MICROBIAL DEGRADATION OF THREE NOVEL BISPHENOL COMPOUNDS

By

KENNIDA POLANCO

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ABSTRACT OF THE THESIS

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KENNIDA POLANCO

Thesis Director

Dr. Max M. Häggblom

Bisphenol A, 2-2-bis (4 hydroxypenyl propane) (BPA) is a synthetic compound used in the production of polycarbonate, epoxy resins and in the synthesis of the flame retardant tetrabromobisphenol A. It is found in everyday products such as plastic water bottles, food containers, furniture and textiles. The discovery of its endocrine disrupting properties in animals, as well as its persistence in anaerobic environments has motivated an active search for alternative compounds to substitute BPA in the production of food and water containers. The objective of this study was to evaluate the potential for biodegradation of three novel bisphenol analogs, which show chemical proprieties that made them suitable for substituting BPA in industrial usage. These compounds are 2-6'-dimethyl, 4-4'-dimethoxy bisphenol; 1-1'-ethyl, 2-6-dimethyl, 4-4-dimethoxy bisphenol; and 1-1'-propyl, 2-6-dimethyl, 4-4-dimethoxy bisphenol. Enrichment cultures using Arthur Kill sediment as inoculum were set under aerobic and anaerobic conditions. Analytical

methods based in liquid chromatography and gas chromatography-mass spectrometry (HPLC and GC-MS) were developed. Primary enrichment cultures degraded the three test compounds under methanogenic (after 15 days), sulfate reducing (after 44 days) and aerobic conditions (after 52 days). No substrate loss was observed after 60 days under nitrate reducing conditions or in sterile controls. With transfer of the primary cultures to fresh medium (50% or 10% of the active culture) degradation continued at a higher rate. There was no loss of bisphenol A under anaerobic conditions over an incubation period of 6 months. These data suggest that the new bisphenol analogs are biodegradable under anoxic and oxic conditions where BPA is recalcitrant.

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Introduction

1.1 BPA in the environment

Bisphenol A [4,4'-(propane-2,2-diyl) diphenol] is a synthetic compound consisting of two phenol rings connected by a propane bridge. It is synthetized by the reaction of acetone with phenol and it is used in the production of polycarbonate plastics, phenolic resins and epoxy resins, as well as the flame retardant, tetrabromobisphenol A. BPA can be found in everyday products such as water bottles, textiles, printed paper, food containers and in varnish used to protect the interior of food cans. First synthesized in 1891, its current global annual production has been estimated in 3.8 millions of tons (Michalowick, 2014).

As a consequence of its extensive use, BPA has been detected in agricultural soils, freshwater, seawater, river and marine sediments and in air samples in indoor environments as well as in the atmosphere (Yang et al., 2015; Flint et al., 2012; Artham and Doble, 2012). The releases of BPA to the environment in the USA exceed one million of pounds per year (EPA, 2010). This occurrence in natural environments is a concern due its toxic and endocrine disrupting properties, generating a risk to wildlife and human health.

BPA can be released to the atmosphere though the emissions from industry, burning of plastic waste at the domestic scale or at high scale during the incineration of municipal waste; by the application of aerosol paints or by the suspension of soil particles in the air. BPA is ubiquitous in

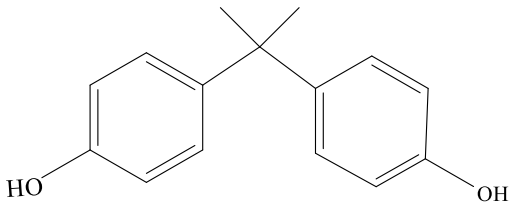
the atmosphere and has been found in rural and urban areas around the world and in polar regions (Pingqing and Kawamura, 2010). In the atmosphere, BPA can react by photo-catalyzed oxidation via the attack of hydroxyl radicals with a half-life of 0.74-7.4 h (Cousin et al., 2002). Although BPA has the potential for photo-oxidation, it is possible that the atmosphere can acts as reservoir or play a role in BPA transport.

The major sources of BPA in aquatic environments are discharge from domestic and industrial wastewater treatment plants, leachate from landfills and runoff from agriculture and urban soils (Kang et al., 2007). In aquatic environments, the moderate solubility and high octanol-water coefficient (Log K_{ow} 3.4) of BPA favors its partition distribution between the water and the sediment, with a higher proportion of BPA binding to the sediment (Table 1). The water-octanol partitioning coefficient is used to infer the behavior of xenobiotic compounds in aquatic environments. The water immiscible organic solvent, n-octanol, acts as a surrogate for organisms or organic matter present in the sediment, allowing to infer how able is the compound of binding to the membrane of the microorganism or to organic matter present in natural soil or sediments (Schwarzenbach et al., 1993).

In terrestrial environments, BPA can leach from waste products containing epoxy resins. The application of biosolids from wastewater treatment plants as fertilizer in agriculture is an important source of introduction of BPA and other endocrine disruptors to the soil. In wastewater treatment plants, BPA is partially degraded, with a removal percent range of 55-99% (Lee et al., 2004). The direct application of biosolids to soil can result

in the dispersal of the BPA fraction that was not degraded during the treatment process (Zhang et al., 2015; Flint et al., 2012). The half-life of BPA in aerobic soils is 7 days, but it is persistent under anoxic conditions, with no degradation after 70 days (Ying and Rai, 2005). In soils, BPA can be transformed or mineralized by plants, fungi or bacteria.

Table 1. Bisphenol A chemical features at 25° C.

Chemical nomenclature 4,4'-(propane-2,2-diyl)diphenol p,p'-isopropylidenebisphenol, 2,2-bis(4-hydroxyphenyl)propane	
Molecular weight	228.29 g/mol
Solubility	300 g/m^3
Melting point	155 ° C
Log Kow	3.4
Koc	880

Source: Cousin, 2002. A multimedia Assessment of the Environmental Fate of Bisphenol A.

Environmental conditions such as salinity, temperature and pH can affect the Log K_{ow} of BPA. Borrirukisitsak et al. (2012) reported an increase in the Log K_{ow} in BPA at higher salinity, with a major proportion of BPA binding to sediments at a salinity range similar to that of estuaries and seas; as well as a reduction of Log K_{ow} at higher pH and temperature. Once in contact with sediments, the abundance of particulate organic matter can contribute to the

sorption of BPA to sediments; and the low level of oxygen can inhibit its degradation, resulting in the accumulation of BPA in the anoxic zones. Several studies have reported that the concentration of BPA in sediments is three orders of magnitude higher than the concentrations found in river water (Lee et al., 2013) (Table 2).

The lower levels of BPA in river water, in contrast to that found in sediments, can be also explained by the dilution process due the continuously recharge of the river by aquifers. High concentrations of dissolved oxygen in water can also contribute to its faster degradation by microorganisms. In the photic zone, where light and oxygen is available, BPA can be photo-degraded by the combined action of light and oxygen reactive species (ROS) (Kang and Kondo, 2005).

Another source of bisphenol A in aquatic sediments is the anaerobic dehalogenation of chlorinated and brominated BPA derivatives. Tetrabromobisphenol A is used as a flame retardant in the production of electric circuits, while chlorinated bisphenols are present in wastewater from the paper industry. These compounds can be used as electron acceptors for respiration by anaerobic bacteria that inhabit anoxic sediments, leading to the production and accumulation of bisphenol A under methanogenic and sulfate reducing conditions (Ronen & Abeliovich, 2000; Voordeckers et al., 2002; Ravit et al., 2005; Häggblom et al., 2006; Liu et al., 2013).

1.2 Environmental fate

In natural environments microorganisms can play an important role in pollutant transformations. Particularly important are the transformations that result in a reduction in the toxicity of the contaminants or lead to complete mineralization. The occurrence and extent of microbial degradation depend on factors such as the existence of microorganisms with the metabolic capacity to transform it, the bioavailability of the contaminant, and the existence of environmental conditions that allow the proliferation of the potentially active microorganisms (Alexander, 1999).

Microbial mediated degradation of bisphenol A in freshwater, seawater and sediments under aerobic conditions has been reported. Loss of BPA in aerobic sediments was observed after 15 days (Robinson and Hellou, 2009) while a consortium of bacteria previously acclimated to the compound was able of mineralize it in 6-28 hours (Peng et al., 2015) (Table 2).

In freshwater BPA has a half-life of 4-8 days (Dornet al., 1987, Staplest et al., 1998; Kang and Kondo, 2002b; Klêcka et al., 2011). In fresh water the factors that have more influence on the rate and extent of degradation are temperature and bacterial density (Kang and Kondo, 2002b). Kang and Kondo (2005) reported a long acclimation phase (30 days) in aerobic seawater microcosms followed by rapid BPA loss. It is interesting that the observed BPA loss was not related to an increase in the number of bacteria in the cultures, even when in the sterile control the BPA concentration did not

change. The authors suggested the existence of a possible link between the generation of ROS by the microbial population and the oxidation of the parent compound. Later studies on microbial degradation of BPA in seawater obtained a positive relationship between BPA degradation and bacterial growth (Danzl et al., 2009).

Under anaerobic conditions BPA has been reported to be highly recalcitrant in water, soil and sediments in several studies, with no loss after 10 days, or up to 3 - 6.5 months of incubation under methanogenic and sulfate respiring conditions (Ronen & Abeliovich, 2000; Voordeckers et al., 2002; Kang and Kondo, 2002b; Liu et al., 2013). The higher levels of BPA found in benthos detritus as well as the higher concentration of BPA in filter organisms and in fish tissue suggest that the recalcitrant proprieties of BPA in anaerobic environments can cause its incorporation in the trophic chain (Kang and Katamaya, 2007).

Two recent studies reported the degradation of BPA under anaerobic conditions. Yang et al. (2015) reported the loss of BPA under sulfate and nitrate reducing conditions in sediment microcosms from the Beiyun Canal, China. The substrate concentration was assessed by high performance liquid chromatography. Under nitrate-reducing conditions a 93% of substrate loss was observed in 120 days in contrast to the 20% substrate loss in sterile controls, and a half-live of 75 days. Under sulfate-reducing conditions an 89% loss of BPA was observed versus a loss of 17% in the sterile controls. No intermediaries were documented as a result of the degradation process, and the link of BPA loss to either denitrification of sulfate-reduction was also not

demonstrated. Li et al. (2012) reported on BPA degradation by *Bacillus* sp. strain GZB under iron reducing conditions. This strain was isolated from the sediment of a creek in an electronic-waste recycling site in Guiyu, China. This strain was not able to degrade BPA under any other anaerobic conditions. Nonetheless, the extent of anaerobic BPA degradation reported in these studies is not conclusive due that the rates reported for microbial growth under iron reducing conditions are unusual high, and the incapacity to demonstrate that the process of BPA loss from the media is not a result of bioaccumulation process.

In soil and sediments, BPA can react with MnO_2 by an oxidation reaction that produces 4-hydroxycumyl alcohol (HCA) as a major intermediate. This compound shows a higher estrogenic activity (10-fold higher) than BPA and it is also recalcitrant under anaerobic conditions. Due to the low Log K_{ow} of HCA (0.76) and its higher water solubility, it is more prone to migrate to the aqueous phase than BPA, where oxygen is available, and the microbial community may mineralize it. In vitro experiments have demonstrated that HCA can react with MnO_2 promoting its mineralization. Nevertheless, the rate of this reaction is lower than the rate of the reaction of BPA and MnO_2 (Im et al., 2015). These findings have environmental relevance, due the fast consumption of oxygen in the superficial layer of the sediments, the higher concentration of MnII in marine sediments and soil, and the presence of bacteria that can use MnII as electron donor and oxygen as electron acceptor releasing MnO_2 to the environment. Then, MnO_2 can interact with BPA in the anoxic zone and produce HCA. This suggests that

BPA can undergo chemical transformations in anaerobic environments that make it more bioavailable for microbial degradation.

1.3 Microorganisms capable of degrading BPA under aerobic conditions

Diverse microorganisms with the capacity to degrade or transform BPA have been isolated from river water, seawater, soil, and leachate of landfill and wastewater sludge (Zhang et al., 2013). They are mainly bacteria, although some species of fungi, microscopic algae and diatoms have been reported (Hirooka et al., 2005; Hirano et al., 2000; Shimoda and Hamada, 2009). In some cases, these transformations proceed to mineralization or to the formation of less complex intermediates. In others cases, the transformation consists of the conjunction of BPA, reducing its estrogenic properties (Table 2; Figure 1).

Table 2. Fate of BPA in natural environments

Phase	**Range of concentration (ppb)***	**Conditions**	**Half life**	**Reference**
Freshwater sediment	1.40 to 140	Aerobic	7-9 days	Peng, Chen et al., 2015
Marine sediment	1.5 to 5.0	Aerobic	In aerobic condiitons15 days	Robinson and Hellow, 2009
Surface water	0.009 to 12	Aerobic	Freshwater, 4-7 days seawater, >30 days	Kang and Kondo, 2002; Kang and Kondo 2005
Groundwater	0.006 to 2.5	Anaerobic or microaerobic conditions	----------	----------
Soil	4 to 14	Aerobic	4-9 days in oxygenated soils; in anoxic soil >70 days	
Drinking water	< 0.1 to 0.42	Aerobic	----------	

*Source BPA action plan, Environmental Protection Agency of USA 2010.

