Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 20(2008) 745–752
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Effects of Cd(II) and Cu(II) on microbial characteristics in 2-chlorophenol-degradation anaerobic bioreactors

HUANG Aiqun[1], CHEN Hao[1], CHEN Ling[1,*], DAI Yalei[2], ZHAO Jianfu[1]

1. State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, China. E-mail: huangaiqun2033@sina.com
2. Department of Immunology, School of Basic Medical Science, Tongji University, Shanghai 200092, China

Received 14 July 2007; revised 20 August 2007; accepted 30 September 2007

Abstract

The effects of Cd^{2+} and Cu^{2+} at 300 mg/L on anaerobic microbial communities that degrade 2-cholorophenol (2-CP) were examined. Based on the polymerase chain reaction (PCR) of 16S rDNA, bacterial community diversity and archaeal community structure were analyzed with denaturing gradient gel electrophoresis (DGGE) and cloning, respectively. Degradation capabilities of the anaerobic microbial community were drastically abated and the degradation efficiency of 2-CP was reduced to 60% after shock by Cu^{2+} and Cd^{2+}, respectively. The bacterial community structure was disturbed and the biodiversity was reduced after shock by Cu^{2+} and Cd^{2+} for 3 d. Some new metal-resistant microbes which could cope with the new condition appeared. The sequence analysis showed that there existed common *Archaea* species in control sludge and systems when treated with Cu^{2+} and Cd^{2+}, such as *Methanothrix soehngenii*, *Methanosaeta concilii,* uncultured *euryarchaeote*, and so on. Both the abundance and diversity of archaeal species were altered with addition of Cd^{2+} and Cu^{2+} at high concentration. Although the abundance of the predominant archaeal species decreased with Cd^{2+} and Cu^{2+} addition for 3 d, they recovered to some extent after 10 d. The diversity of archaeal species was remarkably reduced after recovery for 10 d and the shift in archaeal composition seemed to be irreversible. The 2-CP-degradation anaerobic system was more sensitive to Cu^{2+} than Cd^{2+}.

Key words: 2-cholorophenol (2-CP); Cd^{2+}; Cu^{2+}; microbial community; diversity; *Bacteria*; *Archaea*; DGGE; cloning

Introduction

The 2-Cholorophenol (2-CP) has been widely used as an important industrial raw material in chemicals, pesticides, medicines, paper-making, antiseptics, and other industries. Because of its adverse effects on the environment and microorganisms, 2-CP was targeted for concern in the environmental priority pollutants by Environmental Protection Agency, USA (USEPA) in 1979 and by China in 1990. In fact, the pollution of toxic organic pollutants and heavy metals usually coexists (Helen, 2005; Tewari *et al*., 2001). Heavy metal toxicity towards microbes is one of environmental concerns because it could suppress the biodegradation of organic compounds and transfer the accumulated metals to higher organisms in the food chain (Kuo and Genthner, 1996; Said and Lewis, 1991). The studies of microbial diversity in environmental pollution provide a useful model for studying bacterial adaptation.

The traditional isolation and incubation techniques often underestimate the bacterial diversity in natural environments (Pace, 1997). Molecular techniques make it feasible to investigate complex microbial communities (Amann *et al*., 1995), especially in anaerobic bioreactors, where the stability and performance are strongly dependent on the complex microbial interactions (Caroline and Colleran, 2006). The use of 16S rDNA-based methods employing denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1993), molecular cloning and sequencing and fluorescent *in situ* hybridization (Raskin *et al.*, 1994) could improve understanding of the role and dynamics of microorganisms (Kong *et al*., 2001) and diversity in a complex community.

Recently, most microbial molecular studies on the transformation, degradation of chlorophenol and polychlorinated biphenyl in the anaerobic systems have mainly focused on the deoxidization-dechlorination process of the microorganism in sediment, anaerobic sludge (Cord *et al*., 2002; Ren *et al*., 2005), and enrichment in the incubation substrates (Wu *et al*., 2002; Cutter *et al*., 2001). There are many studies on the effects of heavy metals on the structure and diversity of microbial communities in soils and other environments (Dell'Amico *et al.*, 2007; Li *et al*., 2006; Liao *et al*., 2005; Müller *et al*., 2001; Ranjard *et al*., 2006; Sandaa *et al*., 1999; Smit *et al*., 1997), however, the related studies in anaerobic bioreactors are not enough. The effects of Cu^{2+} and Cd^{2+} on *Bacteria* and *Archaea* in 2-CP-degradation anaerobic system, respectively were

* Corresponding author. E-mail: chenling@mail.tongji.edu.cn.

investigated in present paper to study the connections of the addition of heavy metal ions, the microbial community structure, and the degradation capability of the 2-CP-degrading anaerobic system.

1 Materials and methods

1.1 Experimental setup

The experimental setup used in the present study was an upflow anaerobic sludge blanket (UASB) reactor, including one big set and two small sets. The size of the big reactor, which was used for acclimation of the anaerobic granular sludge for 2-CP-degradation, was as follows: the inner and external diameter was 150 and 250 mm, respectively; the total height was 1,000 mm and the net volume was 13 L. The size of the small reactors, which were used for treatment with Cd^{2+} and Cu^{2+}, was as follows: the inner and outer diameter was 50 and 80 mm, respectively; the total height was 400 mm and the net volume was 0.75 L.

