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Anaerobic biodegradability of alkylphenols and fuel oxygenates in the presence of alternative electron acceptors

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Abstract Alkylphenols and fuel oxygenates are important environmental pollutants produced by the petrochemical industry. A batch biodegradability test was conducted with selected *ortho*-substituted alkylphenols (2-cresol, 2,6-dimethylphenol and 2-ethylphenol), fuel oxygenates (methyl *tert*-butyl ether, ethyl *tert*-butyl ether and *tert*-amylmethyl ether) and *tert*-butyl alcohol (TBA) as model compounds. The *ortho*-substituted alkylphenols were not biodegraded after 100 days of incubation under methanogenic, sulfate-, or nitrate-reducing conditions. However, biodegradation of 2-cresol and 2-ethylphenol (150 mg l^{-1}) was observed in the presence of Mn (IV) as electron acceptor. The biodegradation of these two compounds took place in less than 15 days and more than 90% removal was observed for both compounds. Mineralization was indicated since no UV-absorbing metabolites accumulated after 23 days of incubation. These alkylphenols were also slowly chemically oxidized by Mn (IV). No biodegradation of fuel oxygenates or TBA (1 g l^{-1}) was observed after 80 or more days of incubation under methanogenic, Fe (III)-, or Mn (IV)-reducing conditions, suggesting that these compounds are recalcitrant under anaerobic conditions. The fuel oxygenates caused no toxicity towards acetoclastic methanogens activity in anaerobic granular sludge.

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Introduction

Fuel oxygenates and alkylphenols are important environmental pollutants generated by the petrochemical industry. Phenolic compounds are important constituents of petrochemical effluents arising from processes like spent caustic and coal gasification. Phenolic compounds may amount to 2–10 g l^{-1} in spent caustic liquors. Phenol and alkylphenols like cresols, xylenols and ethylphenols are the main constituents. There is ample evidence for the biodegradation of alkylphenols in the absence of oxygen (Heider and Fuchs 1997). However, *ortho*-substituted alkylphenols are less readily biodegraded.

As a result of the 1990 Clean Air Act Amendment in the United States, fuel oxygenates have been used in gasoline at levels up to 15% by volume. Methyl *tert*-butyl ether (MTBE) is the most popular additive. This compound, together with other ethers such as *tert*-amyl methyl ether (TAME) and ethyl *tert*-butyl ether (ETBE), is part of the oxygenated gasoline used today in cities with high atmospheric pollution. Due to their high water solubility and abundance, fuel oxygenates migrate into the groundwater when aquifers are contaminated either by direct spills of gasoline or by tank leakage. MTBE has been detected in shallow groundwater at concentrations as high as 2,000 μg l^{-1}. In the United States, MTBE is the second most important aquifer pollutant (Steffan et al. 1997). A study performed in different cities in the United States has shown that 66% of rain water samples contain MTBE (0.2–8.7 μg l^{-1}), indicating that urban stormwater is a source of MTBE contamination in the shallow groundwater (Delzer et al. 1996).

In polluted environments where oxygen is not present, microorganisms can utilize compounds like nitrate, sulfate, carbon dioxide, manganese, iron and humic acids as electron acceptors (Lovley and Phillips 1988; Holliger and Zehnder 1996; Langenhoff 1997a). Information concerning the anaerobic degradation of *ortho*-substituted alkylphenols and fuel oxygenates with

different electron acceptors is scarce. Many studies have demonstrated that these compounds are largely resistant to anaerobic biodegradation (Blum et al. 1986; Suflita and Mormille 1993; Yeh and Novak 1994; Razo-Flores et al. 1996). Therefore, in order to gain more insight on the biodegradability potential of these kinds of pollutants, 2-cresol, 2,6-dimethylphenol, 2-ethylphenol, MTBE, ETBE, TAME and *tert*-butyl alcohol (TBA) were chosen as model compounds for study under several microbial redox conditions.