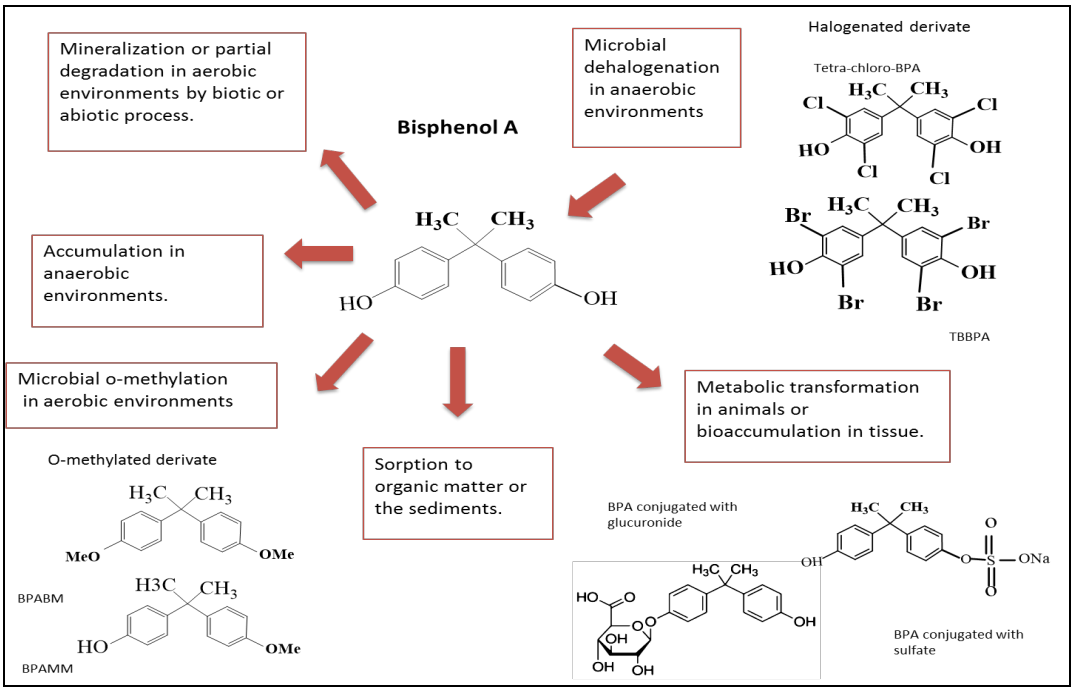


Figure1. Major transformations that impact the fate of bisphenol A in aquatic environments.

Although the capability for BPA biotransformation is widely distributed in bacteria that inhabit soil and freshwater environments, the rate and extent of this degradation varies. A limited number of species can tolerate high concentrations of BPA or possess a rate of degradation that make it important for removal of this compound from natural environments. An interesting aspect is that the bacteria with BPA degradation capacity belong to diverse phylogenetic groups (Table 3).

Species with highest BPA degrading capacity include *Achromobacter xylosoxidans*, *Sphingomonas bisphenolicum*, *Sphingomonas paucimobilis, Sphingomonas* sp. MV1, *Enterobacter gergoviae,* and *Bacillus* sp. GBZ

(Zhang et al., 2013). *Sphingomonas bisphenolicum* (strain A01) shows the most rapid degradation rate and tolerance to high BPA concentrations. This species was isolated from agricultural soil and it is able to degrade 100 mg/l of BPA in 6 hours under aerobic conditions (Oshiman et al., 2006).

BPA can also be metabolized by mixed cultures, in some cases resulting in a higher rate of consumption. Sakai et al. (2007) reported that *Sphingomonas* sp. strain BP7 was able to degrade 100 ppm of BPA in 40 days, while a mixed culture of this *Sphingomonas* BP7 and *Pseudomonas* BP-14, a non-BPA degrading strain, could mineralize the same concentration of BPA in 7 days. The authors suggested that the *Pseudomonas* BP-14 could supply some nutrients required by *Sphingomonas* BP7, increasing its growth rate. This hypothesis was based on the observation that the *Pseudomonas* strain is not able to degrade 4-HAP, the mainly identified intermediates in the pathway, and the fact that the addition of yeast extract, beef extract and peptone increased the rate of BPA degradation to the same levels that was observed in the mixed cultures. The authors suggested that this syntrophic relationship could be a strategy to overcome the low level of nutrient in seawater.

The transformation of xenobiotic compounds can be performed by a microbial consortium, in which several species with different metabolic capacities interact leading to the final mineralization of the contaminant. Eio et al. (2014) studied the diverse metabolic pathways found in a consortium from a wastewater treatment plant in Tokyo, and suggested that four different metabolic pathways for BPA degradation were active in the slurry. This study

also evaluated the effect of light and EDTA concentration on the BPA degradation rate when the consortium was exposed to the light or kept in the darkness. Because EDTA is a metal chelator with wide use in the industry; it is widely detected in wastewater. The authors found that the reaction of EDTA and iron in the presence of light decreased the BPA degradation rate and hypothesized that it was due to a substrate competition. This hypothesis is based on the fact that the Fe-EDTA complex could be degraded and used as a carbon source for the microbial community while EDTA was not.

Peng et al. (2015) reported changes in the microbial community from river sediments in response to BPA enrichment conditions. The changes included a reduction in the number of species from the time zero to the moment at which BPA was almost depleted. Using denaturing gradient gel electrophoresis, the authors identified the presence *of Pseudomonas knackmussii* and *Methylomonas clara* as the dominant species in this microbial consortium. Even though not all the species in the consortium showed BPA degradation capacity, the diversity of species was crucial to support the rapid decomposition of the contaminant. A similar study by Yang et al. (2014) reported changes in the community of river sediments after spiking with BPA. A shift of in the abundance of the major bacterial taxonomic groups was observed after 3-4 days of BPA incubation, with an increase in Gammaproteobacteria and Alphaproteobacteria.

The changes in the microbial community can be the result of differential growth between the species that are able to use BPA as carbon source and those than cannot. In the initial period, the organic matter present

in the sediment can be used by bacteria that cannot degrade BPA; once the indigenous organic matter is totally consumed, just the species able to use BPA can continue growing.

The algae *Chlorella fusca* can transform BPA in a light dependent reaction at concentrations of 10-80 μM with an effectiveness of 95%. Monohydroxy-bisphenol A was detected as a primary intermediate in this process, and the metabolite does not exhibit estrogenic activity (Hirooka et al., 2005). The algae *Pavlova sp.* is able to glycosylate BPA reducing its estrogenic activity in 80% in 5 days (Shimoda and Hamada, 2009).

Several species of wood-decay fungi are able to degrade BPA. This capacity is due to the activity of the enzymes manganese peroxidase and laccase enzyme, which play a role in lignin degradation (Table 4). Hirano et al. (2000) reported BPA degradation by the fungi *Pleurotus ostreatus* with 80% loss in 12 days. In this study the authors tested the effects of pure enzyme extract over the compound generating several aromatic intermediaries as the result of the random break of the C-C bridge between the phenol rings.

Table 3. Microorganisms capable of degrading BPA under aerobic conditions.

Species	**Source of isolation**	**Partial degradation (PD) / Mineralization (M)**	**Reference**
Algae			
Chlorella fusca	Culture collection	Biotransformation. Monohydroxy BPA as major product	Hirooka et al., 2005
Pavlova sp.	Culture collection	Biotransformation. Glycosylated BPA as final product	Shimoda and Hiroka, 2009
Diatoms			
Chaetocero gracilis	Culture collection		Ishihara and Nakajima, 2003
Fungi			
Pleurotus ostreatus O-48	Culture collection	Partial degradation to less complex and non-estrogenic aromatic product	Hirano et al., 2000
Phanaerochaete chrysosporium ME-446	NR		Tsutsumi et al., 2001
Trametes versicolor IFO-7043	NR		Tsutsumi et al., 2001
Trametes villosa	NR		Fukuda et al., 2011
Aspergillus fumigatus	Culture collection		Yim et al., 2003
Fusarium moniliformie 2-2	Culture collection		Chai et al., 2005

Species	Source of isolation	Partial degradation (PD) / Mineralization (M)	Reference
Aspergillus terreus	Culture collection		Chai et al., 2005
Emericella nidulans MT-98	Culture collection		Chai et al., 2005
Stereum hirsutum	Culture collection		Lee et al., 2005
Heterobasidium insulare	Culture collection		Lee et al, 2005
Coriolopsis polyzona	Culture collection		Cabana et al., 2007
Bacteria			
Shingomonas bisphenolicum A01	Agriculture soil	Mineralization	Oshiman et al., 2007
Sphingomonas paucimobilis FJ-4	Sludge in sewage plant		Ike et al., 1995, 2002
Sphingomonas sp. MV1	Sludge	Mineralization	Zang, Yin and Chen, 2013.
Sphingomonas sp. strains SOII, SOIa and SO4a	Sediment		Matsumura, 2009
Sphingomonas sp. BP-7	Seawater	Mineralization	Sakai, Yamanaka et al., 2007
Sphingomonas sp. TTNP3	Sludge		
Sphingomonas yanoikuyae	water		
Achromobacter xylosoxidans B-16	Compost leachate	Mineralization	Zhang et al., 2007
Alcaligenes sp. O1T7	Soil		Matsumura et al., 2009

Species	Source of isolation	Partial degradation (PD) / Mineralization (M)	Reference
Bacillus cereus	Plant rhizosphere		
Bacillus sp. GBZ	Sediment	Mineralization	Li et al., 2007
Bacillus pumilus BP-2CK, BP-21DK, BP-22DK	Foof of kimchi		
Bacillus sp. NO13, NO15, YA27			Matsumura et al., 2009
Bordetella sp. OS17			Matsumura el al., 2009
Pseudomonas sp. LBC1			
Pseudomonas sp. SU1, SU4, NAR11, FU12, NO14, KA16, SU19, FU20, HUK21 and HUK22	River water		Matsumura, 2009
Pseudomonas sp. KA4	River water		Kang and Kondo, 2002A and B.
Pseudomonas paucimobilis FJ-4			
Pseudomonas monteilii	Sewage	Mineralization	Masuda et al, 2006
Pseudomonas putida KA5	Water		Kanda and Kondo, 2002 and B.
Pandorea sp.			
Pandorea sp. HY06			Matsumura, 2009
Enterobacter sp. H19, HA18			Matsumura, 2009
Enterobacter gergoviae strain BYK-7	Petrochemical Wastewater		

Species	Source of isolation	Partial degradation (PD) / Mineralization (M)	Reference
Enterobacter sp. BPR1 and BPW5	Plant Rhizosphere		
Serratia sp. HI10			Matsumura, 2009
Klebsiella sp. NE2, SU3 and SU5			Matsumura, 2009
Streptomycetes sp.	River water		Kang et al., 2004
Cupriavidus basilensis JF1			Fisher, 2010.
Nitrosomonas europaea			Roh et al, 2009
Novosphigobium sp. TYA-1			Toyama et al, 2009

Source: Adapted from Zhang et al., 2013 and Kang and Katayama, 2007.

Table 4. Enzymes that catalyze initial BPA transformation. Adapted from Kang & Katayama (2007).

Enzyme	Source	Function
Manganese peroxidase	Fungi	Catalyze the oxidation of C-C bond cleavage between the two phenolic rings
Laccase	Fungi	
Horseradish peroxidase	Plant roots	
Tyrosine		
Cytochrome P450	Bacteria	Catalyze the hydroxylation of a methyl group
UDP-glucuronosyltransferase	Vertebrates	Conjugated BPA with glucuronide in a detoxification pathway
Sulfotrasferase	Vertebrates	Conjugated BPA with sulfate in a detoxification pathway

1.4 Proposed pathways for BPA degradation by bacteria

In the degradation of aromatic compounds by bacteria two main types of pathways can be identified: a. Peripheral pathways, which remove functional groups from the aromatic ring and generate a reduced number of aromatic intermediaries and b. Central pathways that have as a goal the break of the aromatic intermediary generated in the peripheral pathways and the formation of molecules such as acetyl-coA or succinate. In aerobic microorganisms the peripheral pathways typically involve the activity monooxygenase and dioxygenase enzymes, while the ring cleavage is preforming by dioxygenase enzymes (Heider and Fuchs, 1997). During the metabolism of xenobiotic compounds, the transformation of the pollutant can be the result of the direct use of the compound as source of nutrient, as electron acceptor or transformation as a result of co-metabolism. BPA can be used as only source of carbon by a diverse number of bacteria species. *Sphingomonas* strains are the most frequently isolated BPA degrading bacteria (Zhang et al., 2013).

Through the analysis of the metabolites produced during degradation of BPA by pure cultures of bacteria, diverse pathways for BPA degradation have been proposed. The first pathway to be proposed correspond to the strain *Sphingomonas* sp. MV1 (Spivack et al., 1994). The authors reported the oxidation of the aliphatic methyl group as initial transformation step, producing after a skeletal rearrangement triol 1,2-bis (4-hydroxyphenyl)-2 propanol, which undergoes rapid cleavage by oxidation to 4-hydroxybenzaldehyde and 4-hydroxyacetophenone. Then,

hydroxybenzaldehyde is oxidized to hydroxyl benzoic acid. Both daughter compounds are further degraded and mineralized. A minor product of the initial hydroxylation, 2-2-bis (4-hydroxyphenyl)-2-propanol, is transformed in 4-hydroxyphenalcyl alcohol.

During BPA degradation by *Sphingomonas bisphenolicum* strain A01, some of these intermediates have been detected (Sasaki et al., 2005): 1,2-bis(4-hydroxyphenyl)-2-propanol and 2,2-bis(4-hydroxyphenyl)-1-propanol, and 4-hydroxy acetophenone. In addition, the authors suggested that the initial step in this pathway is catalyzed by a cytochrome P450 monooxygenase. This was demonstrated by the inhibition of BPA degradation after addition of metyrapone and proadifen, two cytochrome P450 inhibitors. The genes and proteins that encode for this enzyme complex were also identified (Sasaki et al., 2005; Sasaki et al., 2008).

The pathway for BPA by *Achromobacter xylosoxidans* has been proposed by the analysis of putative intermediates in the process. Zhang et al. (2007) suggested that *A. xylosoxidans* cleaves the propane bridge generating p-hydroxyacetophenone, p-hydroxybenzaldehyde, and p-isopropenylphenol as the main intermediates. Then, hydroxyacetophenone is transformed to benzoic acid and p-isopropenylphenol is transformed to p-hydroquinone. All of these intermediates are subsequently mineralized.

During BPA degradation by *Bacillus* sp. GZB hydroxybenzaldehyde, 4-(2 hydroxypropan-2-yl) phenol, 4-(prop-1-en-2yl) phenol, 1-(4-hydroxyphenyl)

ethanone, 4-hydroxybenzaldehyde, benzoic acid, 2-hydrozypropanoic acid, 2-methylbutanoic acid have been identified as putative intermediates.

1.5 BPA in wastewater treatment facilities

The techniques used to remove biodegradable organic compounds include biotic and abiotic techniques. A primary process consists in the removal of a portion of the suspended solid. A secondary process consists in the removal of the biodegradable organic matter in solution or suspension and nutrients such as nitrogen and phosphorus. A tertiary or advanced treatment is used for further removed of BOD, nutrient, pathogens and toxic substances (Tchobanoglous et al., 2004; Bitton, 2011). The most common techniques include chemical assisted primary treatment, aerated lagoons, facultative lagoons, conventional activated sludge, trickling filter and rotating biological contractors (Bitton, 2011).

