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Anaerobic biodegradability of phthalic acid isomers and related compounds

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Abstract

All three phthalic acid isomers (*ortho*, *meta* and *para* benzene dicarboxylic acid) are produced in massive amounts, and used in the chemical industry as plasticizers or for the production of polyester. Wastestreams generated during the production of phthalate isomers generally contain high concentrations of aromatic acids. To study the potential biodegradability of these primarily anthropogenic compounds in anaerobic bioreactors, biodegradability studies were performed. Compounds tested were benzoate, *ortho*-phthalate, isophthalate, terephthalate, dimethyl phthalate, dimethyl terephthalate, *para*-toluate and *para*-xylene. Seed materials tested were two types of granular sludge and digested sewage sludge. It was found that all phthalate isomers and their corresponding dimethyl-esters, could be completely mineralized by all seed materials studied. Lag phases required for 50% degradation of these compounds, ranged from 17 to 156 days. The observed degradation curves could be explained by growth of an initially small amount of organisms in the inoculum with the specific ability to degrade one phthalate isomer. The observed order in the length of the lag phases for the phthalate isomers is: phthalate < terephthalate < isophthalate. This order appears to be related to the environmental abundancy of the different phthalate isomers. The initial step in the degradation pathway of both dimethyl phthalate esters was hydrolysis of the ester sidechain, resulting in the formation of the corresponding mono-methyl-phthalate isomer and phthalate isomer. The rate limiting step in mineralization of both dimethyl phthalate and dimethyl terephthalate was found to be fermentation of the phthalate isomer. *Para*-toluate was degraded only by digested sewage sludge after a lag phase of 425 days. The observed degradation rates of this compound were very low. No mineralization of *para*-xylene was observed. In general, the differences in the lag phases between different seed materials were relatively small. These results indicate that the time needed for the start-up of anaerobic bioreactors treating wastewaters containing phthalic acid isomers, depends little on the microbial composition of the seed material applied, but may take several months.

Introduction

Besides natural occurrence, human activities have added additional aromatic compounds to nature through manufacture, mining and combustion. In order to assess the fate of these compounds in methanogenic environments, anaerobic degradation of aromatic compounds has been studied extensively in recent years. Anaerobic mineralization of a large range of mono-aromatic compounds has been demonstrated using inocula from various origins. Most mono-aromatic compounds with at least one phenolic or carboxylic functional groups, and even aromatic hydrocarbons like toluene and *ortho*-xylene, were found to be mineralized under methanogenic conditions (Battersby & Wilson 1989; Boyd et al. 1983; Edwards & Grbic-Galic 1994; Healy & Young 1979; Horowitz et al. 1982; Long-de Valliere et al. 1989).

A distinct group of primarily anthropogenic and environmentally ubiquitous aromatic compounds are Phthalic Acid Esters (PAE's). Phthalic acid esters are used as plasticizers in polyvinyl chloride (PVC)

plastics. Interest in the environmental fate of these compounds arises from their ubiquity in the environment and their potential carcinogenic and pseudo-oestrogenic properties (Giam et al. 1984). The most frequently identified phthalic acid esters in the environment are bis(2-ethylhexyl) phthalate and di-n-butyl phthalate. These compounds have been found to be widely distributed in sediments, natural waters, plants and aquatic organisms (Atlas & Giam 1980; Giam et al. 1984). Because the abiotic removal of phthalic acid esters proceeds very slow, with half lives for chemical hydrolysis ranging from 4 months to over 100 years (Wolfe et al. 1980a and 1980b), the principal method for removal is microbial activity. In both aerobic and anaerobic environments several phthalic acid esters can be metabolised. Complete anaerobic mineralization has been demonstrated for phthalate, dimethyl phthalate, diethyl phthalate, dibutyl phthalate and butyl benzyl phthalate. No anaerobic degradation, or only partial conversion was established for dioctyl phthalate and bis(2-ethylhexyl) phthalate. Anaerobic degradation was established using digested sewage sludge (O'Connor et al. 1989; Shelton et al. 1984; Ziogou et al. 1989), anaerobic freshwater or salt marsh sediments (Johnson & Heitkamp 1984; Painter & Jones 1990) and municipal solid waste under landfilling conditions (Ejlertsson et al. 1996).

The initial step in both the aerobic and anaerobic mineralization of phthalic acid esters is hydrolysis of the ester side chains, resulting in formation of monoalkyl phthalate and phthalate (Benckiser & Ottow 1982; Engelhardt & Wallnöfer 1978; Ribbons et al. 1984; Shelton et al. 1984; Taylor & Ribbons 1983). The most common pathway for aerobic degradation of phthalate is through the protocatechuate pathway, followed by ring cleavage and complete mineralization to carbon dioxide and water (Ribbons et al. 1984). Mineralization of phthalate under anaerobic conditions has only been described for denitrifying cultures. These cultures decarboxylate phthalate under formation of benzoate (Aftring et al. 1981; Aftring & Taylor 1981; Nozawa & Maruyama 1988a and 1988b; Taylor & Ribbons 1983). Benzoate is anaerobically reduced to carboxy-cyclohexene level, followed by ring cleavage (Schink et al. 1992). No detailed information concerning the mineralization of phthalate under methanogenic conditions is currently available.

Terephthalic acid and isophthalic acid are used for the production of polyester. With an annual production of 14.4 million ton (1993), terephthalic acid and its dimethyl ester are world-wide among the 50 most produced chemicals (Savostianoff & Didier 1993). The most well known application of these compounds is in polyethylene terephthalate (PET) bottles, which are widely used for carbonated drinks. The environmental occurrence of terephthalate and isophthalate is less abundant than phthalic acid esters (Giam et al. 1984). Due to their limited ecological significance and low toxicity compared to PAE's, the anaerobic biodegradability of terephthalic acid and isophthalic acid have been only scarcely studied.

