Microbial anaerobic degradation of polychlorinated biphenyls in the culture containing burnt inorganic materials

Daisuke Baba¹, Hiroshi Okumura², Arata Katayama¹

1. EcoTopia Science Institute, Nagoya University, Nagoya, Japan
2. Environmental Materials Research & Development Center, TYK corp., Tajima, Japan

Abstract: Anaerobic activity for degrading mixture of Kanechlor-300 and -400 was obtained from Japanese PCB-free paddy soils, and was maintained by serial transferring for more than three years. The wide spectrum of congeners in the PCB mixture was degraded without accumulation of less chlorinated congeners. The anaerobic PCB-degrading activity was enhanced about 2.5-fold higher degradation percentage in the culture containing burnt soil, which was prepared by calcining the paddy soil at 550°C for 24 hours until organic matters in the soils were oxidized completely, where total degraded PCBs was 39.7±5.2 mol% for 56 days of incubation. The maximum PCB-degrading activity was 238 ng-total-PCBs/ml-culture/day. Some degradation activities for less chlorinated biphenyls were correlated with sulfate reduction, although PCB-degrading microorganisms were not identified by analyzing polymerase chain reaction-denaturing gradient gel electrophoresis profile based on 16S rRNA gene. This activity was able to rejuvenate by addition of nutrients for long period of incubation. Analysis of the microbial consortium in the enhanced culture showed the predominance of anaerobic Firmicutes, Deltaproteobacteria, and Chloroflexi.

Keywords: Polychlorinated biphenyl, Dechlorination, Degradation, Anaerobic, Microorganism

1. INTRODUCTION

Polychlorinated biphenyls (PCBs) are a class of 209 synthetic congeners that were sold as commercial PCB mixture, such as Kaneclor and Aroclor, to industry and widely used for a significant portion of the past century. Because of their hydrophobicity and chemical stability, PCBs are often present at low concentrations in anaerobic environments such as sediments and estuaries. The fate of PCBs in the environment is of public concern because these compounds are relatively persistent, toxic and tend to bioaccumulate. There is substantial evidence that under reducing conditions some PCBs in contaminated sediments are dechlorinated [1]. Laboratory evidence has shown that natural populations of bacteria are responsible. Recently, Dehalococcoides ethenogenes 195 [2] and two anaerobic PCB-dechlorinating uncultured microorganisms, DF-1 [3] and o-17 [4], have been reported their dechlorination activity for single congeners. Besides, some anaerobic consortia containing Dehalococcoides sp. and/or uncultured bacteria within Chloroflexi were also shown to link their growth to the reductive dechlorination of PCBs [5]. It is also essential in bioremediation technology to dechlorinate broad varieties of congeners in commercial PCB mixtures, such as Kaneclor and Aroclors, which contain many congeners. However, only a few mixed cultures that can dechlorinate PCB mixture have been reported and maintained under sediment-free conditions [6, 7]. In some instances, enrichments of either pure of mixed cultures have been found to lose their activity when transferred to another matrix. Thus, there is no proven PCB bioremediation technology currently available, largely due to the low bioavailability of PCBs, the requirement for inducing substrates for biostimulation, and the need for a high population density of degrading microorganisms.

The objective of this study was to develop the anaerobic biodegradation activity for PCB mixture and to evaluate the capacity of the PCB-degrading culture containing burnt materials as bio-carriers for the possibility of applications on contaminated sites. Herein, we show that the anaerobic PCB-degrading activity in Japanese paddy soils with no history of PCB contamination was highly enhanced and maintained by using burnt soils in the culture. The relationships among PCB degradation, consumption of electron donors and acceptors, and microbial growth were investigated. We also characterized the anaerobic microbial consortium in the BS culture by the effects of inhibitors on the activity, PCR-DGGE analysis of partial 16S rRNA genes, and specific PCR targeting members of dechlorinating microorganisms.

2. MATERIALS AND METHODS

2.1. PCBs

A PCB mixture was used in this investigation by mixing Kanechlor-300 and -400 (Kanegafuchi Chemical Industry (Kaneka corp.), Osaka, Japan) for 1:1 (w/w) as an acetone solution of 1 g/l. The PCB mixture contained tri-chlorinated biphenyls (39.9% w/w) and tetrachlorinated biphenyls (44.4% w/w) mainly.