The conventional activated sludge process consists of an aeration tank, a clarifier container and ducts that made possible the flow of the treated effluent and the recycling of the solid phase, containing the active microorganisms. In the aeration tank the microbial and chemical oxidation is promoted, then the liquid phase and part of the solid phase is transferred to the clarifier tank where the solid is induced to precipitation. The water is released to the environment or goes to a tertiary treatment process, while the solid phase is recycling returning to the aeration tank. The residence of liquid phase is the 4-8 hours (Britton, 2011), while the residence time of the sludge can vary according to the system in a range of 3-20 days (Stasinakis et al.,

2010).

Biological Aerated Filter system (BAF) employs a bioreactor with media in suspension, to which biomass are attached. A variation of the BAF is the trickling filter; it consists in an aerobic attached growth process in which microorganisms are attached to media in a packed tower (Guerra et al., 2015).

The efficiency in BPA removal varies from one technique to another. More pioneering techniques include photo-degradation, and chemical oxidation by the induction of hydroxyl and sulfate radicals or sonication (Qayyum and Qayyum, 2013; Lin et al., 2013; Lu et al., 2013). Biotic techniques include the bio-sorption into plant tissue, application of enzymatic extract from fungi or plants, and stimulation of aerobic microbial degradation.

Guerra et al. (2015) studied the efficiency in removal of BPA by twenty five wastewater treatment plants operating under different systems: aerated lagoons, facultative lagoons, chemically-assisted primary treatment plants, conventional activated sludge, trickling filter, biological aerated filter processes, and membrane bioreactor. This study found that the highest efficiency in the removal of BPA under different wastewater treatment correspond to biological aerated filter process, with a mean removal percent of 95% and the lowest efficiency percent correspond to trickling filter with less than 5%; the activated slurry had a mean removal efficiency of 68%.

Stasinakis et al. (2010) studied the effects in BPA removal of the sludge retention time (SRT) for activated sludge systems. Non-significant

effect of SRT in the sorption process or in microbial biodegradation was reported for artificial continuous flow activated sludge with SRT of 3, 10 and 20 days. In addition, the efficiency of removal for this EDC was over 90% in all of the cases. Similar results were reported by Nakada et al. (2006) with 92 % of efficiency of BPA removal by activated sludge in WWTP in Japan. Kim et al. (2007) reported the highest rate of BPA degradation in activated sludge that had been enrichment for ammonium oxidizing bacteria compared to activated sludge previously enriched for a nitrite oxidizing community. In both cases the authors suggested a minor role of heterotrophic bacteria in BPA degradation in this system. In this system both type of microorganisms can growth by using ammonium or nitrite as electron donor and BPA as carbon source. The use of allylthiourea, an inhibitor of ammonium oxidation, generated a 87% reduction in BPA degradation.

More pioneering techniques for BPA removal include photo-degradation, chemical oxidation by the induction of hydroxyl and sulfate radicals and sonification (Qayyum and Qayyum, 2013; Lin et al., 2013; Lu et al., 2013). Alternative biotic techniques for BPA degradation include the application on enzymes extracted from fungi or bioaccumulation in plants tissue.

1.6 Effects of BPA on wildlife

BPA is categorized as an endocrine disruptor compound (EDC) (Rochester, 2013; Husain and Qayyu, 2013). BPA is commonly thought to exert its effects by acting as a weak estrogen receptor (ER) agonist (Kinch et

al., 2014). In addition, its antagonist capacity against androgen receptors (ARs) and thyroids receptors (ThRs) has also been demonstrated (Zoeller et al., 2005). BPA is also capable to interfere with the lipogenesis by affecting the concentration of the hormone adiponectin and lectin (Michalowick, 20014).

BPA can be toxic to aquatic organisms at concentrations that range from 1,000 to 10,000 mg/l (Alexander et al., 1988). BPA at a concentration range of 0.1 -0.08 µg/L produces feminization in freshwater snail and affects the development of copepod larvae (Flint et al., 2012). Other negative effects of BPA in invertebrates are related to alteration in the reproductive cells and organs, alteration in the oogenesis or changes in female and male proportion in the offspring (Kang et al., 2007; Flint et al., 2012).

Due to its endocrine disruptor proprieties, BPA can generate alteration in the growth and reproductive capacity in fish at lower concentrations, including the inhibition of growth in males at 640 and 1280 µg/l. BPA can also decrease the number of eggs in hatching, inhibition of spermatogenesis, embryo deformation and feminization (Kang et al., 2007). McCormick et al. (2011) reported a LC50 (the concentration required to kill the 50% the individuals used in the test) for embryos of zebra fish (*Danio rerio*) of 5 mg/l at 5 days after fertilization and 1.8 mg/l at 28 days after the fertilization. In amphibians BPA acts as a thyroid antagonist inhibiting the metamorphosis when tadpoles are exposed to 2.28 µg/l of BPA for 9 days. Also sex reversion has been reported, from male to female, in amphibians exposed to 22.8 µg/l for two weeks (Flint et al., 2012).

Some microbial transformations of BPA can generate products with higher toxic or higher estrogenic activity than BPA. The microbial O-methylation of BPA by *Mycobacterium vanbaaleni, M. chlorophenolium, M. smegmatis and M. fortuitum* generates monomethyl BPA and dimethyl BPA. The exposure of fish to these metabolites can generate deformation such as pericardial and yolk sac edema and hemorrhage and curved tail at concentration of 10 mg/l (McCormick et al., 2011).

In vertebrates BPA is metabolized producing the conjugated forms BPA-sulfate and BPA-glucuronide (Kang et al., 2006). In fish, metabolism of this compound mainly occurs in the cell of the intestine by the action of the enzyme (UGT). Then, BPA-glucuronide is excreted in feces. The conjugation of BPA to sulfate is catalyzed by the enzyme sulfotransferase, and represents a minor pathway for BPA detoxification in fish. Analog enzymes are found in humans, but the conjugation in mammals occurs in the liver (Kang and Katayama, 2007).

BPA can accumulate in animal tissue when the metabolic capacity has been saturated. Lee et al. (2015) reported accumulation of BPA in muscular tissue in fish that inhabit freshwater, marine environments and aquaculture facilities, with higher levels of BPA in tissue where detected in fish from freshwater natural environments.

1.7 BPA and humans

BPA has been identified in human fluids such as urine, sweat, blood, serum and maternal milk. BPA may also accumulate in adipose tissue and in

placenta (Rochester, 2013; Michalowick, 2014; Troisi, 2014). The main source of human exposure to BPA is the diet; canned food is considered the predominant source of BPA (Geens et al., 2012). BPA has been detected in canned vegetables, canned soup and fish; as well as in beverages, dairy products and infant formula (Kang et al., 2006). Another source of human exposition include thermal paper, dental amalgams, dust of indoor environment, medical care devices and baby bottles (Geens et al., 2012).

Cytological studies using human hepatic cells have shown that low concentrations of BPA can affect oxygen consumption, ATP production and cause mitochondrial dysfunction (Michalowick, 2014). Long-term exposure to BPA has been related to polycystic ovary syndrome, breast cancer, miscarriage, premature delivery, low birth weight, testicular cancer and low fertility in men (Kang et al., 2006; Rochester, 2013, Troisi, 2014). BPA exposure has been also associated to type II diabetes, cardiovascular disorder and hypertension in adults (Rochester, 2013).

In order to reduce the risk to the human health, different approaches have been taken: a. Regulatory approach, which include ban of the use of BPA in baby bottles and containers for infant formula, the inclusion of BPA in the list of hazardous chemicals, the determination of daily intake limits, and the regulation of BPA concentration in wastewater discharge from industry; b. The continuing innovation in techniques that improve the removal of BPA from industrial and municipal wastewater treatment plants; and c. The active search for chemicals capable to substitute BPA in the industry.

1.8 BPA substitutes

The emerging data on the negative effects of BPA on wildlife and humans has motivated several regulations on the use of BPA in the production of food and beverage containers. The usage of BPA in the production of baby bottles was prohibited in the European Union since 2011, while the specific migration limit of BPA (SML) for food containers have been fixed to 0.6 mg/kg since 2004 (Geens et al., 2012). France prohibited the use of BPA in food containers since January 2015 (Eladak, 2015). In United Stated the use of BPA in baby bottles was prohibited by the Food and Drug Administration Act since July 2012. In addition, intake limits of BPA per day have been established, using as guide a concentration 1000 times lower that the doses capable to generate negative effects in rat (Kang et al., 2006). The daily intake limit for adult is 0.05 mg/kg of body weight per day (EPA, 2010). Limits for BPA release in wastewater from industrial sources have been established in Canada, with a limit of 1.75 µg/L for emission from manufactures and users (Flint, 2012).

The increase public concern about the safety of product containing BPA, has motivated the active search for compounds that can substitute BPA in industrial usage. Bisphenol F (4-4' sulfonyldiphenol), bisphenol S, bis (4 hydroxyphenyl-methane) (Fig. 2) are the major substitutes of bisphenol A in BPA free products. These compounds have been considered safer for humans because they migrate at a lower rate from cans than BPA. Jordäková et al. (2003) reported lower migration of BPF and BFDEE from food cans than the levels obtained for BPA and BADGE. Nonetheless, human exposure to

bisphenol S has been documented by its presence in 80% of urinary samples out of 315 samples from adults from 7 different Asiatic countries and USA in concentration that range from 0.02 ng/ml to 21 ng/ml (Liao et al, 2012).

The endocrine disrupting activity of both BPA analogs have been reported for human and mice embryos cells. Eladak et al. (2015) evaluated the effect in the testosterone production by embryo testicular cell after exposition to BPA, BPS and BPF. These compounds trigger an endocrine response similar to BPA at doses of 1000 nM with a reduction in testosterone secretion.

Bisphenol S has similar neurological effects as BPA at a concentration of 0.006 µg in embryos of zebra fish (*Danio rerio*) after 16-24 hours post fertilization and 24-36 hours post fertilization; exposure before and after these period does not have effects on locomotion. This suggests a period of hyper sensibility during the embryo develop coinciding with the stage at which neurogenesis take place (Kinch et al., 2014).

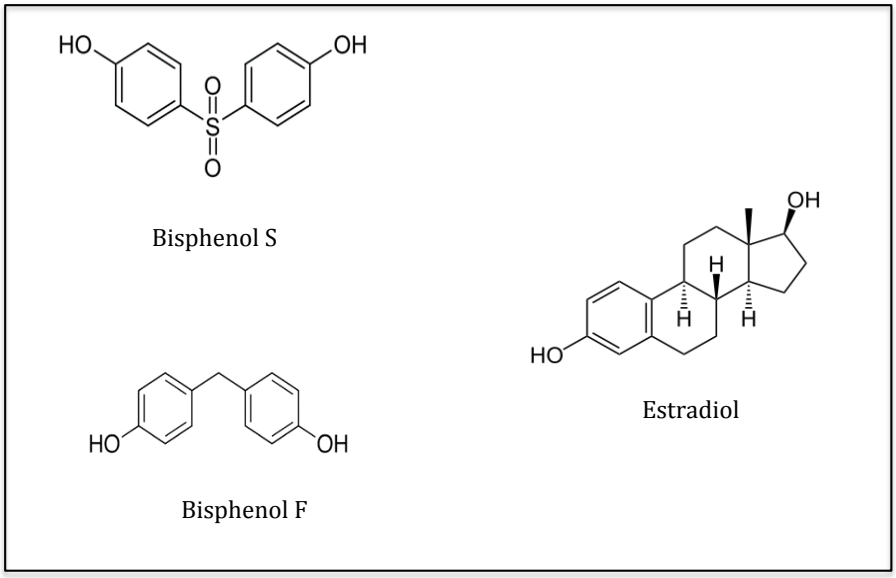


Figure 2. Molecular structure of bisphenol F (4-4'dihydroxydiphenylmethane) and Bisphenol S (4-4' sulfonyldiphenol), two of the major substitutes for BPA and the structure of Estradiol, the sexual hormone that these compounds mimic.

The various BPA analogs have been found in effluent of wastewater treatment plants in Korea. Lee et al. (2015) found higher levels of BPF in domestic wastewater, while higher levels of BPS were associated with industrial usage. Danzl et al. (2009) reported that BPS was not degraded after 60 days in aerobic seawater microcosms. Although the degradation of Bisphenol F in aerobic seawater and river was reported (Inoue et al., 2008; Danzl et al., 2009) its presence in slurry from the wastewater plants suggests that it is recalcitrant under anaerobic conditions. In addition, their persistence in anaerobic environments can trigger similar deleterious effects to wildlife as BPA. The estrogenic activity of BPF and BPS as well as their recalcitrant behavior under anaerobic conditions make them questionable substitutes for BPA.

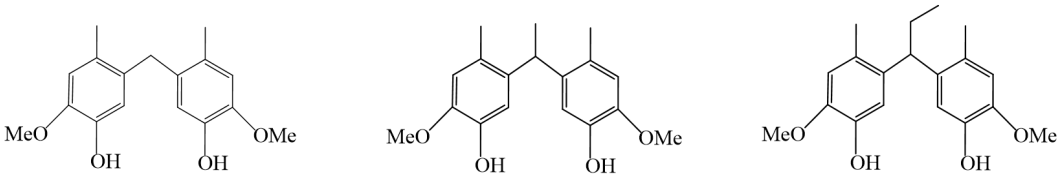
1.9 Objective and aims of the thesis

My main objectives were to: 1. Assess the microbial biotransformation of the bisphenol A analogs, 2-6'-dimethyl, 4-4'-dimethoxybisphenol (DDB); 1-1'-ethyl, 2-6'-dimethyl, 4-4-dimethoxybisphenol (EDDB), and 1-1'-propyl, 2-6-dimethyl, 4-4-dimethoxybisphenol (PDDB) (Figure 3), under aerobic and three anaerobic (nitrate reducing, sulfate reducing, methanogenic) conditions; and 2. Characterize the microbial community that mediates the biodegradation/biotransformation of the bisphenol A analogs. Secondary goals included the characterization of the microbial community involved in the degradation and identification of possible intermediates generated.

