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ORIGINAL ARTICLE

Microbial communities associated with anaerobic degradation of polybrominated diphenyl ethers in river sediment

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KEYWORDS
anaerobic degradation;
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BDE-209;
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Abstract *Background/purpose:* Polybrominated diphenyl ethers (PBDEs) are extensively used as a class of flame retardants and have become ubiquitous environmental pollutants. We aimed to uncover the changes in microbial community with PBDE anaerobic degradation with and without zero-valent iron in sediment from the Erren River, considered one of the most heavily contaminated rivers in Taiwan.
Methods: PBDE anaerobic degradation in sediment was analyzed by gas chromatography with an electron capture detector. Microbial community composition was analyzed by a pyrosequencing-based metagenomic approach.
Results: The anaerobic degradation rate of BDE-209 was higher than BDE-28 in sediment; the addition of zero-valent iron enhanced the degradation rates of both. In total, 19 known bacterial genera (4 major genera: *Clostridium*, *Lysinibacillus*, *Rummeliibacillus*, and *Brevundimonas*) were considered PBDE degradation-associated bacteria (sequence frequency negatively correlated with PBDE remaining percentage) as were four known archaea genera (*Methanobacterium*, *Methanosarcina*, *Methanocorpusculum*, and *Halalkalicoccus*; sequence frequency positively correlated with PBDE remaining percentage).
Conclusion: The composition of bacteria and that of archaea affected the anaerobic degradation of BDE-28 and BDE-209. The addition of zero-valent iron further decreased the archaea content to undetectable levels.

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Introduction

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in various industrial products[1] and their residues are found in a wide variety of environments, with their concentrations increasing exponentially.[2] Increasing evidence shows that PBDEs are bioaccumulated and biomagnified and the compounds have been listed as the new persistent organic pollutants, and their fate and transport in ecosystems have received worldwide attention.

Microbial degradation is believed to be one of the major processes that may be used in remediating PBDE-contaminated sediment. PBDEs can be reductively debrominated by anaerobic microbes.[3] High-brominated PBDE congeners can be transformed to low-brominated PBDEs.[4] To enhance the efficiency of biodegradation, three remedial strategies—natural attenuation, bioaugmentation, and biostimulation—have been proposed.[5] Our previous study showed that BDE-209 can be debrominated successively to BDE-3 by anaerobic microbes from sediment; the addition of brij 30, brij 35, rhamnolipid, surfactin, vitamin B_{12}, zero-valent iron, acetate, lactate, and pyruvate enhanced the anaerobic degradation, with zero-valent iron yielding the highest BDE-209 anaerobic degradation.[6] However, little is known about the microbial communities involved in PBDE anaerobic degradation in sediment.

Molecular-biological methods have allowed for studies of microbial diversity in environmental samples. Metagenomic approaches have revolutionized our ability to explore the microbial world, revealing at higher resolution the structure of complex microbial communities that conventional cloning and sequencing methods have not been able to achieve. The pyrosequencing of 16S ribosomal RNA (rRNA) genes has been developed as a high-throughput metagenomic technology for profiling microbial communities in a resolution at the genus level.[7,8]

We aimed to assess the anaerobic degradation of high- and low-brominated PBDEs with zero-valent iron in sediment from the Erren River, considered one of the most heavily contaminated rivers in Taiwan. We used pyrosequencing to examine the phylogenetic diversity, composition, and structure of the microbial community associated with PBDE degradation. The target PBDEs were BDE-28 and BDE-209.

Methods

Chemicals

BDE-28 and BDE-209 were from Sigma Aldrich (St. Louis, MO, USA). Solvents were from Mallinckrodt, Inc. (Paris, KY, USA). All other chemicals were from Sigma Aldrich.

Sampling and medium

Sediment samples were collected from the Erren River in Taiwan. A detailed description of the sampling site was previously described.[6,9] Anaerobic BDE-adaption was achieved by the addition of 50 μg/g BDE-209 at 14-day intervals under static incubation at 30°C in the dark for 2 years. In this article, such sediment refers to anaerobic BDE-adapted sediment. The anaerobic medium consisted of (in g/L): KH_2PO_4, 0.27; K_2HPO_4, 0.35; NH_4Cl, 1.7; $FeCl_2.4H_2O$, 0.01; $CaCl_2.2H_2O$, 0.1; and $MgCl_2.6H_2O$, 0.1. The pH was adjusted to 7.0 after autoclaving; 0.9mM titanium citrate was added as a reducing reagent.

