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**[Keywords]**

Anaerobic; Biodegradation; DBP; Fluorescent In Situ Hybridization; Sludge; Single Strand Conformation Polymorphism

**[Abstract]**

Although anaerobic biodegradation of di-n-butyl phthalic acid ester (DBP) has been studied over the past decade, only a little is known about the microorganisms involved in the biological anaerobic degradation pathways. The aim of this work is to characterize the microbial community dynamics in enrichment cultures degrading phthalic acid esters under methanogenic conditions. A selection pressure was applied by adding DBP at 10 and 200 mg $L^{-1}$ in semi-continuous anaerobic reactors. The microbial dynamics were monitored by Single Strand Conformation Polymorphism (SSCP). While only limited abiotic losses were observed in the sterile controls (20 % - 22 %), substantial DBP biodegradation was shown in the enrichment cultures (90 % - 99 %). In addition, significant population changes were observed. The dominant bacterial species in the DBP-degrading cultures was affiliated to *Soehngenia saccharolytica*, a microbe previously described as anaerobic benzaldehyde degrader. Within the archaeal community, there was a shift between two different species of the genus *Methanosaeta* sp., indicating a highly specific impact of DBP or degradation products on archaeal species. RNA directed probes were designed from SSCP sequences, and Fluorescence *In Situ* Hybridization (FISH) observations confirmed the dominance of *Soehngenia saccharolytica*, and indicated floccular microstructures, likely providing favourable conditions for DBP degradation.

**[Introduction]**

Phthalic acid esters (PAEs) are specifically addressed by EU regulations because of their increasing amounts released in the environment. Over the past years, more than 900,000 tons of PAEs have been produced each year in Europe (ECPI, 2004). Most PAEs are used as plasticizers to increase the flexibility of the polyvinylchloride (PVC) resins, and also as additives in other resins such as polyvinyl acetates, celluloses, and polyurethanes (Staples *et al.*, 1997). The Di-n-Butyl Phthalic acid ester (DBP) is used as additive in epoxy resins, cellulose esters, specialized adhesive formulations, and also as a solvent for dyes, insecticides and other organic compounds (Staples *et al.*, 1997). Widely found in urban wastewaters, DBP is surface active and highly hydrophobic, and readily adsorbs onto sludge organic particles during wastewater clarification. Therefore, DBP up concentrates to orders of magnitude above the values in the original wastewaters. By spreading sludge, DBP may not only accumulate in soils (Hu *et al.*, 2003; Mougin *et al.*, 2006; Patureau *et al.*, 2007), but also readily transfer to plants and animals along the food chain (Yin *et al.*, 2003; Jarosova, 2006). Long term exposure to DBP can adversely affect human reproduction and development, as well as on plants and animals (CEHR, 2000; Kim *et al.*, 2002). Therefore, limitation of DBP contents in sludge before land disposal is strongly recommended. To date, only Denmark within the European Union has fixed a limit of 50 mg $kg_{TS}^{-1}$ for the phthalic acid ester concentration in sludge. Future European Union legislation will probably fix a target limit value of 100 mg $kg_{TS}^{-1}$ for PAEs (Directive 2455/2001/EC).

Several studies have been conducted to assess PAE biodegradability under aerobic (Angelidaki *et al.*, 2000; CEHR, 2000; Yuan *et al.*, 2002), denitrifying (Benckriser & Ottow, 1982), sulfate-reducing (Chauret *et al.*, 1996) and methanogenic conditions (Angelidaki *et al.*, 2000; Wang *et al.*, 2000; Gavala *et al.*, 2003; Chang *et al.*, 2005). DBP is generally described as one of the most readily biodegradable phthalic acid esters because of the shortness of the alkyl branching chain (CEHR, 2000; Yuan *et al.*, 2002; Gavala *et al.*, 2003). Anaerobic conditions remain however less favourable to DBP degradation than aerobic conditions where biodegradation rates are up to ten fold higher (Staples *et al.*, 1997; Yuan *et al.*, 2002). Strong inhibition of methanogenesis was reported after addition of 200 mg $L^{-1}$ of DBP in organic waste-treating reactors (Angelidaki *et al.*, 2000; Gavala *et al.*, 2003). Furthermore, Staples *et al.* (1997) proposed an anaerobic PAE biodegradation pathway based on theoretical considerations. This pathway suggests a first hydrolytic step of one ester bond to form a monoester phthalate and a corresponding alcohol. The second ester bond is hydrolysed and lead to the formation of phthalic acid. Then, the anaerobic mineralization of the phthalic acid by syntrophic methanogenic consortia is possible via the benzoate degradation pathway (Kleerebezem *et al.*, 1999; Qiu *et al.*, 2004). Although several DBP-degrading bacteria have been isolated under aerobic conditions (CEHR, 2000), little is known about the microorganisms involved in DBP biodegradation under anaerobic conditions. Despite the evidence of efficient DBP biodegradation under methanogenic conditions (O'Connor *et al.*, 1989; Ejlertsson *et al.*, 1996; Angelidaki *et al.*, 2000; Gavala *et al.*, 2003), no highly enriched culture or pure cultures have been obtained and characterized in the literature, likely because of the complex syntrophic relationships occurring in anaerobic reactors.

The aim of this work is to characterize microbial dynamics in enrichment cultures degrading DBP under methanogenic conditions. An adapted anaerobic ecosystem was enriched in DBP degraders, and microbial dynamics were monitored by molecular methods.

## [Material and methods]

**Source of methanogenic inoculum.** The methanogenic ecosystem used to inoculate the enrichment reactors was sampled from a full-scale anaerobic sludge digester at Lynetten wastewater treatment plant (Denmark) treating household and industrial wastewaters from the Copenhagen area. The plant treated a number of upstream sources constantly contaminated by phthalic acid esters. The anaerobic digester had been fed with a mixture of primary and secondary sewage sludge, and operated at 35ºC. After sampling, residual biodegradable organic carbon was depleted by storing the inoculum for 15 days at 37°C.

**Chemicals and preparation of the medium.** All chemicals were of analytical grade (>98%). The Pentane and Diethyl-ether solvents as well as the Di-n-Butyl Phthalate solutions were provided by Sigma Aldrich (Saint-Louis, USA). Borosilicate glassware and experimental apparatus were treated overnight at 200°C to remove trace contaminants. Basal anaerobic (BA) medium was prepared according to Angelidaki *et al.* (2000). The BA medium was supplemented with 2 g $L^{-1}$ of yeast extract, flushed with $N_2$:$CO_2$ (80:20, v/v) for 10 min, and autoclaved (120°C, 30 min.). Vitamins were added under sterile conditions, according to Angelidaki *et al.* (2000). Before feeding the reactors, 200 mL of BA medium were freshly amended with 2 mL of DBP solutions at 0 g $L^{-1}$, 1 g $L^{-1}$ or 20 g $L^{-1}$ in Pentane : Diethyl ether (15:85, v/v). The final concentrations of DBP in BA medium were of 0, 10 or 200 mg $L^{-1}$, respectively.

**Experimental enrichment procedure.** Five enrichment reactors were operated: Three were performed with DBP at 0, 10 and 200 mg.$L^{-1}$, called **Blank**, **R10**, and **R200,** respectively. Two sterile control reactors were performed with 1.4% (w/v) sodium azide, 2 % (w/v) formaldehyde and DBP at 10 and 200 mg.$L^{-1}$, called **CTRL10** and **CTRL200,** respectively. The enrichments were carried out in 250 mL serum bottles sealed with Teflon coated rubber stoppers and aluminium caps. Inoculation corresponded to 200 mL of anaerobic digested sludge flushed with a mixture of $N_2$:$CO_2$ (80:20, v/v) for 10 min. Enrichments were carried out for 100 days under semi-continuous conditions with daily manual feeding corresponding to an average hydraulic retention time of 20 days. A two step feeding procedure was performed: It involved sampling of 10 mL reactor content followed, by the addition of 10 mL BA medium supplemented with DBP at 0, 10 or 200 mg.$L^{-1}$, respectively. No DBP was initially added to the inoculum. The sampling procedure was carried out under vigorous agitation to obtain homogeneous outlet sample from the reactor. Reactors were operated in a temperature controlled room (37°C), under continuous magnetic stirring. Biogas production was daily measured, and biogas composition of the headspace was weekly analysed.