Microcosms were established using Arthur Kill sediment as inoculum under aerobic, methanogenic, sulfate reducing and nitrate reducing conditions. The loss of the parent compounds and possible products generated during the degradation process was monitored using high performance liquid chromatography and gas chromatography-mass spectrometry. Microbial community analysis was performed using 16S RNA general primers for bacteria, 341FGC and 534R, in a PCR reaction of 50 ml and 30 cycles: 1.5 minutes, 94ºC, 2 minutes at 48ºC and 2 minutes at 72ºC, finally 5 minutes at 72ºC. In order to identify the potential members of the community a r Denaturing Gradient Gel Electrophoresis was preforming using a 40-60% of denaturing agent in a acrylamide gel essay (Fig. 4).

My overall hypothesis was that Arthur Kill sediments harbor a microbial community capable of degrading/transforming various bisphenol analogs

under either aerobic or anaerobic conditions. I selected Arthur Kill sediments as inoculum because this area has been affected by the influx of contaminants for decades, and in these conditions is feasible to find microbial species with the capacity to degrade xenobiotic compounds. Diverse bacterial species degrading BPA have been isolated from sediment of contaminated areas and the microbial community from areas exposed for decades to BPA, are likely enriched in BPA degrading capability (Zhang et al., 2007; Yang et al., 2014; Li et al., 2012; Zhang et al., 2015). Due to the similarities in the chemical structure of these new bisphenol analogues to BPA, we expected that bacteria with the capacity to degrade BPA would be also able to remove the test compound from the environment.



2-6' dimethyl, 4-4' dimethoxy bisphenol (DDB)

1-1' ethyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol (EDDB)

1-1' propyl, 2-6' dimethyl, 4-4' dimethoxy bisphenol (PDDB)

Figure 3.Chemical structure of test compounds

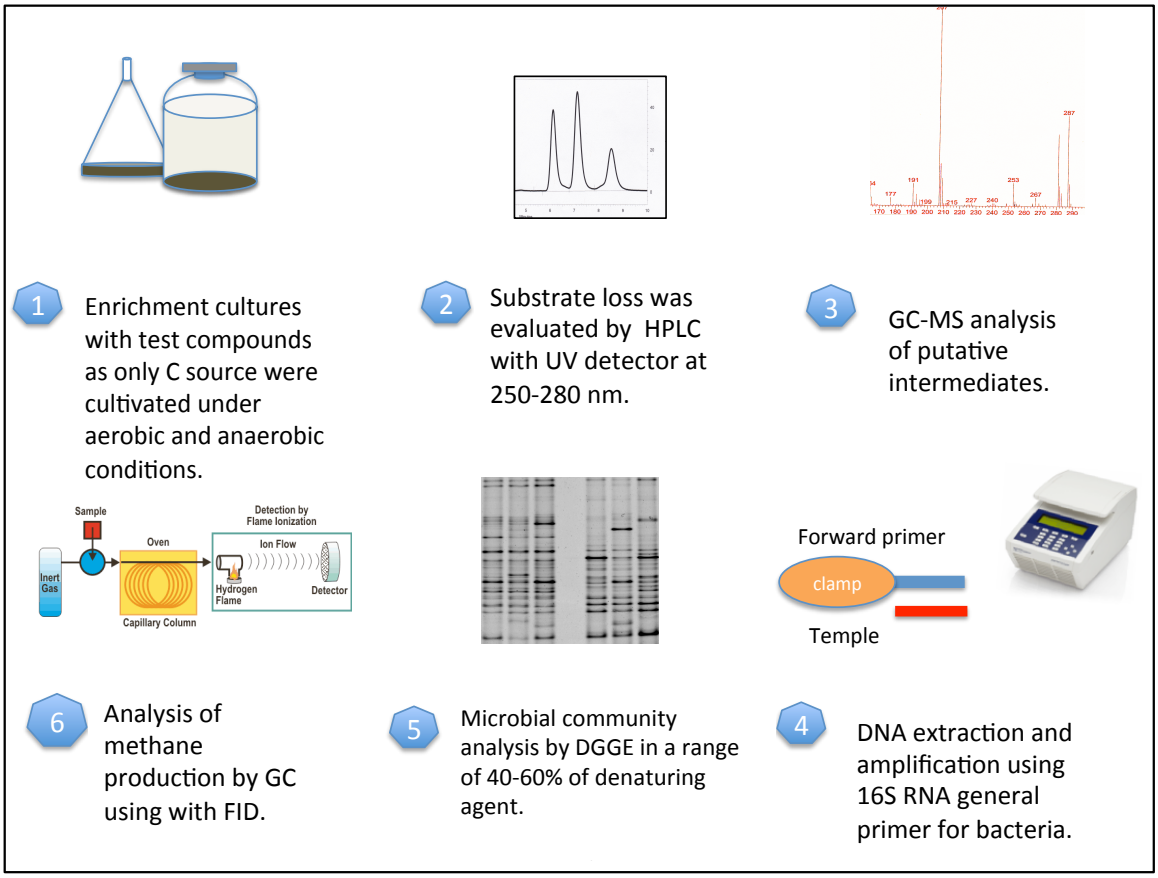


Figure 4. Summary of the methodology used in this study.

2. Methodology

2.1 Culture techniques

Enrichment cultures were established using Arthur Kill (an intertidal strait between New Jersey and Staten Island, NY) sediment as inoculum. Cultures were spiked with 200 µM of one of the test compounds: 2-6-dimethyl, 4-4'-dimethoxybisphenol (DDB); 1-1'-ethyl, 2-6-dimethyl, 4-4'-dimethoxybisphenol (EDDB); or 1-1'-propyl, 2-6'-dimethyl, 4-4'-dimethoxybisphenol (PDDB). An additional set of enrichment cultures were spiked with bisphenol A as a control, as well as cultures without any external

carbon source were also set up. Sterile culture controls were autoclaved three consecutive days at 121˚C for 30 minutes.

Since the test compounds are not water soluble, the substrates were delivered to the culture using dry sediment impregnated with the test compounds. First, 1 gram of Arthur Kill air dried sediment was added to 50 ml culture bottles and autoclaved for 3 consecutive days at 121°C for 30 minutes. Then, a stock solution of 10 mM of the test compounds was prepared in acetone, 1 ml of the solution was added to the 50 ml culture bottle to yield a final concentration of 200 µM of the test compound. Acetone was allowed to evaporate for 6 hours in a fume hood before addition of the sediment slurry. Arthur Kill sediment was mixed in a 9:1 proportion with the medium. Then, 50 ml of this mix was delivered in each culture bottle previously containing the test compounds.

The aerobic medium used was an adaptation from George and Häggblom (2008), prepared as follows: K_2HPO_4 (2.4 g/l), KH2PO4 (2.1 g/l), $MgSO_4.7H_2O$ (0.4 g/l) and NaCl (0.03 g/l). Once the mix containing the inoculum and medium was delivered to the culture bottle, it was covered with an aluminum foil and incubated on a shaker at 150 rpm in the dark.

For setting up the anaerobic enrichments, the mixture containing the culture medium and inoculum was kept under a flux of nitrogen gas to ensure an oxygen-free environment. The basic mineral anaerobic medium was prepared as follows: 1.3 g/l KCl, 0.2 g/l KH_2PO_4, 23 g/l NaCl, 0.5 g/l NH4Cl, 0.1 g/l $CaCl_2.6H_2O$, 2.5 g/l $NaHCO_3$. The medium was supplemented with

trace salts and vitamin solution. Then, the electron acceptor for the different growth conditions was added as follows: nitrate respiring condition was supplied as 3.3 g/l KNO_3. The electron acceptor for sulfate respiring condition was added to the medium as 2.84 g/l Na_2SO_4. After setup, the bottles were sealed and incubated at room temperature in the dark without shaking.

After the concentration of the test compounds declined under the detection limit for the analytic method used in this study, 50% of the degrading culture was transferred to new medium containing 200 µM of the respective test compound supplied using the same procedure used to setup the initial enrichment.

In order to maintain the sterility the medium, the flask and rubber septum were previous sterilized for 45 minutes at 121 ºC. The substrate and the medium were delivered to the culture bottle using sterile pipette and sterile needles and syringes.

2.2 Sampling

To evaluate the loss of substrate over time, 0.5 or 0.7 ml samples were removed from the cultures periodically. Then, a methanol extraction was performed. For extraction, methanol was added as a ratio of 1:1 sample-methanol, mixed by shaking for 30 minutes and then centrifuged to pellet the sediment. The liquid phase was removed and filtered using a 0.45 µm Millipore HPLC syringe filter. The samples were analyzed the same day or stored in the freezer at -20°C until analyzed. This extraction technique had an efficiency of approximately 80% (data not shown).

2.3 Analytic methods

2.3.1 Reverse Phase High Performance Liquid Chromatography

The substrate loss was monitoring by reverse phase high performance liquid chromatography (HPLC). A Shimadzu (SPD-M10A VP) HPLC equipped with a non-polar Phenomenex Sphereclone *ODS* column (250 mm x 4.6 mm, particle size 5 µm) and an absorbance detector set at 280 nm was used. The mobile phase consisted of 70% methanol, 29% deionized water and 1% acetic acid at flow rate of 1ml per minute. The retention times of the test and reference compounds for this method are listed in Table 5.

The three bisphenol A analogs have distinct retention times by this method; DDB elutes at 5.9 minutes; 1-1' ethyl, EDDB elutes at 6.9 minutes, while PDDB elutes at 8.3 minutes. This bisphenol analog was an impure resin containing some remains of the original reagents used in its synthesis, this impurity eluted at 4.1 minutes (Figure 5)

Table 5. Retention time of analytes used in this study

Compound	**Retention time**	**Detection limit in 10 µl injection**
4,4"-(propane-2,2-diyl) (bisphenol A) (BPA)	6.0	5 µmol
2-6 dimethyl, 4-4'dimethoxy bisphenol (DDB)	5.9	2.5 µmol
1-1-ethyl, 2-6 dimethyl, 4-4 dimethoxy bisphenol (EDDB)	6.9	2.5 µmol
1-1' propyl, 2-6 dimethyl, 4-4 dimethoxy bisphenol (PDDB)	8.3	5 µmol

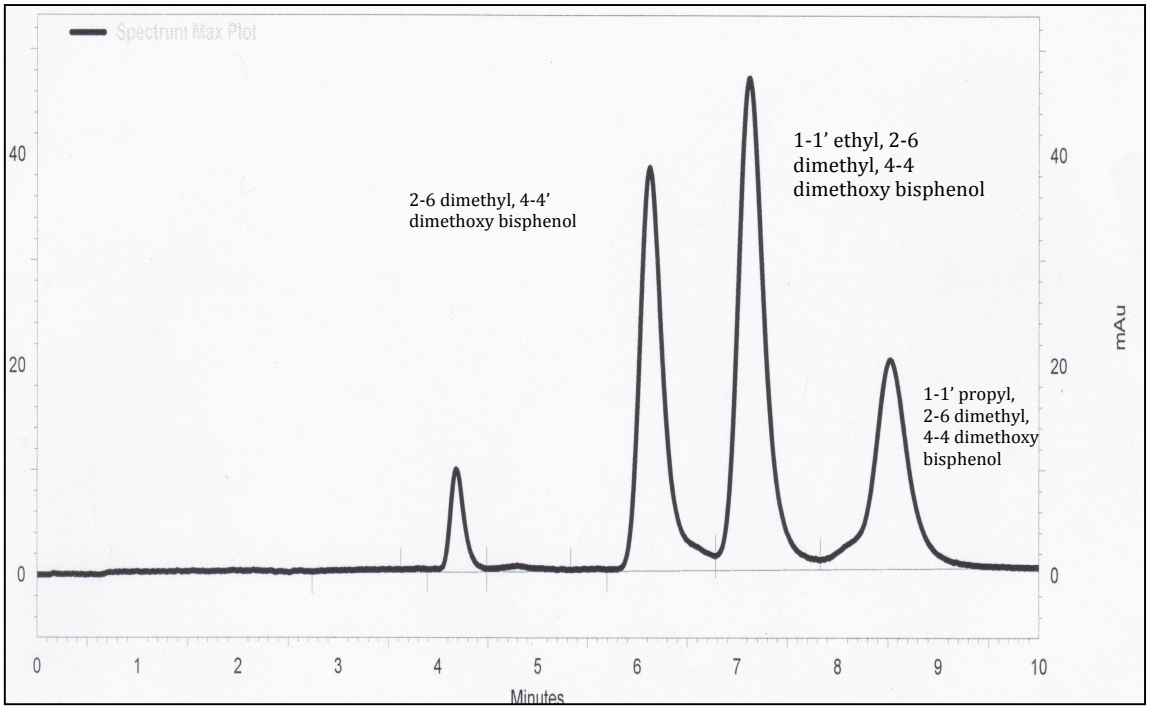


Figure 5. HPLC chromatogram of a mixed standard (200 µM) of three bisphenol analogs.

To calculate the concentrations of the test compounds, a set of standards from 200 µMol to 5 µMol for each compound was prepared and analyzed by HPLC. A standard curve was prepared with the result obtained (peak area versus concentration) from the analysis of the standard and the equation of the curve was used to calculate the concentration of test compounds in the samples.

2.3.2 Gas Chromatography-Mass spectrometry

A GC-MS protocol was developed to analyze the putative intermediates detected by HPLC. In order to be considered for GC-MS

analysis the peak must fill the following requirements: a. Absence at time zero. b. Appearance after an appreciable time of degradation of the parent compound was observed. C. Absence in the sterile control or in the culture that was not fed with the test compounds.

In order to prepare the samples for GC-MS an extraction with 1:1 in ethyl acetate was prepared, mixed several times by shaking and then spinning for 1 minute. The upper layer consisting of the ethyl acetate fraction with the putative intermediates dissolved in it, was then transfer to a 1.5 ml GC vial.

The equipment used for this analysis was an Agilent 6890 Gas Chromatograph equipped with a HP-5ms capillary column and an Agilent 5973 Mass Detector. The carrier gas was helium at a flow rate of 1 ml per minute. The temperature ramp was 50-280 °C with an increment of 10 °C per minute.

