PCP degradation in anaerobic soil has received increased attention during recent decades. Until now, most of the previous reports have focused on reductive dechlorination of CPs. However, during degradation of PCP, the aromatic intermediates are also toxic and bio-accumulative [5]. Therefore, the incubation of degradation microcosms for complete mineralization of PCP to CO_2 is necessary, and the biological mechanism of complete mineralization remains to be further explored. In previous studies, a number of microorganisms were linked to PCP degradation in pure or complex conditions [6–8], and several bacteria were shown to have the potential to mineralize PCP [9–11] in addition to methanogenic archaea with syntrophic associations between several archaea [12,13]. Additional knowledge of the microbial communities that can mineralize CPs in environmental samples is needed to enhance our understanding of this process.

The DNA-based SIP method is a powerful tool for identifying specific functional groups of microorganisms that participate in the metabolic processes of ^{13}C labeled substances [14]. This method involves the extraction and separation of labeled DNA and a combination of molecular-based methods, including terminal restriction fragment length polymorphism (T-RFLP), clone libraries, denaturing gradient gel electrophoresis (DGGE), and others. Until now, the microorganisms responsible for the degradation of a variety of organic pollutants have been identified via SIP, such as phenol [15], 2,4-dichlorophenoxyacetic acid [16], 2,4-dichlorophenol [17], toluene [18], and polycyclic aromatic hydrocarbons [19]. Recently, SIP was used to determine the PCP degradation microorganisms using the DGGE method in grassland soil under aerobic conditions [20]. In another study, PCP 4-monooxygenase (*pcpB*) genes were detected in the ^{13}C-heavy fractions DNA, and the hypothesis was that several microorganisms were linked with PCP biodegradation [21]. However, no study has reported on PCP anaerobic biodegradation in paddy soil using SIP. In addition, the fate of ^{13}C i.e., mineralization, biomass or residue was not investigated in previous work and identification of the particular microorganisms (both bacteria and archaea) that are directly responsible for ^{13}C label uptake from PCP degradation has not been clearly completed.

In this work, we take the advantage of the DNA-based SIP with ^{13}C PCP as a substrate to explore the key microorganisms for PCP mineralization in paddy soil under anaerobic conditions. The methods involve DNA extraction, ultracentrifugation, and fractionation, which separate labeled DNA from unlabeled DNA. Each fractional analysis was carried out via TRFLP and quantitative PCR. Both bacteria and archaea were targeted which might incorporate ^{13}C PCP or its breakdown products into their DNA. The mineralization products of $^{13}CH_4$ and $^{13}CO_2$ were determined to evaluate the mineralization rate. Our results provide direct evidence for the active microorganisms linked with mineralization of PCP and offer a new insight into the anaerobic biodegradation of PCP in paddy soil.

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). The [$^{13}C_6$]-PCP (99% atom $^{13}C_6$) was obtained from Cambridge Isotope Laboratories (Cambridge, MA, USA). All other analytical grade chemicals were obtained from the Guangzhou Chemical Co. (Guangzhou, China). Deionized water (18.2 MΩ) was prepared in an ultrapure water system (Easy Pure II RF/UV, ThermoScientific, USA) and used in all experiments.

2.2. Microcosm experiments for PCP degradation in paddy soil

Soil sample was collected in a rice paddy field in Nanchang village (22°01′33.6″N, 112°47′25.7″E), Taishan City, PR China. The paddy soil was formed by complex river sediment deposition in the bay with the typical characteristics of an alluvial plain. No chlorinated phenols were detected in soil and our previous studies have found the chlorinated compounds can be degraded by the microbial communities in this soil [22,23]. The method for soil collection was described previously [24]. The basic physicochemical properties of the soil were analyzed with a previously described methods [25], and the results were as follows: pH (4.64), cation exchange capacity (CEC) (8.82 cmol kg^{-1}), organic matter (17.4 g kg^{-1}), complex–Fe (1.23 g kg^{-1}), dithionite-citrate-bicarbonate (DCB) Fe (16.8 g kg^{-1}), amorphous–Fe (4.99 g kg^{-1}), SiO_2 (52.6 %), Al_2O_3 (21.1%).

Batch microcosm experiments in 100 ml serum bottles were carried out in an anaerobic chamber in triplicate at a constant temperature of 25 ± 1 °C and pH 7.0 ± 0.1. Neutral or slightly acidic conditions were the optimum pH for PCP biodegradation in soils [26]. Each bottle contained ~5 g soil (wet weight) in 25 ml medium, with or without 10 mM lactate [24,27], 30 mM PIPES buffer, and 8 mg L^{-1} labeled [^{13}C]-PCP (99% atom $^{13}C_6$) or unlabeled PCP. After addition of chemicals, the bottles were sealed with butyl rubber stoppers and aluminum crimp seals and finally wrapped with foil. The bottle was subsequently incubated in an anaerobic chamber. The controlled experiments were conducted using the same procedures using chemicals sterilized by γ-irradiation at 50 kGy. At given time intervals, samples in triplicate bottles were taken out for DNA extraction and measurements of PCP, CH_4, CO_2, and biomass.