2.2. Soils and materials

A PCB-free paddy soil mainly used in this investigation was collected by plowing layers of paddy soil: a gray lowland soil from the Kuridashi area, Aichi, Japan. The other soils were described previously [8]. The paddy soil (abbreviated as PS) was passed through a 2 mm sieve and stored under water-flooded at 22°C in the dark until use. The dried PS was prepared by air drying. The burnt soils (abbreviated as BS) were prepared by calcining the dried PS at 550°C for 24 h for organic-free in the soil. The

Corresponding author: D. Baba, dababa@esi.nagoya-u.ac.jp
2.3. Maintenance of PCB-degrading activity

Kuridashi PS was employed as the PCB-degrading microbial source, which was described previously [9]. The PCB-degrading activity was maintained in the culture in 100 ml glass vials with the following composition: 4 ml of sterilized PS slurry containing approximately 3.5 g (dry weight) of PS or 4 g of the dried PS, the ground PS, BS or the ground BS (autoclaved at 121°C for 30 min for three consecutive days), 100 µl of 1 g/l PCB mixture as acetone solution, and an acetate/lactate (AL) medium [9] was added under anaerobic condition. Then, the vial was filled with 100% nitrogen and sealed with a Teflon-coated butyl rubber stopper. The prepared medium containing AL medium and PS, the dried PS, the ground PS, BS and the ground BS are designated as PS medium, dried PS medium, ground PS medium, BS medium and ground BS medium, respectively.

The microbial source described above was inoculated into each medium anaerobically at a transfer rate of 5% (v/v) and incubated statically at 30°C in the dark. The incubated samples were designated as cultures, such as PS culture for PS medium. After 56 d of incubation, the culture was transferred to new medium at a rate of 5% (v/v) for maintenance of the activity, and the PCB residues were determined.

To examine the effects of additional electron donors on the degrading activity, 1 mmol each of sodium acetate and sodium lactate were added into the Kuridashi BS culture after 14, 28, 56, and 112 d over 196 d of incubation. The effects of inhibitors on the consortium were examined by addition of 2 mM of sodium molybdate, or 0.1% paraformaldehyde.

2.4. Analysis of the PCB residues

The detailed procedures for the determination and analysis of PCB residues were described in the previous paper [9]. Briefly, the PCB residues were extracted from the culture after the incubation with chloroform-methanol (2:1). PCBs were further cleaned up using a silica gel column and a deactivated Florisil column with (2:1). PCBs were further cleaned up using a silica gel column and deactivated Florisil column with (2:1). The concentrations of most of the PCB congeners in the culture spiked with 100 µg of the PCB mixture were determined, including congeners overlapped on the chromatogram. The recovery of total PCBs was 92.9%±5.5% (w/w) among all the cultures, where monochlorinated biphenyls and biphenyl were not detected under this condition. The PCB-degradation activity was corrected by the recovery of total PCB and the individual congeners, by comparing the PCB residues remaining in the culture with those remaining in the sterilized control after incubation. No degradation was observed in the sterilized control as shown by the total PCB residues of 99.1±3.4 mol%.

2.5. Measurement of chlorine, sulfate, fatty acids in the culture

Samples for determination of organic acids, chlorine, and sulfate in the cultures were prepared by filtering the cultures through a membrane filter and diluting with pure water. Organic acids were analyzed using a high performance liquid chromatograph (HPLC) with an ultraviolet detector. The chlorinated and sulfate measurement was carried out with an ion analyzer. Details were described previously [8].

2.7. DNA extraction and PCR-DGGE analysis

Genomic DNAs were extracted from the microbial consortia in the cultures by using DNA extraction kit. To amplify partial 16S rRNA genes of the extracted DNA for the denaturing gradient gel electrophoresis (DGGE) analysis, the polymerase chain reaction (PCR) was carried out using a primer set for bacterial 16S rRNA gene (341F/518R) with a thermal cycler. The amplified PCR products were subjected to the DGGE analysis, and the gel was stained with SYBR green I.

For phylogenetic analysis of the community, the PCR products for DGGE were also subcloned in random order with a cloning kit. The cloned fragments were sequenced and analyzed, with aligned to 16S rRNA sequences obtained from the database of NCBI-BLAST database using the BLAST search program.

Detection of dechlorinating bacteria reported with dechlorinating activity, Dehalococcoides sp., the uncultured bacteium o-17 and/or DF-1 in Chloroflexi, Desulfito-bacterium sp., and Dehalobacter sp., was carried out by PCR targeting genera-specific 16S rRNA gene sequences. The conditions and primer sequences used were reported previously [10].