1.2 Acclimation of anaerobic granular sludge for 2-CP-degradation

The anaerobic granular sludge was obtained from Wuxi Wastewater Treatment Plant in Jiangsu Province, China. The sludge was inoculated in a continuous flow anaerobic bioreactor at mesophilic conditions (37°C). The basic properties of the inoculated sludge were: particle diameter ca. 0.3–1.0 mm; MLSS 70.8 g/L; VSS 65.7 g/L; C_{Cu} and C_{Cd} 0.01 mg/g (Chen, 2005). The influent organic concentration was 2,000 mg COD/L, using glucose and 2-CP as substrates to culture. Some necessary nutrient salts were proportionally supplemented to the inflow (mg/L): $MgSO_4$ 85.2; K_2HPO_4 50.0; $Ca(HCO_3)_2$ 23.2; KH_2PO_4 20.0; $NiSO_4$ 8.4; $FeSO_4$ 6.8; $NaHCO_3$ 1,220; NH_4HCO_3 780. Hydraulic retention time (HRT) was kept at 12 h and pH was controlled at 7.5–8.0. Influent glucose concentration was reduced and influent 2-CP concentration was raised stepwise until it reached 25 mg/L. The inoculated sludge was acclimated successfully when the removal efficiency of COD and 2-CP lasted stably with more than 90%, and the acclimation period took 68 d in total. COD was measured according to the standard method (GB3838-2002). 2-CP was measured using high performance liquid chromatography (HPLC, HP1050 system, Agilent, USA) and the concentrations of Cu and Cd in the sludge were measured by inductive coupled plasma emission spectrometer (ICP, Optima 2100DV, Perkin Elmer, USA) (Chen, 2005).

1.3 Shock by Cu^{2+} and Cd^{2+} and system recovery

Successfully acclimated 2-CP sludge was used for the shock test. The sludge was transferred from the big bioreactor into the two small bioreactors after rinsing and sieving, then was shocked with influent outlined as follows: 2-CP at 25 mg/L, necessary nutrient salts as described above, 300 mg/L Cu^{2+} and Cd^{2+} as the sole shocking factor according to the previous experiments (Chen, 2005). During the period of shock, the concentration of 2-CP in the effluent was monitored every day (data not shown). The results indicated that the concentration of 2-CP almost kept stable without obvious degradation. Therefore the shock was ceased after 3 d and the anaerobic systems began to be acclimated for recovery with the same substrate mentioned in Section 1.2. Analysis indicated that 2-CP in anaerobic systems had not been degraded until day 10. During the whole process, HRT was kept at 12 h.

1.4 Analysis of microbial community structure in 2-CP-degradation anaerobic granular sludge shock by Cu^{2+} and Cd^{2+}

1.4.1 Source of samples

The sludge samples for DGGE analysis and cloning were taken from the small bioreactors shocked by 300 mg/L Cu^{2+} and Cd^{2+} for 3 d, respectively. Then the systems were recovered for 10 d after shocking. About 0.2 g sludge (wet weight) was washed thrice with distilled ultrapure water (Simplicity, Millipore Co., USA) to eliminate the originally adsorbed metal ions and organic substances. Then it was centrifuged at 5000 r/min for 2 min, and poured off the supernatant.

1.4.2 DNA extraction and PCR reaction

Whole community DNA was extracted using the protocol of Zhou *et al.* (1996). The extracted DNA was dissolved in 100 μl distilled ultrapure water and was verified for size and concentration by 0.8% (*W/V*) agarose gel electrophoresis staining with ethidium bromide (data not shown) and a UV spectrophotometer (BioPhotometor, Eppendorf Co., Germany), respectively. Primer set GC338F-518R for *Bacteria* was used for DGGE analysis and ARC21F-ARC958R for *Archaea* was used for cloning. The information for the primers was outlined in Table 1 and all of the primers were synthesized by Invitrogen Co., Shanghai, China.

The PCR reaction mixtures (50 μl) contained 2 μl DNA template (10–100 ng), 5 μl of 10× PCR buffer (20 mmol/L Mg^{2+}; 100 mmol/L KCl; 80 mmol/L $(NH_4)_2SO_4$; 100 mmol/L Tris-HCl, pH 9.0; 0.5% NP-40), 2 U of *Taq*

Table 1 Primers used in PCR experiments

Primer	Sequence (5′→3′)	Specificity	*E.coli* numbering	Reference
338F	CCT ACG GGA GGC AGC AG	*Bacteria*	341–357	Muyzer *et al*., 1993
518R	ATT ACC GCG GCT GCT GG	Universal	518–534	Muyzer *et al*., 1993
ARC21F	TTC CGG TTG ATC CYG CCG GA	*Archaea*	2–21	DeLong, 1992
ARC958R	YCC GGC GTT GAM TCC AAT T	*Archaea*	940–958	DeLong, 1992

A GC clamp of 40 bp was attached to the 5′ ends of the 338F primers for DGGE analysis (Muyzer *et al*., 1993).

polymerase (Sangon Biol Engineering Tech & Services Co., Shanghai, China), 1 μl of each deoxynucleoside triphosphate (dNTPs, 10 mmol/L) and 1 μl of each primer (10 μmol/L).

DNA was amplified with Thermolyne Ampliton^{R} II (Barnstead Thermolyne Co., USA). PCR amplification procedure of *Bacteria* included: an initial denaturation step of 2 min at 94°C, followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 60°C, elongation for 90 s at 72°C, and a final extension step of 10 min at 72°C. PCR amplification parameters for *Archaea* included an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 94°C, for 45 s, 51°C for 60 s, 72°C for 90 s, followed by 72°C for 10 min.

The PCR products were verified for size and yield by 1.2% (*W/V*) agarose gel electrophoresis staining with ethidium bromide (data not shown). Bands in the proper size range were excised and purified with the Wizard SV gel and PCR cleanup system (Promega Co., USA), then measured with the UV spectrophotometer.