**Analytical procedure.** Gas components (methane, carbon-dioxide and nitrogen) analysis and volatile fatty acid (VFA) analysis were performed by GC-TCD and GC-FID respectively (Sorensen *et al.*, 1991). Total Solids (TS) and Volatile Solids (VS) were analysed in triplicates according to the standard methods for examination of wastewater (APHA, 1995). Phthalic acid isomers and benzoic acid concentrations were quantified by high pressure liquid chromatography coupled to UV detection, as described elsewhere (Kleerebezem *et al.*, 1999).

**DBP analysis.** 1 mL of sample was diluted in 9 mL of ultrapure water pH 12, and added to 2 mL of extraction solvent {Pentane : Diethyl ether (15:85 v/v) with 6.6 mg $L^{-1}$ Fluoranthene-d10 (Cambridge Isotope Laboratories, Andover, MA) as internal extraction standard}. Extraction was performed in 15 mL Pyrex tubes capped with a Teflon lined stopper. The tubes were shaken at room temperature in a tube rotator for 24 hours at 170 rpm (Struers, Gerhardt, Germany). The extract was then centrifuged (1500 *g*, 15 min.) and 0.5 mL of supernatant was added to 0.5 mL of GC injection standard {1 mg $L^{-1}$ Phenanthrene-d10 (Cambridge Isotope Laboratories, Andover, MA) in Pentane : Diethyl ether (15:85 v/v)}. DBP concentrations in the extract were quantified by gas chromatography (Agilent Technologies 6890N) coupled to mass spectrometry (Agilent Technologies 5973N) (Christensen *et al.*, 2004).

**Single Strand Conformation Polymorphism (SSCP) procedure.** The procedure of DNA fingerprinting of environmental communities by single strand conformation polymorphism was performed according to Delbes *et al.* (2000), except the following: An aliquot of 2 mL of sludge sample was first centrifuged (6000 *g*, 10 min.), and the pellet was resuspended in 2 mL of 4 M guanidine thiocyanate-tris HCl pH 7.5 0.1 M and 600 µL of 10 % (w/v) N-Lauroyl-Sarcosine. Extraction and purification of bacterial genomic DNA was performed with a QIAAmp DNA stool Mini Kit (Quiagen, Hilden, Germany). The V3 region of the bacterial Small SubUnit rDNA was amplified by PCR with the primers EF330-FUR500 (Table 1). Because of the low amount of *Archaea* in the enrichment cultures, the whole Small SubUnit rDNA of this group was first amplified by PCR using the primers AF333-UR1492 (Table 1). The V3 region of the Small SubUnit rDNA of the *Archaea* was then amplified with the primers AF333-FUR500. The PCR products were analysed by SSCP by addition of a size standard (Genescan-400 Rox; Applied Biosystems), electrophoresis, and computing correction (Genescan software, Applied Biosystems), according to Delbes *et al* (2000).

[TABLE 1]

**Construction of 16S rDNA clone library and phylogenetic analysis.** The V3 region of the total 16S rDNA was amplified with the primers AF333-UR500 for *Archaea* and EF330-UR500 for *Bacteria* (Table1). The PCR products were then cloned according to the TOPO TA cloning kit recommendations (Invitrogen). The clones were then selected to identify individual peaks of SSCP profiles and sequenced (Delbes *et al.*, 2000). An equal portion of rRNA gene (*E.coli* position 326 to 450) was used for the sequence analysis. Sequences were submitted to Genbank for preliminary analysis. The NCBI Blast Software was used to identify putative close phylogenetic relatives. Sequences were aligned to their nearest neighbour with the automated alignment tool of the ARB software package, and manually checked. The sequences have been submitted to the Genbank database under the accession numbers **EF380210** to **EF380215**.

**Fluorescent *In Situ* Hybridization (FISH) procedure and microscopy observations.** The method of Hugenholtz *et al.* (2001) was used for fixation and *in situ* hybridization of the samples. In this study, several 16S rDNA probes were designed and tested for their specificity in targeting the microorganisms identified as potentially involved in DBP degradation (Table 2). The probes were optimised with a hybridisation temperature of 46°C, and wash temperature of 48°C, using blanks as negative controls (Hugenholtz *et al.*, 2001). There was no response to non-target microbes at any formamide concentration, and strongest emission was found at 0 % and 20 % formamide (v/v). The slides were examined using a Zeiss LSM 510 confocal laser scanning microscope (CLSM) with an upright Axioplan 2 microscope and ApoChromat 63/1.4 aperture. Appropriate excitation lasers and emission filters were used for indocarbocyanine (CY3) and fluorescein (FITC) labels. In general, the target bacterial cells were labelled using CY3, all bacteria in FITC, and *Archaea* in CY3.

[TABLE 2]

**[Results]**

**Methanogenic activity of the enrichment cultures**. All active cultures showed significant methanogenic activity during the 110 days experiments (Table 3). The sterile control reactors produced no gas. The biologically active reactors contained approximately 60 % of methane in the biogas. No significant inhibition of methanogenesis by DBP at 10 mg $L^{-1}$ was observed compared to the blank containing no DBP (t value of a t-test = 1.34 < 4.3 at 95 % confidence). In contrast, total biogas production was significantly lower in reactor R200 than in the blank, indicating an inhibitory effect of DBP at 200 mg $L^{-1}$ (value of a t-test =11.1 > 4.3 at 95 % confidence). Furthermore, no VFA accumulation was detected in R200 (< 5 mM), suggesting that degradation of VFA was not specifically inhibited, and that inhibition was rather affecting initial biodegradation steps. No phthalic acid or benzoic acid accumulation was observed in the biological reactors. In contrast, because of dilution in the reactors, the concentration of total biomass (VS) constantly decreased in the sterile control reactors over enrichment time from 7.2 ± 0.6 g $L^{-1}$ to 1.51 ± 0.04 g $L^{-1}$ (CTRL10) and 1.34 ± 0.03 g $L^{-1}$ (CTRL200) at steady state. The VS content of these reactors corresponded to the remaining yeast extract and DBP in reactor outlet. The higher VS amounts in the controls were likely due to a lack of biological hydrolytic activity on the remaining solids. The lowest VS value was found in the blank (0.80 ± 0.02 g $L^{-1}$). The methanogenic activity in the blank resulted from degradation of residual particulate substrate, yeast extract, or from autotrophic decay. Final VS contents in the DBP-degrading biological reactors, i.e. R10 and R200, were similar with an average value of 0.89 ± 0.02 g $L^{-1}$ (F value of ANOVA-test =2.4 < 7.71 at 95% confidence). Since no DBP degradation was observed in previous enrichment attempts in absence of yeast extract, the addition of yeast extract could not be avoided (data not shown).