The spectra of the standards of the test compounds using this technique are shown in Figures 6-8. The elution time for 2-6 dimethyl, 4-4 dimethoxy bisphenol was 21.0 min, the elution time for 1-1' ethyl, 2-6 dimethyl, 4-4 dimethoxy bisphenol was 21.2 min; while the elution time of 1-1' propyl, 2-6 dimethyl, 4-4 dimethoxy bisphenol was 21.6 min. The last compound had low purity, generating a response of 3 peaks in the chromatogram at the time 20.7, 21.2 and 21.6 min. The spectrum shown corresponds to the main compound.

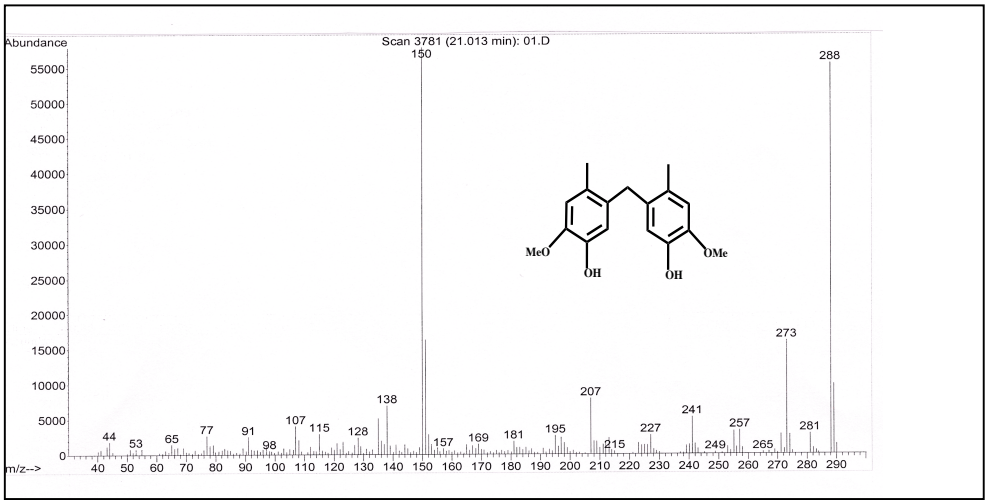


Figure 6. Mass spectrum of 2-6’ dimethyl, 4-4’ dimethoxy bisphenol.

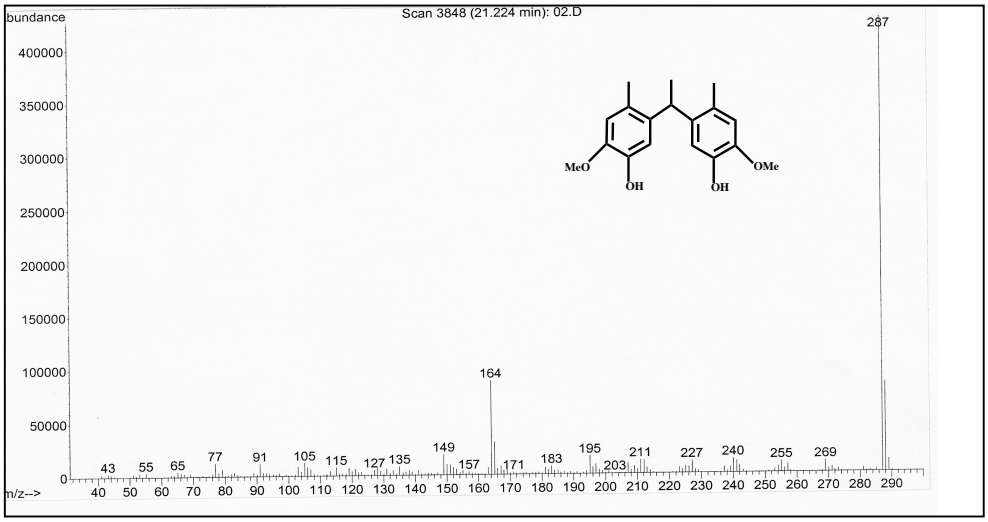


Figure 7. Mass spectrum of 1-1 ethyl, 2-6’ dimethyl, 4-4’ dimethoxy bisphenol.

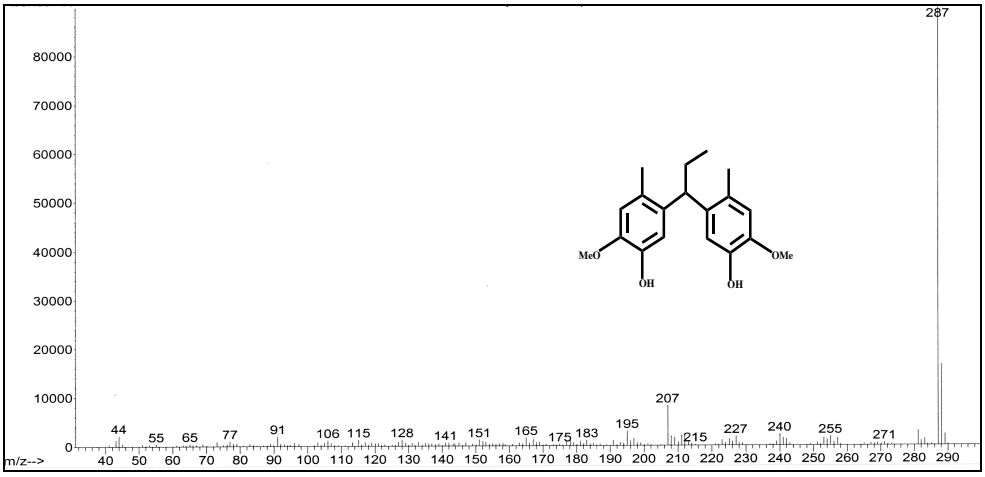


Figure 8. Mass spectrum of 1-1 propyl, 2-6' dimethyl, 4-4' dimethoxy bisphenol.

2.3.3 Methane production

With the objective to identify methane production, 30% of a culture that was actively degrading the test compounds under methanogenic condition was transferred to a new methanogenic medium and fed with 200 µM of the respective substrate. The control for this experiment consisted of 30% of the active microbial community in medium without any additional carbon source.

After 16 days, the methane content in the total volume of gas produced was accessed, aliquots of 2 µl were taken and analyzed by Gas Chromatography using an Agilent 6890N equipped with a flame detector and a capillary column GSPro 30m X 320 µm X 250 µm. The oven temperature was set at 150 °C and the detector heater at 250 °C. The carrier gas was helium and a mix of H_2 and air at a flow rate of 40 ml/min and 45 ml/min, respectively. To facilitate the interpretation of the data obtained, standards for methane concentration from 2%, 3%, 5%, 10% 20% and 40% were prepared

and a standard curve was prepared and the equation used to analyze the data obtained for the samples.

The expected gas production was calculated in order to compare the observed gas production with the expected amount, using the Buswell equation.

$$C_nH_aO_b + (n\text{-}a/4\text{-}b/2)\ H_2O \rightarrow (n/2\text{-}a/8\text{+}b/4)\ CO_2 + (n/2\text{+}a/8\text{-}b/4)\ CH_4$$

2.4 Microbial community analysis

To identify the members of the microbial community that could be involved in the degradation of the three novel bisphenol compounds under methanogenic conditions, 3 ml of the culture was removed from each bottle. First, biomass was pelleted by centrifugation for 1 minute, and the liquid phase was discarded. Then, DNA was extracted using the PowerSoil Kit protocol manufactured by MO BIO.

DNA was amplified using the general primers for bacterial 16S RNA 341F and 534R in a 50 ml PCR reaction. In a reaction of 30 cycles: 1.5 minutes, 94 ºC, 2 min 48 ºC, and 2 min 48 ºC and 2 min 72 ºC, finally 5 min 72 ºC. The DNA template was 3 microliter. The PCR product was analyzed using Denaturing Gradient Gel Electrophoresis in a gradient from 40%-60% of denaturing agent, as proposed by Logemann and Jetten (1998).

3. Results

3.1 Degradation of 2-6'dimethyl, 4-4' dimethoxy bisphenol

Under aerobic conditions initial loss of 200 μM 2-6 dimethyl, 4-4' dimethoxy bisphenol was observed after 30 days in initial Arthur Kill sediment enrichment cultures (Figure 9). No substrate loss was observed in killed controls. With transfer (10%) of the of the initial culture substrate loss was observed in 8 days. Putative intermediates with a shorter retention time, 3.6 and 3.8 minutes, were detected by HPLC after 41 days of incubation in the initial enrichment (Fig. 10) and after 30 days in the first transfer cultures. In the second transfer cultures the peaks appeared within an hour of incubation.

Under methanogenic conditions complete loss of the bisphenol analogs was observed in 14 days in the initial enrichments (Figure 11), with an increase in the rate of disappearance to 3 days and 2 days after the second and third transfer. No intermediates in the methanogenic degradation were detected by UV absorbance, which suggests the degradation of the aromatic ring under this condition.

Loss of 2-6 dimethyl, 4-4 dimethoxy bisphenol under nitrate reducing conditions was not observed after two months. Under sulfate reducing conditions initial lost of the substrate was observed after 24 days reaching a non-detectable level after 44 days (Figure 12). No aromatic intermediates were detected under sulfate reducing conditions.

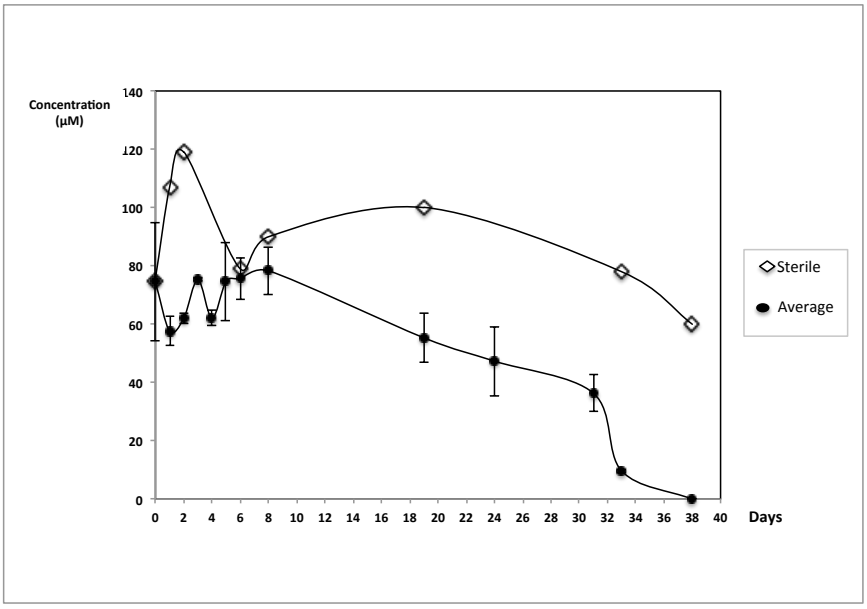


Figure 9. Loss of 2-6 dimethyl, 4-4 dimethoxy bisphenol under aerobic condition (primary enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

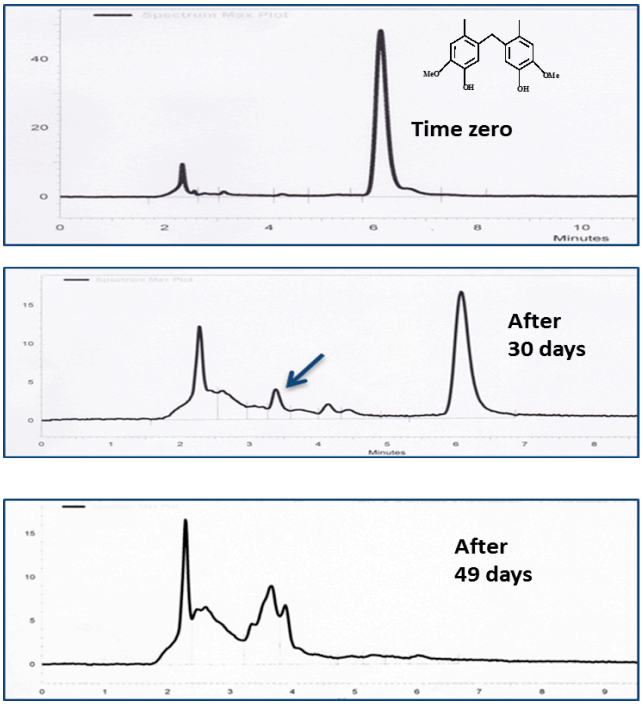


Figure 10. Aerobic cultures of 2-6’ dimethyl, 4-4’ dimethoxy bisphenol showing putative intermediates.