However, there exist a number of point sources releasing large amounts of terephthalic and isophthalic acid into the environment. The main source consists of the concentrated waste generated during the production of terephthalic acid and isophthalic acid. Also accidental spills may occur around chemical production plants. Specific characteristics of the wastestream generated during terephthalic acid production are the high temperature (40–50 °C) and the high concentration of organic pollutants (5–20 kg-Chemical-Oxygen-Demand(COD)(m^{-3}). Main compounds in this wastestream are, in decreasing order of concentration, terephthalic acid, acetic acid, benzoic acid and *para*-toluic acid (Duffel 1993; Liangming et al. 1991; Macarie et al. 1992; Pereboom et al. 1994). Wastestreams generated during production of dimethyl terephthalate contain, besides the previously mentioned compounds, methanol, formaldehyde, formic acid and dimethyl terephthalate (Leenheer et al. 1976; Liangming et al. 1991; Reule 1990). Wastewater produced during isophthalate production may contain high concentrations isophthalate (Duffel 1993). Besides these main compounds, several side products of the production process can be present in these wastewaters, including *para*-xylene; the raw material of which terephthalic acid and dimethyl terephthalate are produced (Bemis et al. 1982; Roffia et al. 1984).

The traditional treatment method of the waste generated during production of these aromatic acids comprises the activated sludge system, often combined with incineration of the solid waste produced (Lau 1978). Anaerobic pre-treatment combined with aerobic post treatment may represent an attractive alternative. However, so far only limited information is available concerning the anaerobic biodegradability of the aromatic compounds in these effluents. Despite this limited information, several full scale anaerobic bioreactors are currently in operation for pre-treatment of terephthalic and isophthalic acid wastewater (Duffel 1993; Macarie et al. 1992; Pereboom et al. 1994). A

general characteristic of all these anaerobic reactors is a very long start-up time of up to two years.

This study focuses on the ability of different seed-materials to degrade the aromatic compounds in terephthalic acid and isophthalic acid wastewater. Considering the fact that the UASB-process is worldwide the most applied anaerobic treatment process (Lettinga 1995; Macarie 1996), two seed materials originating from full-scale applications of this type of reactor were tested. To date, most anaerobic biodegradability studies have been performed with digested sewage sludge, that we therefore used as a reference seed material. The anaerobic mineralization of phthalic acid and dimethyl phthalate were tested in order to asses whether these environmentally more abundant compounds were degraded more rapidly than their *meta* and *para* isomers.

Material and methods

Source and characteristics of the anaerobic inocula applied. Biomass from three different full scale reactors was tested for their ability to degrade aromatic compounds present in terephthalic acid wastewater. For this purpose, 25 litre sludge samples were taken from the reactors and stored at 4 °C until use. General characteristics of the different types of biomass applied are summarized in Table 1.

Medium and substrate preparation. The basal medium used in the biodegradability studies contained the following (in mg $\cdot$ l^{-1} liquid volume): $NaHCO_3$ (4000), NH_4Cl (280), K_2HPO_4 (250), $MgSO_4.7H_2O$ (100), $CaCl_2.2H_2O$ (10), yeast extract (18) and one millilitre of a trace element stock solution as described by Huser et al. (Huser et al. 1980). Stock solutions of disodium phthalate, isophthalate and terephthalate, and sodium *para*-toluate were prepared in demineralized water. Dissolution and neutralisation of these poorly soluble acids, particularly terephthalic acid, could be enhanced through addition of a slight overdose of NaOH resulting in a pH of 11–12 at an elevated temperature of 80–90 °C. After cooling down, the stock solutions were neutralised using 1 M HCl. Dimethyl phthalate, dimethyl terephthalate and p-xylene were dosed in the concentrated form. Dimethyl terephthalate is a water insoluble solid and was weighed out and added to the serumbottles as solid. Dimethyl phthalate and *para*-xylene are water insoluble liquids and were dosed to the serumbottles using a 100 μl syringe. Para-xylene was dosed after flushing the headspace of the serumbottle to avoid volatilisation. The final concentration of all substrates amounted 500 mg-COD $\cdot$ l^{-1}. Equal COD-concentrations were applied for easy comparison of methane production values.

Preparation of the test bottles. Nutrients, sludge and substrate (except para-xylene) were dosed to the serumbottles. Granular sludge was sieved prior to dosage to the bottles and digested sewage sludge was added directly from a well mixed sample. The initial biomass concentration in all experiments amounted to 5.0 $\pm$ 0.5 gVS $\cdot$ l^{-1}. Serumbottles were sealed with 2 cm thick butylrubber septa (Emergo, Schiedam, The Netherlands) and capped with aluminium screw caps. The headspace was replaced by a mixture of N_2/CO_2 in a ratio of 70 : 30, and 1 ml of a 30 g$Na_2S.7$–$9H_2O \cdot l^{-1}$ was dosed to the medium to ensure anaerobic conditions in the bottle. All experiments were performed in duplicate, except for the blanks (no substrate dosed) for which triplicates were applied. Bottles were incubated stationary in the dark at 37 °C. No sterile controls were included in the experimental procedure, because the compounds tested are known to be highly stable in abiotic environments (Wolfe et al. 1980a and 1980b). The method applied in this study is essentially comparable to the method described by (Shelton & Tiedje 1984).