1.4.3 Bacterial community analysis with DGGE

DGGE was performed as described by Muyzer *et al*. (1993) and modified by Watts *et al*. (2001), using the Bio-Rad Dcode^{TM} Universal Mutation System (Bio-Rad Laboratories, Hercules, USA). DGGE was performed with 8% (*W/V*) polyacrylamide gels that contained a 40%–60% linear chemical gradient of denaturants (100 ml of 100% denaturant containing 42 g urea, 40 ml deionized formamide, 20 ml of 40% acrylamide/bis solution (37.5:1), 2 ml of 50×TAE buffer), and electrophoresis was performed for 5 h at 60°C in 1×TAE at a constant voltage of 120 V. The reproducibility of the results and analysis methods were checked by loading each sample in duplicate. After electrophoresis, the gels were stained for 15 min in ethidium bromide gel (200 ml 1×TAE, 25 μl ethidium bromide (10 mg/ml)). The stained gel was immediately photographed on a UV transillumination table with a video camera module (FR-980 Electrophoresis image analysis system, Shanghai FURI Sci & Tech Co., China).

1.4.4 Cloning and sequencing

The purified PCR products amplified with primer ARC21F-ARC958R were used for cloning. According to the instruction manual, the purified PCR products were ligated into the pMD19-T Vector (Takara Co., Japan) and then were transferred into *E. coli* DH5α competent cells with ampicillin selection and blue/white screening. All of the white colonies were further verified by PCR-amplification with primer ARC21F-ARC958R and screened by 1.2% (*W/V*) agarose gel electrophoresis staining with ethidium bromide. Only plasmids with the proper size (about 900 bp) were further sequenced by Sangon Co., Shanghai, China.

1.4.5 Data analysis

The DGGE profiles were analyzed with Smart View (Shanghai FURI Co., China) and the bacterial diversity was calculated with the Shannon diversity index (H'), as described in the following equation (Cox, 1979):

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \quad (1)$$

where, S is the total number of species; p_i is the relative abundance of each species, calculated as the proportion of individuals of a given species to the total number of individuals in the community. An equal portion (about 900 bp) of small subunit ribosomal DNA (SSU rDNA) was used for sequence analysis. The sequences were checked for chimeric artifacts with the Check-Chimera service from the Ribosomal Database Project, comparing the alignments at the beginning and the end of each sequence, and alignmenting of the entire sequence. Each sequence was compared with the sequences of the nucleotide database of GeneBank and BLAST (National Center for Biotechnology Information). Sequences with more than 94% homology were taxonomically aligned with the Vector NTI, advanced 9.0 program (Invitrogen Co., USA). Phylogenetic and molecular evolutionary analyses were conducted with MEGA 3.1 (Kumar *et al*., 2004), based on the Kimura 2-parameter model (Kimura, 1980) and Neighbor-Joining (NJ) algorithms (Saitou and Nei, 1987). The letters in phylogenetic trees correspond to the clone name and the numeral corresponds to the clone number of each sample. The accession numbers of blast genes are shown in parenthesis.

2 Results and discussion

2.1 Degradation efficiency of 2-CP

The anaerobic granular sludge in small reactors was singly shocked by 300 mg/L Cu^{2+} and Cd^{2+} for 3 d and was then recovered for 10 d. The concentration of 2-CP in the effluent was monitored every day (data not shown). The 2-CP concentration in the effluent rose to 15–20 mg/L and the degradation efficiency of 2-CP decreased to 60% due to the shock by Cu^{2+} and Cd^{2+}. These indicated that the degradation capabilities of the microbial community were drastically decreased in the metal-contaminated samples, which was consistent with other studies (Kuo and Genthner, 1996; Dobler *et al*., 2000; Fabienne and Antonis, 2004). The anaerobic system shocked by Cu^{2+} needed longer time to recover its degradation capability for 2-CP than that of Cd^{2+}.

2.2 *Bacteria* community structure

DGGE profiles of the PCR-amplified bacterial community in anaerobic systems shocked by metal ions and Shannon diversity index of these samples are shown in Figs.1 and 2, respectively. It could be recognized that both bands in sample Cd_A and Cu_A were reduced after shock by Cd^{2+} and Cu^{2+} for 3 d. The Shannon diversity index decreased from 2.86 of the control sample to 2.73 after shock by Cd^{2+}, then restored to 2.69 after recovery for 10 d. The index decreased from 2.86 of the control sample to 2.58 after shock by Cu^{2+}, then restored to 2.65 after recovery for 10 d. It is widely accepted that the number and

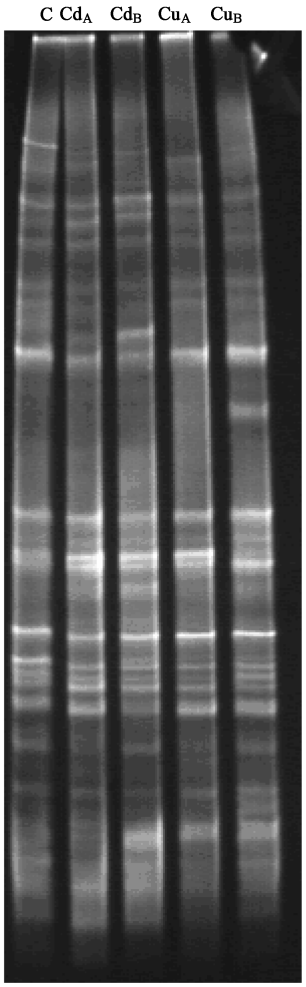

Fig. 1 DGGE profiles of PCR-amplified 16S rDNA V3 fragments of anaerobic bacterial community shocked by Cd^{2+} and Cu^{2+}. Cd_A and Cu_A stand for sludge samples shocked by Cd and Cu respectively for 3 d, while Cd_B and Cu_B stand for sludge samples recovered for 10 d after shock by Cd and Cu, respectively, and C stands for control sludge sample, which was 2-CP-acclimated granular sludge in the big anaerobic bioreactor.