Materials and methods

Inoculum

Two kinds of inoculum were used during the experiments. The first was an anaerobic granular sludge (GS) treating distillery wastewater obtained from Nedalco (The Netherlands). It contained 8.56% volatile suspended solids (VSS) and had an acetoclastic methanogenic activity of 0.95 g chemical oxygen demand (COD)-CH_4 g^{-1} VSS day^{-1}. The second inoculum was anaerobic Rhine sediment (ARS; Wageningen, The Netherlands) with a VSS content of 4.47%.

Basal medium

The basal medium used during the biodegradation assay contained the following compounds (mg l^{-1}): $NaHCO_3$ (5,000), NH_4Cl (280), $CaCl_2 \cdot 2\ H_2O$ (10), K_2HPO_4 (250), $MgSO_4$ (100), yeast extract (100) and 1 ml of micronutrients stock solution containing (mg l^{-1}): $FeCl_2 \cdot 4\ H_2O$ (2,000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl_2 \cdot 2\ H_2O$ (38), $MnCl_2 \cdot 4\ H_2O$ (500), $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ (50), $AlCl_3 \cdot 6\ H_2O$ (90), $CoCl_2 \cdot 6\ H_2O$ (2,000), $NiCl_2 \cdot 6\ H_2O$ (142), $Na_2SeO \cdot 5\ H_2O$ (164), EDTA (1,000), resazurin (200) and 36% HCl (1 ml).

Batch biodegradability assays

The batch anaerobic biodegradability assay was conducted in 120-ml glass serum bottles. Either 2 g VSS l^{-1} GS or a 10% volume of ARS was transferred to serum bottles containing basal medium: 25 ml in the case of fuel oxygenates and 50 ml for the *ortho*-substituted alkylphenols. The serum bottles were sealed with 12-mm thick butyl rubber stoppers and flushed with a gas mixture of 30% CO_2 and 70% N_2 for 5 min. The serum bottles were incubated overnight at 30 °C to allow the biological consumption of residual oxygen. One day later, the desired amount of test compound was added to triplicate serum bottles, using a concentrated stock solution. All compounds were used as the only carbon and energy source with the following concentrations: *ortho*-substituted alkylphenols (2-cresol, 2,6-dimethylphenol and 2-ethylphenol), 150 mg l^{-1}; fuel oxygenates (MTBE, ETBE, TAME and TBA), 1 g l^{-1}. The incubation periods were up to 116 days for the fuel oxygenates and TBA, and up to 100 days for the alkylphenols. The studies with *ortho*-substituted alkylphenols were conducted under methanogenic, sulfate-reduction, denitrification, Fe (III)- and Mn (IV)-reducing conditions. The studies with fuel oxygenates were conducted under methanogenic, Fe (III)- and Mn (IV)-reducing conditions. Additionally, the effect of the model humic substance, anthraquinone 2,6-disulfonate (AQDS), was tested in certain experiments. Nitrate and sulfate were used at a concentration of 2 g l^{-1}. Amorphous hydroxide forms of Fe (III) and Mn (IV) were used at 80 g l^{-1} and 68 g l^{-1}, respectively, for the fuel oxygenates; and 4.44 g Mn (IV) l^{-1} and 8.88 g Fe (III) l^{-1} were used for the *ortho*-substituted alkylphenols. In the case of AQDS as electron acceptor and mediator, the concentration was 206 mg anthraquinone 2,6-disulfonate (disodium salt) l^{-1}. The serum bottles were incubated at 30 °C for a period of more than 100 days. Duplicate samples of GS without any treatment were autoclaved (40 min at 100 kPa) and used as a control for abiotic removal mechanisms. Background levels of methane production, sulfate reduction and nitrate reduction were monitored in controls lacking any added test compound. The concentration of the compounds was monitored during the duration of the experiment.

