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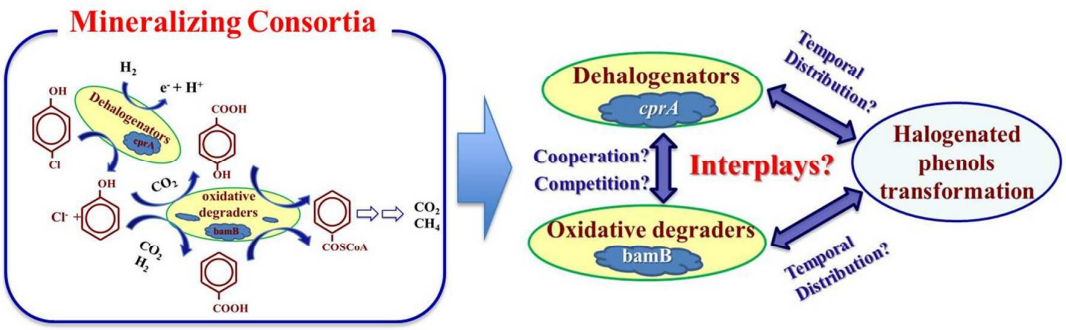

Growth interactions of functional dehalogenators, degraders and genes (*cprA* and *bamB*) during anaerobic mineralization of HACs in an enriched consortium

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Title:

Temporal distributions of functional microbes and putative genes associated with halogenated phenol anaerobic dehalogenation and further mineralization

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Abstract:

Several anaerobic systems have been established for mineralizing halogenated aromatics (HACs) by synthesizing dehalogenation and oxidative degradation consortium. However, functional interplays of bacterial and genetic activity during HAC mineralization are poorly understood. In this study, temporal distributions of potential dehalogenators, oxidative degraders and relevant functional genes were investigated in an anaerobic consortium for mineralizing HACs. The consortium held the wide mineralization potential, and was able to mineralize various types of HACs with halogens on *ortho*- and *meta*-positions, such as 4-chloropheol (4-CP) and 2,4,6-trichlorophenol (2,4,6-TCP). Copy numbers of potential dehalogenators (*Dehalobacterium* and *Sulfurospirillum* spp.) and phenol degraders (*Geobacter* spp.) proliferated enormously (10-10^2 copies·ml^{-1}) and maintained at high lever (10^5-10^7 copies·ml^{-1}) throughout 4-CP and 2,4,6-TCP mineralization processes (60 days). Meanwhile, growth of two functional genes, putative chlorophenol reductive dehalogenase *cprA* and benzoyl-CoA reductase *bamB* genes showed the similar trends, with numbers increased over 10 copies·ml^{-1} averagely. In comparison, under condition without addition of HAC, no significant growth of potential dehalogenators, degraders and functional genes was observed. Distribution trends of functional bacteria and genes revealed the simultaneous satisfaction of redox niches of both dehalogenation and oxidative degradation by an enriched consortium. The study proposed the complete mineralization of a wide range of HACs by supplying one consortium containing multiple functional microbes and simple nutrient supplement under anaerobic conditions.

Keywords: Anaerobic mineralization; Halogenated aromatic compounds; Temporal distribution; Functional genes; Dehalogenation; Phenol oxidative degradation

1. Introduction

Halogenated aromatic compounds (HACs) are a kind of human synthesized organic compounds that widely applied in industry and agriculture. Based on the large application, most of them are frequently detected in river sediment and subsurface zones [1]. Because of high toxicity and bioaccumulation properties, they have posed severe threaten to human health and ecological safety. Therefore, several types of HACs are listed as priority pollutants by US Environmental Protection Agency [2].

Detoxification of HACs with microorganism has addressed an increasing attention as a promising and cost-effective remedaition approach. Under aerobic conditions, several aerobic isolates, such as *Pseudomonas* or *Burkholderia* sp., have been reported that mineralized HACs through oxidative/hydrolytic dehalogenation and oxygenolysis. Meanwhile, the function of enzymatic genes were well elucidated [3,4]. Under subsurface zones where anaerobic environment prevalent, reductive dehalogenation by organohalide-respiring bacteria plays a vital role to decompose HACs to less/none halogenated ones [5]. Next, an oxidative degradation step of dehalogenated compounds to non-toxic is obligatory to completely remove the toxicity of HACs [6]. Since dehalogenation and degradation processes involve the different redox niches and nutrient requirement, simultaneous proceeding of dehalogenation and degradation was traditionally considered difficult to be realized [5,7].

