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Biodegradation of alkylates under less agitated aquifer conditions

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Abstract

The biodegradability of three alkylates (2,3-dimethylpentane, 2,4-dimethylpentane and 2,2,4-trimethylpentane) under less agitated aquifer conditions was investigated in this study. All three alkylates biodegraded completely under these conditions regardless of the presence or absence of ethanol or benzene, toluene, ethylbenzene, and xylenes (BTEX) in the feed. In the presence of ethanol, alkylates degradation was not inhibited by ethanol. However, alkylates degraded more slowly in the presence of BTEX suggesting competitive inhibition to microbial utilization of alkylates. In the sterile controls, alkylates concentrations remained unchanged throughout the experiments.

Key words: biodegradation; alkylates; inhibition; BTEX; ethanol
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Introduction

To improve fuel combustion efficiency and decrease exhaust emissions, fuel oxygenates such as methyl t-butyl ether (MTBE) are added to gasoline to replace lead organic compounds because of their high octane value and ease of blending with gasoline (Mormile et al., 1994; Siminiceanu, 2007; Wallner et al., 2009; Kim et al., 2012). However, use of MTBE has resulted in widespread groundwater contamination due to its high water solubility and low affinity to soil particles (Freitas and Barker, 2011). MTBE has become one of the most frequently detected volatile organic compounds in groundwater and has raised public health concerns (Squillace et al., 1996; Landmeyer et al., 1998; Rosell et al., 2007; Wang and Deshusses, 2007; Kim et al., 2012). Furthermore, MTBE poses an environmental challenge when it is inadvertently released to drinking water resources from leaking underground storage tanks at gas stations throughout the country (Squillace et al., 1996; Hartley et al., 1999; Zein et al., 2004). MTBE is often found with other gasoline contaminants such as benzene, toluene, ethylbenzene, and xylenes (BTEX) in groundwater, and it tends to persist in the environment. It has an estimated half-life in groundwater of at least 2 years due to its molecular structure, i.e. the tertiary carbon atom, the ether bond, and the absence of long alkyl chains (Deeb et al., 2001; Ferreira et al., 2006; Siminiceanu, 2007; Wang and Deshusses, 2007). Because of its low taste and odor threshold in drinking water and the fact that data support the conclusion that MTBE is a potential human carcinogen at high doses, the U.S. Environmental Protection Agency (EPA) in 2002 drafted plans to phase out the use of MTBE nationwide over four years. As a result, MTBE was replaced with less toxic alternatives such as ethanol (Mackay et al., 2006; Wang and Deshusses, 2007).

Ethanol rapidly replaced MTBE because it is a renewable, biomass-based source of fuel and its lower environmental impact when compared to MTBE (Rice, 1999; Zhang et al., 2006). Ethanol is also expected to degrade rapidly by microorganisms without any acclimation period under most redox conditions (Powers et al., 2001a; Mackay et al., 2007; Feris et al., 2008). Ethanol concentrations in groundwater typically are not reported at leaking underground storage tank sites because it is an unregulated contaminant, has low toxicity and persistence and is difficult to measure at trace concentrations (Zhang et al., 2006). For this reason, ethanol has largely been assumed to be an innocuous replacement for MTBE (Feris et al., 2008).

However, ethanol is expected to influence the *in-situ* biodegradation of BTEX and other fuel components since it may cause the depletion of electron acceptors needed

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for the degradation of the fuel hydrocarbons and/or decrease the microbial community able to degrade BTEX (Corseuil and Alvarez, 1996; Powers et al., 2001a, 2001b; Da Silva and Alvarez, 2002; Mackay et al., 2006, 2007). Several laboratory studies have shown that BTEX compounds persist in the presence of ethanol and sometimes for considerable periods of time after ethanol is degraded (Corseuil et al., 1998; Ruiz-Aguilar et al., 2002; Osterreicher-Cunha et al., 2004, 2007; Mackay et al., 2006). Ethanol has been reported to increase the solubility and migration of BTEX and other gasoline constituents (Corseuil and Alvarez, 1996; Beller et al., 2002; Da Silva and Alvarez, 2002; Lovanh et al., 2002; Ruiz-Aguilar et al., 2002; Williams et al., 2003; An and Lee, 2008; Feris et al., 2008; Freitas and Barker, 2011). Ethanol and ethanol blends of gasoline conduct electricity. In contrast, unblended gasoline is an electrical insulator. For this reason, pure ethanol is more corrosive than gasoline, and materials-compatibility must be considered when designing large-volume, bulk-ethanol storage tanks (Marchetti et al., 1999; Rice, 1999).