Experimental design

Experiments involved 12.5-mL serum bottles containing 4.5 mL medium, 0.5 g sediment, 50 μg/g PBDEs (BDE-28 or BDE-209), and zero-valent iron (1 g/L). Anaerobic experiments were conducted in an anaerobic glove box (Forma Scientific, Model 1025S/N, USA (Thermo Fisher Scientific Inc, 81 Wyman StreetWaltham, MA 02451, USA)) filled with N_2 (85%), H_2 (10%), and CO_2 (5%). Bottles were capped with butyl rubber stoppers and crimp seals and wrapped in aluminum foil to prevent photolysis, then incubated without shaking at 30°C in the dark. Each treatment was performed in triplicate. Samples were collected to measure residual PBDEs at 0 days, 30 days and 60 days and to detect bacterial communities at 60 days.

Analysis of PBDEs

PBDEs were extracted twice from whole bottles by use of hexane and acetone (9:1), then extracted again for 20 minutes with use of a Branson 5200 ultrasonic cleaner (Branson Ultrasonics, Americas Headquarters, 41 Eagle Road, Danbury, CT 06810, USA). Extracts were analyzed by use of a gas chromatograph (Hewlett Packard 6890 (Hewlett-Packard Company, 2850 Centerville Road, Wilmington, DE 19808-1610, USA)) equipped with an electron capture detector and Stx-500 capillary column. The initial column temperature was set at 170°C, increased by 10°C/min to 300°C, then increased by 2.5°C/min to 340°C. Injector and detector temperatures were set at 350°C and 370°C, respectively. Nitrogen was used as both a carrier gas (flow rate 4.0 mL/min) and makeup gas (flow rate 16.2 mL/min). The recovery percentages for BDE-28 and BDE-209 were 98.4% and 94.9%, respectively.

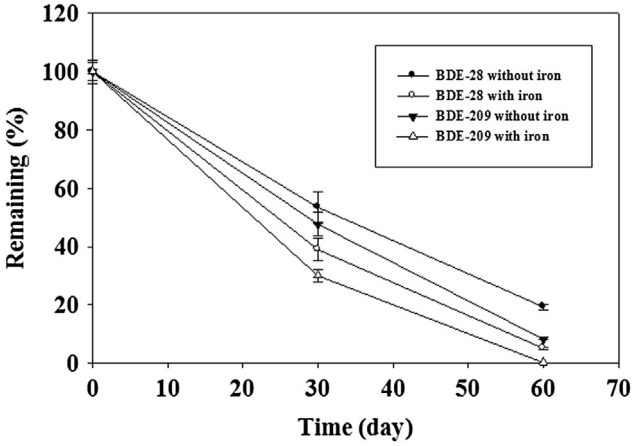

Figure 1. Anaerobic degradation of BDE-28 and BDE-209 with or without zero-valent iron in sediments. BDE = brominated diphenyl ether.

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Table 1 Number of sequences, operational taxonomic units, classification, and diversity indexes for each sediment sample

Exp.	Samples	No. of sequences	OTUs	Phylum	Class	Order	Family	Genus	Shannon index	Chao1
A1	No PBDE (d 0)	1967	87	9	16	18	21	16	2.4	152.0
A2	No PBDE (d 60)	1431	40	2	4	4	9	10	1.2	75.0
A3	BDE-28 (d 60)	2249	245	5	9	11	20	20	3.8	330.6
A4	BDE-28 & Fe (d 60)	1415	298	3	8	11	19	19	4.8	431.4
A5	BDE-209 (d 60)	2195	109	3	8	8	16	16	2.7	154.0
A6	BDE-209 & Fe (d 60)	1045	299	3	7	9	20	19	5.0	448.4

BDE = brominated diphenyl ether; d = day; OTU = operational taxonomic units; PBDE = polybrominated diphenyl ether.

DNA extraction, polymerase chain reaction, and pyrosequencing

Total DNA for each enriched microcosm was extracted by the traditional Cetyl trimethylammonium bromide (CTAB) method. Partial 16S rRNA genes containing variable V5-V8 regions were amplified from the extracted DNA. The sequence for the 5′ primer comprised a 454 pyrosequencing adaptor, a unique 4-mer tag for each sample and 787F (5′-ATTAGATACCCNGGTAG-3′) for 16S rRNA genes. The sequence for the 3′ primer comprised a 454 pyrosequencing adaptor and 1391R (5′-ACGGGCGGTGWGTRC-3′) for 16S rRNA genes. Each polymerase chain reaction (PCR) reaction was performed in 50 μL reaction mixture containing 10 ng DNA, 0.25mM deoxynucleotide triphosphate, 0.2μM each primer, 5 μL 10 × buffer, and 1.25 unit Pyrobest (Takara Bio Inc., Seta 3-4-1, Otsu, Shiga 520-2193, Japan) DNA polymerase. Reactions were cycled with an initial denaturation at 95°C for 10 minutes; 30 cycles of 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes.