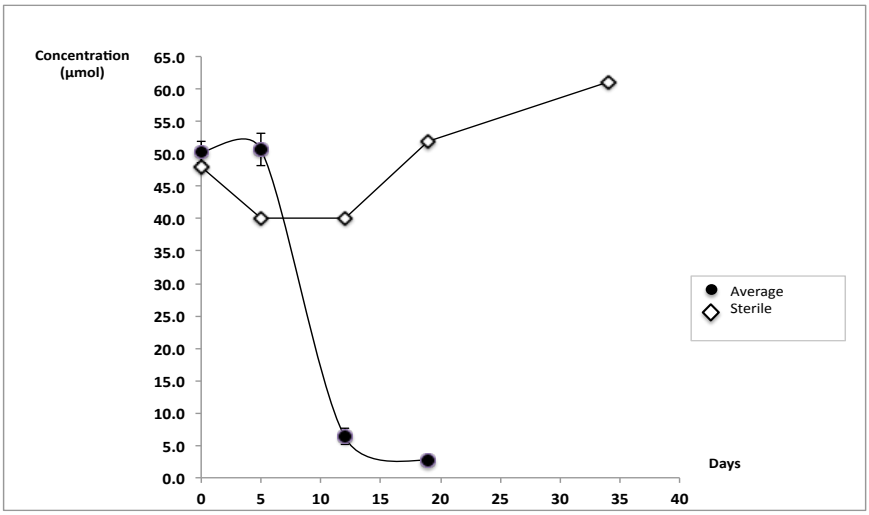


Figure 11. Loss of 2-6 dimethyl, 4-4 dimethoxy bisphenol under methanogenic condition (primary enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

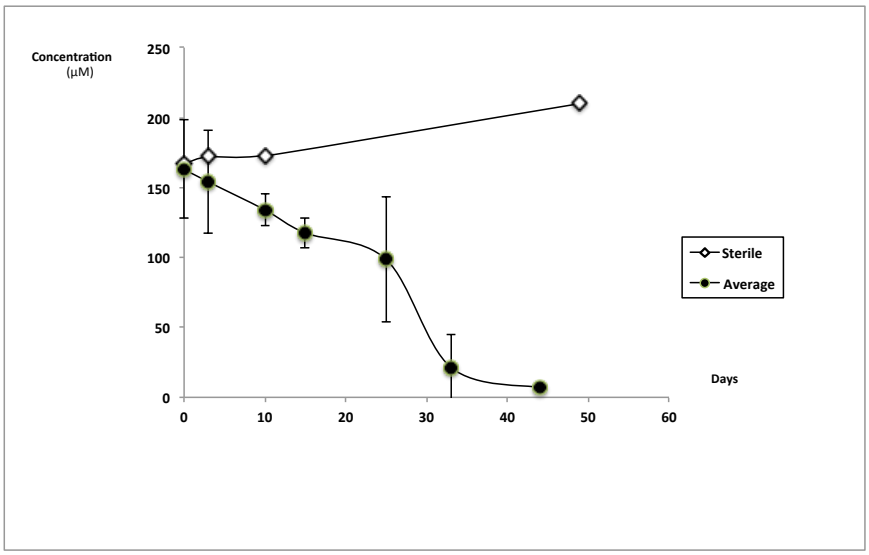


Figure 12. Loss of 2-6 dimethyl, 4-4 dimethoxy bisphenol under sulfate reducing condition (first enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

3.2 Degradation of 1-1 ethyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol

Under aerobic conditions loss of 200 µM 1-1'ethyl, 2-6 dimethyl 4-4 dimethoxy was observed after 49 days. After 51 days the levels of substrate was reduced to ~70% of the original concentration (Figure 13). The rate of degradation increased with subsequent transfer of 10% of the initial culture to new medium. The transferred cultures consumed 38% of the substrate after 28 days.

Using GC-MS a putative intermediate from the aerobic degradation of 1-1' ethyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol was identify based on its mass spectrum as 1-methyl, 2-propane, 4-methoxyphenol (Figure 14-15).

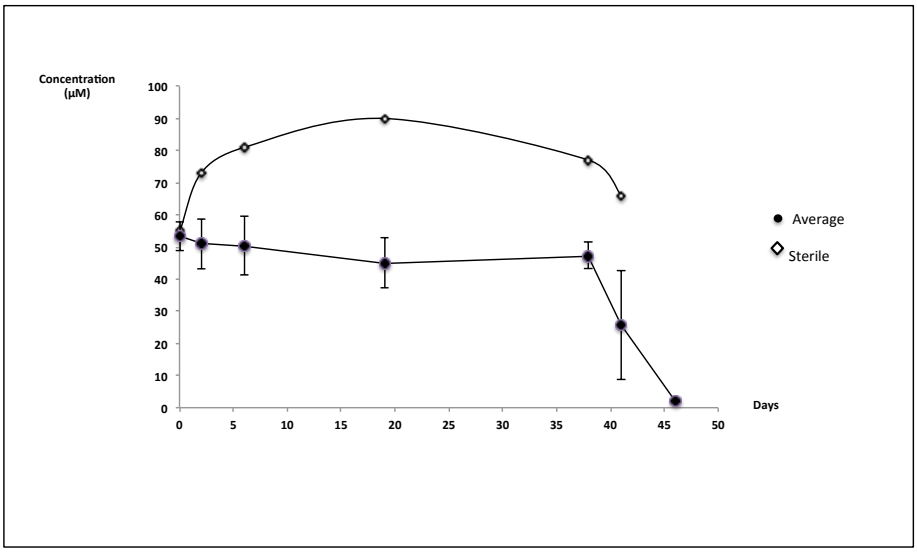


Figure 13. 1-1' ethyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol under aerobic condition (primary enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

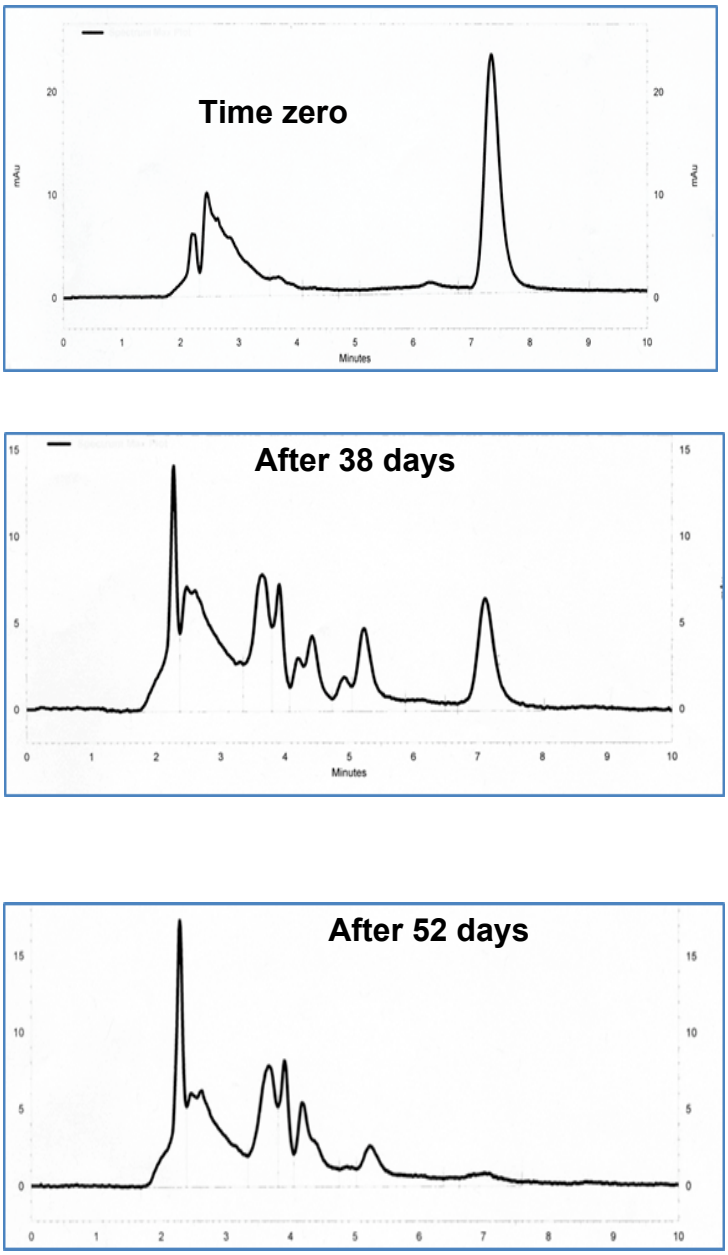


Figure 14. New peaks detected during the aerobic degradation of 1-1’ ethyl, 2-6 dimethyl, 4-4’ dimethoxy bisphenol.

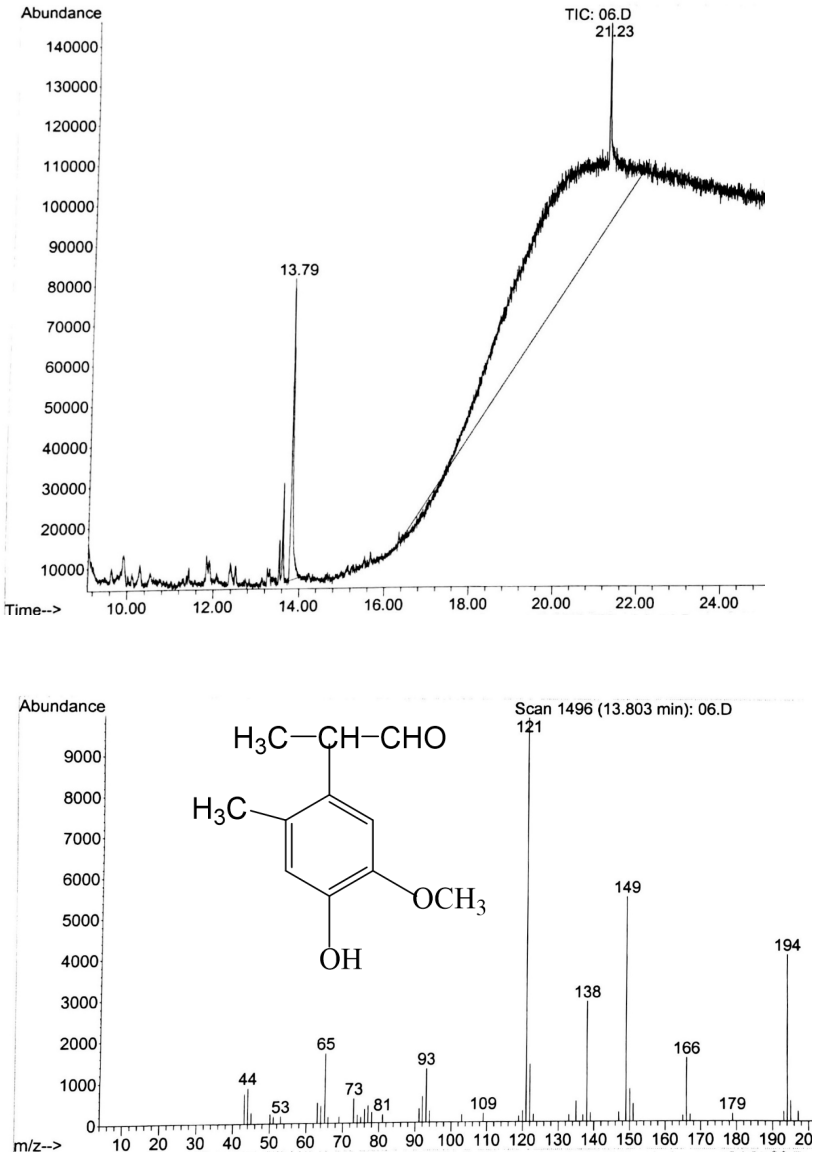


Figure 15. Total ion chromatogram and mass spectrum of a putative intermediate during the aerobic degradation of 1-1' ethyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol.

Under methanogenic conditions initial loss (~4%) of 200 µM of 1-1' ethyl, 2-6 dimethyl, 4-4 dimethoxy bisphenol was observed after 5 days. After 12 days the substrate loss was near 90% in all the replicates, reaching a concentration below the detection limit after 19 days. Previous acclimated

culture degraded the compounds at a higher rate, with a loss of 14% of the compound after 3 days. A posterior transferred culture consumed the 16% and 78% of the compound in 15 and 39 hours respectively (Figure 16).

Under sulfate reducing conditions culture fed with 1-1' ethyl, 2-6' dimethyl 4-4 dimethoxy bisphenol showed an initial degradation detected after 33 days with a substrate loss of 37%. After 44 days of incubation the substrate levels decrease by over 90% (Figure 17).

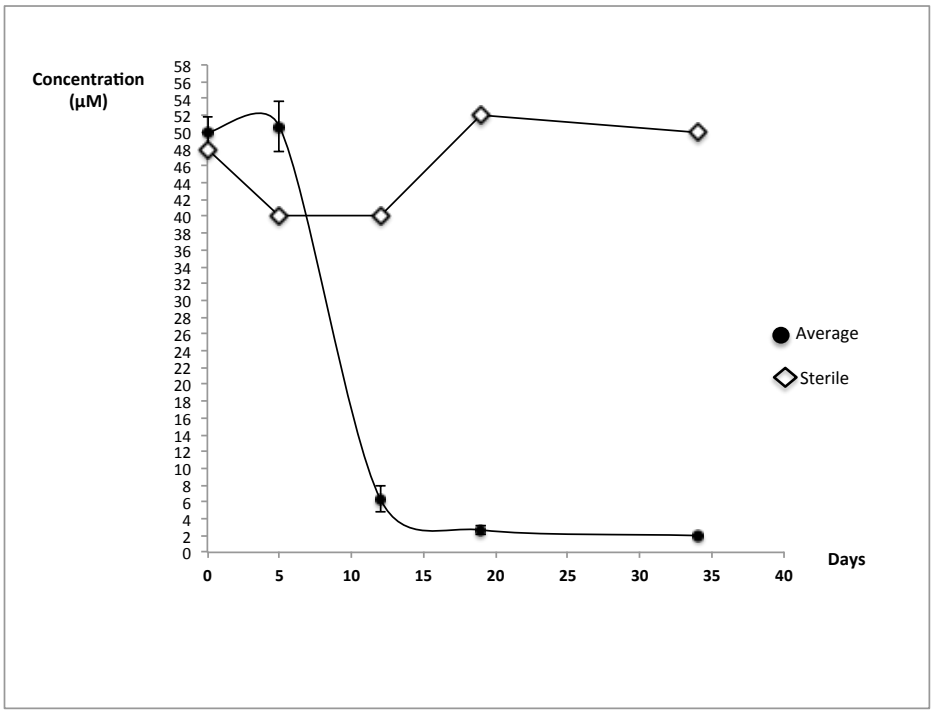


Figure 16. Loss of 1-1' ethyl, 2-6 dimethyl, 4-4 dimethoxy bisphenol in methanogenic Arthur Kill sediment cultures feed with 200 µM. Data points show the mean of triplicate cultures ± standard deviation.