Recently it is found under given synthesis conditions, HAC anaerobic mineralization could be achieved by sequential applying or enriching dehalogenating and anaerobic degrading consortia [6,8-10]. Li et al [6,8] constructed pentachlorophenol (PCP) anaerobic mineralization systems by either sequential combination or gradual acclimation of dechlorinating and degrading cultures. A tribromophenol anaerobic

mineralization consortium was synthesized by optimizing the initial concentration of strain/species and nutrients [10]. However, most constructed systems were highly specific to only one of HACs and the mineralization activity was restrictive to nutrient competition between the different bacterial species [6,8,10]. Also, bacterial interplays involved working mechanism was little understood. Clarification of growth law of functional species during HAC transformation would facilitate the understanding of the competitive or cooperative interrelations between species during mineralization process. The study would give suggestion for the adaptability and regulation strategy for complete mineralization of HACs by the enriched consortium.

Previously, an anaerobic consortium capable of mineralizing 4-chlorophenol (4-CP) under amorphous FeOOH reducing condition was enriched and steadily maintained [8]. 4-CP mineralization pathway was elaborated through dechlorination and oxidative degradation of phenol to completely CO_2 and CH_4 with benzoate and 4-hydroxybenzoate (4-OHB) as the intermediate metabolites. In this study, the mineralization spectrum by this consortium was investigated. Also, temporal distributions of involved bacteria (*Dehalobacterium*, *Sulfurospirillum* and *Geobacter* spp.) and functional genes (chlorophenol reductive dehalogenase *cprA* and benzoyl-CoA reductase/ring-cleavage hydrolasegenes *bamB*) during 4-CP and 2,4,6-trichlorophenol (2,4,6-TCP) decomposition were extensively investigated. Objects of this study were to reveal the competitive or cooperative interrelations of functional species during HAC mineralization process and better understand the working spectrum and mechanism of the enriched mineralizing consortium.

2. Materials and Methods

2.1. 4-CP mineralization consortium: culturing condition and bacterial composition

The consortium was initially enriched from paddy soil and acclimated with phenol by periodic spiking with 100 mM of phenol for more than one year. Then the acclimated soil was taken and introduced to the prepared medium containing certain amount of 4-CP (50 μM) and FeOOH (10 mM). Once 4-CP degradation was observed, about 10% culture was transferred to new medium. The stable consortium was obtained after continuous transferring for over twentieth generation [9]. The culture inoculation method was described as follows: 20 mL of mineral medium was stored in 60 mL serum bottles capped with rubber stoppers and sealed using aluminium crimp caps. The headspace was flushed and maintained with nitrogen and carbon dioxide (5:1). Consortium were inoculated with 10% of consortium suspension with the stable 4-CP decomposition activity after amended with 20 μM or 50 μM of halogenated phenols, 5 mM of FeOOH stock solution, 10 % of mixed vitamin solution, 0.01 g L^{-1} of yeast extract and 10% of titanium (III)-trinitriloacetic acid (NTA) solution. The culture was incubated in dark at 30°C without agitation. Introduction of ^{14}C-U-ring-labeled 4-CP showed 4-CP was mineralized into CO_2 (25.1%), CH_4 (37.2%) and nonhalogenated compounds (phenol, benzoate, 4-OHB) (30.3%) after incubation for 60 days [6]. 16S rRNA gene clone library analysis revealed *Dehalobacterium* and *Sulfurospirillum* were the potential genus that involved in reductive dechloriantion, and while, *Geobacter* was possibly associated with the further oxidative decomposition of nonhalogenated aromatic compounds. Decomposition activities of halogenated/nonhalogenated aromatic compounds other than 4-CP were also testified in this study, with the same way for cultural incubation and inoculation as described above, except for the introduction of other HACs instead of 4-CP.

2.2. Chemical analysis of aromatic organic compounds and chloride ion

Concentration of halogenated phenols and phenol were determined by a gas chromatograph-mass spectrometer (GC-MS QP5050A, Shimadzu, Kyoto, Japan) equipped with a DB-5MS column (Shimadzu, Kyoto, Japan) and an AOC-20i autosampler (Shimadzu, Kyoto, Japan) [6]. Concentration of benzoate and 4-hydroxybenzoate (OHB) were determined with high-performance liquid chromatography (HPLC) (CTO-10A; Shimadzu, Kyoto, Japan) equipped with an ultraviolet detector (UV 280 nm) and PuresilTM C18 column (4.6 mm inner diameter, 250 mm in length, Waters, MA). The applied mobile phase consisted of CH_3CN: H_2O: CH_3COOH with the proportion of 65:34:1. Cl^- was measured by a Metrohm Compaction Chromatograph 761 (Metrohm, Herisau, Switzerland) equipped with an SI-90 4E column. To measure Cl^- release, Ti (III)-NTA was replaced with Na_2S (0.2mM). The background Cl^- came from introduction of FeOOH stock solution.