Pyrosequencing was performed at the Genome Center, National Yang-Ming University, Taipei, Taiwan, R.O.C. Briefly, the quantity and quality of amplicon from each experiment sample was determined by use of the Quant-it PicoGreen dsDNA Kit (Invitrogen (Life Technologies, 3175 Staley Road, Grand Island, NY 14072, USA)) and Agilent DNA 1000 chip on a BioAnalyzer 2100 (Agilent Technologies, 5301 Stevens Creek Blvd., Santa Clara, CA, 95051, USA), respectively. Each amplicon sample was purified separately with Agencourt AMPure XP beads (Beckman Coulter, Inc., Life Science Division Headquarters, 5350 Lakeview Parkway S Drive, Indianapolis IN 46268, USA) according to the

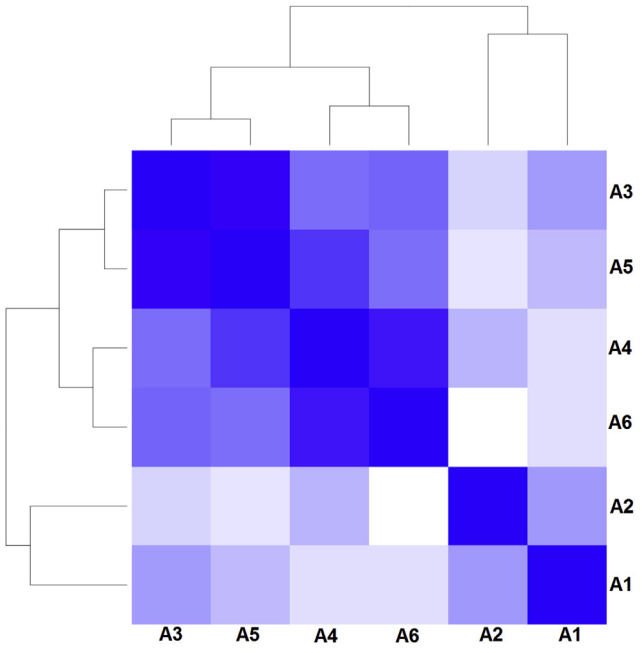

Figure 2. Bacterial community composition with six experimental samples. The Jaccard & Sørensen distance matrix-based clustering trees and heat maps (0.05 cutoff) were generated at the RDPipeline website. High intensity of blue indicates greater similarity between samples. Descriptions for experiments A1—A6 are provided in Table 1.

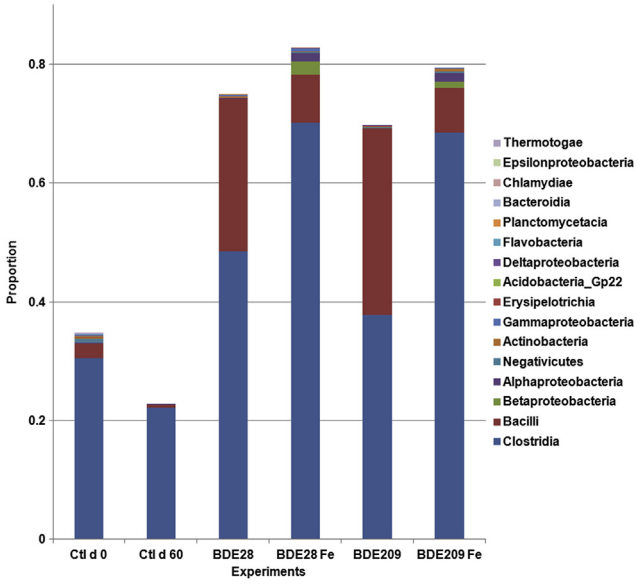

Figure 3. Bacterial community composition (known bacterial classes) in six anaerobic sediment samples. Y-axis indicates proportion of 16S ribosomal RNA gene sequences of each class in each experiment. BDE = brominated diphenyl ether; BDE28 = with BDE-28 (d 60); BDE28 Fe = with BDE-28 & Fe (d 60); BDE209 = with BDE-209 (d 60); BDE209 Fe = BDE-209 & Fe (d 60); Ctl d 0 = without PBDEs (d 0); Ctl d 60 = without PBDEs (d 60); d = day; Fe = iron; PBDE = polybrominated diphenyl ether.