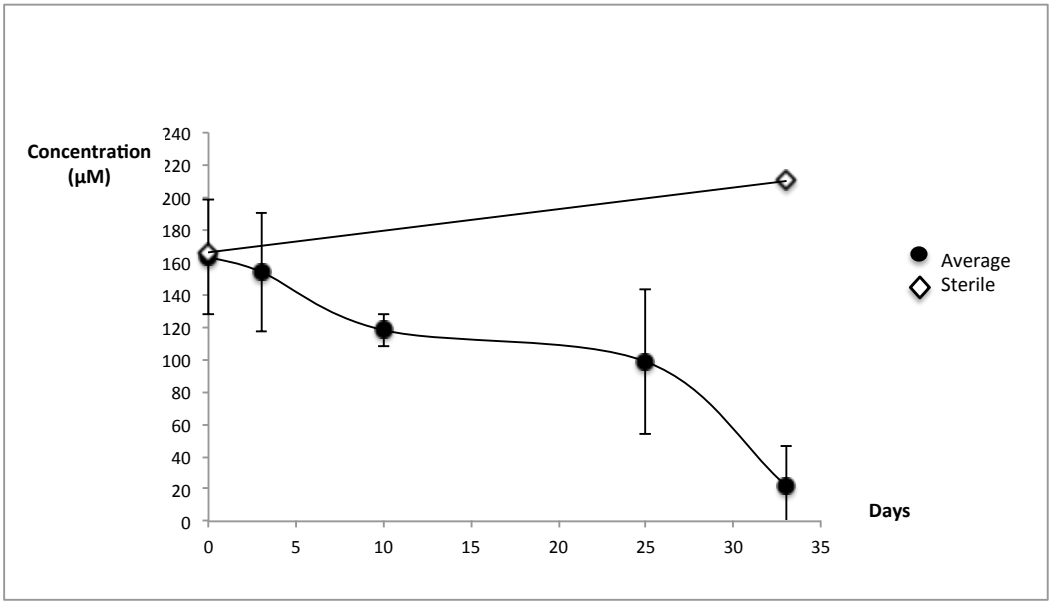


Figure 17. 1-1' ethyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol under sulfate reducing conditions (primary enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

3.3 Degradation of 1-1 propyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol

Enrichment cultures fed with 1-1' propyl, 2-6 dimethyl, 4-4 dimethoxy, bisphenol cultivated under aerobic conditions showed an acclimation phase longer than 20 days, with substrate loss of 41% after 24 days of incubation; after 33 days the mean of substrate los was 84% and after 44 days the concentration had decreased below the detection limit (Figure 18).

The methanogenic culture enrichment with 1-1' propyl, 2-6 dimethyl, 4-4 dimethoxy, bisphenol showed a mean reduction of 90% of the substrate after 5 days and not detectable levels after 12 days of incubation. A previous acclimated culture (50% of the original culture) showed a reduction of a 29% of the substrate in 3 day and concentration under the detection limit (5 µM)

after 6 days (Figure 19). Under sulfate reducing conditions the loss of the 90% of the substrate occurred after 44 days (Figure 20).

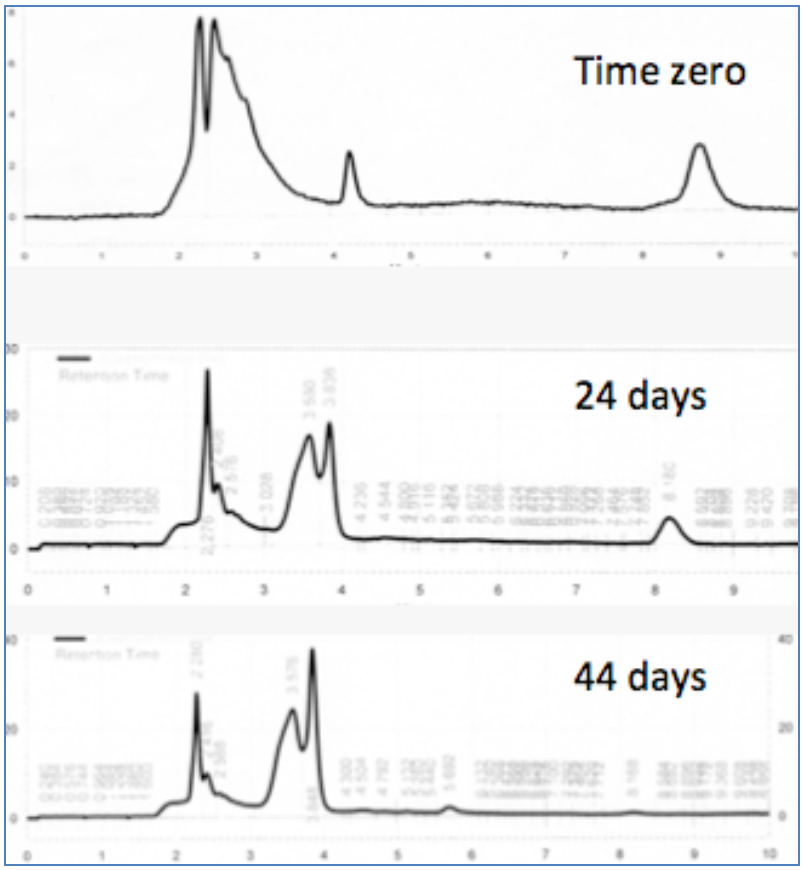


Figure 18. New peaks detected during the degradation of 1-1’ propyl, 2-6 dimethyl, 4-4’ dimethoxy bisphenol.

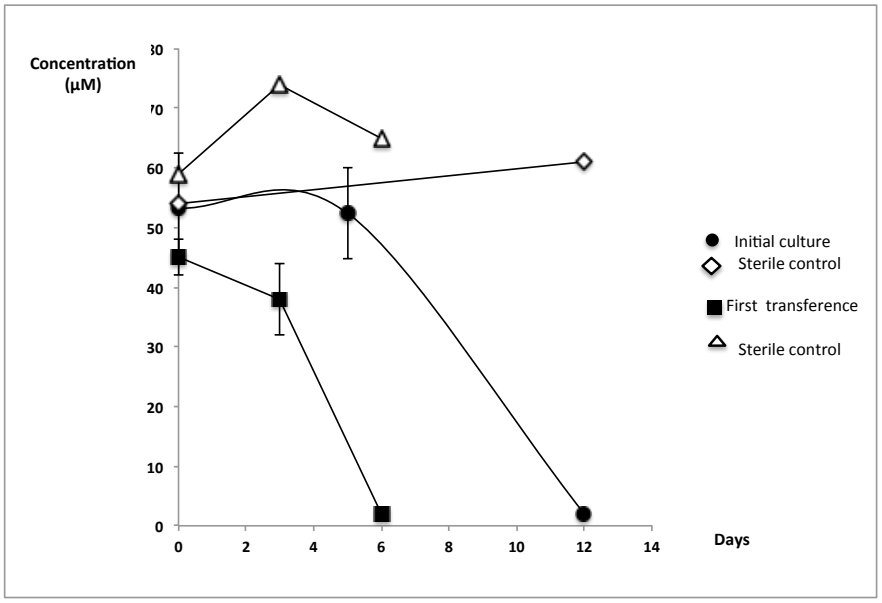


Figure 19. 1-1' propyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol under methanogenic conditions (primary enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

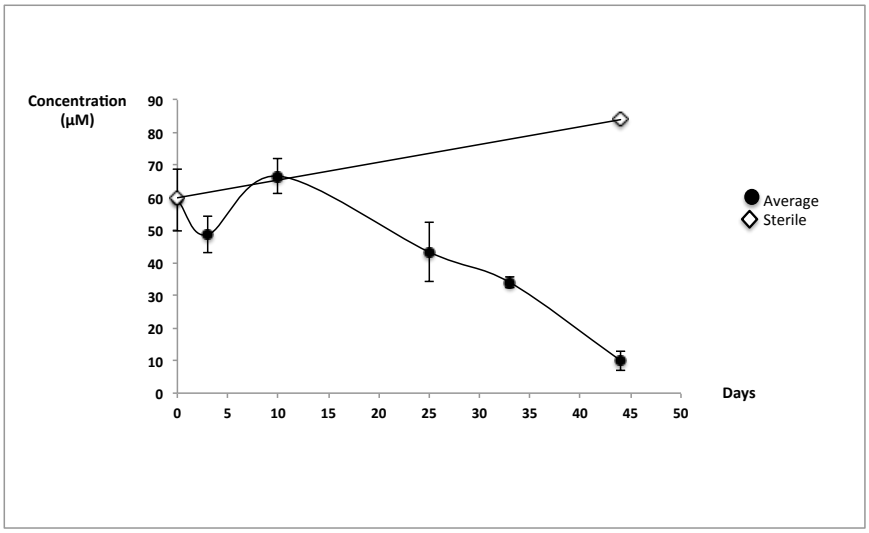


Figure 20. 1-1' propyl, 2-6 dimethyl, 4-4' dimethoxy bisphenol under sulfate reducing conditions (primary enrichment culture). Data points show the mean of triplicate cultures ± standard deviation.

3.4 Methane production

The methane production was evaluated in cultures fed bisphenol analogs and cultured under methanogenic conditions. An active microbial community growing on the test compounds was transferred to two new sets of serum bottles containing medium with the test compounds and medium with no additional source of carbon. The volume of gas produced after 16 days was evaluated using a glass syringe and the proportion of methane in the heat space was determine by GC with a FID detector.

Even when the substrate concentration dropped below to the detection limit after 3 days, the methane production in both the substrate fed and control cultures was similar, suggesting that substrate utilization cannot be directly related to the amount of methane produced. Due that the dried sediment used to deliver the substrate to the new medium is rich in organic matter (Arthur Kill sediment), it is feasible that the microbial community used indigenous organic compound for methane production.

3.5 Bacterial community analysis

In order to determine the possible group of bacteria that can play a role in the degradation of the test compounds under methanogenic conditions, culture samples were taking from the different enrichments, DNA extracted and 16S rRNA genes amplified using general primers for bacteria for DGGE analysis. This analysis was done for DNA samples from the initial enrichment culture with the capacity to degrade the compounds in 15-18 days, as well as

of the community from successive culture transfers with the capacity to degrade the test compounds in 40 hours. Fig. 21 represents the structure of these bacterial communities.

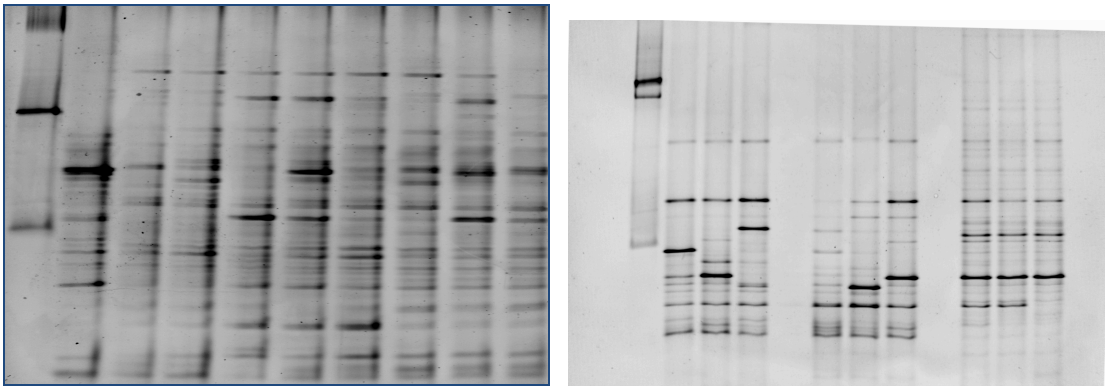


Figure 21. Acrylamide gels showing the bacteria members of the methanogenic consortium. The left gel represents the initial culture (with a rate of degradation of 15-18 days), while the right gel represent the enrichments after third transference. Lane 1 represents the standards in both gels. Lanes 2-4 the enrichment for 2-6' dimethyl, 4-4' dimethoxy bisphenol. Lanes 5-7 enrichment of 1-1' ethyl, 2-6' dimethyl, 4-4' dimethoxy bisphenol and Lanes 8-10 correspond to the enrichment of 1-1' propyl, 2-6' dimethyl, 4-4' dimethoxy bisphenol.

After separation, the individual DGGE bands were collected and eluded from the gel in order to be sequenced. However, due to the poor quality of the sequences obtained this data is not shown.

3.5 Limits and problems in the methodology

When autoclaving the sterile controls, the heating increased the solubility of the bisphenol analogs, generating a "false" increase in the concentration of substrate in the sterile control in the time initial time point

samples. In addition, the source of inoculum used in this study was anaerobic sediment. Hence, aerobic degradation rates were lower than what would be expected in aerobic environments.

The production of methane in methanogenic cultures fed with the test compounds can not be totally discarded, it is possible that the conversion of the intermediates in the degradation can take a long period between the lost of the parent compound and the mineralization of it to carbon dioxide.

4. Discussion

The three bisphenol analogs [2-6'-dimethyl, 4-4'-dimethoxybisphenol (DDB); 1-1'-ethyl, 2-6'-dimethyl, 4-4-dimethoxybisphenol (EDDB), and 1-1'-propyl, 2-6-dimethyl, 4-4-dimethoxybisphenol (PDDB)] are degradable under aerobic and anaerobic (sulfate reducing and methanogenic) conditions. Maintenance of the degradation capability of the culture in successive transfers and the increase in the degradation rate implies that the microbial community is growing on the bisphenol analogs. The fact that not intermediates have been detected using UV, suggests a possible de-aromatization of the compound under sulfate reducing and methanogenic conditions.

The degradation of these bisphenol analogs under anaerobic conditions suggested that they behave differently than BPA under these conditions. It is possible that the presence of the methyl group in the C6 position of the aromatic ring facilitated the degradation of the compound under anaerobic conditions to generated a carboxylic acid derivative, and

then making feasible its later mineralization without oxygen. No intermediates detected by UV absorbance accumulated in methanogenic cultures, even when the sample period was reduced to 6 hours, suggesting a ready de-aromatization of the compounds under this conditions.

Although the community analysis of the methanogenic cultures did not allow the identification of particular genera or species, the banding pattern in the DGGE gel showed a reduction in the number of species of the microbial community through the successive transference. In addition, similar bands in each replicate culture suggests an effective enrichment process to species with the capacity to tolerate or degrade the test compounds.

Under aerobic conditions, Intermediates with similar retention time were detected during the degradation of all of these three bisphenol compounds, suggesting a possible common pathway. All of the intermediates had a shorter retention time in HPLC, suggesting the formation of more hydrophilic mono-aromatic intermediates. A putative mono-aromatic metabolite from 1-1'-ethyl, 2-6-dimethyl, 4-4-dimethoxybisphenol was identified by GC-MS suggesting the attack of one of the aromatic ring as a putative initial steps in the pathway. This is a different rute than the suggested pathways for BPA degradation under aerobic conditions which first step is the attack of the carbon bridge. Future studies are needed to identify the intermediates in the degradation of these compounds and the microbial species that performance this transformation.

The long lag phase during aerobic conditions can be explained by the anaerobic nature of the Arthur Kill sediment used as inoculum where the incidence of aerobic degrader could be low. Another possible cause of the long adaptation phase can be explained by the substrate competition with organic matter present in the sediment. The finding of methane production in non-fed cultures supports that hypothesis.