2.3. Analyses of PCP and intermediates

The PCP concentrations were determined by highperformance liquid chromatography (HPLC). The PCP in the soil suspension with 2 ml was extracted with a water/ethanol mixtures (1:1 in volume) by shaking on a horizontal shaker at 180 rpm for 1 h [28]. The filtrate from the 0.45 μm syringe filters (Millipore, MA, USA) was collected for quantitative analyses of PCP with an HPLC instrument. In batch extraction experiments, the recovery of PCP was higher than 88.57% with sterile soil (Table S1). PCP was analyzed 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) [29].

2.4. The ratio of $^{13}C/^{12}C$ in CO_2, CH_4 and soil microbial biomass C

The CO_2 and CH_4 concentrations in headspace, taking 0.2 ml with Hamilton gas-tight syringes, were measured with GC9700 gas chromatograph (Techcomp Instruments, Shanghai, China) with a flame ionization detector [30]. The concentrations of CO_2 and CH_4 in the aqueous solution has been previously described [31]. The ^{13}C atom percentage of CO_2 and CH_4 in the gas samples were analyzed using gas chromatography combustion isotope ratio mass spectrometry (IRMS) system (MAT-253, Finnigan, Bremen, Germany). The headspace samples were removed with a gas-tight syringe

(Hamilton, Switzerland) and then injected into a vacuumed bottle for analyzing ^{13}C atom percentage. Helium was used as the carrier gas, and high-purity compressed CO_2 was used as the working standard gas. The measurement principle was described previously [32,33]. The $\delta^{13}C$ values of the samples were calculated relative to the international Vienna Pee Dee Belemnite (V-PDB) standard (Eq. (1)):

$$\delta^{13}C[‰] = \left[\frac{(^{13}C_{sample}/^{12}C_{sample} - {}^{13}C_{V-PDB}/^{12}C_{V-PDB})}{(^{13}C_{V-PDB}/^{12}C_{V-PDB})}\right] \times 1000, \quad (1)$$

where $^{13}C_{V\text{-}PDB}/^{12}C_{V\text{-}PDB} = 0.011802$ [34,35].

The supernatant was removed from the incubated samples, and the microbial biomass C of the residual soil was measured with the chloroform fumigation extraction method [36]. The extracts were freeze-dried and measured for $\delta^{13}C$ by the IRMS system and the $\delta^{13}C$ values of the samples were calculated relative to Eq. (1). The calculation of ^{13}C-soil microbial biomass method was the same as reported previously [35,37,38]. The method for ^{13}C residual in the incubated samples was similar with biomass C. The suspensions were freeze-dried, and then measured TOC with a TOC analyzer. $\delta^{13}C$ in the residue was measured by the IRMS system.

2.5. Soil DNA extraction and ultracentrifugation

The sample suspension was centrifuged for collection ~0.25 g soil, and then the DNA in the soil was extracted from ^{13}C-labeled or unlabeled PCP experiments using a PowerSoil™ DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA was quantified by a Qubit 2.0 fluorometer DNA (Invitrogen, NY, USA), and approximately 10 μg of DNA was loaded into Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 ml, Beckman Coulter) together with a Tris-EDTA (TE, pH 8.0)-CsCl solution. Before the tubes were sealed, the buoyant densities (BD) were measured with a model AR200 digital refractometer (Reichert, Inc., USA). The centrifugation was performed at 178,000 × *g* (20 °C) for 48 h in a Stepsaver 70 V6 vertical titanium rotor (eight tubes, 5.1 ml capacity each) [18]. Gradients of DNA were removed by using a Beckman fraction recovery system (Beckman Coulter). The DNA was retrieved from each gradient fraction with the EZNA™ MicroElute DNA Clean Up kit (OMEGA Biotek, USA).

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

The ultracentrifugation fractions of DNA from ^{12}C- and ^{13}C PCP addition experiments were used as a template to recover the 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′) for bacteria and A109F-FAM (5′-ACKGCTCAGTAACACGT-3′, 5′ end-labeled with carboxyfluorescein) and A934R (5′-GTGCTCCCCCGCCAATTCCT-3′) for archaea; 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 [39–41].

The purified heavy fraction of PCR products were cloned into vector *p*GEM-T Easy (Promega, USA), and then transformed into *E. coli* DH5α 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 the mothur software [40].

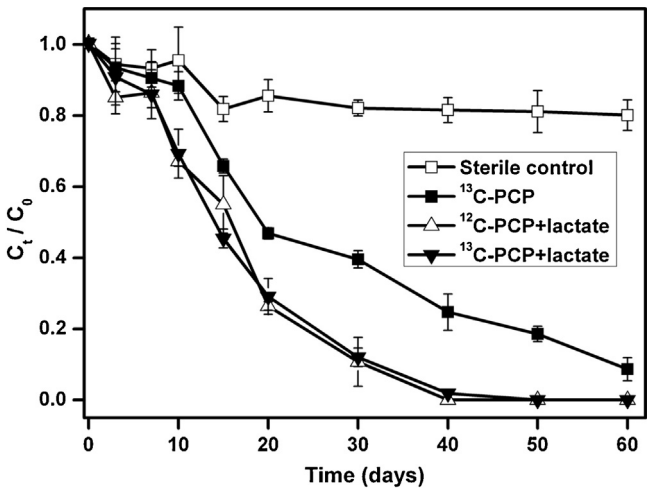

Fig. 1. The PCP (8 mg L^{-1}) transformation rates in soil samples with or without 10 mM lactate under anaerobic conditions at 25 ± 1 °C and pH 7.0 ± 0.1.