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manufacturer's protocol. The purified amplicon was quantified and diluted to 1×10^9 molecules/μL and a second purification involved Agencourt AMPure XP beads. The double-purified amplicons with different sample-specific barcode sequences were quantified and diluted to 1×10^8 molecules/μL and pooled proportionally. The quality of the amplicon pool was assessed by Agilent High Sensitivity DNA chip, and subsequent emulsion PCR involved the GS emPCR Lib-L Kit (Roche Diagnostics Corporation, 9115 Hague Rd, Indianapolis, IN 46256-1045, USA). Pyrosequencing was performed on a GS Junior System (Roche Diagnostics Corp) according to the manufacturer's instructions with titanium chemistry.

Data analysis

Calculations were for PBDE remaining percentage [%] = (residue PBDE concentration / initial PBDE concentration) × 100; and PBDE degradation rate [%] = [1− (residue PBDE concentration/initial PBDE concentration)] × 100. A p value < 0.05 was considered statistically significant. Data analysis involved analysis of variance with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

The 16S rRNA gene sequence data were analyzed using RDPipeline (http://pyro.cme.msu.edu/). First, sequences were analyzed by Chimera Check to remove chimeric sequences.[10] Analyses with RDPipeline involved 16S rRNA gene sequence alignment (Aligner), 16S rRNA gene sequence clustering (Complete Linkage Clustering), α-diversity index (Shannon Index and Chao1 estimator), β-diversity comparison (Jaccard & Sørensen distance matrix), rarefaction curve, and phylogenetic analysis (RPD classifier).[11] Pearson correlation of sequence frequencies and PBDE remaining percentage data involved the function PEARSON in Microsoft Excel. For testing H_0: $\rho = 0$, bacterial classes and genera with $p < 0.05$ were considered PBDE degradation-associated bacteria.

Results

Anaerobic degradation of PBDEs in the sediment

The remaining percentage of BDE-28 and BDE-209 were 19.3% and 8.3% without zero-valent iron and 5.1% and 0.2% with zero-valent iron after 60-day incubation (Fig. 1). The rate of anaerobic degradation was higher for BDE-209 than BDE-28, and the addition of zero-valent iron increased the anaerobic degradation rate of BDE-28 and BDE-209 in sediment.

Diversity of microbial community in PBDEs anaerobic degradation experiments

In total, 10,302 16S rRNA gene sequences were produced from six experimental samples. Analysis with RDP classifier revealed nine phyla, 16 classes, 26 orders, 46 families, and 47 genera of known bacteria in the samples. The number of biological classification groups in the six samples are presented in Table 1. The difference in microbial community composition between samples is shown in Fig. 2. Two main clusters correspond to the presence and absence of PBDEs (Fig 2). The first cluster is composed of samples A1 and A2 for anaerobic sediment without PBDEs. The second cluster is composed of samples A3—A6 for anaerobic degradation of BDE-28 and BDE-209. This cluster is further divided into two subclusters that correspond to the presence and absence of zero-valent iron. Thus, the bacterial composition distinguished between anaerobic degradation of BDE-28 and BDE-209 and anaerobic sediment without PBDEs.