Anaerobic toxicity assay

The anaerobic toxicity assay was performed in 120-ml glass serum bottles. Samples of 2 g VSS GS l^{-1} were transferred to serum bottles containing 25 ml basal medium and acetate from a neutralized stock solution to yield a final concentration of 2.5 g COD l^{-1}. The serum bottles were sealed with 12-mm-thick butyl rubber stoppers and flushed with a gas mixture of 30% CO_2 and 70% N_2 for 5 min. The serum bottles were incubated overnight at 30 °C to allow the biological consumption of residual oxygen. One day later, 1 g l^{-1} of MTBE, ETBE, TAME, or TBA was added to triplicate serum bottles, using a concentrated stock solution. Triplicate controls were prepared where no toxicant was added. Incubations were done at 30 °C. After 3 days of exposure to the toxicant, the acetate concentration was re-established to 1 g COD l^{-1} to assess the methanogenic acetoclastic activity. The headspace was re-flushed with a gas mixture of 30% CO_2 and 70% N_2 and the test bottles were re-incubated in order to determine the methane production rate. The methane was measured every hour during a 6–8 h incubation period.

The maximum acetoclastic methanogenic activity was calculated from the slope of the methane production versus time curve. To determine the degree of inhibition, the methanogenic activities of the control and samples containing inhibitory compounds were determined and compared.

Preparation of amorphous Fe (III) and Mn (IV)

Amorphous Fe (III) oxide was prepared by neutralizing a solution of 0.4 M $FeCl_3$ with NaOH (Lovley and Lonergan 1990; Langenhoff et al. 1996). Amorphous Mn (IV) oxide was prepared by mixing equal amounts of 0.4 M $KMnO_4$ and 0.4 M $MnCl_2$ and adjusting the pH to 10 by adding NaOH (Lovley and Phillips 1988; Langenhoff et al. 1996). Both metals were washed four times with demineralized water.

Analytical methods

The depletion of MTBE, ETBE and TAME was determined by gas chromatography (GC). Each compound was measured in the headspace using a pressure-lock gas syringe (100 µl). The GC was an Interscience GC8000 with a flame ionization detector (FID), equipped with a CP-select column 624 (30 m × 0.53 mm × 3 µm) and a split-flow system (103 ml min^{-1}). The temperatures of the column, injector and the FID were 40, 200 and 240 °C, respectively. The carrier gas was helium at a flow rate of 50 ml min^{-1}. The retention times for the MTBE, ETBE and TAME were 2.5, 3.02 and 4.98 min, respectively. Standards of each compound were prepared with 25 ml basal medium equilibrated in 120-ml glass serum bottles under the same anaerobic conditions used in the experiments.

Quantification of TBA and *ortho*-substituted alkylphenols was carried out in the aqueous phase after centrifuging the aliquot with a Hewlett-Packard 5890, GC-FID system. A glass column (2 m × 6 mm × 2 mm) was used with 10% Fluorad 431 on Supelco-port (100–200 mesh). The temperature of the oven was 70 °C for TBA and 130 °C for *ortho*-substituted alkylphenols. The temperature of the injector and detector were 200 °C and 280 °C, respectively. The carrier gas was nitrogen saturated with formic acid at a flow rate of 40 ml min^{-1}. Samples of 1 µl were analyzed. The retention time for TBA was 1.01 min and the retention times for 2-cresol, 2,6-dimethylphenol and 2-ethylphenol were 9.85, 7.35 and

14.32 min, respectively. The methane production was determined by GC in the headspace according to the method described by Razo-Flores et al. (1996).

The nitrate and sulfate ions were determined in the aqueous phase, after centrifuging the aliquot, by high-performance liquid chromatography with a Vydac-anion exchange 5-μm column (250 mm × 4.6 mm) and a Waters 431 conductivity detector. The mobile phase was 0.009 M potassium hydrogen phthalate at a flow rate of 1.2 ml min^{-1}. The size of the sample was 20 μl. The retention time was 7.50 min for nitrate and 11.75 min for sulfate. All other analytical determinations were performed according to "Standard methods for examination of water and wastewater" (APHA 1985).