The abundances of the *mcrA* gene in the suspension cultures were determined by qPCR on a MyiQTM 2 Optics Module (BIO-RAD, USA) with the primers *mcrA*-F (5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′) and *mcrA*-R (5′-TTCATTGCRTAGTTWGGRTAGTT-3′) (*M* = A/C, *R* = A/G, *Y* = C/T, *W* = A/T) [42]. 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 [43].

The nucleotide sequence data were deposited in GenBank under accession numbers KM581062 to KM581223.

3. Results

3.1. Anaerobic degradation of PCP in the paddy soil

The PCP degradation processes in the soil of the microcosm experiments under different conditions were presented in Fig. 1, and the calculated parameters of the first-order kinetics were listed in Table S2. For both ^{12}C- and ^{13}C PCP, the PCP concentrations declined rapidly in unsterile soil, whereas only a low percentage (~15%) of PCP removal was obtained after reaction for 60 days in the sterile control experiments, which was likely due to soil sorption because the removal rate of 15% fell into the maximum adsorption of 19.5% in the experiments (Fig. S1). The difference between the sterile and unsterile soils suggested the microbial degradation of PCP. Compared with the process in the absence of lactate, the PCP degradation was obviously accelerated with the addition of lactate, and the first-order kinetic constant *k* values increased from 0.039 day^{-1} to 0.075 day^{-1}. In the absence of lactate, PCP was still not completely degraded at end of the incubation period (60 days), whereas the completely degradation of PCP was achieved even after reaction for 40 days in the presence of lactate.

Two degradation mechanisms involving dechlorination and ring-cleavage were expected for PCP degradation. During the microbial degradation of PCP, several intermediates were detected, including 2,3,4,5-tetrachlorophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol, 2,4-dichlorophenol and 4-chlorophenol (Fig. S2). With or without the presence of lactate, no $^{13}CO_2$ or $^{13}CH_4$ was detected in the first 10 days, suggesting that no PCP was completely mineralized in the beginning. After that, PCP degradation was accelerated and even metabolized to produce $^{13}CH_4$, $^{13}CO_2$ and biomass

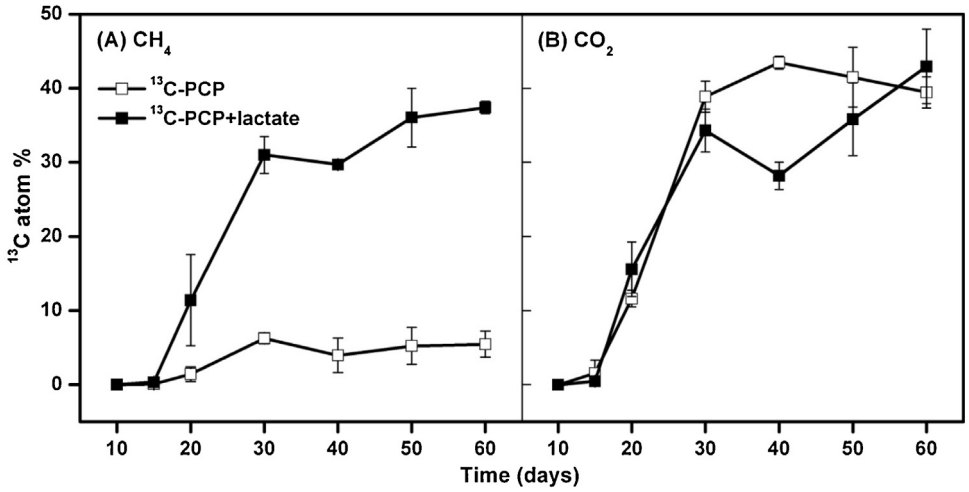

Fig. 2. Formation of (A) $^{13}CH_4$ and (B) $^{13}CO_2$ from the microcosm with ^{13}C PCP and ^{13}C PCP + lactate added.

Table 1
Distributions and percentages of carbon in the gas and slurry phases from A (soil + PCP) and B (soil + PCP + lactate) treatments.

			Gas phase		Slurry phase		Total recovery rate (%)
			$^{13}CO_2$	$^{13}CH_4$	^{13}C biomass	^{13}C residue	
^{13}C distribution percentage (%)	A	10 days	0	0	0.61 ± 0.12	99.91 ± 0.53	100.52
		30 days	38.86 ± 2.09	1.42 ± 0.98	2.70 ± 0.76	58.84 ± 1.31	101.82
		50 days	41.49 ± 4.04	5.22 ± 2.47	5.67 ± 0.56	50.41 ± 2.43	102.97
	B	10 days	0	0	0.79 ± 0.17	98.25 ± 0.58	99.04
		30 days	34.31 ± 2.89	30.98 ± 2.48	1.02 ± 0.15	36.78 ± 1.59	103.09
		50 days	35.81 ± 4.91	36.03 ± 3.67	6.65 ± 0.41	25.38 ± 1.26	103.87