[TABLE 3]

**DBP biodegradation.** DBP concentrations in biological reactors, as well as DBP removal efficiencies are presented in Figure 1. Both R10 and R200 reached similar final effluent DBP concentrations of 1.01 ± 0.07 mg $L^{-1}$ and 1.3 ± 0.65 mg $L^{-1}$, respectively. The theoretical DBP concentration was based on a mass balance model, indicating DBP accumulation without degradation (Fig.1). In the sterile reactors, DBP losses, as compared to the accumulation model, occurred during the first 40 days to reach a maximum of 65 % at 10 mg $L^{-1}$ DBP. Since residual biogas production was also observed (approx. 3 mL $week^{-1}$), DBP removal was attributed to incomplete sterility of the reactor. Thenceforth, the sodium azide concentration was increased from 7 to 14 g $L^{-1}$ at day 41 (still 2 % (w/v) formaldehyde). The biogas production then stopped and the DBP level increased towards the theoretical concentration. At the final points, both sterile control reactors had approximately 20 % DBP losses (21.8 ±7.5 % at 10 mg $L^{-1}$, and 21.4 ±7.8 % at 200 mg $L^{-1}$), attributed to abiotic removal. Considering that DBP is a volatile compound, the highest abiotic loss was probably due to volatilization rather than sorption to the glass parts of the reactor but this needs further investigations. No DBP was detected in the blank, indicating no external contamination. High and constant DBP removal rates were observed in the biological reactors fed with DBP at 10 mg $L^{-1}$ (89.7 ±0.8 %) and at 200 mg $L^{-1}$ (99.3 ±0.3 % ) (Fig. 1). In addition, reactor steady-state was defined as the stable period of time where DBP removal variations were lower than 5 % around the final average value. It was observed that sterile reactors were not as stable as the biological reactors, likely because of the higher DBP concentrations causing spatial heterogeneity. The reactor at 10 mg $L^{-1}$ reached a DBP-removal steady state after 70 days of enrichment, while DBP removal stability was earlier achieved at 200 mg $L^{-1}$ (16 days). Based on a mass balance kinetic model, estimated removal rates at steady state were assessed in R10 and R200 at 0.46 ±0.01 $mg_{DBPdeg}$ $(L\ day)^{-1}$ and 9.97 ±0.1 $mg_{DBPdeg}$ $(L\ day)^{-1}$, respectively. According to VS contents in R10 and R200, specific DBP degradation rates were assessed to be 0.52 ±0.02 $mg_{DBPdeg}$ $(g_{VS}\ day)^{-1}$ and 11.1 ±0.35 $mg_{DBPdeg}$ $(g_{VS}\ day)^{-1}$, respectively.

[FIGURE 1]

**Dynamics of SSCP microbial profiles in the enrichment cultures.** Both bacterial and archaeal communities were characterized in the biological reactors. Sterile control reactors did not contain enough DNA material to perform suitable SSCP analysis without introducing unspecific PCR amplification. Figure 2 compares SSCP profiles of the inoculum and after 100 days of enrichment for blank, R10, and R200. Area of the SSCP peaks was representative of the abundance of the associated 16S rDNA sequence. For *Archaea*, the blank maintained its dominant peak (Arc3, 76 % of peak area) with several sub-dominant species (Arc1, Arc2 and Arc4, 5 %, 8 %, and 7 % of peak area respectively) (Fig.2). At 10 mg $L^{-1}$ of DBP, the population shifted to a bipolar dominance of Arc3 and Arc4 with approximately equal relative abundance (43 % and 37 % respectively). At 200 mg $L^{-1}$ of DBP, species Arc4 was highly dominant (83 %) whereas Arc3 was only found at trace levels (approx. 6%). Comparatively, *Bacteria* presented more complex profiles (Fig. 2). Nonetheless, a lower number of SSCP peaks (< 20) were observed in the final *Bacteria* profiles compared to the inoculum. Owing to the use of synthetic medium in the inlet, the enrichment procedure led to simplify the total bacterial community in the reactors. In the blank reactor, the species Bac3 was dominant with approx. 29 % of relative abundance. The abundance of the other sub-dominant peaks was lower than 5 %. At 10 mg $L^{-1}$ DBP, additional dominant peaks appeared, and especially, Bac1 was found at the same level as Bac3 in the final enrichment culture (17 % and 19 % respectively of relative abundance). At 200 mg $L^{-1}$ DBP, the SSCP profile was highly simplified with only four main peaks: Bac1, Bac2, Bac3, and Bac4 with respectively 23 %, 5 %, 15 %, and 11 % of relative abundance.

[FIGURE 2]

Microbial SSCP profiles dynamics were evaluated over the entire enrichment procedure and are presented in Figure 3. Although the archaeal community was phylogenetically stable in the blank reactor, a progressive shift from Arc3 to Arc4 was observed slowly (after 10 weeks) at 10 mg $L^{-1}$, and more rapidly (within 2 weeks) at 200 mg $L^{-1}$ of DBP. Concerning the bacterial community, the increasing dominance of species Bac1 to Bac4, as well as the disappearance of a group of intermediary peaks located between Bac1 and Bac2 occurred (Fig.3). In the blank, Bac3 species preferentially developed and was mainly dominant after 100 days of experimentation. At 10 mg $L^{-1}$ of DBP, Bac3 also developed but the main dominance in the final enrichment culture was supported by Bac1. The Bac1 dominance occurred after approximately 8 to 9 weeks of enrichment (Fig. 3). At 200 mg $L^{-1}$ of DBP, Bac1 developed more rapidly to dominance. In both reactors, the emergence of Bac1 occurred simultaneously with the achievement of a stationary phase in term of DBP removal. As well, the system was considered as phylogenetically stable with regard to no major changes of the microbial relative abundance, after 8 to 9 weeks at 10 mg $L^{-1}$, and only after 2 weeks at 200 mg $L^{-1}$.

[FIGURE 3]

**Phylogenetic identification.** The phylogenetic trees representing the affiliation of individual SSCP clones are shown in Figure 4. Arc1 and Arc2 were not identified because of their low abundance in the final enrichment cultures. Although a significant shift of the archaeal population was observed by addition of 10 and 200 mg $L^{-1}$ of DBP, both Arc3 and Arc4 belong to the *Methanosaeta* genus. The divergence between 16S rDNA fragments of the two microorganisms was of 3.9 %. Although these two clones were phylogenetically close, Arc4 was even closer to *Methanosaeta concilii* strain GP6 than Arc3 (2.5 % and 4.7 % divergence, respectively).

The dominant clones within *Bacteria* phylum in the final DBP enrichments were also phylogenetically similar (Fig.4). The most dominant species -Bac1- was closely related to *Soehngenia saccharolytica*. In contrast, the main dominant species Bac3 found in the blank and in the enrichment cultures was related to the genus *Bacteroides*. Bac2 and Bac4 peaks corresponded to an identical species belonging to the phylum *Bacteroidetes*, and probably to the order *Bacteroidales*. Additionally, Bac2 and Bac4 SSCP peaks corresponded to two isomers of the same

16S rDNA fragment belonging to one species. A similar artefact of double peaks corresponding to one 16S rDNA sequence was previously observed under the same analytical conditions (Delbes *et al.*, 2000).

[FIGURE 4]

***In situ* characterization of the enrichment cultures by FISH.** The probes SOE01-432 and BCT01-409 were designed to specifically target *Soehngenia saccharolytica* (Bac1), and *Bacteroides* sp. (Bac2-Bac4). The FISH images are shown in Figure 5. In all samples, microbial material was flocculant (10-100 μm). No microbes in the blank or inoculum were detected responding to the probes SOE01-432 or BCT01-409, but organisms responding to both SOE01-432 and BCT01-409 were observed in both enrichment cultures. Organisms responding to SOE01-432 (Bac1) were abundantly present throughout flocs, as well as *Archaea* responding to ARC-915 (approximately 10% of total microorganisms in the enrichment cultures). Based on visual estimation, Bac1 represented 70 % of the total *Bacteria* community in the flocs, which is significantly higher than in the SSCP profiles representing the distribution of flocculating and non-flocculating bacteria (approximately 25 %). The remainder of bacteria (EUB338/338+), as observed by FISH appeared to be Bac2-Bac4 (BCT01-409) with even, but non structural distribution throughout the flocs.