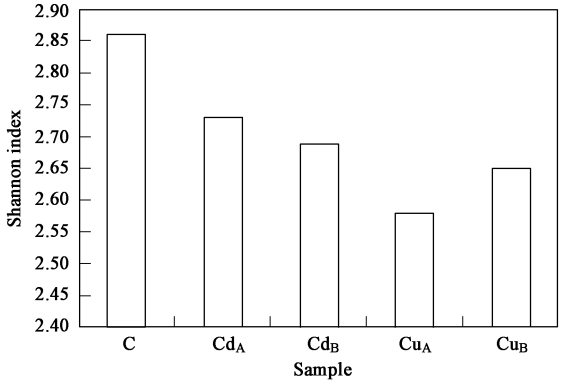

Fig. 2 Shannon diversity index of *Bacteria* community in DGGE profiles.

intensity of bands in DGGE-profiles should be regarded as semi-quantitative information due to existing PCR-biases (Cilia *et al*., 1996; Wintzingerode *et al*., 1997). DGGE profiles and Shannon diversity index in Figs.1 and 2 clearly revealed that the bacterial community structure was altered and the bacterial diversity was reduced with the addition of Cd^{2+} and Cu^{2+}. This was in accordance with other studies which reported that the size and diversity of bacterial populations were reduced by metal contamination using DGGE and other molecular techniques (Dell'Amico *et al*., 2007; Li *et al*., 2006; Liao *et al*., 2005; Müller *et al*., 2001; Smit *et al*., 1997).

Some bands were strengthened and some were abated and even disappeared, while other new bands were recognized after recovery for 10 d in sample Cd_B and Cd_B. This indicated that bacteria sensitive to metal ions were abated (David, 2004) or replaced by some metal-resistant but probably were ineffective to populations (Reber, 1992; Takashi *et al*., 1999; Wenderoth *et al*., 2001). There were also common bands in all of the samples, which might be bacteria that could survive in both metal-contaminated and metal-free environments. It is possible that they were multiple metal-resistant, which could resist both 2-CP and high doses of metal ions.

2.3 *Archaea* community structure

Thirty clones for each sample were analyzed by sequencing. The sequence analysis showed that there were common *Archaea* in all the samples, including *Methanothrix soehngenii*, *Methanosaeta concilii* and uncultured *euryarchaeote* and so on. (Table 2). The abundance of *M. soehngenii* decreased from 93.9% to 79.3% in control sludge after shocking by Cd and then restored to 96.3% after recovery for 10 d. It decreased from 93.9% in control sludge to 76.7% after shock by Cu and then restored to 86.7% after recovery for 10 d. Similar changes were observed in uncultured *archaeon* TA05 and uncultured *archaeon* TA04. *M. soehngenii* was reported to be the predominant species in UASB reactors and other anaerobic systems (Ren *et al*., 2005), our result was in accordance with their studies. Although the abundance of predominant archaeal species decreased after shock by Cd^{2+} and Cu^{2+} for 3 d, they recovered to some extent after recovery for 10 d.

The diversity of archaeal species varied remarkably (Figs.3–7). Some archaeal species that originally existed in the control sludge disappeared after shock by Cd^{2+} and Cu^{2+}, such as uncultured *archaeon* TA02, uncultured *Methanomicrobiaceae archaeon* MRR49, and *Methanosaeta concilii*, and so on, some new archaeal species were found after shock by Cd^{2+} and Cu^{2+}, such as uncultured *archaeon* 72-1, *Methanosaeta harundinacea*, Toluene-degrading methanogenic consortium, and so on. Compared to the 23 kinds of archaeal species originally existed in the control sludge, the number of archaeal species increased to 30 after shock by Cd^{2+} and then decreased to 14 after recovery for 10 d. It decreased to 18 after shock by Cu^{2+} and then further decreased to 9 after recovery for 10 d. The results indicated that the diversity of *archaeal* species was reduced with addition of Cu^{2+} at high concentration, but it increased with addition of Cd^{2+}. Both of these were reduced after recovery for 10 d. The shifts in *archaeal* composition seemed to be irreversible, which was consistent with other investigators (Sandaa *et al*., 1999).

In this study, the effects of Cu^{2+} and Cd^{2+} on *Bacteria*

Table 2 Sequence and closest phylogenetics of common *Archaea* in all of the sludge samples

Closest relative in GeneBank	Abundance in control (%)	Abundance in Cd_A (%)	Abundance in Cd_B (%)	Abundance in Cu_A (%)	Abundance in Cu_B (%)	Accession number	Homology (%)
Methanothrix soehngenii 1	93.9	79.3	96.3	76.7	86.7	X51423.1	99
Methanothrix soehngenii 2	93.9	79.3	96.3	76.7	86.7	X16932.1	99
Uncultured *archaeon* TA05	93.9	79.3	96.3	76.7	86.7	AF229778.1	99
Uncultured *archaeon* TA04	90.9	75.9	96.3	76.7	86.7	AF229777.1	98
Uncultured *archaeon* TA01	57.6	34.5	33.3	60	46.7	AF229774.1	99
Uncultured *euryarchaeote* EHB109	39.4	27.6	29.6	40	26.7	AF374281.1	98