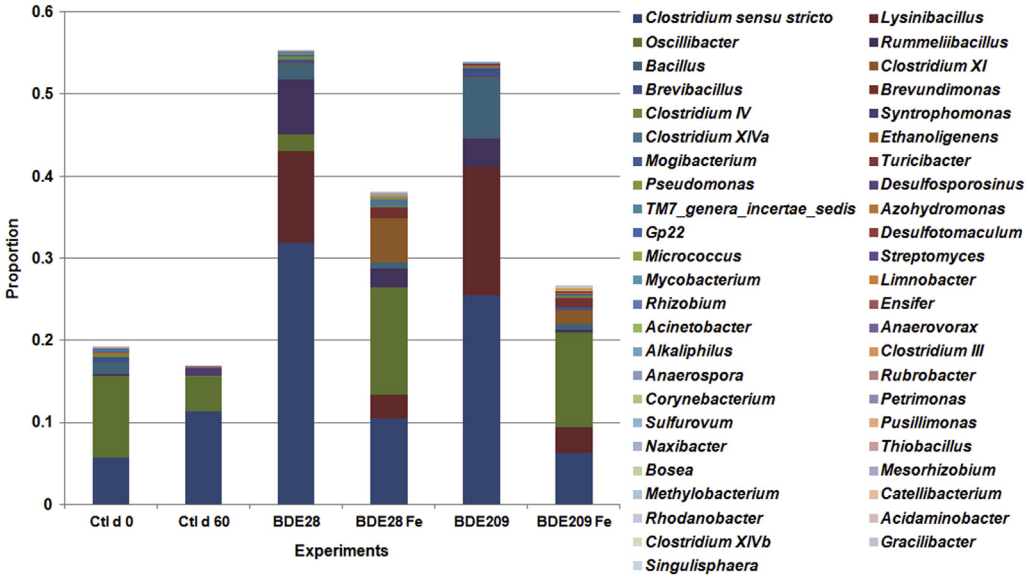

Figure 4. Bacterial community composition (known bacterial genera) in six anaerobic sediment samples. Y-axis indicates proportion of 16S ribosomal RNA gene sequences of each genus in each experiment. BDE = brominated diphenyl ether; BDE28 = with BDE-28 (d 60); BDE28 Fe = with BDE-28 & Fe (d 60); BDE209 = with BDE-209 (d 60); BDE209 Fe = with BDE-209 & Fe (d 60); Ctl d 0 = without PBDEs (d 0); Ctl d 60 = without PBDEs (d 60); d = day; Fe = iron; PBDE = polybrominated diphenyl ether.

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The addition of zero-valent iron further changed the microbial composition.

Bacteria associated with anaerobic degradation of PBDEs

The microbial community composition at class and genus levels are shown in Figs. 3 and 4, respectively. The major known bacterial classes were Clostridia and Bacilli (22～78%) and the major known bacterial genera were *Clostridium*, *Lysinibacillus*, *Oscillibacter*, *Rummeliibacillus*, and *Bacillus* (15.7—54%). To identify bacteria associated with anaerobic degradation of PBDEs, proportions of each class and genus in the samples were used to compute the Pearson correlation coefficient with PBDE remaining percentages in degradation experiments. Five known bacterial classes (Clostridia, Bacilli, Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria) that have negative correlations with PBDE remaining percentages (increasing as PBDE degradation) were identified (Fig. 5A). However, four known archaeal classes (Methanobacteria, Methanomicrobia, Halobacteria, and Thermoprotei) that have positive correlations with PBDE remaining percentages (decreasing as PBDE degradation) were identified (Fig. 5B). Similarly, 19 known bacterial genera (four major genera: *Clostridium*, *Lysinibacillus*, *Rummeliibacillus*, and *Brevundimonas*) that have negative correlations with PBDE remaining percentages (increasing as PBDE degradation) were identified (Fig. 6A). Four archaeal genera (*Methanobacterium*, *Methanosarcina*, *Methanocorpusculum*, and *Halalkalicoccus*) that have positive correlations with PBDE remaining percentages (decreasing as PBDE degradation) were identified (Fig. 6B).