[FIGURE 5]

**[Discussion]**

**DBP biodegradation under strict anaerobic conditions.** Although DBP biodegradation has previously been observed in anaerobic environments (Angelidaki *et al.*, 2000; Wang *et al.*, 2000; Gavala *et al.*, 2003; Chang *et al.*, 2005), the present study reports for the first time, the possibility to enrich microbial cultures with highly efficient DBP biodegradation ability under strict anaerobic conditions. Working with volatile and hydrophobic organic compounds requires the consideration of the potential abiotic losses caused by the experimental setup, such as volatilization or adsorption on experimental glassware. In the present study, abiotic disappearance of DBP was estimated at 20 % in both sterile controls. The significant differences between controls and biological reactors indicated effective biological degradation of DBP. Mass balance did not provide evidence of full mineralization of DBP, since yeast extract was necessary to be added and mineralization could not be separately evaluated.

Nevertheless, primary attack of DBP was clearly shown in both biological reactors. At both 10 mg $L^{-1}$ and 200 mg $L^{-1}$ inlet concentrations, the effluent DBP concentrations were of approximately 1.1 mg $L^{-1}$. This value likely corresponds to a threshold concentration where DBP biodegradation was limited by bioavailability.

Because the DBP solvents (pentane and diethyl ether) were added at the same concentration in all experiments, inhibition due to these specifically would have been the same. Considering this, specific and significant impact of DBP was observed at 200 mg $L^{-1}$ compared to lower concentrations. Previous study reported methanogenesis inhibition at a similar level (Angelidaki *et al.*, 2000). In contrast, O'Connor *et al.* (1989) reported no toxic effect on methanogenic activity for concentration above 300 mg $L^{-1}$. This suggests that the nature and the composition of the microbial community impact on its own sensitivity to phthalic acid ester inhibition. In our enrichment cultures, since the DBP concentrations were relatively low in the enrichment reactors, direct inhibition is unlikely. It is more likely that selection of DBP degrading microorganisms and the resulting specialization of the bacterial population caused a decrease in overall methanogenic activity. Since no by-product accumulation was observed (VFA or possible aromatic intermediates), this decrease of activity was due to a decrease in hydrolytic activity rather than methanogenic activity.

**Microbial dynamics and phylogenetic affiliation of the microorganisms involved in DBP biodegradation.** In all biological cultures, a decrease in diversity was observed by SSCP throughout the enrichment procedure.  This was mainly due to the wash out of non-growing microorganisms, such as in the blank. The simplicity of the SSCP profiles showed that only few microbial species from the inoculum were able to grow on yeast extract under the applied operating conditions.  Moreover, the application of DBP selection pressure in inlet favoured the specialization of the DBP-degrading consortium by improving the growth of DBP-degrading microorganisms. Because of the low in-reactor DBP concentration in the enrichment cultures, DBP growth inhibition did probably not occur.  This is consistent with the VS contents in the biological reactors, which were significantly higher than in the blank without DBP.  Phylogenetic stability of the consortium was observed at steady state, i.e. no major changes in the DBP-degrading microbial population. Furthermore, the emergence of the final bacterial profile was very slow at 10 mg $L^{-1}$ and faster at 200 mg $L^{-1}$.  The early profile of microbial dynamics at 200 mg $L^{-1}$ corresponded to the final profiles at lower concentration (10 mg $L^{-1}$), suggesting that selection of microorganisms followed similar steps over time, and that higher DBP concentrations speed up selection of the specific degrading microbial consortium.  Additionally, the emergence of the final microbial profiles correlated well with the time to reach a stationary phase for DBP removal.  All these results are consistent with direct involvement of the microbial consortium in DBP biodegradation pathway. Interestingly, the experimental setup of the enrichment procedure only influenced the dynamics of the microbial communities that finally tend to a similar consortium whatever the DBP selection pressure.

In this study, a population shift within A*rchaea* kingdom occurred.  This suggested a rapid adaptation of the methanogens to DBP (2 weeks at 200 mg $L^{-1}$ DBP).  The selected methanogen (Arc4) likely had lower sensitivity to DBP compared to the original species (Arc3) found in the blank.  Co-dominance of both species at 10 mg $L^{-1}$ indicated that low DBP concentrations slightly favour Arc4 emergence, which outcompete Arc3.  These results support the involvement of Arc4 as a partner of DBP degraders at high DBP concentration.  Surprisingly, although the abundance shift was clear according to the increasing DBP concentrations, the phylogenetic shift was only very limited, with Arc3 and Arc4 both being members of the genus *Methanosaeta*. It was therefore concluded that the final DBP-degrading methanogenic consortium was highly specific to their local environment, either linked to acetate affinity since both *Methanosaeta* species carried the same function, or due to physicochemical properties (e.g., surface properties) suitable for DBP degradation by other microbes necessarily involved in the process.

Nevertheless, the presence of *Methanosaeta* sp. in anaerobic enrichment culture was unsurprising. Leclerc *et al.* (2004) previously reported that *Methanosaeta* sp. represented more than 75 % in abundance of the archaeal species amongst 44 different anaerobic digesters, and was found in 84 % of the anaerobic reactors. Although physiological properties would rather favour the implementation of fast growing hydrogenotrophs (Leclerc *et al.*, 2004), the hydrophobic properties and the high affinity for acetate as substrate favour mainly the implementation of *Methanosaeta* sp. in flocs and granules in anaerobic reactors (Grotenhuis *et al.*, 1991; Schmidt & Ahring, 1996; Sekiguchi *et al.*, 1999; Leclerc *et al.*, 2004). Moreover, *Methanosaeta* sp. outcompete other fast-growing acetate users, such as *Methanosarcina* sp., at low acetate concentrations (Conklin *et al.*, 2006). Considering that phthalic acid esters are hydrophobic compounds and most of the potential DBP-degraders concentrated within flocs in the enrichment cultures as observed by FISH, the presence of *Methanosaeta* sp. moreover likely favoured local hydrophobic environment within flocs. Although the two identified archaeal species found in blank and enrichments were closely related, Arc4 was even closer to several microbes (**AF229777**- **AF229778**- **AF229774**) previously found in methanogenic consortium degrading terephthalate, an isomer of the probable intermediate orthophthalate in DBP degradation pathway (Wu *et al.*, 2001).

In addition, among the two bacteria –Bac1 and Bac2/Bac4 - identified as DBP-degrading candidates, Bac1 was phylogenetically affiliated to *Soehngenia saccharolytica*, an anaerobic benzaldehyde degrader (Parshina *et al.*, 2003). The emergence of *Soehngenia saccharolytica* as dominant bacteria in the enrichment cultures correlated well with the stationary phase for DBP removal in both biological reactors. This is consistent with direct involvement of Bac1 in DBP biodegradation pathway. One strain of *Soehngenia saccharolytica* was previously reported to require yeast extract for growth coupled to detoxification by dismutation of benzaldehyde to benzoate and benzylalcohol (strain BOR), but was not reported to perform aromatic ring fission (Parshina *et al.*, 2000). No intermediate was detected in our enrichment cultures suggesting that complete mineralization occurred. This implied the occurrence of primary attack likely performed by Bac1 affiliated to *Soehngenia saccharolytica*, followed by a ring fission and further oxidation steps carried by other emerging bacteria (e.g. Bac2/Bac4). According to Staples *et al.* (1997), phthalic acid, a probable intermediate, is a central intermediate in the biological degradation under methanogenic conditions of phthalate esters and is then converted to $CH_4$ (Kleerebezem *et al.*, 1999).

The second emerging group (Bac2/Bac4) belonged to the genus *Bacteroides*, commonly found in anaerobic environments. Because of this sub-dominant group appeared later over the enrichment procedure, it was concluded that Bac2/Bac4 probably corresponded to bacteria growing on by-products, especially aromatic rings. Chen *et al.* (2004) reported that clones related to *Bacteroides* sp. may be involved at a lower extent in terephthalate degradation under thermophilic conditions. Additionally, molecular tools may present biases, especially with regards to the retrieved sequences that are only representative of dominant species, as well as the limited specificity of PCR primers (Delbes *et al.*, 2000; Leclerc *et al.*, 2004). Nevertheless, the FISH observations presented here, using probes developed from SSCP sequence information, confirmed the SSCP results. At last, the expression of the degradation function by sub-dominant species, as previously discussed by Delbes *et al* (2000), was unlikely here because of the low complexity of the SSCP profiles after enrichment and the high specialization of the degrading consortium.