Chemicals

All compounds were purchased from either Jansen Chimica (Tilburg, The Netherlands), Merck (Darmstadt, Germany), or Sigma (Bornem, Belgium). All the chemicals were of the highest purity commercially available.

Results

Biodegradability assay for the *ortho*-substituted alkylphenolic compounds

No biodegradation of any of the *ortho*-substituted alkylphenols was observed under methanogenic, sulfate-reducing, or denitrifying conditions after up to 100 days of incubation. There was no decrease in the concentration of the test compounds. Also, no net methane production and no loss in sulfate or nitrate ions was detected. Biodegradation of 2-cresol and 2-ethylphenol occurred in the presence of Mn (IV) as electron acceptor, both in the presence and absence of the redox mediator, AQDS. However, in comparable experiments with either Fe (III) or AQDS as sole electron acceptor, no degradation was observed in ARS during a 20-day incubation period.

The degradation of 2-cresol and 2-ethylphenol took place within 15 days (Fig. 1A, B). The 2-cresol was 96% consumed in less than 3 days at a rate of 9.81 ± 1.57 mg l^{-1} day^{-1}. The biodegradation of 2-ethylphenol presented the same pattern with 92% of the compound being degraded at a rate of 8.33 ± 0.39 mg l^{-1} day^{-1}. A fraction of the *ortho*-substituted compounds reacted chemically with Mn (IV) in the abiotic controls, which did not occur in the abiotic controls lacking the metal oxide. The 2,6-dimethylphenol was rapidly depleted in both treatment bottles and abiotic sterile control bottles, indicating that this compound was largely converted by chemical oxidation with Mn (IV). AQDS was utilized with the objective of facilitating the transfer of electrons from the bacteria to Mn (IV). However contrary to expectations, the combination of AQDS and Mn (IV) caused a decrease in the degradation rate to 6.67 mg l^{-1} day^{-1} and 7.29 ± 1.32 mg l day^{-1} for 2-cresol and 2-ethylphenol, respectively.

A second feed of Mn (IV) and the *ortho*-substituted alkylphenols was conducted at the original concentration in both treatment and sterile control bottles. The initial biodegradation rate of 2-cresol and 2-ethylphenol in the second feed was comparable to that of the first feed (Fig. 1A, B). However, the sterilized controls presented a ten-fold increase in the rate of chemical oxidation as compared with the first feed. An excess of unreacted Mn (IV) remaining from the previous feed in the serum bottles could be the reason for the observed increase in the chemical oxidation rate.