Alkylation is an industrial synthesis process that is used to produce a high-octane solution of branched alkanes, called isoalkanes (Marchetti et al., 1999; Rice, 1999). These alkylate solutions are used as a blending component for gasoline. During World War II, alkylate production was increased to meet the demand for high-octane gasoline used by fighter planes (Marchetti et al., 1999; Rice, 1999). After the war, the demand for high octane components for aviation fuels diminished, and the use of alkylates shifted towards the automotive market (Marchetti et al., 1999; Rice, 1999). Alkylates are branched alkanes, such as isooctane (2,2,4-trimethylpentane), and have low water solubility and high octanol-water partition coefficient. Thus, they would likely be retained in the aquifer material and be much less mobile through the groundwater (Marchetti et al., 1999; Rice, 1999; Cho et al., 2007).

The percentage of alkylates in gasoline will be increased significantly in the future to compensate for the loss in octane number, resulting from either the removal of MTBE and use of ethanol or no oxygenates in fuel (Marchetti et al., 1999; Rice, 1999). For example, although ethanol also has a high-octane level, its oxygen content is about twice that of MTBE; consequently, less ethanol is required to meet a specified oxygen content (e.g., 2.5 wt% oxygen) (Marchetti et al., 1999; Rice, 1999). California's oxygenated gasoline program limits oxygen content to a maximum of 2.2% to limit increases in nitrogen oxides (NOx) emissions that occur from adding oxygen to gasoline (Marchetti et al., 1999; Rice, 1999). To compensate for the resulting octane deficit, petroleum companies could add more alkylates as the high-octane blending components. If an oxygen requirement is eliminated altogether, even greater amounts of high-octane components, such as alkylates, must be added to gasoline (Marchetti et al., 1999; Rice, 1999). Since the physicochemical properties of alkylates resemble those of other hydrocarbons present in gasoline (low solubility in water, high octanol-water partition coefficient, and high Henry's law constant), an increase in the alkylate percentage of gasoline may not affect significantly the way gasoline behaves in environmental releases (Marchetti et al., 1999; Rice, 1999). From an environmental standpoint, alkylates have some of the more desirable properties for a blending component for gasoline. Therefore, as environmental regulations move toward cleaner burning fuels, it is likely that the use of alkylates as blending stock for gasoline could rise (Marchetti et al., 1999; Rice, 1999). However, only limited studies have been performed to evaluate the degradation of alkylates.

A laboratory study by Solano-Serena et al. (1998) showed that all gasoline components were biodegraded to below detection limit after 28 days with the exception of 2,2,4-trimethylpentane (isooctane), 2,3,4-trimethylpentane, and cyclohexane. The percentages of degradation for these compounds were 18% for 2,2,4-trimethylpentane, 9% for 2,3,4-trimethylpentane, and 100% for benzene after 28 days of incubation. The low biodegradation rates of 2,2,4-trimethylpentane and 2,3,4-trimethylpentane were likely due to a deficiency in microorganisms that can degrade these hydrocarbons and not to any inhibition by the alkanes (Solano-Serena et al., 1998; Marchetti et al., 1999). In contrast, biomass that was acclimated to three alkylates, 2,3-dimethylpentane (2,3-DMP), 2,4-dimethylpentane (2,4-DMP) and 2,2,4-trimethylpentane (2,2,4-TMP) in porous pot reactors, completely mineralized all three alkylates within 10 days (Cho et al., 2007) under agitated conditions. The objective of this study was to evaluate the biodegradability of three alkylates, 2,3-DMP, 2,4-DMP, and 2,2,4-TMP, under less agitated conditions typical of groundwater aquifers in the presence and absence of ethanol and BTEX.

1 Materials and methods

1.1 Chemicals and columns

The alkylates, 2,3-DMP, 2,4-DMP, and 2,2,4-TMP, BTEX compounds, and sodium azide were purchased from Sigma-Aldrich, USA. Dichloromethane (DCM) and ethanol were purchased from Fisher Scientific, USA. All the chemicals including nutrients had greater than 99.0% purity. The 25-mm diameter glass tubes used for this study were purchased from Aceglass Inc., USA.