2.3. QPCR analysis of the potential dechlorinators and phenol degraders

DNA was extracted with 2 mL of enriched consortium sample with a DNA extraction ISOPLANT kit (Nippon Gene Co., Tokyo, Japan). Quantitive real-time PCR (*q*PCR) was performed by a LightCycler system (Roche Diagnostics, Mannheim, Germany) and LightCycler Fast-start DNA Master SYBR green I kit (Roche Molecular Biochemicals, Indianapolis, India) [10]. Bacteria, *Sulfurospirillum* sp., and *Geobacter* sp. were determined with primer sets of Eub341f and Eub534r [10], Sulfuro114f and Sulfuro421r [11], and Geo494f and Geo825r [12], respectively. Primer set of Dhbr1162f and Dhbr1376r was specially designed by alignment of 16S rRNA gene sequences of *Dehalobacterium* sp. in this consortium, *Dehalobacterium formicoaceticum* strain DMC, all reported uncultured *Dehalobacterium* spp. and the closest related strains with *Dehalobacterium formicoaceticum* strain DMC in BLAST program

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) in GenBank. The comparison was conducted using BioEdit Sequence Alignment Editor software (version 7.0.5.3, Ibis Biosciences, Carlsbad, CA). Calibration curve (log DNA concentration versus an arbitrarily set cycle threshold value) for Bacteria was constructed using serial dilutions of amplicons of *Escherichia coli* K12. Calibration curves for *Dehalobacterium* sp., *Geobacter* spp., and *Sulfurospirillum* sp., were constructed using the serial dilutions of the single colony amplicons of *Dehalobacterium*, *Geobacter* and *Sulfurospirillum*, respectively, after setting up the 16S rRNA clone library with bacterial consensus primers (27f and 1492r). Single colonies of *Dehalobacterium* and *Geobacter* were obtained in this consortium and single colony of *Sulfurospirillum* sp. was obtained in a 3-CP reductive dechlorination consortium (unpublished). Detection limit for Bacteria, *Dehalobacterium* spp. *Sulfurospirillum* sp. and *Geobacter* spp. were 2.8×10^2, 6.5×10^2, 1.1×10^2 and 1.5×10^2 copies ml^{-1}, respectively.

2.4. Primer design and qPCR analysis of cprA and bamB genes

The primer pair specific for chlorophenol reductive dehalogenase *cprA* gene fragments was designed as f4-m3-g1 (5'-CRGAACYCTYGGYTAYAWTGC-3', position 774-794) and r3-m3g1 (5'-CCATAVCCRAAGATATCATC-3', position 1255-1274) targeting for binding motifs of two iron-sulfur clusters, modified by combining the recently released *cprA* gene sequences [5,13,14]. The gene sequences applied for primer design included *cprA* (*Desulfitobacterium dehalogenans* ATCC 51507, *Desulfitobacterium* sp.Viet-1, *Desulfitobacterium hafniense* DCB-2, and *Dehalobacter restrictus* with accession numbers of AF115542, AAG49544, AAG46192, and CAC37166, respectively), *cprA1* (*Desulfitobacterium* sp. PCE1, *Desulfitobacterium chlororespirans* and *Desulfitobacterium hafniense* DCB-2 with accession numbers of

AAG46187, AAL84925, AAG46192, and AF403182, respectively), *cprA2* (*Desulfitobacterium* sp. PCE1, *Desulfitobacterium chlororespirans* with accession numbers of AAG49543 and AAG43483 and *Desulfitobacterium hafniense* DCB-2 contig 3277), and *cprA3* (*Desulfitobacterium hafniense* DCB-2 contig 3277, 3246 and *Desulfitobacterium hafniense* PCP-1 with accession numbers of YP_002457213). In addition, specific primer pair of *bamB* (BamBf, ATGMGGTAYGSAGARACHGG; BamBr, CCSGCRWRYTTCADYTCCG) targeted for active-site subunit of class II benzoyl-coenzyme reductases, was applied for *bamB* gene amplification involved in phenol oxidative decomposition from obligate anaerobes [7].

Calibration curves (log DNA concentration versus an arbitrarily set cycle threshold value) for putative functional genes (*cprA* and *bamB* genes) were constructed using serial dilutions of amplicons of single colonies, obtained from setting up the 16S rRNA clone library with the bacterial consensus primers (27f and 1492r) of DNAs from 4-CP mineralization consortium. The *q*PCR detection limits for *cprA* and *bamB* genes were 2.5×10^2 and 3.4×10^2 copies mL^{-1}, respectively. Triplicate cultures were examined for each condition.