**Enrichment cultures of anaerobic phthalate ester degraders.** Development of enrichment cultures under strict anaerobic conditions is subject to scientific and technical constraints that were addressed in this study. First, the PAEs are only found at trace levels in the environment, and the selection and adaptation of an efficient PAE-degrading ecosystem is very time-consuming (Kleerebezem *et al.*, 1999; Hayes *et al.*, 1999; Qiu *et al.*, 2004). Edwards & Grbic-Galic (1994) showed that *ex-situ* adaptation of anaerobic ecosystems to single aromatic compounds, such as toluene and o-xylene, required more than 100 and 200 days of adaptation in lab systems, respectively. The levels of exposure (Yuan *et al.*, 2002) as well as the period of contamination (Hayes *et al.*, 1999) affect the capability of the anaerobic microbial consortium to degrade aromatic compounds. This issue was addressed in the current study by a preliminary screening of several potential inocula. In particular, the ability of the anaerobic ecosystem to degrade DBP was not widely distributed and only long-term naturally contaminated sludge exhibited a substantial potential for DBP degradation.

Second, it is commonly assumed that biodegradation of phthalic acids require syntrophic microbial populations to occur under methanogenic conditions (Kleerebezem *et al.*, 1999; Qiu *et al.*, 2004). Obtaining highly enriched cultures depends on the ability to maintain an active syntrophic consortium of oxidising bacteria, and hydrogen utilising methanogenic *Archaea* throughout the enrichment procedure. In our study, less than 4 months were necessary in semi-continuous reactors to select highly enriched cultures by applying strong selection pressure – both dilution rates, and high loading rates. In contrast, Kleerebezem *et al.* (1999) reported that stable enrichment cultures on phthalates were obtained after a period of more than 1 year and through numerous transfers into fresh medium. Qiu *et al.* (2004) reported that over 2 years of enrichment were required to establish phthalate-degrading enrichment cultures. Such usual enrichment method consisting in successive transfers into fresh medium is therefore time consuming since microbial growth rates of anaerobic cultures are low, within a range from 0.08 to 0.25 day$^{-1}$ with phthalic acids (Kleerebezem *et al.*, 1999; Qiu *et al.*, 2004), and 0.1 day$^{-1}$ with other aromatic compounds (Edwards & Grbic-Galic, 1994). The use of a semi-continuous, rather than transfer system therefore favoured more rapid selection. Nevertheless, the application of higher dilution rates under methanogenic conditions was restricted by the presence of slow-growing methanogens and DBP-degrading bacteria in the degradative consortium.

At last, stability of the enrichment culture was reached with stable DBP degradation rates near the end of the test. Therefore, at this dilution rate, cell growth matched washout and decay of non degrading microorganisms, and 20 days hydraulic retention time was highly suitable to maintain the ability to degrade DBP. In contrast, Kleerebezem *et al.* (1999) reported that phthalate-enriched cultures were unstable at low-rates or when less than 20% of cultures were transferred. Qiu *et al.* (2004) reported similar observations with the possibility of losing the ability to grow on pure phthalate.

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**[Tables]**

**Table 1** Primer sequences used for PCR amplification of the total or partial 16S Small SubUnit rRNA genes

| Name | Specificity | *E.coli* position | Sequence (5'to 3') |
|---|---|---|---|
| UR500 | Universal [a] | 500 | TTA CCG CGG CTG CTG GCA G |
| FUR500 | Universal [a,b] | 500 | 6-FAM- TTA CCG CGG CTG CTG GCA G |
| UR1492 | Universal [a] | 1492 | GNT ACC TTG TTA CGA CTT |
| AF3 | *Archaea* | 3 | ATT CYG GTT GAT CCY GSC RG |
| AF333 | *Archaea* | 333 | TCC AGG CCC TAC GGG G |
| EF330 | *Bacteria* | 330 | ACG GTC CAG ACT CCT ACG GG |

[a] all prokaryotes including *Bacteria* and *Archaea*
[b] 6-FAM= 6-carboxyfluorescein, terminal DNA fluorescent label

**Table 2** Fluorescent labelled oligonucleotides used for Fluorescent *In Situ* Hybridization probing

| Name | Target group | Formamide (%) | Non-group hits NCBI database | Probe sequence (5' to 3') | *E.coli* position | *Reference* |
|---|---|---|---|---|---|---|
| SOE01-432 | *Soehngenia saccharolytica* | 20 | 1[a] | GTCATTATCTTCCCCTAGGACAGAGC | 432 | This study |
| BCT01-409 | *Uncultured Bacteroides* sp. | 20 | 0 | CAACCCTTAGGGCCGCCTTC | 409 | This study |
| EUB-338[b] | *Bacteria – most* | 20 | 0 | GCTGCCTCCCGTAGGAGT | 338 | (Stahl & Ammann, 1991) |
| EUB-338+[b] | *Bacteria-remaining* | 20 | 0 | GCWGCCACCCGTAGGTGT | 338 | (Daims *et al.*, 1999) |
| ARC-915 | *Archaea* | 20 | 0 | GTGCTCCCCCGCCAATTCCT | 915 | (Stahl & Ammann, 1991) |

[a] *Catonia barnesae* (AB38361)
[b] EUB-338/EUB-338+ were used simultaneously to target all *Bacteria* (EUB-Mix).

**Table 3** Methanogenic performances in the biological and control cultures, after 100 days of enrichment

| Bioreactor Name | DBP concentration in inlet (mg $L^{-1}$) | Methane content in the biogas (%) [a] | Average biogas production rate (mL $week^{-1}$) [a] | VS content (g $L^{-1}$) [a] |
|---|---|---|---|---|
| Blank | 0 | 61.3 ± 1.6 | 41.7 ± 1.5 | 0.80 ± 0.02 |
| R10 | 10 | 62.3 ± 0.6 | 38.0 ± 3.5 | 0.88 ± 0.01 |
| CTRL10 | 10 (sterile control) | n.d | ø | 1.51 ± 0.04 |
| R200 | 200 | 60.7 ± 1.1 | 13.7 ± 3.2 | 0.90 ± 0.02 |
| CTRL200 | 200 (sterile control) | n.d | ø | 1.34 ± 0.03 |

[a] Indicated errors corresponded to 95% confidence intervals of triplicate analyses at steady state
ø not measurable production (< 0.5 mL.$week^{-1}$)
n.d. not determined

**[Figure Legends]**

**Fig. 1.** Theoretical and measured DBP concentrations in the sterile controls (CTRL10 and CTRL200) and biological reactors (R10 and R200). DBP removal corresponds to the ratio between the theoretical curve and the measured values. Plain lines indicate biological reactors, while stripped lines correspond to sterile controls. Error bars of the concentration values represent the standard deviation of triplicate analyses. Error bars of the DBP removal curves represent 95 % confidence interval of the calculated value.

**Fig. 2.** SSCP profiles of the archaeal (a) and bacterial (b) communities in the sludge inoculum and after 100 days of enrichment at 0, 10 and 200 mg $L^{-1}$ of DBP. Species of interest are marked by an arrow and numbered.