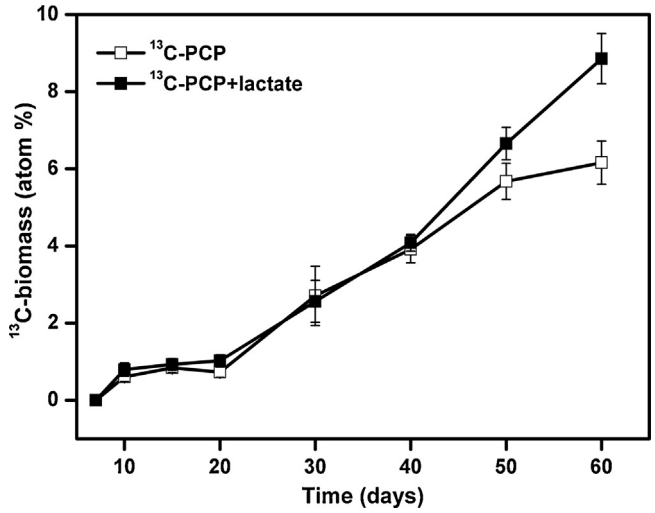

Fig. 3. Formation of ^{13}C-biomass from ^{13}C-labelled PCP and ^{13}C-labelled PCP + lactate.

^{13}C. As Fig. 2 showed, with or without the presence of lactate, $^{13}CO_2$ was formed at the same rate, and the $^{13}CH_4$ produced with lactate (35%) was much higher than that without lactate (5%). The $^{13}CH_4$ and $^{13}CO_2$ products were not detected in the sterile control experiments with PCP (data not shown). The assimilation results of ^{13}C PCP in the biomass were shown in Fig. 3. The two experiments with or without lactate yielded a similar extent of assimilation. To further verify the degradation of PCP, the ^{13}C carbon balance was examined in the two experiments (Table 1). The ^{13}C residue in the samples did not contain biomass ^{13}C. The ^{13}C carbon was primarily observed as produced CO_2, CH_4 and residue in suspension. From the achieved mass balance of ^{13}C, we can conclude that complete mineralization was achieved when lactate was initially present, whereas approximately half of the total ^{13}C residue in PCP or its degradation intermediates was produced in the absence of added lactate.

3.2. TRFLP results for SIP and microbial communities in PCP biodegradation

The DNA extracts from both labeled and unlabeled PCP-supplemented soil reaction systems were analyzed using SIP and TRFLP to profile the bacterial and archaeal communities at three sampling time intervals (10 days, 30 days, and 50 days). The first time point 10 days was chosen for SIP and TRFLP, which was an early time point in terms of formed biomass ^{13}C, and thus bore the potential to truly illuminate distinctions in label distribution to the different consortium members. Fragments were shown throughout the T-RFLP fingerprinting from all gradient fractions for ^{12}C and ^{13}C PCP treatments, and the microorganisms responsible for ^{13}C assimilation were enriched in the ^{13}C heavy fractions but not in the unlabeled heavy fractions. One bacterial T-RF (220 bp) was enriched in ^{13}C heavy fractions in experiments with or without lactate addition, whereas such enrichment was not detected in the ^{12}C heavy fractions (Figs. 4 and 5). In the only PCP-supplemented soil microcosms, the highest relative abundance (RA) was 33.3% at BD 1.725 mg L^{-1}, and the RA was continuously increased during the incubation time (10 days, 30 days, and 50 days). A similarly high RA (31.5%) was also observed for the 220 bp in the PCP and lactate-supplemented soil microcosm at 1.744 mg L^{-1}. To further identify the representative microorganisms via the key T-RF fragments involved in PCP biodegradation, the 16S rRNA clone libraries were investigated (Table S3) in the PCP degradation experiments. With or without lactate, the library composition for bacteria was similar and the most dominant bacteria phylum was determined to be the *Proteobacteria*, and the second most dominant was *Acidobac-*