3. Results and discussion

3.1. Decomposition capacity of halogenated or non-halogenated aromatic compounds

The consortium degraded 65 μM of 4-CP within 60 days, and while, Cl^- release was observed following 4-CP decomposition, considered to be originated from 4-CP dechlorination (Fig.1A). Besides 4-CP, decomposition capacities of the other halogenated or non-halogenated aromatic compounds were also evaluated, as shown in Table 1. The consortium showed the decomposition activities on 2,4,6-TCP, 2,4,6-TBP, 2,4-DCP and 2-CP (Table 1). Both the decomposition spectrum and metabolites

indicated that the dehalogenation was occurred on *ortho* and *para* positions. No decomposition ability was appeared on pentachlorophenol and 3-CP, revealing the incapability of dehalogenation on *meta*-position. It is considered halogen on *meta* position was the most difficult to be removed, attributed to the halogen position farthest from the hydroxyl active site, compared with halogens on *ortho* and *para* positions [9]. Among of them, decomposition rate of 2,4,6-TBP was considered the highest, since 65 μM of 2,4,6-TBP was completely removed after 60 days. Other than that, the consortium showed the decomposition capacity of phenol, 4-OHB and benzoate. The results indicated a wide range of dehalogenating and mineralizing potentials of both HACs with halogens on *ortho* and *para* positions and also non-halogenated aromatic compounds.

3.2. Temporal distribution of potential bacteria during 4-CP degradation

After incubation for 5 days, copy number of bacteria increased about 10^2 times and maintained at 10^7-10^8 copies ml^{-1} later (Fig. 1B). Meanwhile, exponential growth of *Dehalobacterium* and *Sulfurospirillum* spp., the two dehalogenation species [15,16], were observed on the 5th day. Their copies were highly maintained throughout 4-CP degradation process. *Dehalobacterium* and *Sulfurospirillum* spp. occupied 3-34% and 10-53% of total bacterial species, respectively. *Geobacter* spp., the phenol oxidative degrader that utilized FeOOH as electron acceptor [17], was gradually increased as 4-CP degradation carried out. Copies of *Geobacter* spp. approached to the highest at 50-60 days (6×10^6 copies ml^{-1}), occupied 7-10% of the total bacterial species. Under condition without amendment of 4-CP, the growth of *Dehalobacterium*, *Sulfurospirillum*, or *Geobacter* were not evident, about 3, 4 and 2 times in average, respectively (Fig. 1C). During 60 days, average copy numbers of *Dehalobacterium*, *Sulfurospirillum* and

Geobacter spp. corresponded to 1-5%, 2-5% and 1-7% of the total bacterial species, respectively. The proliferation in numbers of *Dehalobacterium*, *Sulfurospirillum* and *Geobacter* spp. confirmed their involvement during 4-CP and 2,4,6-TCP dechlorination and further mineralization. It is noticed the growth trend of *Geobacter* was similar with the growth of both bacteria and dehalogenating population. As phenol was occasionally detected with a low concentration [8], it is speculated that phenol degradation occurred simultaneously with 4-CP dechlorination.

3.3. Temporal distributions of putative functional genes during 4-CP decomposition

Abundance of *cprA* and *bamB* genes were examined with extracted DNAs on the 15^{th} and 40^{th} days during 4-CP decomposition, respectively. With the designed primer for *cprA*, 500 bp lengths of products were successfully amplified. More than 95% of the selectively picked up colonies possessed two-conserved iron-sulfur cluster binding motifs and shared the highest similarity with the reported *cprA* genes from BLAST program of GenBank nucleotide sequence database (data not shown). This indicated the putative *cprA* genes and the validity of the designed primers. By applying the specific primer for *bamB*, about 320 bp lengths of products were successfully amplified. Among of them, over 95% of picked colonies shared the highest similarities with the identified *bamB* genes (data not shown), suggesting the successful amplification of putative *bamB* gene.

Numbers of both *cprA* and *bamB* increased more than 10 times after incubation for 5 days and maintained at about 10^5-10^6 copies ml^{-1} subsequently (Fig. 2A). In contrast, without amended with 4-CP or 2,4,6-TCP, number increase of neither *cprA* nor *bamB* were obvious (Fig. 2B). Trends of temporal distribution of putative *cprA* and *bamB* genes were coincided with those of the potential dechlorinators (*Dehalobacterium* and

Sulfurospirillum spp.) and phenol degrader (*Geobacter* spp.), respectively (Fig. 1B and C). The exponential growth in numbers of potential bacteria and putative genes, suggested their significant roles during 4-CP decomposition. It is noticed copy numbers of *cprA* and *bamB* genes were the most dominant at 30 and 50 days, accounted for 5-17% and 8-56% of the summed potential dechlorinators and degraders, respectively. In fact, one cell usually contained several unique putative functional genes [18]. The reason of the low percentage of functional genes would attribute to the restrictive amplification ability with the designated primers.