**Fig. 3.** Three dimensional representations of the archaeal (a) and bacterial (b) SSCP profiles over enrichment time, and according to DBP concentrations. The SSCP peaks of interest are marked by an arrow and numbered. (A.U.=Arbitrary Unit)

**Fig. 4.** Phylogenetic trees of 16S rDNA fragment of the dominant archaeal (A) and bacterial (B) species in the blank and enrichment cultures. The trees were generated by using neighbour joining distance method in ARB software, with distant microorganisms as roots. Numbers at the nodes indicate the bootstrap values above 50 %, for 1000 bootstrap calculations. The scale bar represents the number of substitutions per nucleotide. The phylogenetic divergence correspond to the comparison of partial sequences from *E.coli* nucleotide 330 to nt 500 (*Bacteria*) and nt 333 to nt 500 (*Archaea*). The sequence roots correspond to *Methanosarcina mazei* (**AF028691**) and *Escherichia coli* (**AJ567617**) for *Archaea* and *Bacteria* trees, respectively. The sequences obtained from the present study are indicated in bold.

**Fig. 5.** Fluorescent In Situ Hybridization microscopic observations of identified DBP degraders in the 200 mg $L^{-1}$ DBP enrichment culture. A: sample hybridized with EUB338-FITC, ARC915-CY3, and the specific probe SOE01-432-CY3 to give target coloured cells yellow, other *Bacteria*, green, and *Archaea* red. B: sample hybridized with EUB338-FITC, ARC915-CY3, and the specific probe BCT01-409-CY3 to give target coloured cells yellow/red, other *Bacteria*, green, and *Archaea* red. In Figure 5B, most of the target cells appear red instead of yellow, due to the very strong response by other cells (presumptively Bac1) to the EUB338 probe. Bar indicates 10 μm.

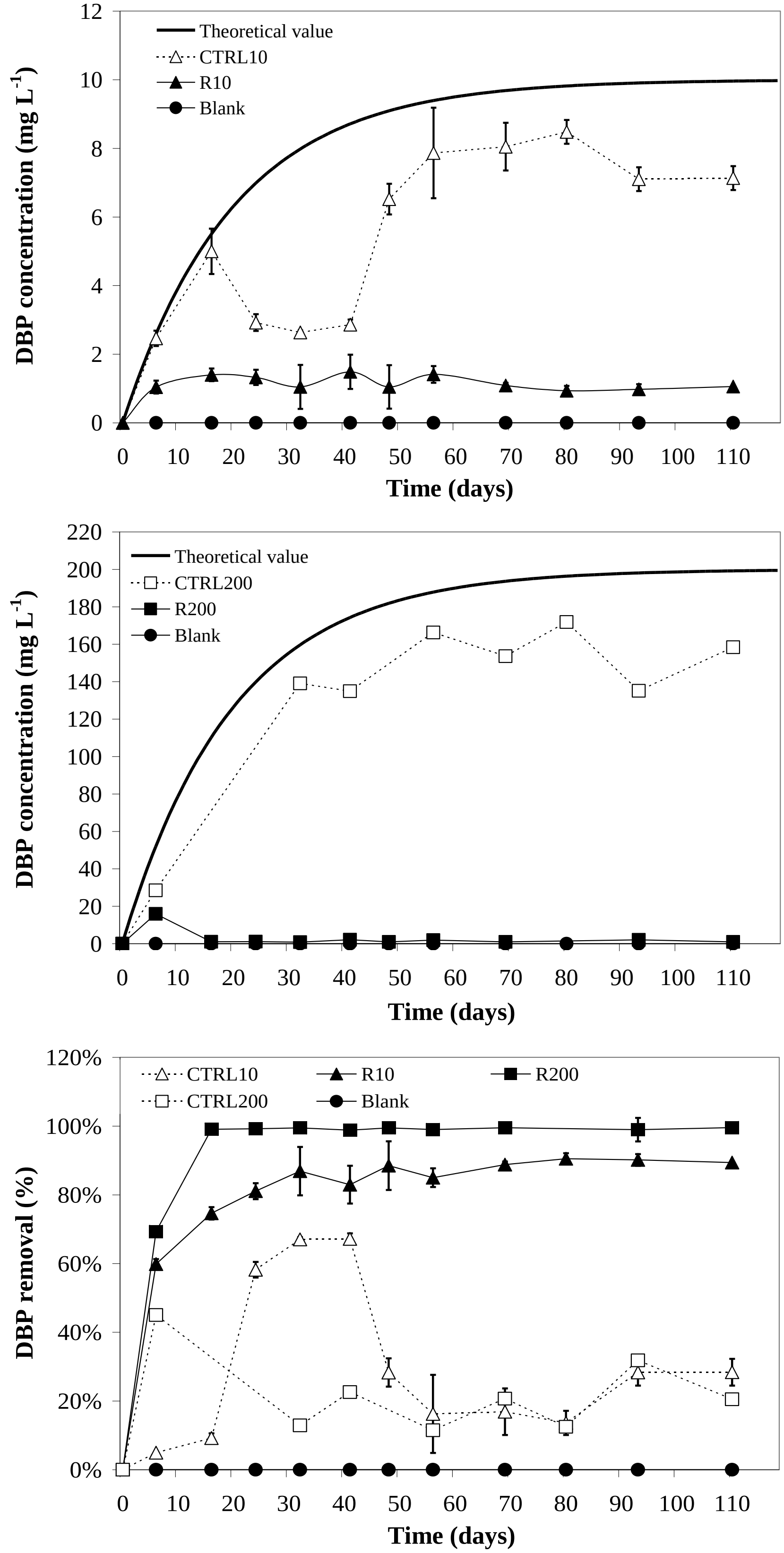

Theoretical value
CTRL10
R10
Blank
DBP concentration (mg L-1)
Time (days)
Theoretical value
CTRL200
R200
Blank
DBP concentration (mg L-1)
Time (days)
CTRL10
R10
R200
CTRL200
Blank
DBP removal (%)
Time (days)