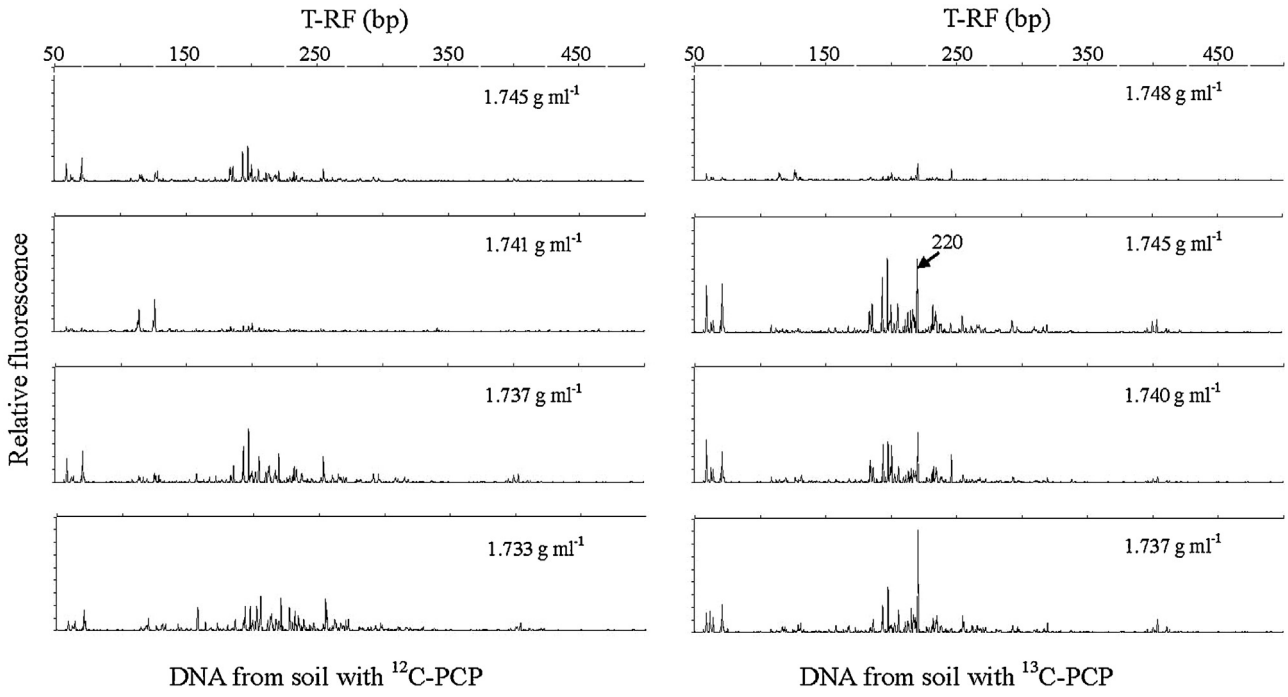

Fig. 4. Comparison of heavy fraction TRFLP profiles from ^{12}C and ^{13}C PCP supplemented soils to illustrate the dominance of 220 bp fragments in the labeled heavy fractions after reaction for 50 days.

teria. The T-RF 220 bp fragment related to bacteria in the heavy fractions belonged to the genera *Dechloromonas* and *Azospira* of the family *Rhodocyclaceae*. To confirm which bacterium was truly responsible for the 220 bp in the ^{13}C DNA heavy fraction, another two restriction enzymes (*Alu*I and *Rsa*I) were used to digest the ^{13}C enriched heavy fractions (Table S5). The slight differences (two or three bases) between the measured fragment lengths and those predicted by the sequence data were also reported in a previous study [44]. From these digests, the genus *Dechloromonas* of the family *Rhodocyclaceae* was found to be directly associated with the ^{13}C uptake from PCP degradation.

The TRFLP profiles for archaea showed that two T-RFs (135 bp and 235 bp) produced the dominant peaks in the ^{13}C heavy fractions in the soil system with PCP and lactate, as shown in Figs. 6 and 7. However, no fragments were detected in ^{13}C heavy fractions for the TRFLP profiles without lactate (data not shown). The maximum RA values of 135 bp and 235 bp were 45.5% (1.733 mg L^{-1}) and 35.8% (1.740 mg L^{-1}), respectively. The 16S rRNA clone libraries of archaea were constructed only for the soil experiments with added PCP and lactate (Table S4). Two phyla in the archaea library were observed to have *Euryarchaeota* as the dominant archaea. The archaea of 135 bp and 235 bp T-RF fragments were classified as the genera of *Methanosaeta* and *Methanocella*, respectively. It is expected that all three fragments (putative degraders) were responsible for PCP or its breakdown products biodegradation during the incubation period.

To investigate whether the archaea played an important role in the anaerobic degradation of PCP, the *mcrA* genes were quantified from both the labeled and unlabeled samples at three time intervals (10 days, 30 days, and 50 days). The qPCR results of

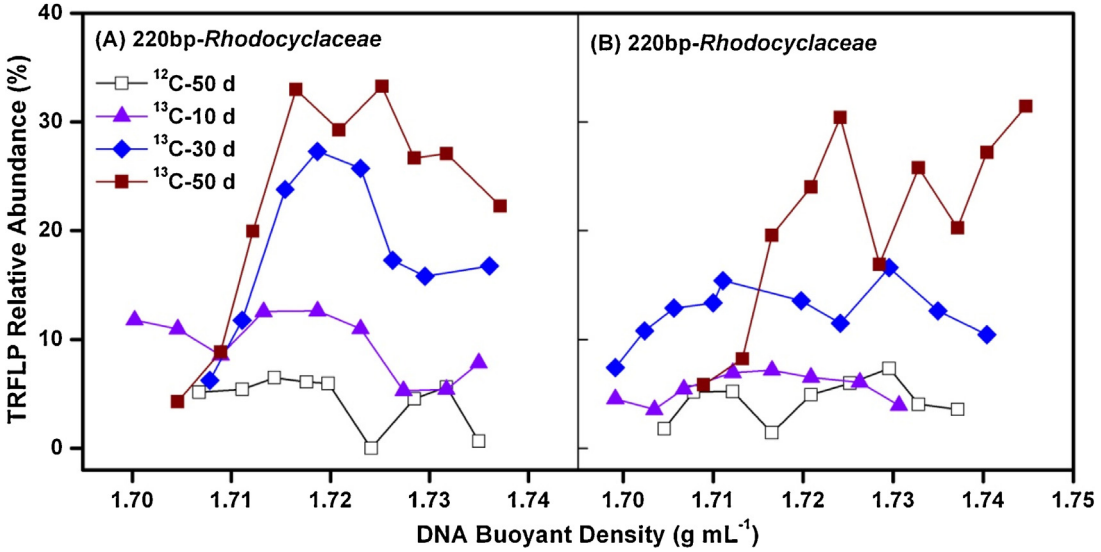

Fig. 5. Percent relative abundance of fragments (digested by *Hae*III) assigned to *Dechloromonas* (220 bp) (A: PCP-added, B: PCP + lactate-added) within the buoyant density gradients of bacterial DNA.