Experimental. The concentration of methane in the headspace of the bottles was used as a primary indicator for degradation of the substrate. At the beginning of the experiment this measurement was performed on a daily base. In time the frequency of analysis was gradually decreased to approximately once every two weeks after 500 days. When based on the methane measurement, mineralization of the substrate could be suspected, liquid samples were withdrawn and substrate concentrations were determined. Analyses of the substrate concentration in the liquid is only possible for the water soluble substrates (benzoate, phthalate, isophthalate, terephthalate and para-toluate). The bottles incubated with non-soluble substrates were analysed for possibly formed water soluble aromatic intermediates. Once degradation of a substrate manifested, liquid samples were analyzed for volatile fatty acids and the gas phase for hydrogen, in order to assess whether non-aromatic intermediates accumulated. After complete mineralization of the substrate, a second substrate feed was supplied to the bottles.

Table 1. General characteristics of the two types of granular biomass (CAB and BER) and digested sewage sludge (DSR) applied

	Unit	DSR	CAB	EER
Waste treated		Sewage sludge	Starch processing	Papermill
Reactor-type[1]		CSTR	IC	UASB
Conc. volatile solids[2]	$gVS \cdot kg^{-1}$	16.2	114	118
Ash content[2]	%	37	31	19
Methanogenic activity[3]	$gCOD \cdot gVS^{-1} \cdot d^{-1}$	0.20	0.59	0.78

[1] CSTR: Completely Stirred Tank Reactor, IC: Internal Circulation reactor (Yspeert et al. 1993), UASB: Upflow Anaerobic Sludge Bed reactor.
[2] Granular sludge was seeved prior to determination of the concentration volatile solids (VS) and ash content. The concentration volatile solids and the ash content of the digested sewage sludge was determined directly in the mixed liquor.
[3] The specific methanogenic activity of the sludge at 30 °C was measured in serum bottles, using 1 $gCOD \cdot l^{-1}$ sodium acetate as substrate and 1.5 $gVS \cdot l^{-1}$ biomass. The methane concentration in the headspace was used as indicator for acetate conversion in time.

Analysis. The methane content of the headspace was determined by gas chromatograph (Hewlett Packard 438/S) equipped with a stainless steel column (2 m · 2 mm) packed with Poropak Q (80–100 mesh). 100 μl samples were injected using a gas-lock syringe (Dynatech, Baton Rouge, La.). Nitrogen was used as a carrier gas. The temperature of the column, injection port and flame ionization detector were 60, 200 and 220 °C, respectively.

The concentration of water soluble aromatic acids was determined by high pressure liquid chromatography (HPLC). Centrifuged liquid samples (3 minutes at 10,000 *g*) were diluted to concentrations smaller than 50 $mg.l^{-1}$ using a Meyvis Dilutor (type no. 401) and a volume of 10 μl was injected by autosampler (Marathon). Separation of the compounds was obtained using a Chromospher 5C18 column (100 · 3 mm). The solvent used as a carrier was a mixture of methanol and a 1% acetic acid solution in water. A methanol-acetic acid ratio of 40–60 was applied at a flow rate of 0.3 $ml \cdot minute^{-1}$. The separated components were detected by UV-detector (Spectroflow 773) at a wavelength of 230 nm.

The concentration and composition of volatile fatty acids in the medium was determined gas chromatographically (Hewlett Packard 5890A), using a glass column (2 m · 4 mm) packed with Supelcoport (100–200 mesh) and coated with 10% Fluorad FC431. The temperature of the column, injection port and flame ionization detector were 130, 200 and 280 °C respectively. Nitrogen gas saturated with formic acid was used as a carrier gas at a flow rate of 50 $ml \cdot min^{-1}$. Prior to analysis the samples were diluted and fixed with a formic acid solution (3% v/v). After formic acid addition, aromatic acids precipitate and samples needed to be centrifuged (3 min, 10,000 *g*).

Hydrogen was determined by gas chromatograph (Hewlett Packard 5890), equipped with a stainless steel column (1.5 m · 6.4 mm), packed with molecular sieve 25H (60–80 mesh). The temperature of the column, injection port and thermal conductivity detector were 40, 110 and 125 °C respectively. Argon was used as a carrier gas at a flow rate of 25 $ml \cdot min^{-1}$.

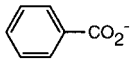
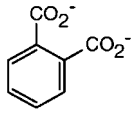
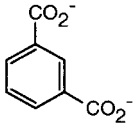
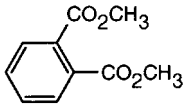
Chemicals. All chemicals applied were of analytical grade (Merck, Darmstadt, Germany) and had a purity of at least 95%.

Results and discussion

Table 2 summarizes the length of the lag periods prior to anaerobic mineralization of the aromatic substrates by the three different sludges. The length of the lag phase was defined as the time needed to convert approximately 50% of the substrate into methane. In the following paragraphs the observed lag phases as well as the anaerobic biodegradability of the various compounds tested will be discussed in detail.

Benzoate. Complete mineralization of benzoate was obtained within two weeks for all seed materials applied. Herewith among all compounds tested, benzoate was most rapidly mineralized. This was not surprising because benzoate plays a key role in methanogenic mineralization of a large range of naturally occurring aromatic compounds (Schink et al. 1992). Anaerobic degradation of benzoate was first demonstrated by

Table 2. Compounds screened for their anaerobic biodegradability by three different types of inocula

Compound name	Structural formula	Initial conc[4] (mM)	Time required to degrade 50% of the aromatic substrate (days)[1]		
			DSR	CAB	EER
benzoate		2.1	10 ± 1	9 ± 1	4 ± 0.5
phthalate		2.1	17 ± 1	49 ± 8	16 ± 2
iso-phthalate		2.1	74 ± 4	156 ± 12	87 ± 4
terephthalate		2.1	55 ± 1	61 ± 7	44 ± 4
dimethyl phthalate		1.5	16 ± 2	38 ± 2	17 ± 1
dimethyl terephthalate		1.5	58 ± 2	39 ± 1	48 6 5
para-toluate		1.7	425 ± 50	>500	>500
para-xylene		1.5	>500	>500	>500

[1] Characteristics of the three types of sludge (DSR, CAB and EFR) are described in Table 1.
[2] Concentrations applied were all equivalent to 500 mg-COD · l^{-1}.