**Fig. 1.**

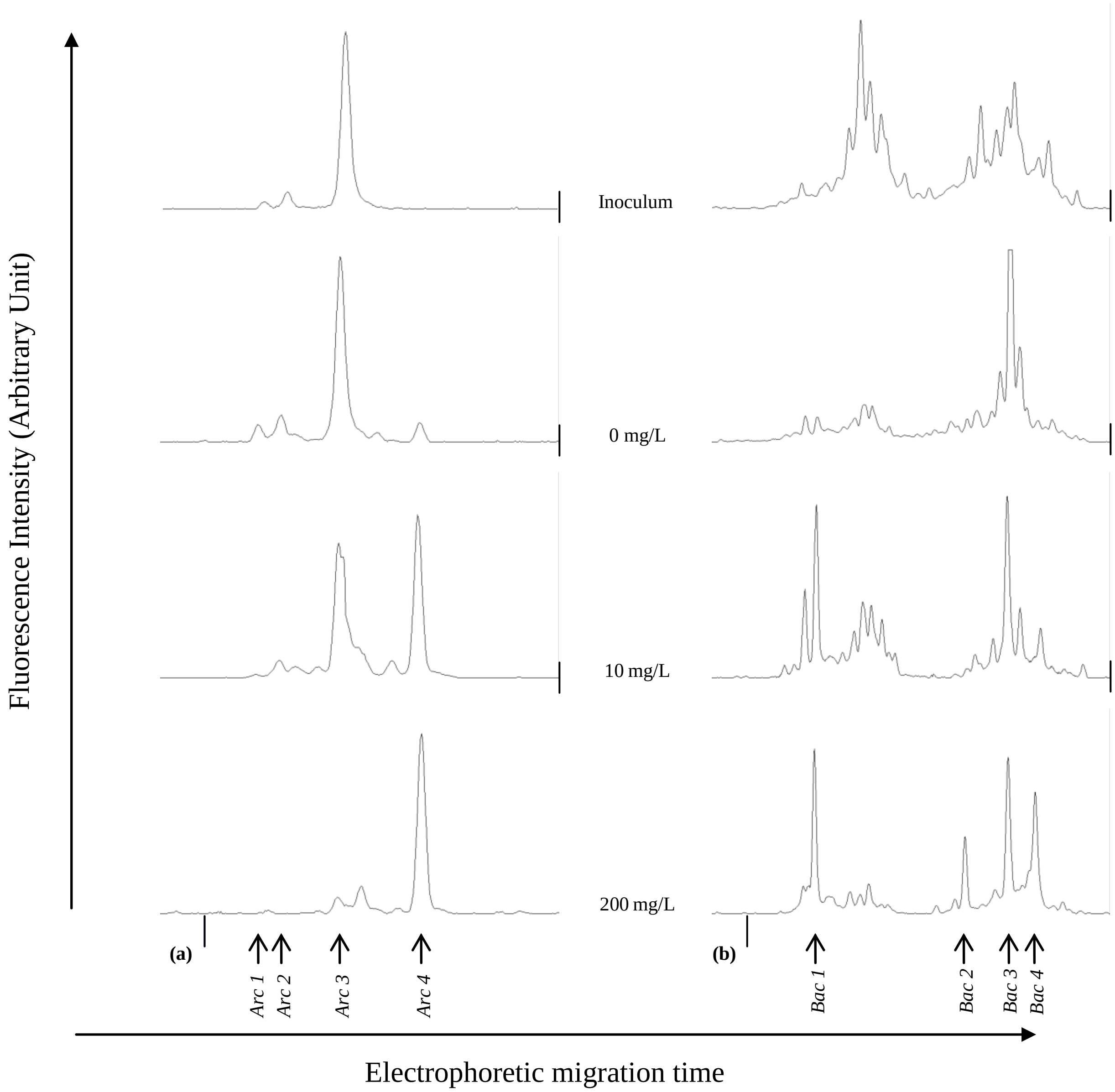

Fluorescence Intensity (Arbitrary Unit)
Inoculum
0 mg/L
10 mg/L
200 mg/L
(a)
(b)
Arc 1
Arc 2
Arc 3
Arc 4
Bac 1
Bac 2
Bac 3
Bac 4
Electrophoretic migration time

**Fig. 2.**

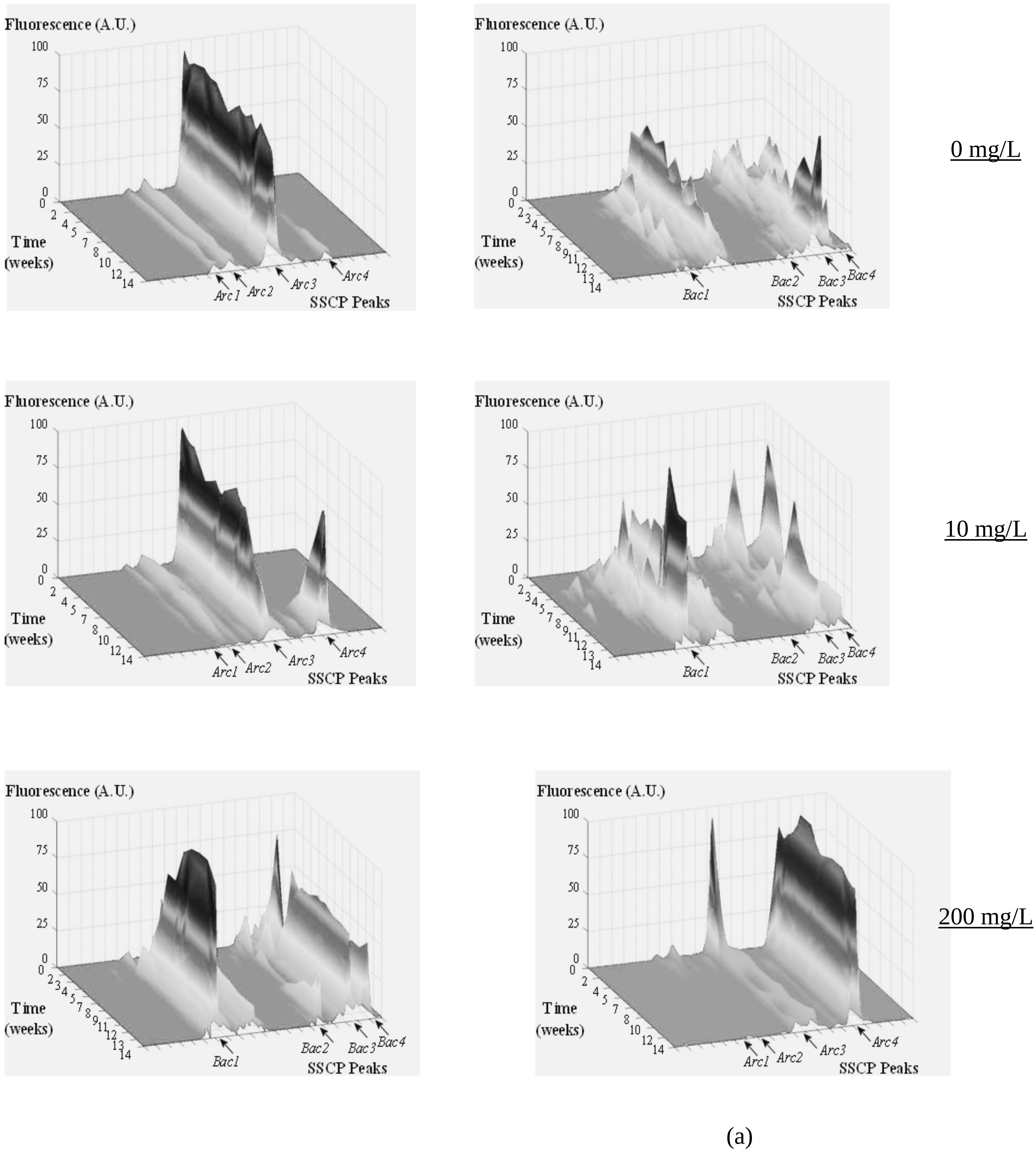
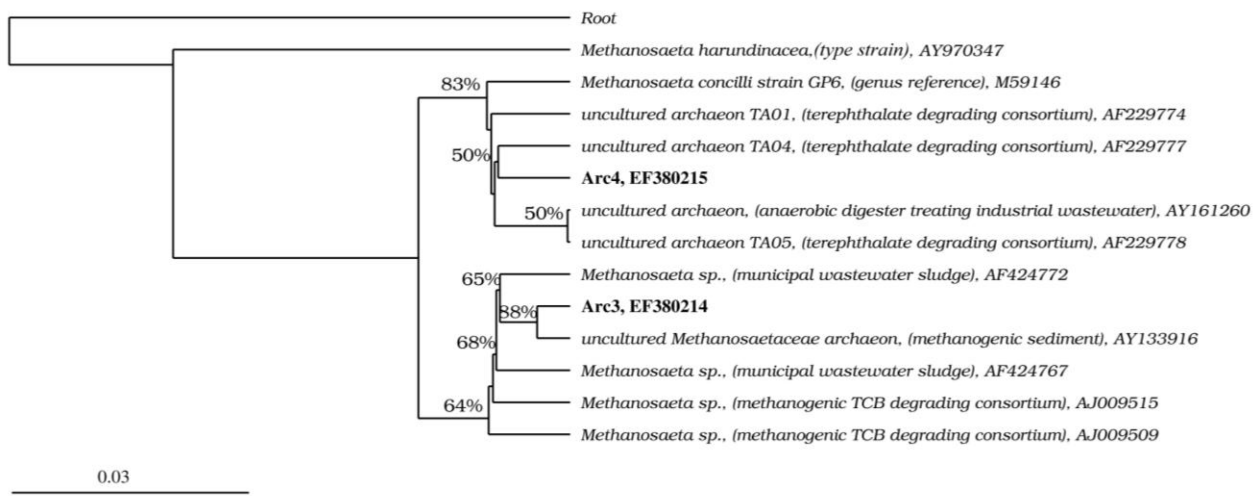
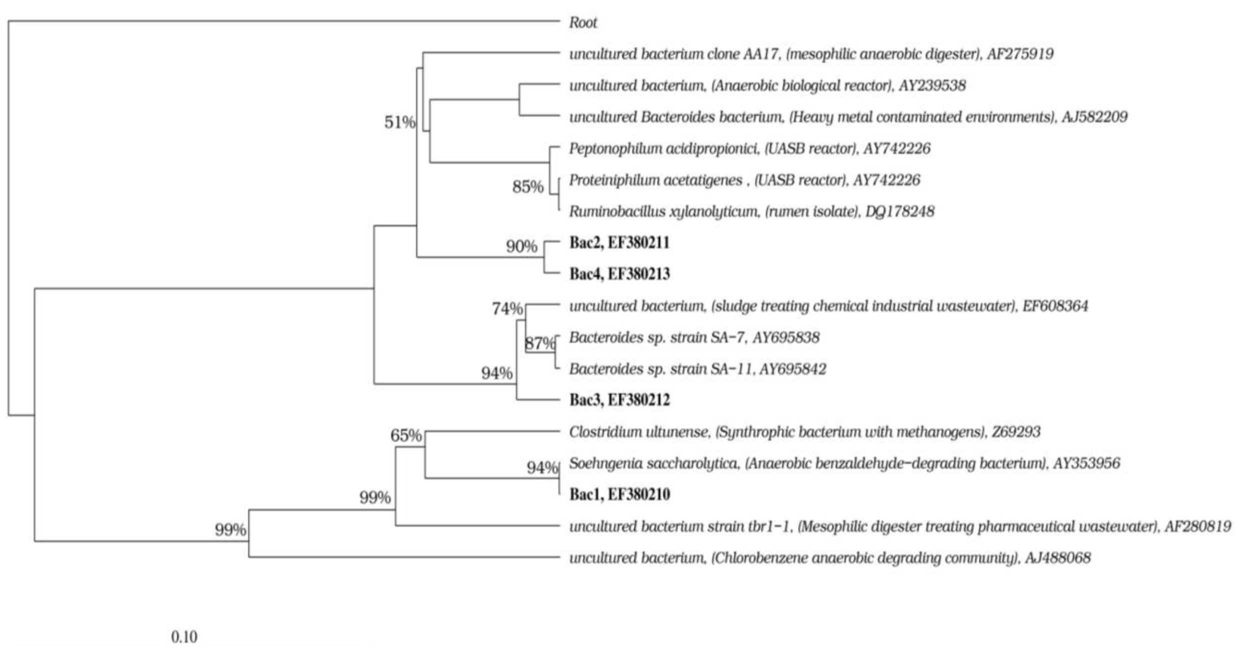