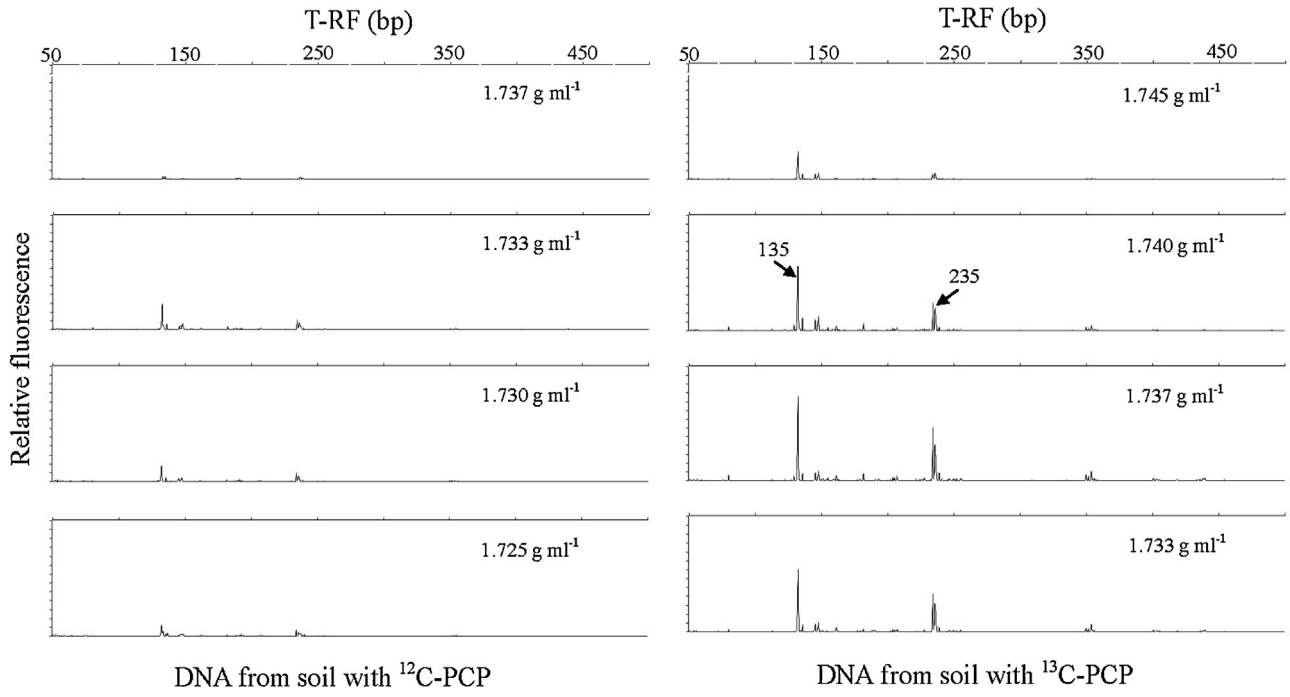

Fig. 6. Comparison of heavy fraction TRFLP profiles from ^{12}C and ^{13}C PCP supplemented soils to illustrate the dominance of 135 bp and 235 bp fragments in the labeled heavy fractions after reaction for 50 days.

the soil system supplemented with PCP and lactate are shown in Fig. 8. The maximum copies showed a significant shift in the heavy fractions between the ^{12}C and ^{13}C samples. For example, in the Fig. 8C, the maximum position occurred at BD of 1.750 g mL^{-1} in the ^{13}C fractions compared with1.739 g mL^{-1} in the ^{12}C fractions. The peak shift indicates that the archaea assimilated the ^{13}C during the anaerobic biodegradation of PCP.

4. Discussion

4.1. Key microorganisms involved in PCP mineralization

In the natural soil environment, PCP may be subjected to reductive dehalogenation by dehalorespiring bacteria to yield energy [45,46]. Under anaerobic conditions in paddy soil, ^{13}C PCP was mineralized to CO_2 and was clearly metabolized by the microorganisms assimilated into their DNA (~10% atom). The SIP can be used to identify organisms that were involved in the degradation of organic matters [14]. And in this work, the microbial degraders with assimilated ^{13}C were indentified using the SIP approach. To assure that the organisms were targeted by sufficient ^{13}C PCP, the incubation time lasted up to 50 days, according to the PCP degradation kinetics. The intermediates of PCP metabolism indicated that carbon ring-cleavage had also taken place after dehalogenation. In the current study, the results indicated the presence of key PCP degrading bacteria which was classified as the genus *Dechloromonas* of the class β *Proteobacteria*. The isolate that presented 220 bp with the highest similarity was *Dechloromonas* sp. JJ, which has been previously isolated as a humic substances oxidizing nitrate reducer [47]. *Dechloromonas* was reported to be related to the microbial mineralization of organic matters, and the *Dechloromonas* strain RCB was proven capable of anaerobic mineralization of benzene coupled with nitrate reduction [47]; further research showed that can also utilize toluene, ethylbenzene and