Tarvin and Buswell (Tarvin & Buswell 1934), and in numerous studies since with seed materials from various origin (Battersby & Wilson, 1989; Horowitz et al. 1982).

***Ortho*-phthalate, isophthalate and terephthalate.** The three phthalate isomers were all degraded by digested sewage sludge and both types of granular sludge. Significant differences were observed in lag-phases between the different phthalate isomers and the different seed materials tested. When after complete degradation of the substrate a second dosage of substrate was supplied, substrate conversion started within one week. The evolution in time of the methane concentration in the headspace and the phthalate isomer concentration in the medium are presented in Figure 1 for CAB granular sludge. From this figure can be seen that despite the relatively high methane production in control experiments (800–1500 mgCH_4-COD · l^{-1} after 50 days), the methane production related to substrate conversion could easily be identified. In literature the occurrence of anaerobic mineralization of *ortho*-phthalate has been reported repeatedly (Battersby & Wilson 1989; Horowitz et al. 1982; O'-Connor et al. 1989). Anaerobic terephthalate degradation has been described in technological studies using anaerobic bioreactors (Duffel 1993; Kleerebezem et al. 1997; Pereboom et al. 1994). Isophthalate mineralization under methanogenic conditions has only been reported by Van Duffel (Duffel 1993).

Comparing the length of the lag periods required to obtain 50% mineralization of the phthalate isomers, the following order was found for all sludge types: *ortho*-phthalate < terephthalate < isophthalate. As microbial degradation is the main removal mechanism for phthalate isomers released into the environment, the environmental abbundancy of organisms with the specific ability to degrade one of the phthalate iso-

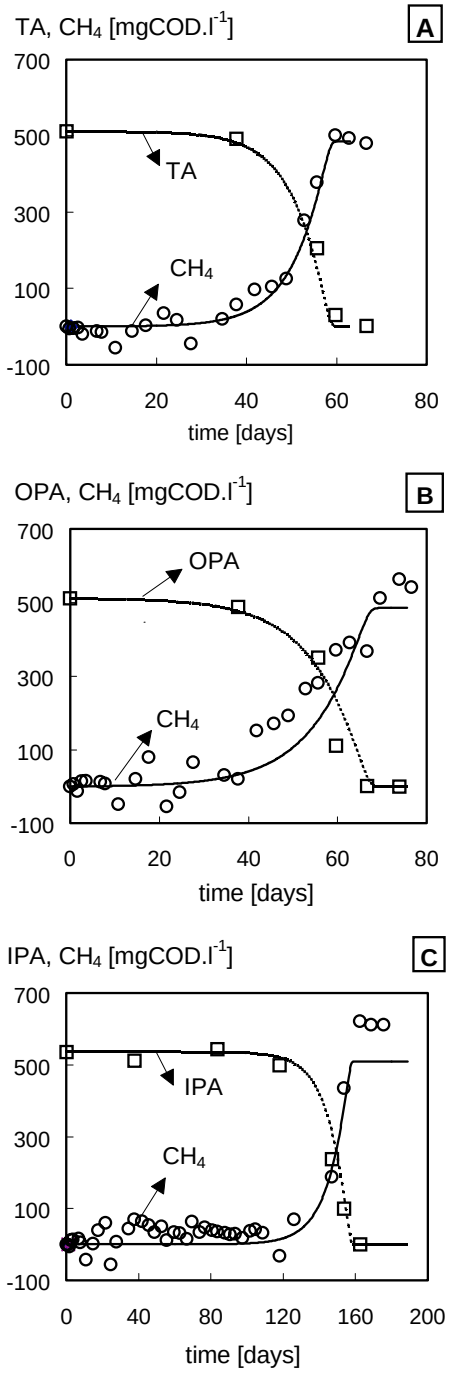


Figure 1. Anaerobic biodegradation of terephthalate (A, TA), *ortho*-phthalate (B, OPA) and isophthalate (C, IPA) by CAB granular sludge. Methane concentrations (CH_4) represent net methane production. Markers indicate measured concentrations and lines were calculated as described in the text.

mers may therefore be directly related to the amounts released into the environment. Although all phthalate isomers are primarily anthropogenic compounds, catabolic pathways have been described that converge at *ortho*-phthalate. This compound for instance was found to be an intermediate in the degradation of polycyclic aromatic hydrocarbons (Kiyohara & Nagao 1978). Besides its natural occurrence, large amounts of *ortho*-phthalate are introduced into the environment anthropogenically in the form of phthalate esters (Giam et al. 1984). Considering that the initial step in abiotic and microbiological conversion of phthalate esters is hydrolysis of the ester side chain resulting in the formation of phthalate, it is a plausible assumption that micro-organisms adapted to *ortho*-phthalate are widely distributed in the environment.

Compared to iso-phthalate, terephthalate is produced in much bigger amounts. Despite the slow hydrolysis of the products in which both compounds are applied, very likely larger amounts of terephthalate than iso-phthalate are introduced into the environment. This is confirmed by measurement of trace concentrations of terephthalate in surface water, municipal wastewater and air (Behret, 1991; Matsumoto 1982), while no reports on environmental occurrence of iso-phthalate occurrence were found. Based on these considerations it is suggested that the differences observed in the length of the lag periods prior to degradation of the individual phthalate isomers is determined primarily by the corresponding environmental turn over.