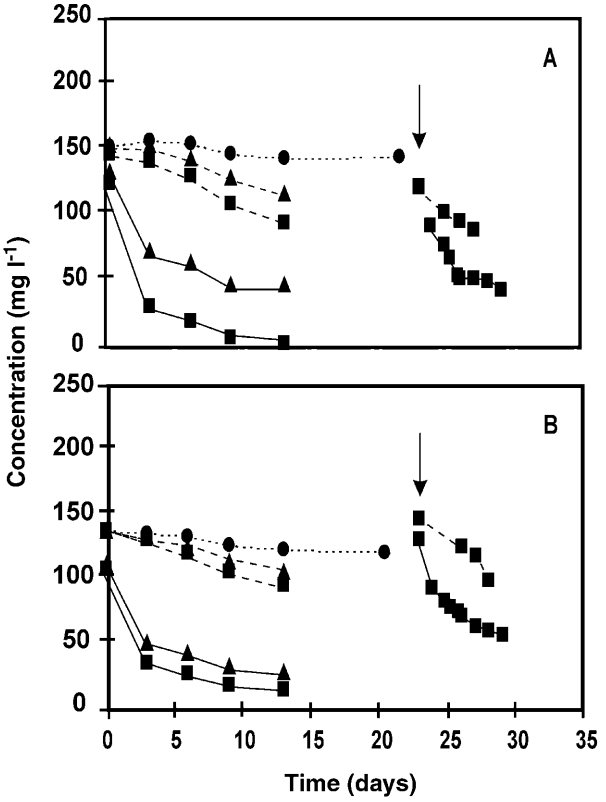

Fig. 1 Profile of biodegradation for 2-cresol (**A**) and 2-ethylphenol (**B**) in the presence of Mn (IV) (–■–) and the combination of Mn (IV) with AQDS (–▲–) as electron acceptors. The sterile controls are (- -■- -) and (- -▲- -) respectively. The symbol (···●···) represents a sterile control without Mn (IV). The *arrow* marks the second feed

Figures 2A and 3A show UV scans of the media in serum bottles treating 2-cresol and 2-ethylphenol at the beginning of the experiment and after 23 days of incubation. The UV scans are compared with those obtained from sterilized controls containing Mn (IV) (Figs. 2B and 3B). The results suggest that both compounds were highly biodegraded as evidenced by the removal of UV absorption, indicating loss of aromatic structure. The UV absorption of these compounds remained unchanged in the sterilized controls. These results confirm that 2-cresol and 2-ethylphenol were mineralized under Mn (IV)-reducing conditions.

Biodegradability assay for the fuel oxygenates

Three of the most important gasoline oxygenates (MTBE, ETBE and TAME) were examined in a

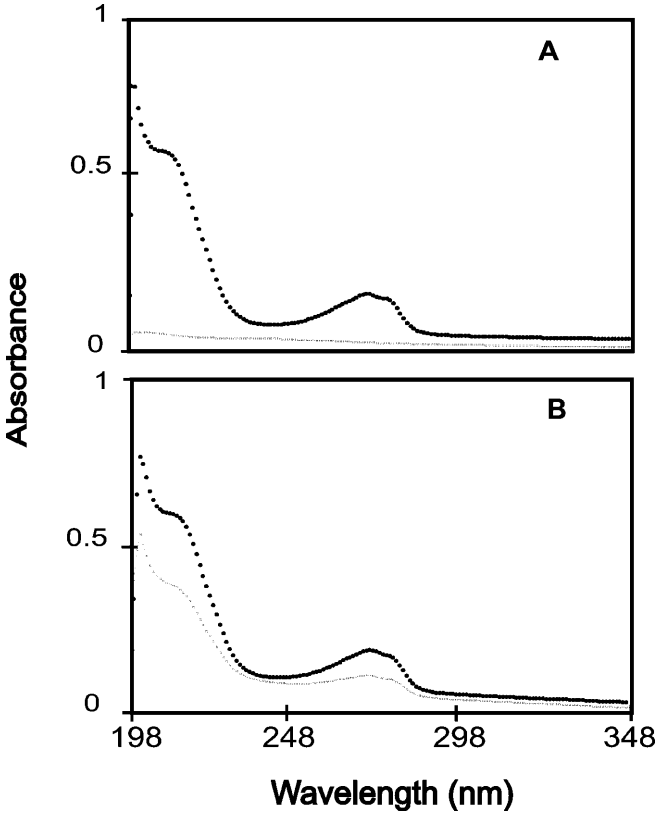

Fig. 2A, B UV scans for the biodegradability assay of 2-cresol (**A**) and the respective sterilized control (**B**) at the beginning of the experiment (——) and after 23 days of incubation (- - - -). The samples were diluted 20 times with phosphate buffer, pH = 7