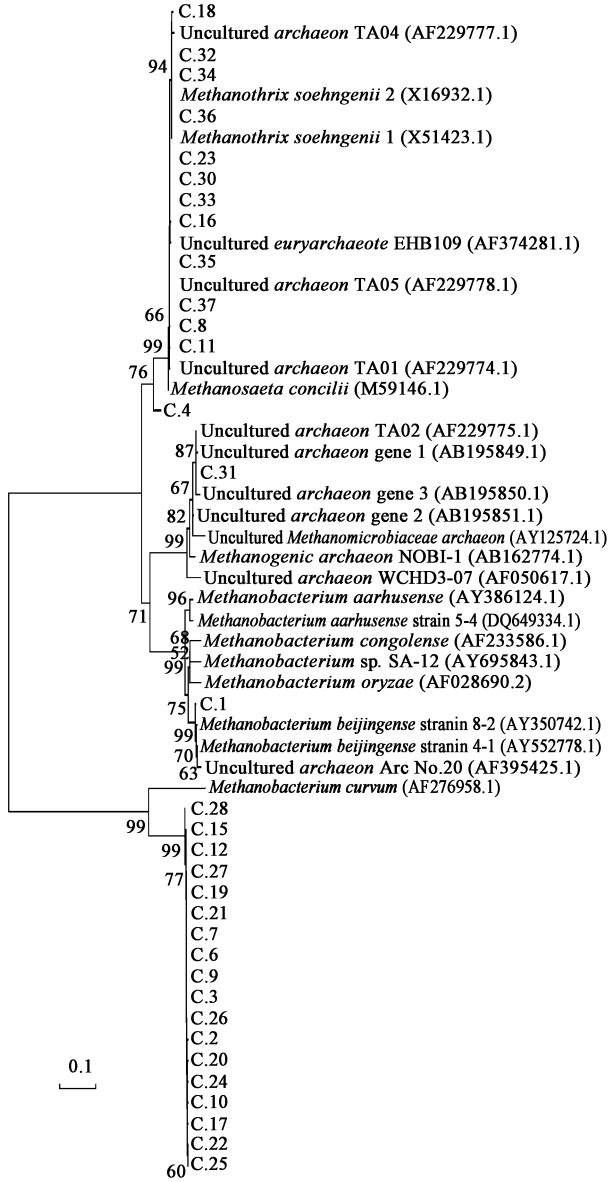

Fig. 3 Phylogenetic tree of archaeal 16S rDNA phylotypes from the control sludge. The scale bar represents the substitution per nucleotide position. Bootstrap analyses with 500 replicates were performed and only values 50 are shown as percentage.

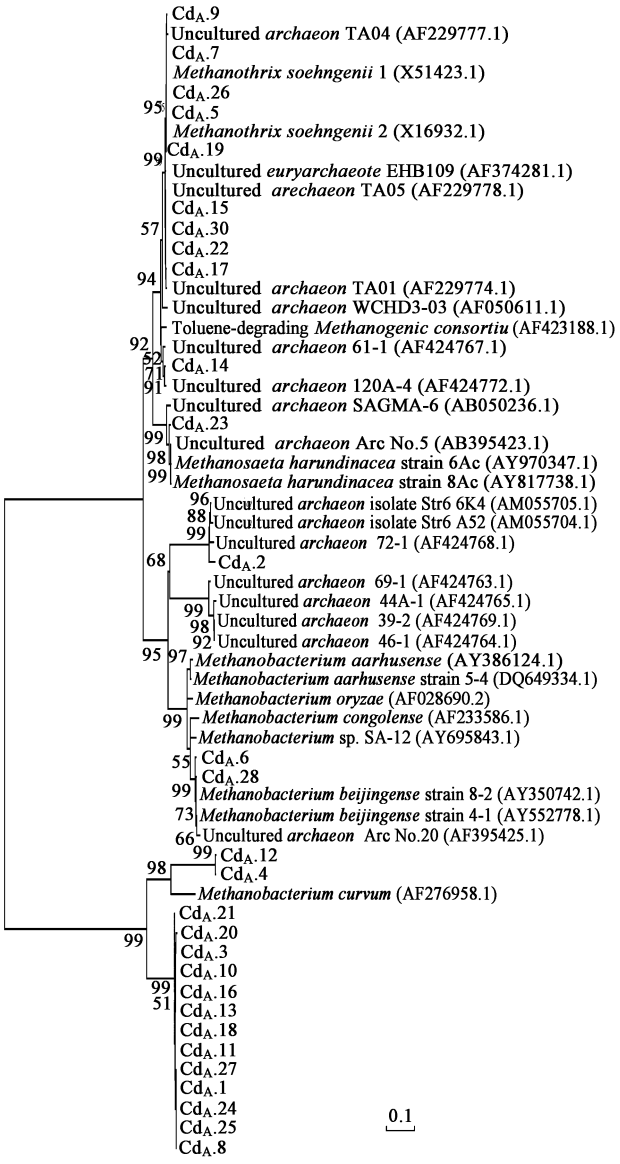

Fig. 4 Phylogenetic tree of archaeal 16S rDNA phenotypes from 2-CP-acclimated anaerobic sludge shocked by Cd^{2+} for 3 d.

and *Archaea* were totally different. It is not consistent with the previous works, which suggested that metal-resistant genes were harbored in the same populations (Huysman *et al*., 1994). This was probably owing to the short-time shock and the fact that metal treatments could select opportunist populations (Ranjard *et al.*, 2006). Many researchers concluded that methanogens were the most sensitive to added toxicants, while other bacterial groups were responsible for detoxification (O'Connor *et al*., 1990; Yang and Speece, 1985). Some microbes have been reported to be resistant to/or capable of transforming heavy metal ions (Lovley, 1993), such as *Sulfolobus metallicus* for Cu(II) resistance (Remonsellez *et al*., 2006), *cadA* and *cadR* in *Pseudomonas putida* for Cd(II) and Zn(II) resistance (Lee *et al*., 2001). As a nonessential metal, cadmium is considered to be the metal having the most