The drawn lines in Figure 1 were calculated assuming exponential growth of an initially small amount of organisms in the inoculum. Since no significant amounts of intermediate compounds were found (benzoate or volatile fatty acids), and the methane produced corresponds reasonably well to the amounts of substrate degraded, apparently the initial fermentation of the phthalate isomers to methanogenic substrates is the rate limiting step. This means that the growth rate of the mixed culture degrading the phthalate isomers can be described by single species kinetics (Powell 1984). Estimated apparent maximum specific growth rates (μ_{max}) ranging from 0.10 to 0.20 day^{-1} resulted in reasonable descriptions of the measured data, as shown in Figure 1. These values for the specific growth rate are in the same order of magnitude as those found for other methanogenic syntrophic consortia degrading propionate or butyrate (Pavlostathis & Giraldo-Gomez 1991). Accurate estimation of specific growth rates based on the data presented here

is not possible, but it may be speculated that growth of a small amount of specific organisms originally present in the inoculum (with the ability to degrade one specific phthalate isomer) is the main mechanism responsible for the observed increase in the substrate conversion rate. However, time dependent adaptation of specific organisms in the seed material through induction of specific enzyme systems can not be excluded.

The large differences observed in the length of the lag phases with the different phthalate isomers, indicate that all three phthalate isomers are fermented by different species of organisms. From aerobic environments several strains of bacteria have been isolated with the ability to degrade at least two phthalate isomers (Ribbons et al. 1984). Recently, a soil bacterium has been isolated (*Pseudomonas* sp. strain P136) capable to utilise all three phthalate isomers through nitrate respiration (Nozawa & Maruyama 1988a and 1988b). Higher substrate specifities of organisms in methanogenic environments, as compared to organisms in aerobic or anoxic environments, are common (Schink et al. 1992).

Dimethtyl phthalate and dimethyl terephthalate. Both dimethyl phthalate and dimethyl terephthalate were completely mineralized by the three seed materials tested. Adaptation periods required for their complete mineralization were comparable to those found for their phthalate analogues.

Liquid samples were analysed by HPLC to assess whether water soluble aromatic compounds accumulated during degradation of dimethyl phthalate and dimethyl terephthalate. Both dimethyl esters could not be detected in the medium, due to their poor solubility and tendency to become adsorbed to the biomass. The results show an accumulation of the mono-methyl ester, as well as the corresponding phthalic acid isomer in all experiments (Figure 2). After complete conversion into the phthalate isomers, conversion into methane occurred. Based on these observations the degradation pathway for dimethyl terephthalate is proposed to be as presented in Figure 3. An equivalent pathway is proposed for degradation of dimethyl phthalate. The degradation pathway shown in Figure 3 is comparable to that proposed for the anoxic mineralization of dibutyl phthalate by *Pseudomonas pseudoalcaligenes* (Benckiser & Ottow 1982) and methanogenic mineralization of butylbenzyl phthalate by digested sewage sludge (Shelton et al. 1984).

Drawn lines in Figure 2 were calculated using a simple metabolic model involving two groups of microorganisms: (i) organisms responsible for the two-step demethylation of dimethyl-terephthalate, resulting in formation of methylterephthalate and terephthalate respectively, and (ii) a mixed culture of organisms converting terephthalate into a mixture of methane and carbondioxide. The methyl-groups removed were assumed to be converted to methane. From Figure 2 it can be seen that a reasonable description of the experimental data could be obtained using this approach, confirming the degradation sequence shown in Figure 3.

No significant difference in the rate of demethylation of dimethyl phthalate and dimethyl terephthalate was observed, suggesting that demethylation proceeds independently of the location of the methyl-ester group on the aromatic ring. Demethylation by acetogenic bacteria has been observed previously for aromatic compounds containing a methoxy group. These bacteria use the methyl group as a methyl donor in the synthesis of acetyl-CoA (Fuchs et al. 1994). In mixed methanogenic cultures, acetate formed by these bacteria will be converted rapidly into methane and carbon dioxide by acetoclastic methanogens.

***Para*-toluate and *para*-xylene.** *Para*-toluate was degraded only by one of the seed materials tested (digested sewage sludge) after an extremely long lag phase of approximately 425 days (Table 2). The degradation of *Para*-toluate has been observed previously by Horowitz (Horowitz et al. 1982), using fresh water sediments as inoculum. No degradation was observed by this author when two different types of digested sewage sludge were used. Macarie et al. (Macarie & Guyot 1992; Macarie & Guyot 1995) observed a slow removal of *para*-toluate in a UASB reactor seeded with digested sewage sludge.

The mineralization of *para*-toluate was found to proceed extremely slowly, even after a second feeding was supplied (Figure 4). The growth rate of the *para*-toluate fermenting culture apparently was extremely low. From Figure 4 it can be seen that a reasonable description of the data of the first substrate feeding can be obtained using an overall growth rate of 0.012 day^{-1}. However, as the conversion rate of *para*-toluate did not increase during the second substrate feeding, the growth rate may be even lower than this value.

No *Para*-xylene degradation was obeserved in any of the experiments, within the 500 days the experiment lasted. The methane production was even 10–25%