3. RESULTS

3.1. Anaerobic PCB degradation in PS culture

The Kuridashi PS examined showed PCB-degrading activity after more than four months incubation anaerobically, indicating that the uncontaminated Kuridashi PS had the potential to degrade the PCB mixture. Effects of different electron donors and acceptors on the cultures were not significant, indicating the PS could supply the essential nutrients for the activity. The anaerobic PCB-degrading activity in Kuridashi PS was successfully maintained by serial transfer, where inoculation rate of 5% coupled with a 56-day interval between transfers was necessary to maintain the activity. The presence of the PS was also required to maintain stable degradation activity. The averaged amount of residual PCBs was 84.8±9.9 mol% compared with the sterilized control (P <0.01) (Fig. 1). The concentrations of most of the PCB congeners in the PCB mixture decreased significantly, although the residual concentration of each congener fluctuated during the transfers. The accumulation of less chlorinated congeners in the culture was observed in the PS cultures.
3.2. Enhancement of PCB-degrading activity

The amounts of total PCB residues that remained in the BS cultures were approximately 15% (w/w) less than those in the Kuridashi PS culture, where the activity was not significant changed in the paddy soil cultures under various incubation conditions such as electron donors, electron acceptors, temperature and pH, demonstrating the enhanced activity in the BS cultures. The total PCB residue in the ground BS culture using Kuridashi PS was also decreased significantly, showing the same extent of enhancement. However, those in the dried PS and ground PS cultures using Kuridashi PS show no enhancement (Fig. 1).

The enhanced PCB-degrading activity was successfully maintained over three years and further enhanced by more than 15 transfers using Kuridashi BS culture with an incubation interval of 56 d and 5% (v/v) of the transfer rate. The amounts of total PCB residues in the culture decreased to 60.3±16.1 mol% compared with the sterilized control (P<0.01) (Fig. 1). Most of the congeners in the PCB mixture were significantly decreased compared with the PS culture, where the accumulation of less chlorinated biphenyls was not observed, corresponding to the decrease in the highly chlorinated biphenyls. These results suggested that the dechlorination activity was highly enhanced in the Kuridashi BS culture.

The addition of molybdate inhibited the PCB degradation as shown by the total PCB residue of 77.6±15.6 mol%, where sulfate reduction was inhibited. The large remaining amounts of PCB congeners were observed in a broad spectrum although the enhanced degradation was observed in a small number of congeners. The most conspicuous inhibition was observed as the accumulation of two less chlorinated biphenyls, which were under the detection limit before incubation. In the BS culture without the addition of molybdate, there was no accumulated congener during the degradation of the PCB mixture, and most of the congeners were degraded at about the same proportion. In the culture containing BES, the activity was also inhibited on total PCB residue (80.6±4.3 mol%), not individual congeners significantly, where methane production was inhibited.

Figure 2 shows the time course of total PCB residue in the Kuridashi BS culture as well as the amount of chlorine. The anaerobic degradation of PCBs was mainly observed from seventh to 56th day of incubation, corresponding to the production of chlorine. The lactate and sulfate in the culture were consumed within first 7 d, and then acetate was consumed until 56 d. The results suggested that the anaerobic PCB degradation correlated with the increase of chlorine, and acetate was the major electron donor and/or carbon source during the degradation of the PCB mixture.

The additions of electron donors resulted in the continuous degradation of the PCB mixture until 196 d of incubation, while the degradation stopped in the BS culture having no additional electron donors after 56 d of incubation (Fig. 2). In the BS culture with additional electron donors, about 10% of the total PCB residue was further decreased from the 56th to 196th day of incubation. However, the PCB-degrading activity was lower than that during the first 56 d of incubation.
3.3. Characterization of the PCB-degrading culture

The consortium of the BS culture after 56 days of incubation was analyzed by PCR-DGGE. Fig. 3 shows the DGGE band patterns of bacterial 16S rRNA genes in the consortium. DGGE clones of partial 16S rRNA genes were classified to *Firmicutes*, *Deltaproteobacteria* and *Chloroflexi* in BC culture. The band patterns of DGGE of the BS cultures were different from that of the PS cultures. Some predominant bands appeared corresponding to the enhancement of the PCB-degrading activity.

Fig. 3 The DGGE patterns of bacterial 16S rRNA genes of the PCB-degrading cultures. Numbers above shows; 1:original Kuridashi paddy soil, 2: PS culture, 3: BS culture, 4: BS culture with molybdate, 5: BS culture with BES

The DGGE band patterns in the consortia amended with inhibitors are also shown, where most of bands in the cultures with inhibitors were observed in the culture without inhibitors. Some weak bands in the consortium amended with inhibitors suggested influence on the activity.

Specific detections of 16S rRNA genes of dechlorinating bacteria were not correlated with the PCB-degrading activity because the degrading activity of the whole culture was stable during the serial transfers as previously reported [10].

4. DISCUSSION

In this study, the anaerobic PCB-degrading activity was obtained from uncontaminated paddy soil, and was enhanced in the BS cultures about 2.5-fold higher degradation percentage than that in the PS culture. The enhancement effect in the BS culture was shown by the nonaccumulation of less chlorinated congeners and the dechlorination not only at *meta*- and *para*-substituted positions but also at *ortho*-substituted position, because most of the congeners contained in Kanecchlor-300 and -400 mixtures were degraded. No accumulation of less chlorinated biphenyls in BS culture indicated the further dechlorination of less chlorinated biphenyls, although dechlorinated products, such as monochlorinated biphenyls and biphenyl, were not detected under the analytical conditions used in this study. These characteristics were different from the other reports on dechlorination activity of highly chlorinated biphenyls and had the new activity for PCB mixture. Further study should be conducted to determine the metabolized products from dichlorinated biphenyls.