In summary, based on the results obtained from these experiments we can expect that the novel bisphenol analogs can have a shorter time of residence in the environment and are more feasible to be degraded using conventional waste water treatment techniques. Nevertheless complementary studies are necessary to determine their possible endocrine proprieties to humans and animals and evaluate the possible toxicological effects to wildlife.

References

Alexander H, Dennis C, Smith L, Guiney P, Phillip D. 1988. Environmental Toxicology and Chemistry, 7, 19-26.

Alexander M. 1999. Biodegradation and Bioremediation. Second Edition. Academic Press.

Artham T, Doble M. 2012. Bisphenol A and metabolites released by biodegradation of polycarbonate in seawater. Environ Chem Lett 10, 29–34.

Bitton G. 2011. Wastewater Microbiology. Fourth edition. Wiley-Blackwell.

Borrirukwisitsak S, KeenanH, Gauchotte-Lindsay C. 2012. Effects of Salinity, pH and Temperature on the Octanol-Water Partition Coefficient of Bisphenol A. International Journal of Environmental Science and Development, Vol. 3, No. 5, 460-464.

Cousin, IT, Staples C, Kleçka, Mackay D. 2002. A multimedia assessment of Environmental Fate of Bisphenol A. Human and Ecological Risk Assessment: 8, 107-1135.

Danzl E, Sei K, Soda S, Ike M, Fujita M. 2009. Biodegradation of Bisphenol A, Bisphenol F and Bisphenol S in Seawater. Int. J. Environ. Res. Public Health 6, 1472-1484.

Eio E, Kawai M, Tsuchiya K, Yamamoto S, Toda, T. 2014. Biodegradation of bisphenol A by bacterial consortia. International Biodeterioration & Biodegradation 96, 166-173.

Eladak S, Grisin T, Moison D, Guerquin M, N'Tumba-Byn T, Pozzi-Gaudin S, Benachi A, Livera C, Rouiller-Fabre V, Habert R. 2015. A new chapter in the bisphenol A story: bisphenol S and bisphenol F are not safe alternatives to this compound. Fertility and Sterility. 103, No. 1, 11-21.

Flint S, Markle T, Thompson S, Wallace E. 2012. Bisphenol A exposure, effects, and policy: A wildlife perspective. Journal of Environmental Management 104, 19-34.

Geens T, Aerts D, Berthot C, Bourguignon J, Goeyens L, Lecomte P, Maghuin-Rogister G, Pironnet A, Pussemier L, Scippo M, Loco J, Covaci A. 2012. Review: A review of dietary and non-dietary exposure to bisphenol-A. Food and Chemical Toxicology 50, 3725–3740.

Häggblom, Fennell D, Ahn Y, Ravit B, Kerkhof L. 2006. Anaerobic dehalogenation of halogenated organic compounds: novel strategies for bioremediation of contaminated sediments. Soil and Water Pollution Monitoring, Protection and Remediation, Volume 69, 505-521.

Heider J, Fuchs G. 1997. Anaerobic methabolisms of aromatic compounds. Review. Eur. J. Biochem. 243, 577-596.

Hirano T, Honda Y, Watanabe T, Kuwahara M. 2000. Degradation of Bisphenol A by the lignin degrading enzyme manganese peroxidase produced by the white-rot basideomycete, Pleuroteus ostreatus. Biosci. Biotechnol. Biochm., 69 (9),1958-1962.

Hirooka T, Nagase H, Uchida K, Hiroshige Y, Ehara Y, Nishikawa J, Nishihara T, Miyamoto K, Hirata Z. 2005. Biodegradation of bisphenol A and disappearance of its estrogenic activity by the green alga Chlorella fusca var. vacuolata. Environmental Toxicology and Chemistry, Vol. 24, No. 8, 1896–1901.

Im J, Prevatte C, Campagna S, Löffler F. 2015. Identification of 4-Hydroxycumyl alcohol as the major MnO2-Mediated Bisphenol A transformation Product and Evaluation of its Environmental Fate. Environ. Sci. Technol. 49 (10), 6214–6221.

Inoue D, Hara S, Kashihara M, Murai Y, Danzl E, Sei K, Tsunoi S, Fujita M, Ike M. 2008. Degradation of Bis (4-Hydroxyphenyl) methane (Bisphenol F) by Sphingobium yanoikuyae Strain FM-2 isolated from River Water. Applied and Environmental Microbiology. 74 (2), 352-358.

Kang J, Aasi D, Katyama Y. 2007. Bisphenol A in Aquatic Environment and its Endocrine-Disruptive Effects on Aquatic Organisms. Critical Reviews in Toxicology, 37: 607-625.

Kang J, Katayama Y. 2007. Biodegradation or metabolism of Bisphenol A in the Environment. Book: Environmental Research Focus. Chapter 2 pp. 49-76.

Kang J, Katayama Y, Kondo F. 2006. Biodegradation or metabolism of bisphenol A: From microorganisms to mammals. Toxicology 217, 81–90.

Kang J, Kondo F, Katayama Y. 2006. Human exposure to bisphenol A. Toxicology 226 79–89.

Kang J, Kondo F. 2005. Bisphenol A degradation in seawater is different from that in river water. Chemosphere 60, 1288–1292.

Kang J, Kondo F. 2002a. Effects of bacterial counts and temperature on the biodegradation of bisphenol A in river water. Chemosphere 49, 493–498.

Kang J, Kondo F. 2002b. Bisphenol A Degradation by Bacteria Isolated from River Water. Arch. Environ. Contam. Toxicol. 43, 265–269.

Kinch C, Ibhazehiebo K, Jeong J, Habibi H, Kurrach M. 2014. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. PNAS Early Edition.

Kolvenbach B, Schlaich N, Raoui Z, Prell J, Zu¨hlke S, Sch¨affer A, Guengerich F, Corvini P. 2007. Degradation Pathway of Bisphenol A: Does ipso Substitution Apply to Phenols Containing a Quaternary α-Carbon Structure in the para Position? Applied and Environmental Microbiology, Volume 76, No. 15, 4776–4784.

Logerman S, Jetten S. M. 1998. DDGGEE, a simplified denaturing gradient gel electrophoresis method for the detection of sequence variation in PCR fragments. Biotechnology Techniques, Vol 12, No. 3, 263-265.

Lee C, Jiang L, Kuo Y, Chen C, Hsieh C, Hung C Tien C. 2015. Characteristic of nonylphenol and bisphenol A accumulation in fish and implications for ecological and human health. Science of the Total Environment 502, 417–425.

Lee C, Jiang L, Kuo Y, Hsieh C, Chen C, Tien C. 2013.The potential role of water quality parameters on occurrence of nonylphenol and bisphenol A and identification of their discharge sources in the river ecosystems. Chemosphere 91, 904–911.

Lee S, Liao C, Song G, Ra K, Kannan K, Moon H. 2015. Emission of bisphenol analogues including bisphenol A and bisphenol F from wastewater treatment plants in Korea. Chemosphere 119, 1000–1006.

Lee H, Peart T, Chan J and Gris G. 2004. Occurrence of Endocrine-Disrupting Chemicals in Sewage and Sludge Samples in Toronto, Canada.Water Qual. Res. J. Canada, Volume 39, No. 1, 57–63.

Li G, Zu L, Wong P, Hui X, Lu Y, Xiong J, An T. Biodegradation and detoxification of bisphenol A with one newly-isolated strain Bacillus sp. GZB: Kinetics, mechanism and estrogenic transition. Bioresource Technology 114, 224–230.

Liao C, Lui F, Alomirah H, Loi V, Mohd M, Moon H, Nakata H, Kannan K. 2012. Bisphenol S in urine from the United Sates and Seven Asian Countries: Ocurrence and Human Exposures. Environm. Sci. Techno. 46, 6860-6866.

Lin H, Wu J, Zhang H. 2013. Degradation of bisphenol A in aqueous solution by a novel electro/Fe3+/peroxydisulfate process. Separation and Purification Technology 117, 18–23.

Liu J, Wang, J, Jiang B, Wang L, Chen J, Guo H, Ji R. 2013. Degradation, Metabolism, and Bound-Residue Formation and Release of

Tetrabromobisphenol A in Soil during Sequential Anoxic− Oxic Incubation. Environ. Sci. Technol., 47, 8348−8354.

Lu N, Lu Y, Liu F, Zhao K, Yuana X, Zhao Y, Li Y, Qin H, Zhu J. 2013. H3PW12O40/TiO2 catalyst-induced photodegradation of bisphenol A (BPA): Kinetics, toxicity and degradation pathways. Chemosphere 91, 1266–1272.

Kim J, Ryu K, Kim E, Choe W, Cha G, Yoo I. 2007. Degradation of bisphenol A and nonylphenol by nitrifying activated sludge. Process Biochemistry 42, 1470–1474.

Masuda M, Yamasaki Y, Ueno S, Inoue A. 2007. Isolation of bisphenol A-tolerant/degrading Pseudomonas monteilii strain N-502. Extremophiles 11:355–362.

Matsumura Y, Hosokawa C, Sasaki-Mori M, Akahira A, Fukunaga K, Ikeuchi T, Oshiman K, Tsuchido T (2009) Isolation and characterization of novel bisphenol-A-degrading bacteria from soils. Biocontrol Sci 14:161–169

McCormick J, Van Es T, Cooper K, White L, Häggblom M. 2011. Microbially Mediated O-Methylation of Bisphenol A results in Metabolites with increased toxicity to the developing zebrafish (Danio rerio) Embryo. Environ. Sci. Technol. 45, 6567–6574.

Michalowick J. 20014. Bisphenol A – Sources, toxicity and biotransformation. Environmental toxicology and pharmacology 37, 738–758.

Nakada N, Tanishima T, Shinohara H, Kiri K, Takada H. 2006. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water research 40, 3297–3303.

Oshiman K, Tsutsumi Y, Nishida T, Matsumura Y. 2006. Isolation and characterization of a novel bacterium, Sphingomonas bisphenolicum strain AO1, that degrades bisphenol A. Biodegradation18:247–255.

Peng Y,1, Chen Y, Chang Y, Shih Y. 2015. Biodegradation of bisphenol A with diverse microorganisms from river sediment. Journal of Hazardous Materials 286, 285–290.

Pingqing F, Kawamura K. 2010. Ubiquity of bisphenol A in the atmosphere. Environmental Pollution 158, 3138-3143).

Qayyum H, Qayyum S. 2013. Biological and enzymatic treatment of bisphenol A and other endocrine disrupting compounds: a review. Critical Reviews in Biotechnology 33(3): 260–292.

Ravit B, Ehrenfeld J, M. Häggblom. 2005. Salt marsh rhizosphere affects microbial biotransformation of the widespread halogenated contaminant tetrabromobisphenol-A (TBBPA). Soil Biology & Biochemistry 37,1049–1057

Robinson B, Hellou J. 2009. Biodegradation of endocrine disrupting compounds in harbour seawater and sediments. Science of the Total Environment 407, 5713–5718.

Rochester, J. 2013. Bisphenol A and human health: A review of the literature. Reproductive Toxicology 42, 132–155.

Ronen Z, Abeliovich A. 2000. Anaerobic-Aerobic Process for Microbial Degradation of Tetrabromobisphenol A. Applied and Environmental Microbiology, Vol 66, No. 6, 2372–2377.

Sasaki M, Akahira A, Oshiman K, Tsuchido T, Matsumura Y.2005. Purification of Cytochrome P450 and Ferredoxin, Involved in Bisphenol A Degradation, from Sphingomonas sp. Strain AO1. Applied and Environmental Microbiology, Vol 71, No. 12, 8024–8030.

Sasaki M, Tsuchido T, Matsumura Y. 2008. Molecular cloning and characterization of cytochrome P450 and ferredoxin genes involved in bisphenol A degradation in Sphingomonas bisphenolicum strain AO1. Journal of Applied Microbiology, Vol 105, 1158-1169.

Shimoda K, Hamada H. 2009. Bioremediation of Bisphenol A and Benzophenone by Glycosylation with Immobilized Marine Microalga Pavlova sp. Environmental Health Insights 2009:3.

Schwarzenbach R, Gschwend P, Imboden D. 1993. Environmental Organic Chemistry. “Wiley-Interscience Publication”. ISBN 0-471-83941-8.

Stasinakis A, Kordoutis C, Tsiouma V, Gatidou G, Thomaidis N. 2010. Removal of selected endocrine disrupters in activated sludge systems: Effect of sludge retention time on their sorption and biodegradation. Bioresource Technology 101, 2090–2095.

Staples C,. Dom P, Klecka G, O’Blook T, Harris L. A review of the environmental fate, effects, and exposures of Bisphenol A.1998. Chemosphere Vol 36, No. 10, pp. 2149-2173.

Troisi J, Mikelson, Richards S, Symes S, Adair D, Zullo F, Guida M. 2014. Placental concentrations of bisphenol A and birth weight from births in the Southeastern U.S. Placenta 35, 947-952.

Unitated State Environmental Protection Agency. Bisphenol A Action Plan. March 29 2010.

Voordeckers J, Fennell D, Jones K, Häggblom. 2002. Anaerobic Biotransformation of Tetrabromobisphenol A, Tetrachlorobisphenol A, and Bisphenol A in Estuarine Sediments. Environ. Sci. Technol. 36, 696-701.

Yang Y, Wang Z, He T, Dai Y, Xie, S. 2015. Sediment Bacterial Communities Associated with Anaerobic Biodegradation of Bisphenol A. Microb Ecol 70:97–104.

Yang Y, Wang Z, Xie S. 2014. Aerobic biodegradation of bisphenol A in river sediment and associated bacterial community change. Science of the Total Environment 470–471, 1184–1188.

Ying G, Kookana R. 2005. Sorption and degradation of estrogen-like-endocrine disrupting chemical in soil. Environmental Toxicology and Chemistry. 24, 10, PorQuest Natural Science Collection pg 2640.

Zhang W, YinK, Chen L. 2013. Bacteria-mediated bisphenol A degradation. Appl Microbiol Biotechnol 97:5681–5689.

Zhang Z, Velly M, Rhind S, Kyle C, Hough R, Duff E, McKenzie C. A. 2015. Study on temporal trends and estimates of fate of Bisphenol A in agricultural soils after sewage sludge amendment. Science of the Total Environment 515–516; 1–11.