Fluorescence (A.U.)
Time (weeks)
SSCP Peaks
Arc1
Arc2
Arc3
Arc4
Bac1
Bac2
Bac3
Bac4
0 mg/L
10 mg/L
200 mg/L
(a)

**Fig. 3.**


Root
Methanosaeta harundinacea,(type strain), AY970347
83%
Methanosaeta concilli strain GP6, (genus reference), M59146
uncultured archaeon TA01, (terephthalate degrading consortium), AF229774
50%
uncultured archaeon TA04, (terephthalate degrading consortium), AF229777
Arc4, EF380215
50%
uncultured archaeon, (anaerobic digester treating industrial wastewater), AY161260
uncultured archaeon TA05, (terephthalate degrading consortium), AF229778
65%
Methanosaeta sp., (municipal wastewater sludge), AF424772
88%
Arc3, EF380214
68%
uncultured Methanosaetaceae archaeon, (methanogenic sediment), AY133916
Methanosaeta sp., (municipal wastewater sludge), AF424767
64%
Methanosaeta sp., (methanogenic TCB degrading consortium), AJ009515
Methanosaeta sp., (methanogenic TCB degrading consortium), AJ009509
0.03

**A.**


Root
uncultured bacterium clone AA17, (mesophilic anaerobic digester), AF275919
uncultured bacterium, (Anaerobic biological reactor), AY239538
51%
uncultured Bacteroides bacterium, (Heavy metal contaminated environments), AJ582209
Peptonophilum acidipropionici, (UASB reactor), AY742226
85%
Proteiniphilum acetatigenes , (UASB reactor), AY742226
Ruminobacillus xylanolyticum, (rumen isolate), DQ178248
90%
Bac2, EF380211
Bac4, EF380213
74%
uncultured bacterium, (sludge treating chemical industrial wastewater), EF608364
87%
Bacteroides sp. strain SA-7, AY695838
94%
Bacteroides sp. strain SA-11, AY695842
Bac3, EF380212
65%
Clostridium ultunense, (Synthrophic bacterium with methanogens), Z69293
94%
Soehngenia saccharolytica, (Anaerobic benzaldehyde-degrading bacterium), AY353956
Bac1, EF380210
99%
uncultured bacterium strain tbr1-1, (Mesophilic digester treating pharmaceutical wastewater), AF280819
99%
uncultured bacterium, (Chlorobenzene anaerobic degrading community), AJ488068
0.10

**B.**

**Fig. 4.**

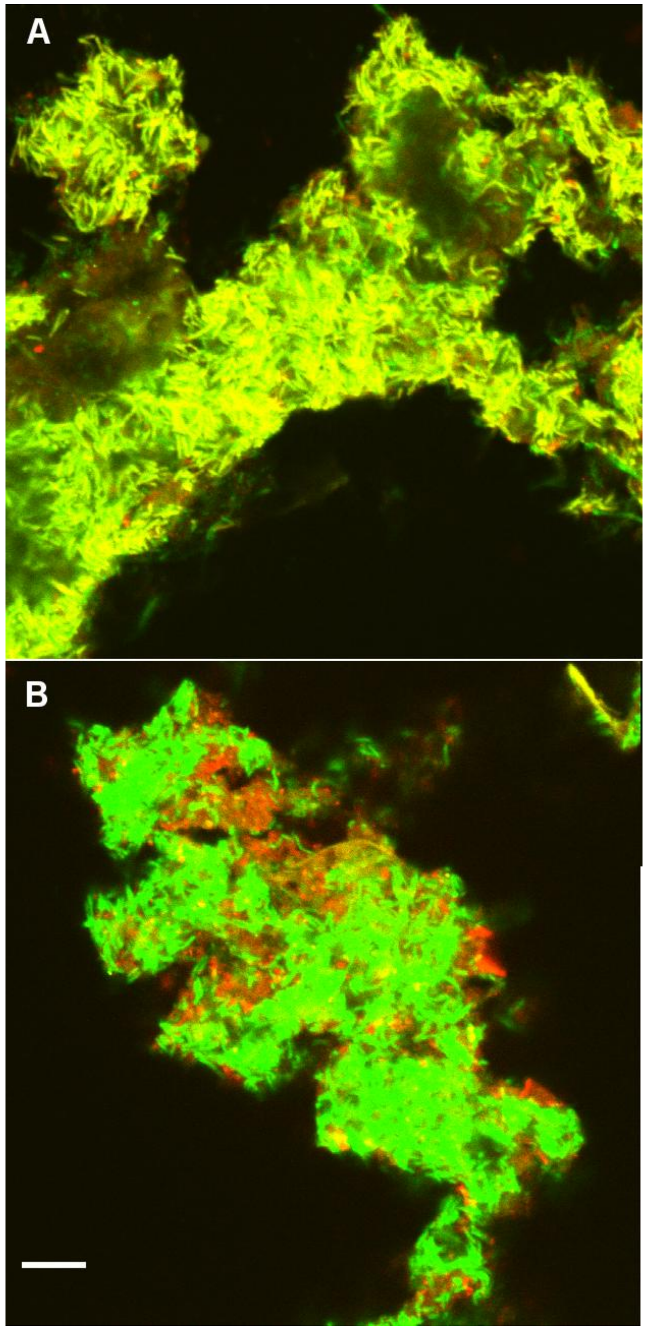



**Fig. 5.**