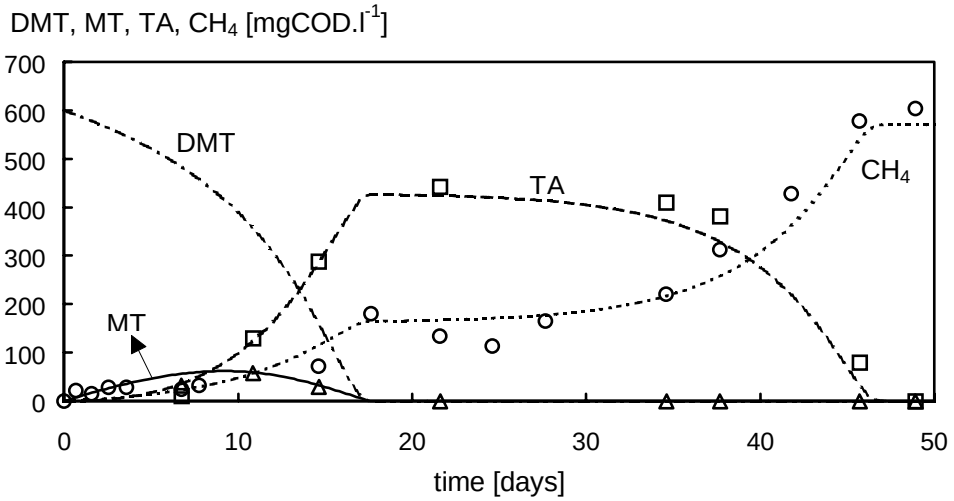


Figure 2. Anaerobic biodegradation of dimethyl terephthalate (DMT) by CAB granular sludge. Methyl terephthalate (MT, △) and terephthalate (TA, □) are intermediate compounds; methane (CH_4, ○) is the endproduct. Markers indicate measured concentrations and lines were calculated with the mathematical model outlined in the text. DMT was not measured. Methane concentrations represent net methane production.

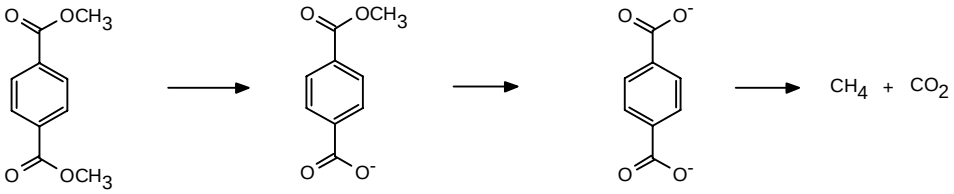


Figure 3. Proposed pathway for anaerobic biodegradation of dimethylterephthalate.

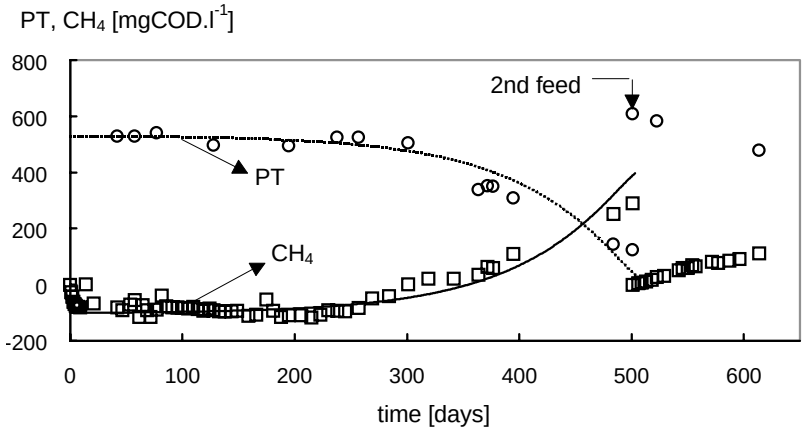


Figure 4. Anaerobic biodegradation of *para*-toluate (PT) by digested sewage sludge (DSR). Markers indicate measured concentrations and lines were calculated based on the stoichiometry of the conversion and simple Monod kinetics. Methane concentrations represent net methane production.

lower compared to reactors where no substrate was dosed (data not shown). This observation suggests that *para*-xylene is toxic to organisms involved in the digestion of the seed material. Blum and Speece (Blum & Speece 1991) found an IC50 value for acetoclastic methanogens of 790 mg-COD $\cdot$ l^{-1}, which is slightly higher than the concentration applied in our experiment (500 mg-COD $\cdot$ l^{-1}). The toxicity of *para*-xylene very likely is the result of its high hydrophobicity (logP is 3.1), which may lead to disturbance of essential membrane functions (Sierra & Lettinga 1991; Sikkema et al. 1994). In literature no reports were found dealing with the mineralization of para-xylene in methanogenic environments. Anoxic mineralization of *para*-xylene has been reported by Häner (Häner et al. 1995).

Technological implications. The relatively small differences between the different seed materials concerning the lag-phases prior to degradation of the phthalate isomers, suggest that they contain a comparable amount of bacteria with the specific ability to degrade phthalate isomers. Therefore it may be presumed that the source of the inoculum has only a limited impact on the time needed for start-up of anaerobic bioreactors for treatment of phthalic acids containing wastewaters. However, as the anaerobic degradation of phthalate isomers is dependent on syntrophic consortia consisting of fermentative bacteria and methanogens, granular biomass with a high specific methanogenic activity will be capable to degrade the fermentation products at high rates, herewith creating optimal micro-environments for growth of the fermentative bacteria. Combined with the excellent settling properties of granular biomass, this suggests that granular sludge is the preferred inoculum over digested sewage sludge for start-up of UASB reactors treating phthalic acids containing wastewaters.

Both granular sludges used in the experiments described here, were grown on “rich substrates”: paper mill wastewater and potato starch processing wastewater. These substrates may contain a variety of aromatic compounds, ranging from lignin derivatives in paper-mill wastewater to aromatic amino acids in potato starch processing wastewater. Methanogenic granular sludge grown on substrates not containing any aromatic compounds, may not be able to degrade the substrates tested here, or only after significantly longer lag periods.