3.4. Distributions of functional bacteria and genes during 2,4,6-TCP decomposition

In the enriched consortium, concentration of 2,4,6-TCP were gradually decreased and disappeared after 35 days, and correspondingly, 2,4-DCP and 4-CP were generated as intermediate metabolites, illustrating dehalogenation as the initial decomposition process (Fig.3A). The obvious decrease of both 4-DCP and 4-CP at 60 days suggested their further degradation. When 2,4,6-TCP decomposition was initialled at 5 day, copies of *Dehalobacterium*, *Sulfurospirillum* and *Geobacter* spp. increased to approximately 10, 10 and 5 times, respectively, and maintained at the relative high levels subsequently (Fig.3B). Meanwhile, both *cprA* and *bamB* genes increased about 10 times after incubation for 5 days and maintained at about 10^5-10^6 copies ml^{-1} after that (Fig. 3C). The high copies of *Dehalobacterium* sp., *Sulfurospirillum* sp. and putative *cprA* gene were maintained throughout the degradation process, coincided with the high dehalogenation activity (Fig. 3A). While, the highest numbers of *Geobacter* spp. and *bamB* genes appeared at 45-60 days, indicated the possible phenol further degradation after 4-CP was further dechlorinated.

3.5. Outlook of this work

Previously, HAC mineralization systems were set up in various anaerobic environments, mainly focused on mineralization capacity and efficiency or optimization strategy [6,9,10]. Kennes et al. [19] and Wu et al. [20] firstly reported the anaerobic mineralization of chlorinated phenols in bioreactors under methanogenic conditions, however, the mineralization involved bioprocess, final metabolites and bacterial population were largely unknown. Li et al [6,8] used to construct pentachlorophenol (PCP) mineralizing anaerobic systems by sequential combining or simultaneous acclimating of dechlorination and phenol degradation cultures. The way of construction achieved the satisfactory mineralizing activity with the high efficiency and non-organic final metabolites. Nutrient competition between acclimated species was observed, however, functional roles of involved species during dechlorination and further mineralization processes was unclear. Also, these constructed systems could only mineralize PCP and the mineralization activity with other HACs was unclear. Li et al [10] synthesized a tribromophenol anaerobic mineralization consortium with isolated strains/species, and found under certain initial concentration of strain/species and nutrients, debromination and 4-CP degradation were sequentially achieved with the exponential growth of *Dehalobactor* sp. and *Desulfatiglans* strain DS. But, the simultaneous maintenance for growth of both dehalogenators and 4-CP degraders could not be realized. In this study, the enriched consortium showed the dehalogenation and further degradation potential of various HACs (Table 1), exhibited the wide mineralization spectrum. In actual *in situ* sites or *ex situ* reactors, HACs are usually coexisted simultaneously in diverse types because of their similar physicochemical properties [1]. Therefore, compared with the previous achieved systems, the consortium reported in this study held a wider capacity of application potential.

In addition, investigation of temporal distributions of chemicals, potential bacteria and functional *cprA* and *bamB* genes (Fig. 1; 2; 3), clearly revealed the simultaneous growth and cooperation function of potential dehalogenators and degraders to achieve the mineralization of 4-CP and 2,4,6-TCP. In fact, optimal ranges of redox environment required for chlorophenol dehalogenation and iron reduction were approximately +250 to +600 mV [18], +200 to +300mV [1], respectively. This indicated the consortium held the capacity to satisfy the redox niches of two different bioprocesses. It is noticed, simple nutrient supplement, like FeOOH and common anaerobic medium, was sufficient for all functional population growth, which simplified the potential operational condition. Also, nutritional competition was effectively avoided during the simultaneous growth of dehalogenators and degraders, indicating the better adaptability and feasibility.

So far, studies on identification and characterization of *cprA* and *bamB* genes are on the beginning and the identified functional genes are still in restricted genomes [7,21]. Usually, several unique putative functional *cprA* and *bamB* genes were present in dechlorinators or phenol degraders [7,18]. Isolation and characterization of these functional bacteria and genes would be further carried out to extend understanding of the genetic functionalization mechanism and the mineralization mechanism of HACs in molecular aspect.

4. Conclusions

Collectively, the study proposed an enriched consortium with the wide mineralization potentials of various types of HACs with halogens on *ortho*- and *meta*-positions. During 4-CP and 2,4,6-TCP mineralization, copies of *Dehalobacterium* sp., *Sulfurospirillum* sp. and *Geobacter* spp. proliferated enormously and maintained at high lever (10^5-10^7 copies·ml^{-1}), indicating their important roles for HAC

mineralization. The simultaneous growth of functional bacteria indicated the simultaneous satisfaction of redox niches of both dehalogenation and oxidative degradation and verified the feasibility of the complete mineralization capacity. Identification of the mineralization spectrum and growth roles of functional bacteria and genes gave suggestions for the efficient mineralization of HAC by the enriched anaerobic consortium in HAC-contaminated sites.

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Figure legends

Figure 1. Temporal distributions of gene copy numbers of the potential bacteria in the

consortium within 60 days. (A): 4-CP degradation and release of chloride ion; (B): Gene copy numbers of Bacteria, *Dehalobacterium* sp., *Sulfurospirillum* sp., and *Geobacter* sp. during 4-CP decomposition; (C): gene copy numbers of *cprA* genes and *bamB* genes without amended with 4-CP. Data was obtained from triplicate cultures with the standard deviations marked with vertical positive error bars.