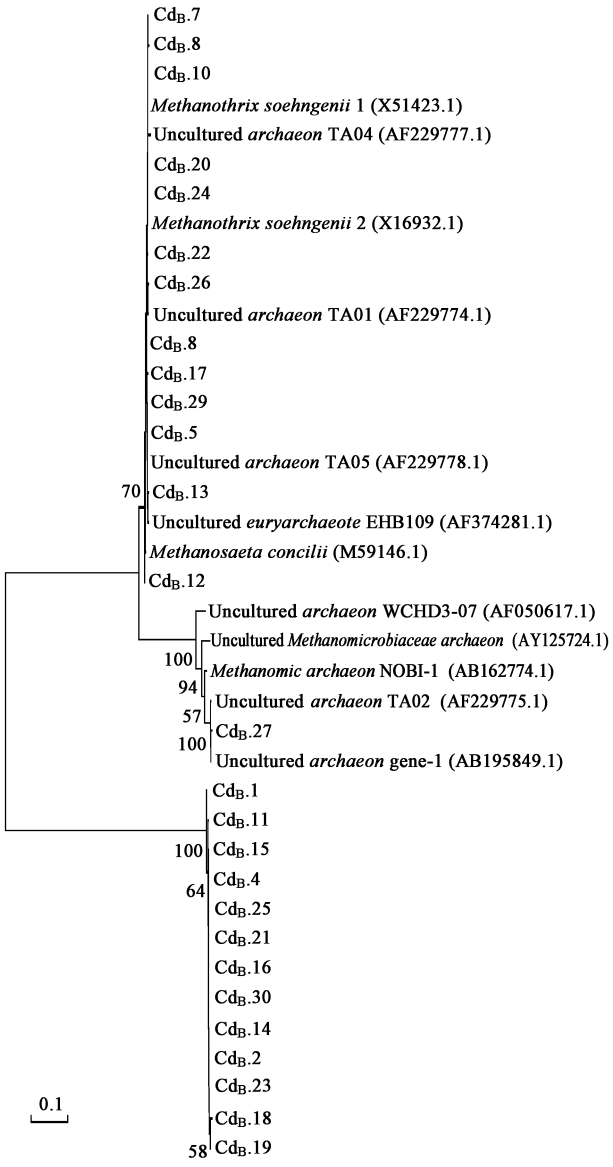

Fig. 5 Phylogenetic tree of archaeal 16S rDNA phenotypes from sludge recovered for 10 d after shock by Cd^{2+}.

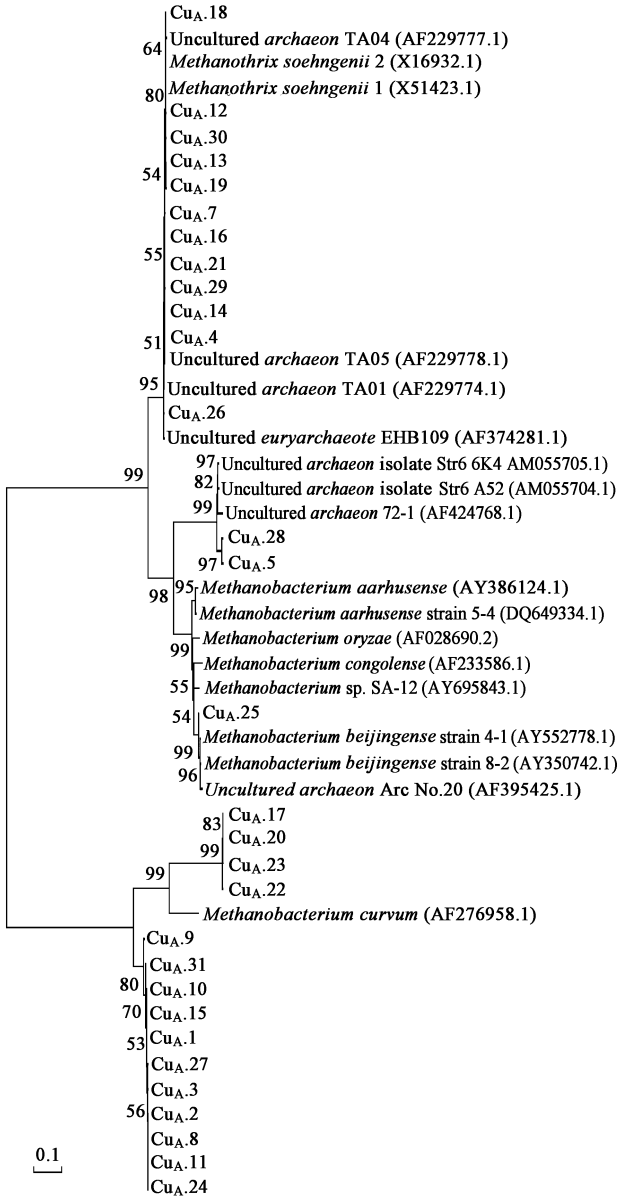

Fig. 6 Phylogenetic tree of archaeal 16S rDNA phenotypes from 2-CP-acclimated anaerobic sludge shocked by Cu^{2+} for 3 d.

adverse effects on microbial processes due to its high mobility and low affinity in soil (Alloway, 1995). The toxicity on the bacterial community was: $Cd^{2+} > Cu^{2+}$ (Ranjard *et al*., 2006). In this study, however, both bacterial and archaeal communities in the 2-CP anaerobic system were more sensitive to Cu^{2+} than Cd^{2+}, which was in accordance with other investigators (Said and Lewis, 1991; Kuo and Genthner, 1996).

Dechlorination of chlorinated compounds in anaerobic mineralization was differentially sensitive to various heavy metal ions (Capone *et al*., 1983), which resulted in different degradation efficiencies of 2-CP. The changes of degradation efficiency reflected the changes of anaerobic sludge activity. In fact, metal ions affected the enzymatic activity of anaerobes or community characteristics in the anaerobic system. A higher concentration of heavy metals was reported to interact with nucleic acids and enzyme active sites. It could inhibit DNA repair processes, cause enzyme denaturation, and inactivation, lead to cell death finally (Cervantes and Gutierrez, 1994; Stohs and Bagchi, 1995). Microbial resistance to Cd^{2+} and Cu^{2+} involves different physiological pathways and genetic determinants, as the *czc* and *cop* operon respectively (Bruins *et al*., 2000). The differences between bacterial populations or the enzymes responsible for dechlorination resulted in the variations in metal sensitivities (Kuo and Genthner, 1996).