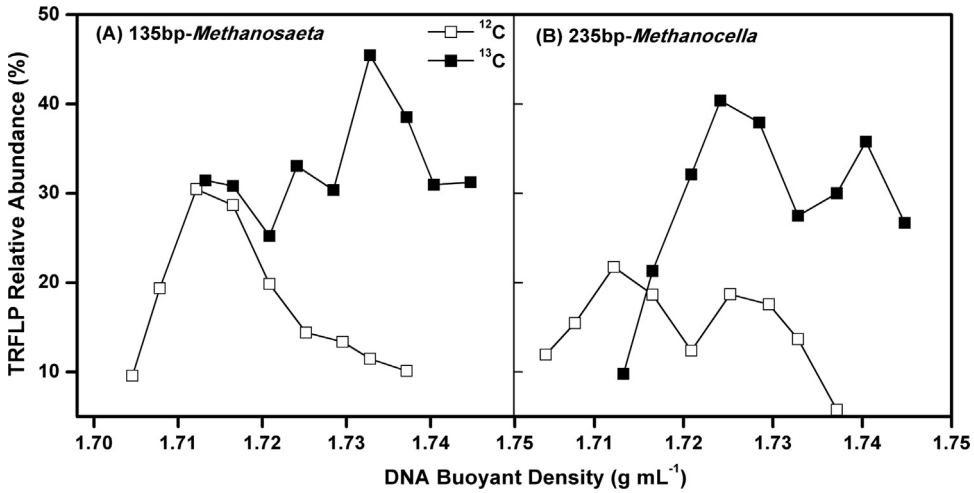

Fig. 7. Percent relative abundance of fragments (digested by *Hae*III) assigned to *Methanosaeta* (135 bp) and *Methanocella* (235 bp) within the buoyant density gradients of archaeal DNA after reaction for 50 days with lactate.

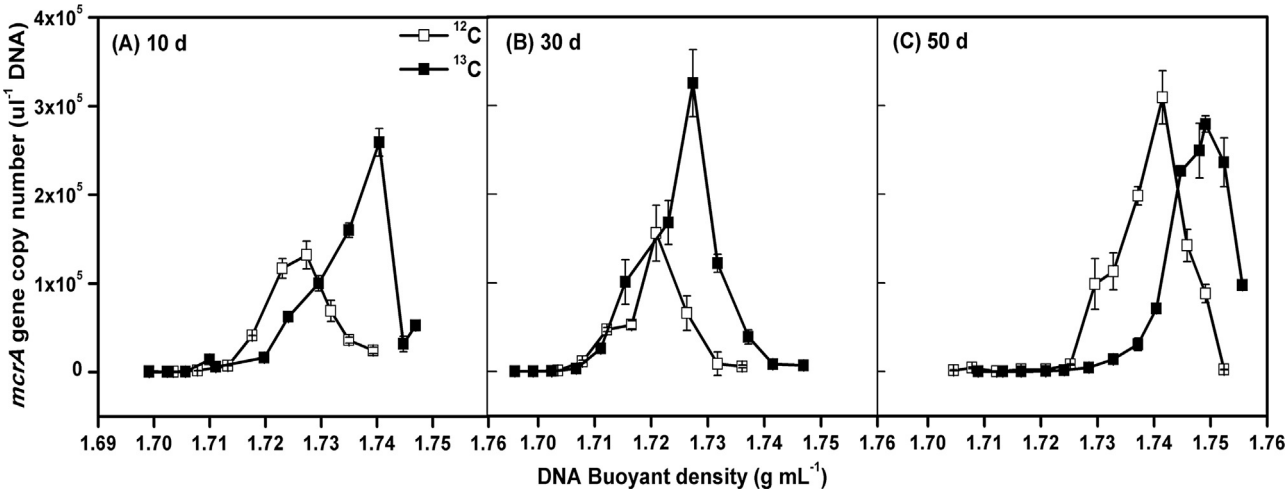

Fig. 8. Abundance of *mcrA* gene copies in ultracentrifugation fractions from unlabeled and labeled (^{13}C-PCP + lactate) PCP-added microcosms for (A) 10 days, (B) 30 days and (C) 50 days.

xylene as carbon sources and completely degrade them into CO_2 [48].

Two steps for ring-cleavage, including hydroxylation and carboxylation, were likely the important degradation steps prior to complete mineralization of PCP by microorganisms [49]. Microorganisms belonging to the family of *Rhodocyclaceae*, i.e., *Azoarcus* or *Thauera* genus, can act as the degraders of these two steps in PCP mineralization [50,51]. The genus *Dechloromonas* belonging to the family of *Rhodocyclaceae* identified in the current study via SIP may also have ability to mineralize PCP in paddy soil. A recent study found a novel anaerobic pathway for aromatic degradation by *Dechloromonas aromatica*, including the benzylsuccinate synthase genes and the central benzoyl-CoA [52]. Generally, the PCP mineralization process consecutively involved hydroxylation, carboxylation, and finally degradation to CO_2.