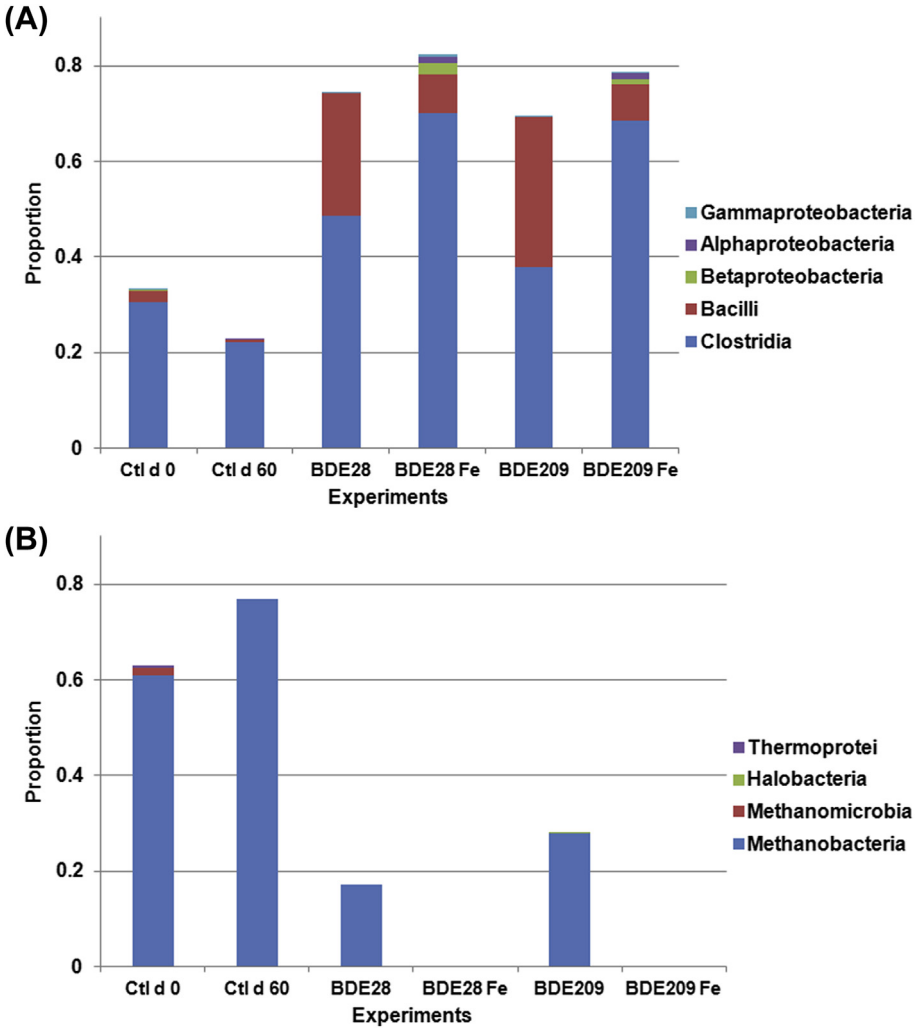

Figure 5. Bacterial and archaeal community composition in anaerobic degradation of BDE-28 and BDE-209. (A) Known bacterial classes have negative correlations between sequence frequencies and PBDE residual proportions. (B) Known archaeal classes have positive correlations between sequence frequencies and PBDE residual proportions. Y-axis indicates proportion of 16S ribosomal RNA gene sequences of each class in each experiment. Correlation coefficients were selected by testing H_0: $\rho = 0$, $p < 0.05$. BDE = brominated diphenyl ether; BDE28 = with BDE-28 (d 60); BDE28 Fe = with BDE-28 & Fe (d 60); BDE209 = with BDE-209 (d 60); BDE209 Fe = with BDE-209 & Fe (d 60); Ctl d 0 = without PBDEs (d 0); Ctl d 60 = without PBDEs (d 60); d = day; Fe = iron; PBDE = polybrominated diphenyl ether.

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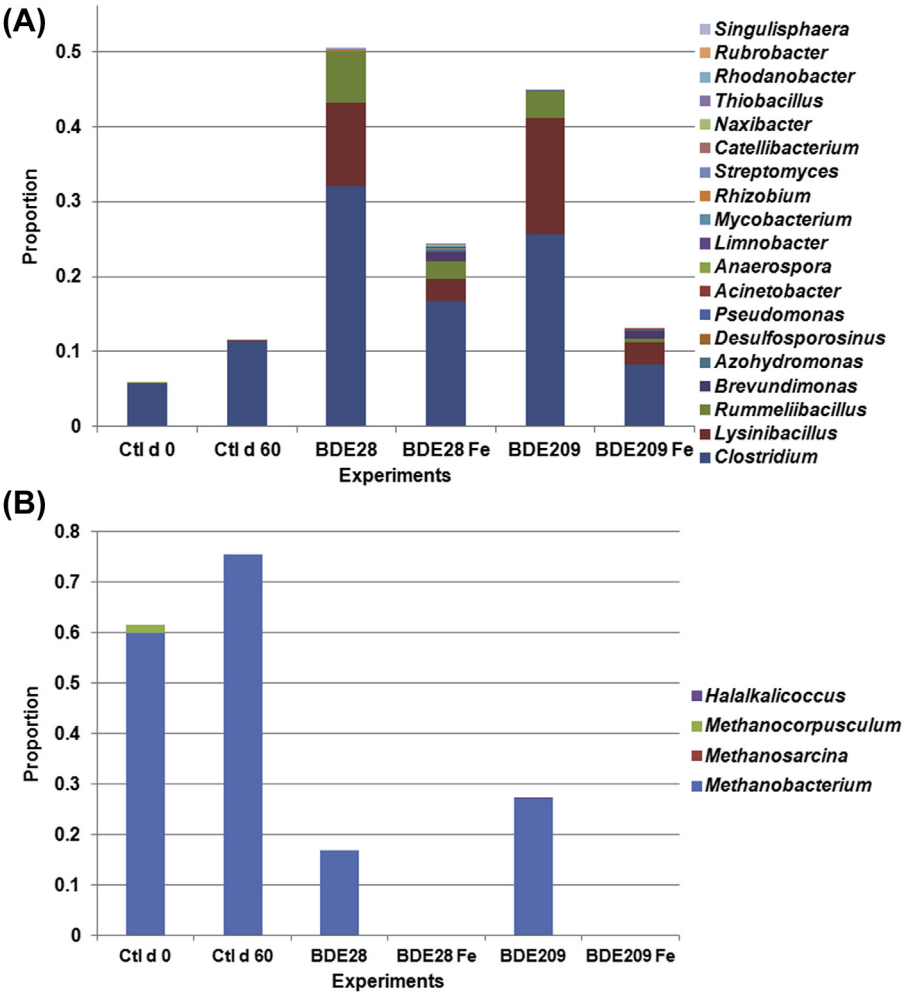