With respect to the application of anaerobic treatment for terephthalic acid wastewater, the long lag periods prior to terephthalate degradation, combined with the observed inhibition of terephthalate degradation by the wastewater constituents acetate and benzoate (Kleerebezem et al. 1997), suggests that the start-up of anaerobic reactors for treatment of terephthalic acid wastewater may take a long time. Start-up times of more than 1 year are indeed more the rule than the exception (Duffel 1993; Kleerebezem et al. 1997; Pereboom et al. 1994). Despite such a long start-up period, anaerobic pre-treatment of wastewaters generated during terephthalic acid production may represent an attractive contribution to conventionally applied aerobic treatment methods, provided that sufficiently high and stable volumetric removal capacities can be achieved after successful start-up. In this respect it should be noted that in anaerobic bioreactors, acetate and benzoate can normally removed to a large extent within a short period of time. Taken into account that these two compounds are important wastewater constituents, treatment efficiencies of around 50% can normally be obtained within weeks of operation. It should furthermore be emphasized that the length of the lag-period prior to terephthalate degradation in anaerobic bioreactors may significantly be reduced by pre-removal of acetate and benzoate in a staged bioreactor concept. Whether the lag period prior to terephthalate degradation through pre-removal of acetate and benzoate is indeed decreased from at least one year in full-scale reactors to approximately 2 months, as observed during this study, will be studied in the near future in our lab.

Mineralization of *para*-toluate can normally not be expected in anaerobic bioreactors for pre-treatment of terephthalic acid or dimethyl terephthalate wastewater. Due to the low growth rate of the specific *para*-toluate degrading biomass, a very long solid retention time needs to be maintained to enable degradation. In general, this means that *para*-toluate needs to be removed during aerobic post-treatment of the anaerobic effluent.

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References

Aftring RP, Chalker BE & Taylor BF (1981) Degradation of phthalic acids by denitrifying, mixed cultures of bacteria. Appl. Environ. Microbiol. 41(5): 1177–1183

Aftring RP & Taylor BF (1981) Aerobic and anaerobic catabolism of phthalic acid by a nitrate respiring bacteria. Arch. Microbiol. 130: 101–104

Atlas E & Giam CS (1980) Global transport of organic pollutants: ambient concentrations in the remote marine atmosphere. Science 211: 163–165

Battersby NS & Wilson V (1989) Survey of the anaerobic biodegradation potential of organic chemicals in digesting sludge. Appl. Environ. Microbiol. 55(2): 433–39

Behret H (1991) Terephthalic acid. In Bayer E (Ed) BUA Reports, Vol 68–70. S. Hirzel, Wissenschaftliche Verlagsgesellschaft. Stuttgart, Germany

Bemis, AG, Dindorf JA, B, H & CS (1982) Phthalic acids and other benzenepolycarboxylic acids. In: Mark HF, Othmer DF, Overberg CG, Seaborg GT, Grayson M & Eckroth D (Eds), Kirk Othmer Encyclopedia of Chemical Technology, Vol 17 (pp 732–777). John Wiley and Sons, New York

Benckiser G & Ottow JCG (1982) Metabolism of the plasticizer di-n-butylphthalate by *Pseudomonas pseudoalcaligenes* under anaerobic conditions with nitrate as the only electron acceptor. Appl. Environ. Microbiol. 44(3): 576–578

Blum DJW & Speece RE (1991) A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Res. J. WPCF 63: 198–207

Boyd SA, Shelton DR, Berry D & Tiedje JM (1983) Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46(1): 50–54

Duffel J v (1993). Anaerobe behandeling van organische zuren. National Conference on Anaerobic treatment of complex wastewaters, Breda, The Netherlands

Edwards EA & Grbic-Galic D (1994) Anaerobic degradation of toluene and o-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60(1): 313–322

Ejlertsson J, Meyerson U & Svensson BH (1996) Anaerobic degradation of phthalic acid esters during digestion of municipal solid waste under landfilling conditions. Biodegradation 7: 345–352

Engelhardt G & Wallnöfer PR (1978) Metabolism of di- and mono-n-butyl phthalate by soil bacteria. Appl. Environ. Microbiol. 35(2): 243–246

Fuchs G, Mohamed MES, Altenschmidt U, Koch J, Lack A, Brackmann R, Lochmeyer C & Oswald B (1994) Biochemistry of anaerobic biodegradation of aromatic compounds. In: Ratledge C (Ed) Biochemistry of Microbial Degradation, (pp 513–553). Kluwer Academic Publishers, The Netherlands.

Giam CS, Atlas E, Powers Jr, MA & Leonard JE (1984). Phthalic acid esters. In: Hutzinger O (Ed) Anthropogenic Compounds, Vol 3, Part C (pp 67–140). Springer-Verlag, Berlin–Heidelberg–New York–Tokyo

Häner A, Höhener P & Zeyer J (1995) Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61(8): 3185–3188

Healy JB & Young Y (1979) Anaerobic degradation of eleven aromatic compounds to methane. Appl. Environ. Microbiol. 38(1): 84–89

Horowitz A, Shelton DR, Cornell CP & Tiedje JM (1982) Anaerobic degradation of aromatic compounds in sediments and digested sludge. Dev. Ind. Microbiol. 23: 435–444

Huser BA, Wuhrmann K & Zehnder ABJ (1980) Methanothrix soehngenii gen. nov. sp. nov., a new acetotrophic non hydrogen oxidizing methane bacterium. Arch. Microbiol. 132: 1–9

Johnson BT & Heitkamp MA (1984) Environmental and chemical factors influencing the biodegradation of phthalic acid esters in freshwater sediments. Environ. Pollution 8: 101–118

Kiyohara H & Nagao K (1978) The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol. 105: 69–75

Kleerebezem R, Mortier J, Hulshoff Pol LW & Lettinga G (1997) Anaerobic pre-treatment of petrochemical effluents: terephthalic acid wastewater. Wat. Sci. Tech. 36(2–3): 237–248

Lau CM (1978) Staging aeration for high-efficiency treatment of aromatic acids plant wastewater. 32nd Ind. Waste. Conf. Purdue University. 63–74