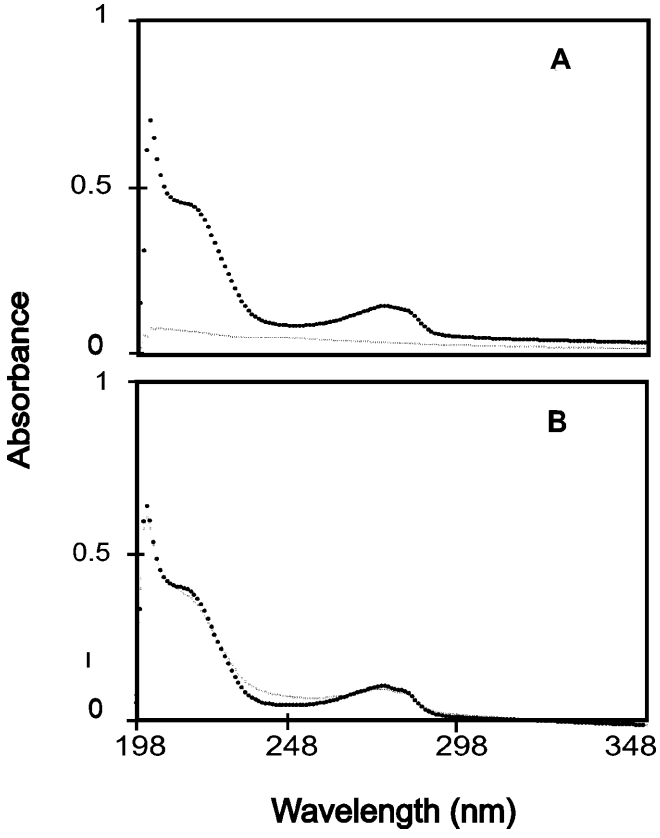

Fig. 3A, B UV scans for the biodegradability assay of 2-ethylphenol (**A**) and the respective sterilized control (**B**) at the beginning of the experiment (——) and after 23 days of incubation (- - - -). The samples were diluted 20 times with phosphate buffer, pH = 7

biodegradability test under several anaerobic conditions, using either GS or ARS as inoculum. There was no detectable loss of any of these compounds after up to 110 days of incubation with GS and up to 96 days of incubation with ARS exposed to different electron acceptors. Also, no net methane production was observed. TBA, a reported metabolite of the degradation of MTBE, also persisted under the conditions tested. To determine if the lack of biodegradation was due to toxicity of the compounds, the acetoclastic methanogenic toxicity of fuel oxygenates and TBA was tested at 1 g l^{-1}. The compounds were not found to be toxic at the concentration tested.

Discussion

The goal of this study was to determine the anaerobic biodegradability of two persistent classes of petrochemical pollutants: *ortho*-substituted alkylphenols and fuel oxygenates. Their biodegradability was tested with two sources of inoculum: granular methanogenic sludge and anaerobic sediment from the Rhine River. Previous studies have indicated that some alkylphenols are recalcitrant under methanogenic conditions. Razo-Flores et al. (1996) showed that 2-cresol, 3-ethylphenol, 2,5-xylenol, 3,4-xylenol and 4-methylcatechol were not degraded after 150 days of incubation. Wang et al. (1988) reported that 2-cresol and all isomers of ethylphenol were not degraded to methane during a 5-month incubation period. In contrast, 3- and 4-cresol were mineralized after incubation for 2 months. Bisaillon et al. (1991) and Tawfiki-Hajii et al. (1999) were able to demonstrate the degradation of 2-cresol under methanogenic conditions with either peptone or whey as cosubstrate. In the case of sulfate-, nitrate- and Fe (III)-reducing conditions, all three cresol isomers can be degraded, but by different pathways (Suflita et al. 1989; Heider and Fuchs 1997). The *ortho*- isomers are the least readily degraded and the *para*- isomers are the most susceptible. The results of the biodegradability assays conducted in the present study indicated that the *ortho*-substituted alkylphenolic compounds were recalcitrant under methanogenic, sulfate-, nitrate- and Fe (III)-reducing conditions.