The enhancement of the activity was observed in all the BS cultures, but not in all the PS cultures examined. It is considered that organic-free materials, such as BS, decreased the sorption of PCBs to soil particles and enhanced the bioavailability of PCBs. Besides it is also known that the physical placement in materials can increase the efficiency of the microbial consortium. Further study should be carried out to elucidate the mechanism enhancing the activity in the culture containing inorganic materials.

The sulfate content can affect the dechlorination of PCBs in the cultures. The addition of molybdate, inhibitor for sulfate reducers, into BS culture resulted in the accumulation of less chlorinated PCB congeners, where sulfate reduction was inhibited completely. On the other hand, the remaining dechlorination activity on some congeners was still observed even in the molybdate amended BS culture. The positive correlation between the consumption of sulfate and the dechlorination activities for less chlorinated biphenyls suggested that the sulfate reducers played important roles for the dechlorination activity in the BS culture.

In the BS medium, which contained acetate and lactate, PCB degradation was observed while acetate was present. This correlation suggested that acetate served as an electron donor and/or carbon source for the dechlorination of the PCB mixture in the culture, although the variety of electron donors did not affect the PCB-degrading activity in the PS cultures. The addition of acetate and lactate as electron donors several times in the BS culture resulted in the rejuvenation of the PCB-degrading activity after 56 d of incubation.

In this study, 6.7 µg/ml-culture of Kanecchlor-300/400 was dechlorinated at a maximum activity of 238 ng-total-PCBs/ml-culture/d in the BS culture after 28 d of incubation. This activity was the same as those in previous reports, although the initial concentration of PCBs was lower in this study [1, 6, 7]. These results suggested that our developed BS culture had a high potential for the anaerobic degradation of PCB mixtures.

Structures of microbial community were compared between BS and PS cultures. By analyzing 16S rRNA gene fragments of PCR-DGGE with cloning at random, clones were classified into *Firmicutes* (67%), *Deltaproteobacteria* (26%), and *Chloroflexi* (6%), suggesting the predominance of *Firmicutes*. The comparison of PCR-DGGE band pattern between PS and BS cultures showed the difference in some of the bands in Bacteria, which were assigned for *Firmicutes*, *Deltaproteobacteria* and *Chloroflexi*, suggesting the changes in members corresponded with the enhanced PCB degradation in BS culture. The members of dechlorinating bacteria, *Dehalococcoides* sp., the uncultured bacterium α-17 and/or *DF-1* in *Chloroflexi*, the *Dehalobacter* sp., and *Desulfotobacterium* sp., were detected sporadically in BS cultures, indicating their low population in the culture. This agreed with the results that all of the DGGE bands were not assigned to the members in any of the dechlorinating bacteria.

The PCB degradation in BS culture was inhibited with addition of inhibitors and the results was compared with BS culture without any inhibitors. Comparison of the DGGE band patterns between BS cultures with and
without inhibitors demonstrated the absent members of "Firmicutes, Deltaproteobacteria (Desulfovibrio sp.) and Chloroflexi," which suggested that these bacteria were related with wide spectrum of PCB dechlorination activity in BS culture. Regardless of the different effects between inhibitors, BES and molybdate, the structure of the community in BS culture with BES was the same as that in BS culture with molybdate, except for a few bands corresponding to "Firmicutes" in PCR-DGGE analysis. The results suggested that some dechlorination activity in BS culture was associated with sulfate reducers undetected in the DGGE profile, confirming sporadic detection of the dechlorinating bacteria at their low population.

In summary, our culture containing inorganic materials, BSs, enhanced the anaerobic PCB-degrading activity significantly. The activity was originated from an uncontaminated Japanese paddy field soil and was maintained for more than three years by serial transfers in the culture containing BS. The enhanced activity had the broad range of congeners and no accumulation of the less chlorinated biphenyls. The degradation activity in the BS culture was at the same high level as those reported previously by other researchers. Moreover, by adding electron donors to the BS culture, the activity could be rejuvenated. The PCB-degrading consortium in BS culture composed of mainly "Firmicutes." The activity was strongly associated with the presence of sulfate reducers. The population density of dechlorinating bacteria in BS culture was likely still low regardless of the highly enhanced degradation activity. These findings suggest that use of burnt inorganic materials will be expected as the bio-carrier for bioaugmentation in polluted site. Further study on the enhancement mechanism, the dechlorinating bacteria, and the examination of other materials should be carried out.

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