Leenheer JA, Malcolm RL & White WR (1976) Investigation of the reactivity and fate of certain organic components of an industrial waste after deep-well injection. Environ. Sci. & Technol. 10(5): 445–451

Lettinga G (1995) Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek 67: 3–28

Liangming X, Yuxiang C & Xiangdong Z (1991) The anaerobic biological treatment of high strength petrochemical wastewater by a hybrid reactor. International conference on Petroleum Refining and Petrochemical Processing, September 11–15, Beijing, China. Int. Acad. Pub. 120–126

Long-de Valliere CL, Petrozzi S, Zurrer D, Baier U & Dunn IJ (1989) Methods of anaerobic degradation of toxic compounds in chemical and industrial wastewaters. In: Mizrahi A (Ed) Biological Waste Treatment, Vol 12 (pp 35–72). Alan R. Liss, Inc. New York

Macarie H (1996). Anaerobic digestion an adequate technology for the treatment of some chemical and petrochemical wastewaters. IV Taller Latinoamericano Sobre Tratiamento Anaerobio de Aguas Residuales. Bucaramanga, Colombia. 313–323

Macarie H & Guyot JP (1992) Inhibition of the methanogenic fermentation of p-toluic acid (4-methylbenzoic acid) by acetate. Appl. Microbiol. Biotechnol. 38: 398–402

Macarie H & Guyot JP (1995) Use of ferrous sulphate to reduce the redox potential and allow the start-up of UASB-reactors treating slowly biodegradable compounds: application to a wastewater containing 4-methylbenzoic acid. Environ. Tech. 16: 1185

Macarie H, Noyola A & Guyot JP (1992) Anaerobic treatment of a petrochemical wastewater from a terephtalic acid plant. Wat. Sci. Tech. 25: 223–235

Matsumoto G (1982) Comparative study on organic constituents in polluted and unpolluted inland aquatic environments; phenols and aromatic acids in polluted and unpolluted waters (III). Wat. Res. 16: 551–557

Nozawa T & Maruyama Y (1988a) Anaerobic metabolism of phthalate and other aromatic compounds by a denitrying bacterium. J. Bacteriol. 170(12): 5778–5784

Nozawa T & Maruyama Y (1988b) Denitrification by a soil bacterium with phthalate and aromatic compounds as substrates. J. Bacteriol. 170(6): 2501–2505

O'Connor OA, Rivera MD & Young LY (1989) Toxicity and biodegradation of phthalic acid esters under methanogenic conditions. Environ. Tox. Chem. 8: 569–576

Painter SE & Jones WJ (1990) Anaerobic bioconversion of phthalic acid esters by natural inocula. Environ. Technol. 11: 1015–1026

Pavlostathis SG & Giraldo-Gomez E (1991) Kinetics of anaerobic treatment: a critical review. Critical Rev. Environ. Control 21(5–6): 411–490

Pereboom JHF, Man d G & Su IT (1994) Start-up of full scale UASB-reactor for the treatment of terephtalic acid waste wa-

ter. 7th Int. Symp. on Anaerobic Digestion, Cape Town, South Africa. 307–312

Powell GE (1984) Equalisation of specific growth rates for syntrophic associations in batch cultures. J. Chem. Tech. Biotechnol. 34B: 97–100

Reule W (1990) Methan aus Chemieabwasser. Chemische Industry, 9

Ribbons DW, Keyser P, Kunz DA, Taylor BF, Eaton RW & Anderson BN (1984) Microbial degradation of phtalates. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds, pp 371–397

Roffia P, Calini P & Motta L (1984) Byproduct Identification in the Terephthalic Acid Production Process and possible mechanisms of their formation. Ind. Eng. Chem. Prod. Res. Dev. 23: 629–634

Savostianoff D & Didier SN (1993) DMT-PTA, Asia advances towards a crushing domination. Informations Chimie, 352: 119–129

Schink B, Brune A & Schnell S (1992) Anaerobic degradation of aromatic compounds. In: Winkelmann VCH (Ed) Microbiol Degradation of Natural Products, (pp 219–242). Weinheim–New York–Basel–Cambridge

Shelton DR, Boyd SA & Tiedje JM (1984) Anaerobic biodegradation of phthalic acid esters in sludge. Environ. Sci. Technol. 18(2): 93–97

Shelton DR & Tiedje JM (1984) General Method for determining anaerobic biodegradation potential. Appl. Environ. Microbiol. 47(4): 850–857

Sierra R & Lettinga G (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl. Microbiol. Biotechnol. 34: 544–550

Sikkema J, Bont de JAM & Poolman B (1994) Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. 269: 8022–8028

Tarvin D & Buswell AM (1934) The methane fermentation of organic acids and carbohydrates. J. Amer. Chem. Soc. 56: 1751–1755

Taylor BF & Ribbons DW (1983) Bacterial decarboxylation of o-phthalic acids. Appl. Environ. Microbiol. 46: 1276–1281

Wolfe NL, Burns LA & Steen WC (1980a) Use of linear free energy relationships and an evaluative model to assess the fate and transport of phthalate esters in the aquatic environment. Chemosphere 9: 393–402

Wolfe NL, Steen WC & Burns LA (1980b) Phthalate ester hydrolysis: linear free energy relationships. Chemosphere 9: 403–408

Yoon H, Klinzing G & Blnch HW (1977) Competition for mixed substrates by microbial populations. Biotechnol. Bioeng. 19: 1193–1210

Yspeert P, Vereijken TLFM, Vellenga S & Vegt d A (1993) The IC-reactor for the anaerobic treatment of industrial wastewater. Food Industry Environmental Conference, Atlanta, USA. 14–16

Ziogou K, Kirk PWW & Lester JN (1989) Behavior of phthalic acid esters during batch anaerobic digestion of sludge. Wat. Res. 23(6): 743–748