The archaea for PCP degradation in paddy soil supplemented with lactate was classified as the genus of *Methanosaeta* of the order *Methanosarcinales* (135 bp) and *Methanocella* of the order *Methanocellales* (235 bp). *Methanosaeta* can be isolated from several cultivation-dependent conditions and were reported to be active in the degradation of organic compounds under methanogenic enrichment [53,54]. For example, in the polycyclic aromatic hydrocarbons degradation system, a microbial community analysis showed that these enrichments were dominated by archaeal members most closely affiliated with *Methanosaeta* and *Methanoculleus* species [55]. Even in the anaerobic biodegradation of the PCP system, *Methanosaeta*-like was found to be the main microorganism at the base of the biofilm [56,57]. Another study also indicated that *Methanosaeta*-like was the dominant methanogenic archaea in PCP degradation [12]. However, these previous studies of microorganisms correlated with PCP biodegradation were based on morphotype, which did not provide direct identification. In the current study, we targeted the functional microorganism *Methanosaeta* with significant ^{13}C via SIP, and the results indicate that *Methanosaeta* can efficiently degrade PCP or its breakdown products associated with other microorganisms under anoxic conditions and may be powerful in complex soil environments.

The isolate that presented 235 bp (*Methanocella*) with the highest similarity was *Methanocella paludicola* isolated from an anaerobic and propionate-degrading enrichment culture [58] and *Methanocella arvoryzae* isolated from a methanogenic consortium [59]. Although no reports correlate *Methanocella* with chlorophenol biodegradation, *Methanocella* may be enriched in heavy fractions through assimilation of the ^{13}C acetate from the byproducts of PCP degradation during the incubation time. Previous report indicated the active microorganisms (containing *Methanocellales* and *Methanosarcina* of the order *Methanosarcinales*) in syntrophic propionate oxidation in anaerobic paddy soil via RNA-SIP [60]. The syntrophic interactions with organisms for transformation of complex organic matters to methane and carbon dioxide under methanogens were represented by Schink [61]. Therefore, the role of *Methanocella* in PCP biodegradation may be syntrophic with other organisms.

4.2. The PCP mineralization under methanogenic (anaerobic) conditions

Due to the physiological characteristics of rice growth, the paddy soil is usually flooded in anoxic conditions during most of the rice-growing season, which typically induced methanogenesis [62]. The roles of bacteria and archaea (mainly methanogens) in anaerobic mineralization of PCP have been reported previously [13,63,64]. However, the anaerobic degradation of PCP coupled with methanogenesis and mineralization in paddy soil has not yet been investigated, particularly with ^{13}C labeled PCP. Our results suggested that the methanogenic cultures derived from anoxic enrichment can mineralize PCP or its breakdown products into CO_2 and CH_4 (Fig. 2), and the additional carbon source of lactate accelerated the reactions (Fig. 1). Lactate, which acted as the electron donor, was important for microorganism metabolism and consequently accelerates microbial PCP degradation in paddy soils under anaerobic conditions. Our previous study also suggested that the microbial community structure can even be changed after biostimulation by the additions of lactate [65].

In this work, the ^{13}C isotope tracer method was used for tracking the anaerobic mineralization of PCP with ^{13}C labeled PCP. The amount of $^{13}CO_2$ in the experiment with added lactate was lower than that without lactate. The slight difference in $^{13}CO_2$ production was resulted from the action of that can use H_2 and CO_2 to produce CH_4 [56,59]. The H_2 was produced as the intermediate from the biodegradation of organic contaminants by fermentative microorganisms under anaerobic conditions [66]. In both of the studies, with or without lactate presence, only one bacterium *Dechloromonas*, was found to be responsible for producing CO_2. Our results show that the amount of produced $^{13}CH_4$ in the treatment with lactate was seven-fold greater that without lactate. The difference in $^{13}CH_4$ production was due to the role of archaea. Two methanogens were detected in the treatment with lactate but were

not detected in the absence of lactate. *Methanosaeta* was an important component of the microbial community of the microcosms in anoxic paddy soil [62]. The chlorophenols can be degraded by methanogens to produce CH_4, and the ring cleavage products were also further assimilated simultaneously by methanogens. In the lactate treatment, the lactate acted as the external carbon source, promoted the growth of indigenous microorganisms growth (Fig. 1) and also likely fermented to produce H_2, acetate and propionate under anaerobic conditions [67]. Therefore, the released H_2 would enhance the mineralization rate of PCP, and additional CH_4 would be produced [27,68]. The relationship between carbon source, methanogen, and CH_4 may play an important role on organic pollutants mineralization. The positive shift of the maximum *mcrA* gene copies in the ^{13}C heavy fractions obtained in the current study confirmed that breakdown products of PCP were assimilated by the methanogen.

5. Conclusions

The use of DNA-based SIP technique allowed us identifying the microorganisms responsible for PCP degradation within microcosms inoculated with paddy soil. Prominent members of the PCP degrading community were *Dechloromonas*, *Methanosaeta* and *Methanocella*. The genus *Dechloromonas* was identified as dominant degrader in microcosms with or without the presence of lactate. However, the archaea of *Methanosaeta* and *Methanocella* were detected only in the soil treatment with lactate, which were favorable for the mineralization of PCP or its breakdown products. The increased production of $^{13}CH_4$ and $^{13}CO_2$ in the presence of lactate indicated that the addition of lactate enhanced the rate of biodegradation and mineralization of PCP. These data are particularly relevant for shedding light on the fate of PCP during microbial degradation and enhancing our understanding of PCP-degrading microorganisms for complex soil environments.

Conflict of interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.05.049

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