Figure 2. Temporal distributions of gene copy numbers of the putative functional genes (A): Gene copy numbers of *cprA* and *bamB* genes during 4-CP decomposition; (B): Gene copy numbers of *cprA* and *bamB* genes without amended with 4-CP. Data was obtained from triplicate cultures with the standard deviations marked with vertical positive error bars.

Figure 3. Temporal distributions of gene copy number of the potential bacteria and putative functional genes during 2,4,6-TCP degradation in the enriched consortium within 60 days. (A) 2,4,6-TCP degradation and release of 2,4-DCP and 4-CP; (B) Gene copy numbers of Bacteria, *Dehalobacterium*, *Sulfurospirillum* sp., and *Geobacter* sp.; (C) gene copy numbers of *cprA* and *bamB* genes. Data was obtained from triplicate cultures with the standard deviations marked with vertical positive error bars.

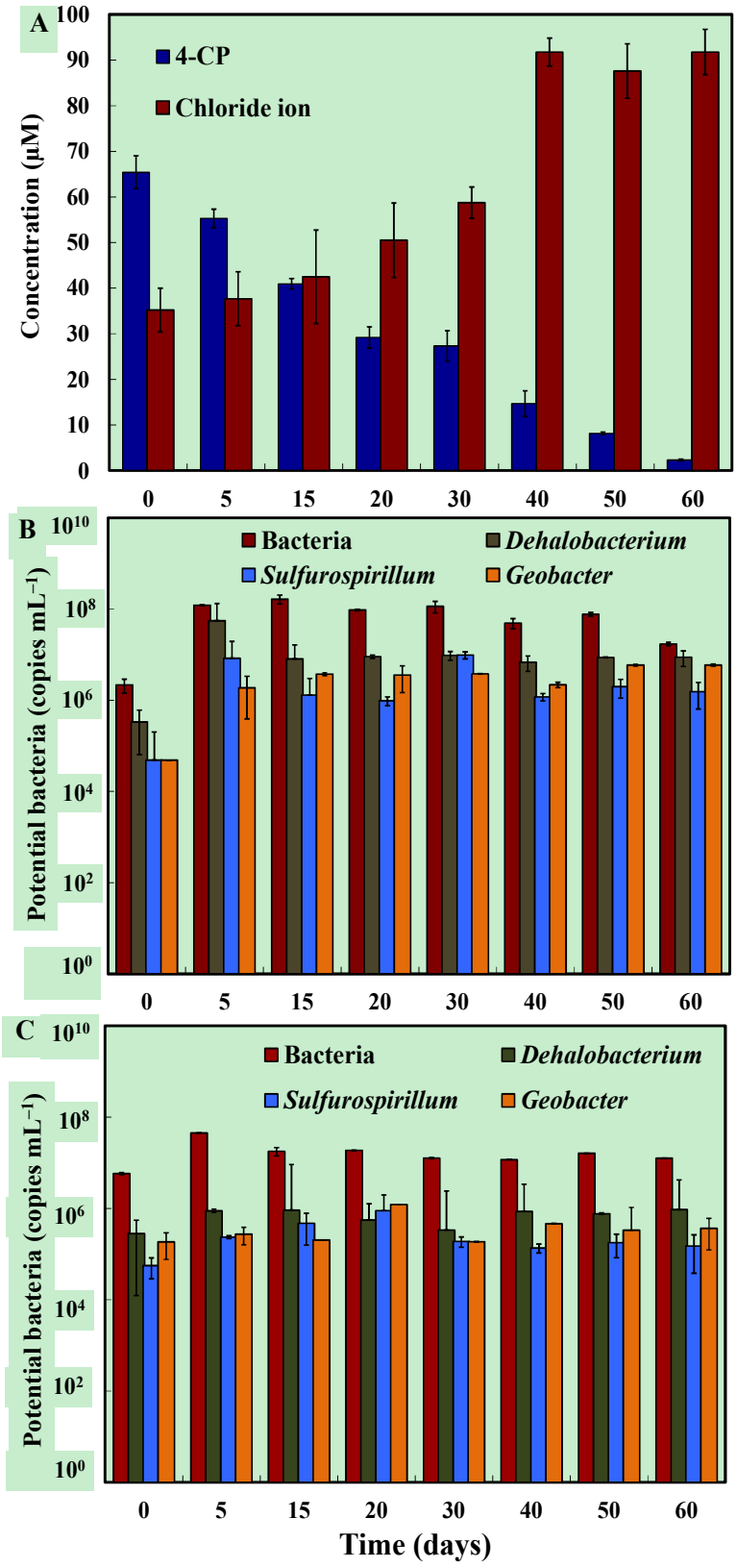

Figure 1.