3 Conclusions

On the basis of the results, the following conclusions can be drawn. (1) The degradation capabilities of the anaerobic microbial community were drastically abated and the degradation efficiency of 2-CP decreased from 90% to 60% after shock by Cu^{2+} and Cd^{2+}, respectively. The biodegradation of 2-CP in the anaerobic system was more sensitive to Cu^{2+} than Cd^{2+}. (2) Both the effect of Cu^{2+}

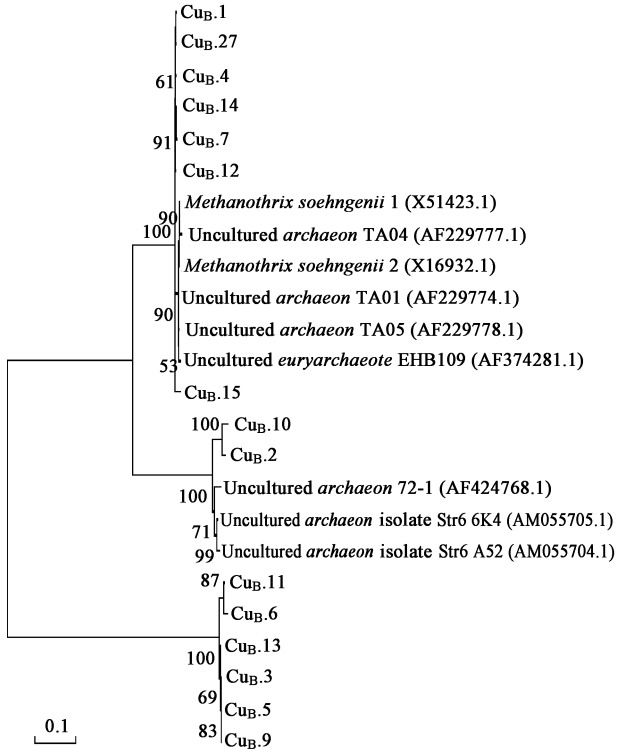

Fig. 7 Phylogenetic tree of archaeal 16S rDNA phenotypes from sludge recovered for 10 d after shock by Cu^{2+}.

and Cd^{2+} on *Bacteria* and *Archaea* in 2-CP-degradation anaerobic systems has been investigated. The bacterial community structure was initially disturbed. Although the biodiversity of the bacteria populations was reduced with the addition of Cu^{2+} and Cd^{2+}, some metal-resistant microbes that could cope with the new condition appeared. (3) There existed common archaeal species in the control sludge and the sludge shocked by metal ions, such as *Methanothrix soehngenii* and uncultured *euryarchaeote*, and so on. Although the abundance of predominant archaeal species decreased after shock by Cd^{2+} and Cu^{2+} respectively for 3 d, they recovered to some extent after recovery for 10 d. The diversity of archaeal species was remarkably reduced after recovery for 10 d and the shifts in archaeal composition seemed to be irreversible.

Acknowledgements

This work was supported by the Hi-Tech Research and Development Program (863) of China (No. 20025AA60127).

References

Alloway B J, 1995. Cadmium. In: Heavy Metals in Soils. Glasgow: Blackie Academic and Professional. 122–151.

Amann R I, Ludwig W, Schleifer K H, 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev*, 59(1): 143–169.

Bruins M R, Kapil S, Ochme W, 2000. Microbial resistance to metals in the environment. *Ecotox Environ Saf*, 45(3): 198–207.

Capone D G, Reese D D, Ronald P K, 1983. Effects of metals on *Methanogenesis*, sulfate reduction, carbon dioxide evolution, and microbial biomass in anoxic salt marsh sediments. *Appl Environ Microbiol*, 45(5): 1586–1591.

Caroline O'Reilly, Colleran E, 2006. Effect of influent COD/SO_4^{2-} ratios on mesophilic anaerobic reactor biomass populations: physico-chemical and microbiological properties. *FEMS Microbiol Ecol*, 56(1): 141–153.

Cervantes C, Gutierrez-Corona F, 1994. Copper resistance mechanisms in bacteria and fungi. *FEMS Microbiol Rev*, 14(2): 121–137.

Chen H, 2005. Study on the heavy metal ions influence on the anaerobic biodegradation of 2-chlorophenol. Shanghai: Tongji University.

Cilia V, Lafay B, Christen R, 1996. Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. *Mol Biol Evol*, 13(3): 451–461.

Cord S, Claudia V W, Regine H, Friedrich V W, Werner H, Ulf B G, 2002. Microbial structure of an anaerobic bioreactor population that continuously dechlorinates 1,2-dichloropropane. *FEMS Microbiol Ecol*, 39(3): 229–237.

Cox G W, 1979. General ecology experimental manual. Beijing: Sciences Press. 120–124.

Cutter L A, Watts J E M, Sowers K R, May H D, 2001. Identification of a microorganism that links its growth to the reductive dechlorination of 2,3,5,6-chlorobiphenyl. *Environ Microbiol*, 3(11): 699–709.

David C G., 2004. The effect of an acute copper exposure on the diversity of a microbial community in North Sea sediments as revealed by DGGE analysis–the importance of the protocol. *Marine Pollu Bull*, 49(5-6): 504–513.

Dell'Amico E, Mazzocchi M, Cavalca L, Allievi L, Andreoni V, 2008. Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. *Microbiol Res*, available online 4 Jan 2007. (in press)

DeLong E F, 1992. *Archaea* in coastal marine environments. *Proc Natl Acad Sci USA*, 89(12): 5685–5689.

Dobler R, Saner M, Bachofen R, 2000. Population changes of soil microbial communities induced by hydrocarbon and heavy metal contamination. *Biorem J*, 4(1): 41–56.

Fabienne G, Antonis C, 2004. Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve-month microcosm experiment. *FEMS Microbiol Ecol*, 48(2): 273–283.