Very little information is available about the oxidation of aromatic compounds coupled to the reduction of Mn (IV). Langenhoff et al. (1997a, b) demonstrated the degradation of toluene to carbon dioxide for an enrichment culture derived from Rhine River sediment, as

well as by a highly purified enrichment culture (LET-13). The LET-13 culture was also able to degrade a variety of substituted monoaromatic compounds like phenol and benzoate with Mn (IV) as electron acceptor. However, the ability of LET-13 to degrade the three cresol isomers under Mn (IV)-reducing conditions could not be demonstrated because these compounds were depleted in the sterile controls suggesting an abiotic reaction (Langenhoff et al. 1997b). Therefore the results of the present study demonstrate for the first time the biodegradation of 2-cresol and 2-ethylphenol coupled to Mn (IV) reduction. The biological activity significantly stimulated the degradation rate of these alkylphenols beyond the slow rate of chemical oxidation by Mn (IV) oxide. The other alkylphenol tested, 2,6-dimethylphenol, was rapidly oxidized abiotically in sterile controls and thus no biodegradation could be demonstrated. The chemical oxidation of phenolic compounds by Mn (III) and Mn (IV) oxide is well known (Stone and Morgan 1984; Stone 1987). Ring substituents influence the reactivity of phenolic compounds with Mn (IV) through steric and resonance effects. Phenols with alkyl substituents are very susceptible to oxidative degradation by Mn (IV) oxide in contrast with carboxyl, nitro-, chloro- and other substituents (Stone 1987).

In some of the experiments with Mn (IV), the quinone, AQDS, was added in order to determine whether it could stimulate the degradation rates by mediating the transfer of electrons from the manganese-reducing bacteria to Mn (IV) as was reported to be the case for iron-reducing bacteria and Fe (III) (Lovley et al. 1996). However, instead of stimulating the degradation rate of *ortho*-substituted alkylphenols, AQDS caused partial inhibition. A plausible explanation should be sought in the reduction of AQDS to its corresponding hydroquinone by endogenous substrate and/or sulfide in the inoculum (which was visually evident, since the corresponding hydroquinone of AQDS is a colored compound). Hydroquinones can react chemically with Mn (IV) (Stone and Morgan 1984), reducing the pool of this electron acceptor.

In the case of fuel oxygenates, no anaerobic degradation was observed in incubations ranging over 80–110 days with either inoculum, regardless of the electron acceptor (methanogenic, Mn (III), Fe (III), or quinone). Similar results were previously reported where the fuel oxygenates and TBA were not degraded after 249 days of incubation under methanogenic, sulfate-, or nitrate-reducing conditions (Suflita and Mormille 1993; Mormille et al. 1994; Yeh and Novak 1994). The reason why these ethers are persistent to anaerobic degradation could be related to their branched molecular structure. In general, these compounds contain a tertiary carbon which provides the high chemical stability required for a high-octane rating in gasoline. A previous study (Suflita and Mormille 1993), highlighted the role of the chemical structure on the biodegradation of these compounds. The linear analog of MTBE, butyl methyl ether (BME) was completely mineralized to methane after 84 days of incubation, whereas MTBE was not (Suflita and Mormille 1993). BME has also been reported to be mineralized under sulfate-reducing conditions (Mormille et al. 1994). Recently, Kang et al. (1999) reported that MTBE was anaerobically degraded after 15 days of incubation, using a fuel-contaminated soil as inoculum. TBA was produced as the main intermediate. In the present study, TBA was monitored and was not detected in incubations with MTBE; nor was TBA found to be degraded. TBA has a more complicated structure compared with the simplest alcohol, methanol, which is rapidly degraded (Suflita and Mormille 1993; Mormille et al. 1994). Suflita and Mormille (1993) compared the degradation of ketones, alcohols and ethers under methanogenic conditions, finding that the latter were the most recalcitrant. The results taken as a whole indicate that fuel oxygenate biodegradation under anaerobic conditions is not a ubiquitous capacity in the environment. Under aerobic conditions, several strains have been isolated that are capable of slow growth on MTBE and ETBE as sole carbon and energy source, producing TBA as an intermediate (Fayolle et al. 1999; Hyman and O'Reilly 1999).

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