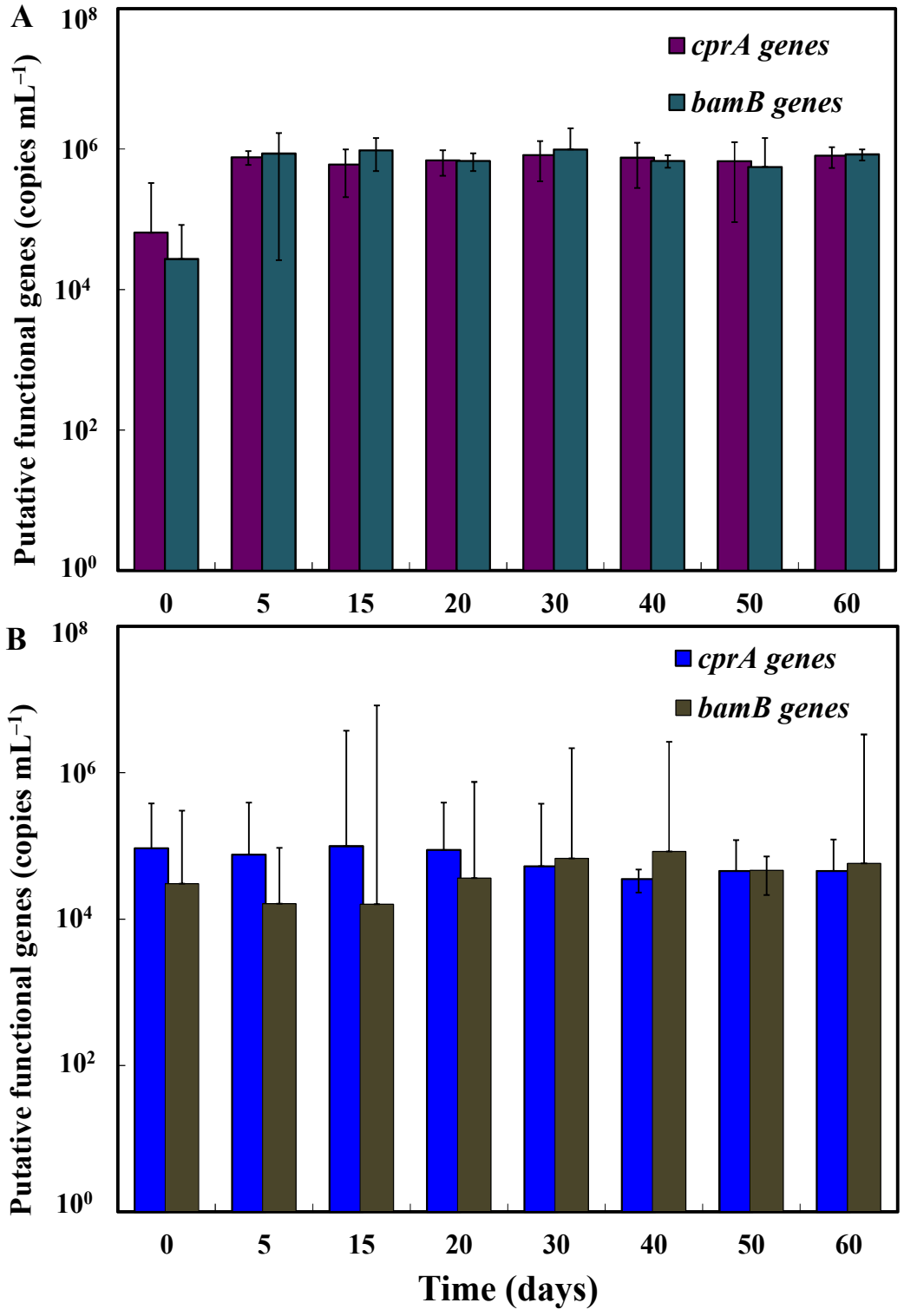

Figure 2.