Helen W, 2005. Oil refineries: a review of their ecological impacts on the aquatic environment. *Estuarine*, *Coastal and Shelf Sci*, 62(1-2): 131–140.

Huysman F, Verstraete W, Brooke P C, 1994. Effect of manuring practices and increased copper concentrations on soil microbial populations. *Soil Biol Biochem*, 26(1): 103–110.

Kimura M, 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol*, 16(2): 111–120.

Kong Y H, Beer M, Seviour R J, Lindrea K C, Rees G N, 2001. Structure and functional analysis of the microbial community in an aerobic: Anaerobic sequencing batch reactor (SBR) with no phosphorus removal. *Syst Appl Microbiol*, 24(4): 597–609.

Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, 5(2):150–163.

Kunito T, Senoo K, Saeki K, Oyaizu H, Matsumoto S, 1999. Usefulness of the sensitivity resistance index to estimate the toxicity of copper on bacteria in copper-contaminated soils.

Ecotoxicol Environ Saf, 44(2): 182–189.

Kuo C W, Genthner B R S, 1996. Effect of added heavy metal ions on biotransformation and biodegradation of 2-chlorophenol and 3-chlorobenzoate in anaerobic bacteria consortia. *Appl Environ Microbiol*, 62(7): 2317–2323.

Lee S W, Glickmann E, Cooksey D A, 2001. Chromosomal locus for cadmium resistance in *Pseudomonas putida* consisting of a cadmium-transporting ATPase and a MerR family response regulator. *Appl Environ Microbiol*, 67(4): 1437–1444.

Li Z J, Xu J M, Tang C X, Wu J J, Muhammada A, Wang H Z, 2006. Application of 16S rDNA-PCR amplification and DGGE fingerprinting for detection of shift in microbial community diversity in Cu-, Zn-, and Cd-contaminated paddy soils. *Chemosphere*, 62(8): 1374–1380.

Liao M, Chen C L, Huang C Y, 2005. Effect of heavy metals on soil microbial activity and diversity in a reclaimed mining wasteland of red soil area. *J Environ Sci*, 17(5): 832–837.

Lovley D R, 1993. Dissimilatory metal reduction. *Annu Rev Microbiol*, 47(1): 263–290.

Müller A K, Westergaard K, Christensen S, Srensen S J, 2001. The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiol Ecol*, 36(1): 11–19.

Muyzer G, Ellen C D W, Andre G U, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*, 59(3): 695–700.

O' Connor O A, Dewan R, Galuzzi P, Young L Y, 1990. Landfill leachate: a study of its anaerobic mineralization and toxicity to methanogenesis. *Arch Environ Contamin Tox*, 19(1):143–147.

Pace N R, 1997. A molecular view of microbial diversity and the biosphere. *Science*, 276(5313): 734–740.

Ranjard L, Lignier L, Chaussod R, 2006. Cumulative effects of short-term polymetal contamination on soil bacterial community structure. *Appl Environ Microbiol*, 72(2): 1684–1687.

Raskin L, Poulsen L K, Noguera D R, Rittmann B E, Stahl D A, 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl Environ Microbiol*, 60(4): 1241–1248.

Reber H H, 1992. Simultaneous estimates of the diversity and the degradative capability of heavy-metal-affected soil bacterial communities. *Biol Fertil Soils*, 13(3): 181–186.

Remonsellez F, Alvaro O, Jerez C A, 2006. Copper tolerance of the thermoacidophilic archaeon *Sulfolobus metallicus*: possible role of polyphosphate metabolism. *Microbiol*, 152(1): 59–66.

Ren Y H, Xu X Y, Tang Q, 2005. Molecular characterization of microbial populations in anaerobic PCP-degrading bioreactor. *Acta Sci Cirtcum*, 25(2): 214–219.

Said W A, Lewis D L, 1991. Quantitative assessment of the effects of metals on microbial degradation of organic chemicals. *Appl Environ Microbiol*, 57(5): 1498–1503.

Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4(4): 406–425.

Sandaa R A, Øivind E, Torsvik V, 1999. Abundance and diversity of *Archaea* in heavy-metal-contaminated soils. *Appl Environ Microbiol*, 65(8): 3293–3297.

Smit E, Leeflang P, Wernars K, 1997. Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. *FEMS Microbiol Ecol*, 23(3): 249–261.

Stohs S J, Bagchi D, 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med*, 18(2): 321–336.

Tewari A, Joshi H V, Trivedi R H, Sravankumar V G, Raghunathan C, Khambhaty Y, Kotiwar O S, Mandal S K, 2001. The effect of ship scrapping industry and its associated wastes on the biomass production and biodiversity of biota *in situ* condition at Alang. *Marine Pollu Bull*, 42(6): 461–468.

Watts J E M, Wu Q Z, Schreier S B, May H D, Sowers K R, 2001. Comparative analyses of PCB dechlorinating communities in enrichment cultures using three different molecular screening techniques. *Environ Microbiol*, 3(11): 710–719.

Wenderoth D F, Stackebrandt E, Reber H H, 2001. Metal stress selects for bacterial ARDRA-types with a reduced catabolic versatility. *Soil Biol Biochem*, 33(4-5): 667–670.

Wintzingerode F V, Gobel U B, Stackebrandt E, 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev*, 21(3): 213–229.

Wu Q Z, Watts J E M, Sowers K R, May H D, 2002. Identification of a bacterium that specifically catalyzes the reductive dechlorination of polychlorinated biphenyls with doubly flanked chlorines. *Appl Environ Microbiol*, 68(2): 807–812.

Yang J, Speece R E, 1985. Effects of engineering controls on. methane fermentation toxicity response. *J Wat Pollut Con Fed*, 57(12): 1134–1141.

Zhou J Z, Mary A B, James M T, 1996. DNA recovery from soils of diverse composition. *Appl Environ Microbiol*, 62(2): 316–322.