Figure 6. Bacterial and archaeal community composition in anaerobic degradation of BDE-28 and BDE-209. (A) Known bacterial genera have negative correlations between sequence frequencies and PBDE residual proportions. (B) Known archaeal genera have positive correlations between sequence frequencies and PBDE residual proportions. Y-axis indicates proportion of 16S rRNA gene sequences of each gene in each experiment. Correlation coefficients were selected by testing H_0: $\rho = 0$, $p < 0.05$. BDE = brominated diphenyl ether; BDE28 = with BDE-28 (d 60); BDE-28 Fe = with BDE-28 & Fe (d 60); BDE20 = with BDE-209 (d 60); BDE209 Fe = BDE-209 & Fe (d 60); Ctl d 0 = without PBDEs (d 0); Ctl d 60 = without PBDEs (d 60); D = day; Fe = iron; PBDE = polybrominated diphenyl ether.

These results suggest not only change of bacteria composition but also decreased archaea composition affecting the anaerobic degradation of BDE-28 and BDE-209.

Bacteria associated with anaerobic degradation of PBDEs in different settings

To obtain more details regarding the bacteria associated with anaerobic degradation of PBDEs in different settings (presence and absence of zero-valent iron for BDE-28 and BDE-209), we examined the distribution of bacterial genera with sequence frequency negatively correlated with PBDE remaining percentage (increased with PBDE degradation). Four bacterial classes (Bacilli, Clostridia, Alphaproteobacteria, and Gammaproteobacteria) were common in four experimental settings (Fig. 7A). The class Betaproteobacteria was identified in three experimental settings except in anaerobic degradation of BDE-209 without zero-valent iron.

At the genus level, three bacterial genera (*Clostridium*, *Lysinibacillus*, and *Rummeliibacillus*) were common to four experimental settings. Two bacterial genera (*Pseudomonas* and *Desulfosporosinus*) were identified in three of the four experimental settings. These bacteria may represent the core bacteria in anaerobic degradation of BDE-28 and BDE-209. Three bacterial genera (*Brevundimonas*, *Azohydromonas*, and *Acinetobacter*) were common in experiments with zero-valent iron. These bacteria extend the core bacteria composition in anaerobic degradation of BDE-28 and BDE-209 in the presence of zero-valent iron. Five bacterial genera (*Rhizobium*, *Rubrobacter*, *Streptomyces*, *Rhodanobacter*, and *Singulisphaera*), associated with anaerobic degradation of BDE-28, were not associated with other experimental settings. Similarly, six bacterial genera (*Naxibacter*, *Thiobacillus*, *Anaerospora*, *Limnobacter*, *Mycobacterium*, and *Catellibacterium*), associated with anaerobic degradation

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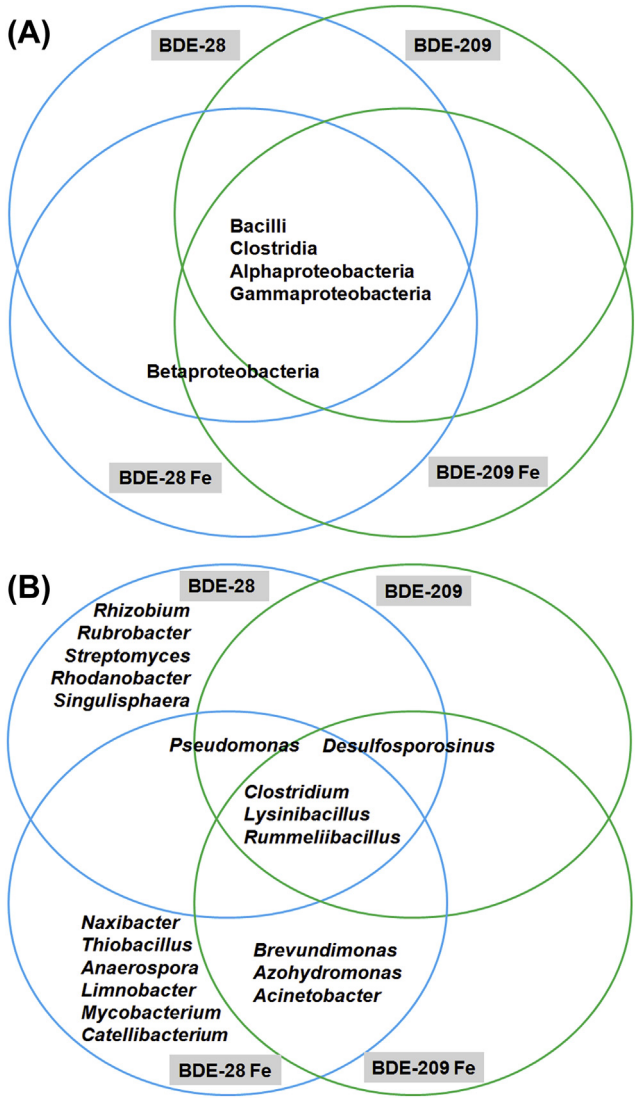