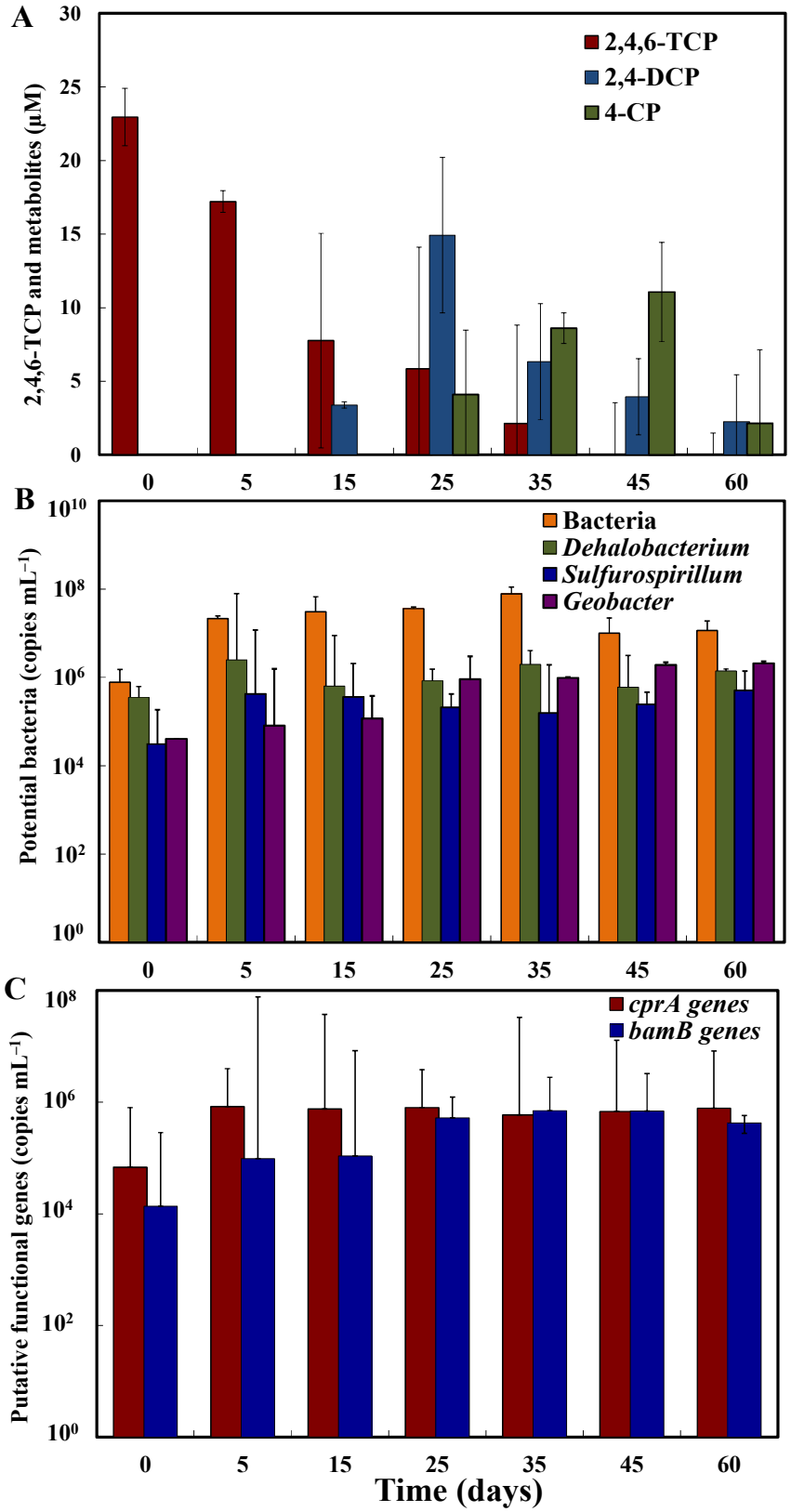

Figure 3.

Table 1. Decomposition capacity of halogenated and non-halogenated aromatic compounds in the enriched 4-CP anaerobic mineralization consortium after incubation for 60 days

Aromatic compounds [a]	Concentrations (μM)		Degradation metabolites
	0 d	60 d	
Pentachlorophenol	51.2 ± 5.5 [b]	52.4 ± 3.8	/ [c]
2,4,6-trichlorophenol	21.6 ± 0.3	N.D. [e]	2,4-DCP and 4-CP [d]
2,4-dichlorophenol	52.3 ± 3.7	15.4 ± 2.6	4-CP [d]
4-chlorophenol	65.4 ± 0.2	8.2± 0.3	Phenol, benzoate and 4-OHB
3-chlorophenol	47.1 ± 2.6	42.4 ± 6.7	/
2-chlorophenol	50.3 ± 1.4	15.5±1.53	Non aromatic compounds
2,4,6-tribromophenol	60.5 ± 3.6	N.D. [e]	4-bromophenol
Phenol	58.9 ± 4.2	N.D.	Non aromatic compounds
4-hydroxybenzoate	50.6 ± 2.5	N.D.	Non aromatic compounds
Benzoate	51.2 ± 4.5	N.D.	Non aromatic compounds

[a] The decomposition capacity of each compound was confirmed with triplicate cultures;

[b] Values on the left-hand side of the "±" symbol indicate the mean results, and values on the right-hand side of the symbol indicate the standard deviation, derived from triplicate cultures;

[c] / indicates the condition when no degradation metabolite was detected;

[d] Carbon balance suggested the further degradation of 4-CP;

[e] N.D. denotes the abbreviation of "not detected" where the concentration was below the detection limit with machine of GC-MS or HPLC.