Figure 7. Comparisons of bacterial classes (A) and genera (B) associated with anaerobic degradation experiments of BDE-28 and BDE-209. BDE = brominated diphenyl ether.

of BDE-28 with zero-valent iron, were not associated with other experimental settings.

Discussion

Here, we aimed to uncover the changes in microbial community composition with PBDE anaerobic degradation with zero-valent iron from sediment of the Erren River. The anaerobic degradation rate was higher for BDE-209 than BDE-28, and the addition of zero-valent iron enhanced the degradation rates of both. In total, 19 bacteria were considered PBDE degradation-associated (sequence frequency negatively correlated with PBDE remaining percentage) as were four known archaeal genera (*Methanobacterium*, *Methanosarcina*, *Methanocorpusculum*, and *Halalkalicoccus*; sequence frequency positively correlated with PBDE degradation). The composition of bacteria and that of archaea may affect the anaerobic degradation of BDE-28 and BDE-209.

High-brominated PBDE congeners are degraded via a reductive debromination process, and their degradation rates are higher than that for low-brominated PBDEs.[12] The observation in this study is consistent with our previous finding that the anaerobic degradation rates of PBDE congeners in sediment were in the order of BDE-209 > BDE-99 > BDE-47 > BDE-28 > BDE-15.[6] The addition of zero-valent iron could reduce PBDEs to less-brominated compounds by anaerobic microbes.[13] Also, sorption could play a role in the BDE-209 anaerobic degradation with the addition of zero-valent iron.[14]

Here we found the classes Gammaproteobacteria, Clostridia, and Betaproteobacteria may be involved in anaerobic degradation of PBDEs. These results are similar to Qiu et al,[15] who reported the effects of electron donors on the anaerobic degradation of PBDEs. The genus *Clostridium* and *Pseudomonas* were present in anaerobic degradation of PBDEs. This finding is consistent with that of Shih et al,[16,17] who studied anaerobic degradation of PBDE with sludge. The addition of zero-valent iron further decreased the composition of archaea to an undetectable level for possibly two reasons. First, BDE-28 and BDE-209 and/or their degradation products such as bromine are toxic to archaea. Second, BDE-28 and BDE-209 and/or their anaerobic products such as bromine change the physical and chemical condition of the sediment. BDE-28 and BDE-209 and/or their degradation products may consume H_2, ammonium, sulfide, or elemental sulfur in the sediment, which are essential for the survival of archaea. As a result, the bacteria composition associated with BDE-28 and BDE-209 degradation was increased, with decreased archaea composition. The addition of zero-valent iron might further enhance these factors. The observation of more known bacterial genera associated with degradation of BDE-28 than BDE-209 (Fig. 7B) regardless of presence or absence of zero-valent iron may be due to the higher toxicity of BDE-209 and its degradation products than BDE-28.[18,19]

In conclusion, the anaerobic degradation rate was higher for BDE-209 than BDE-28 in sediment from the Erren River, one of the most heavily contaminated rivers in Taiwan. The addition of zero-valent iron enhanced BDE-28 and BDE-209 anaerobic degradation rates. A total of 19 known bacterial genera (*Clostridium*, *Lysinibacillus*, *Rummeliibacillus*, and *Brevundimonas*) were associated with PBDE remaining percentage (sequence frequency negatively associated) as were four archaeal genera (*Methanobacterium*, *Methanosarcina*, *Methanocorpusculum*, and *Halalkalicoccus*; sequence frequency positively associated). These results suggested not only the changes in bacterial composition but also decreased archaea composition affecting anaerobic degradation of BDE-28 and BDE-209 and provided an insight into the bacterial community structure and diversity involved in PBDE anaerobic degradation in contaminated river sediment.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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Acknowledgments

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