Feed to the test columns was supplemented with an acidified nutrient solution containing salts and vitamins for biological growth. As shown in **Table 1**, the nutrient solution consisted of 5 macronutrients, 5 micronutrients, and 10 vitamins dissolved in deionized water (Zein et al., 2006). To enhance the dissolution of the nutrient chemicals and to prevent biological growth in the solution, 20 mL of concentrated hydrochloric acid was added to the nutrient

Table 1 Nutrient concentrations

Nutrient type	Nutrients	Concentration (mg/L)	Essential nutrient	Concentration (mg/L)
Macronutrients	$(NH_4)_2SO_4$	93.0	NH_4^+	22.5455
	$MgSO_4{\cdot}7H_2O$	69.6	Mg^{++}	6.8667
	$CaCl_2{\cdot}2H_2O$	22.5	Ca^{++}	6.1302
	K_2HPO_4	6.90	P^{+5}	4.0008
	$FeCl_2{\cdot}4H_2O$	17.25	Fe^{++}	4.8474
Micronutrients	$CuSO_4{\cdot}5H_2O$	0.11	Cu^{++}	0.0286
	$Na_2MoO_4{\cdot}2H_2O$	0.15	Mo^{+6}	0.0595
	$MnSO_4{\cdot}H_2O$	0.13	Mn^{++}	0.0423
	$ZnCl_2$	0.23	Zn^{++}	0.1104
	$CoCl_2{\cdot}6H_2O$	0.42	Co^{++}	0.1041
Vitamins	4-Aminobenzoic acid (99%)	0.01513		
	Biotin	0.00590		
	Cyanocabalamin (B12)	0.00030		
	Folic acid dihydrate (99%)	0.00590		
	Nicotinic acid (98%)	0.01513		
	(+)-Pantothenic acid, Ca salt hydrate (98%)	0.01513		
	Pyridoxine, hydrochloride (98%)	0.03023		
	(–)-Riboflavin (98%)	0.01513		
	Thiamine hydrochloride	0.01513		
	Thioctic acid (98%)	0.01513		

solution (Cho et al., 2007). All columns were continuously supplemented with nutrient and sodium carbonate buffer solutions to support biological growth and maintain the pH in an acceptable range between 7.4 and 8.0.

The soil sample for aquifer material collected from a leaking gasoline underground storage tank site was obtained from EPA, Cincinnati, OH, USA. The soil samples were characterized as sandy clay loam, pH 4.5–4.7, 0.8% of organic matter, moisture content 44.8%–46%, total nitrogen 0.023%, and having a bulk density 1.32 g/cm^3.

The same microbial culture used by Cho et al. (2007) was also used for this study. The biomass was grown in a porous pot cylindrical reactor constructed of 0.48 cm thick filter grade porous polyethylene sheets (Atlas Minerals and Chemicals, USA) with a mean pore size of 18–28 m. The porous pot was placed in a 304-stainless steel chemostat with 21.6 cm in internal diameter and 30.5 cm in height. To mix the contents of the reactor homogeneously, a magnetically coupled variable speed mixer (Autoclave Engineers, USA) was used (Cho et al., 2007).

The porous pot reactor was initially inoculated with 60 mL of a crude-oil-degrading microbial culture originally obtained from EPA. Temperature inside the reactor varied between 20°C and 24°C, and pH of the reactor was maintained between 7.4 and 8.0. To maintain aerobic conditions, pre-humidified air was introduced at the bottom of the porous pot using a diffuser. The reactor feed was supplemented with a sodium carbonate buffer at a flow rate of approximately 7.75 L/day and an acidified nutrient solution at a flow rate of approximately 0.25 L/day designed to maintain a 1-day hydraulic retention time (HRT) in the test columns. The reactors were seeded again with the same culture after 3 weeks of operation. At this time 100 mL of biomass taken from another laboratory bioreactor operated on a feed of MTBE, BTEX, t-amylalcohol, and t-amylmethylether was also added (Zein et al., 2004, 2006).

The three alkylate compounds were introduced into the porous pot reactors in neat form via a syringe that introduced the compounds in the buffer feed line. The syringe pump flow rate was set to 0.23 mL/hr to deliver approximately 1.25 g of each alkylate into the reactor daily.

The biomass from the porous pot reactors was placed in cryo-vials and frozen in a –80°C freezer when volatile suspended solids reached a concentration of approximately 1000 mg/L (Cho et al., 2007). Before the experiment started, the frozen biomass containing vials were thawed, and all the contents were washed with sterile saline solution to remove the glycerol.

1.2 Analytical methods

The three alkylate compounds, ethanol and BTEX in both the killed control and biologically active columns were monitored using a Hewlett Packard 6890 Series II gas chromatograph (GC). The GC/FID was equipped with a DB-5 capillary column (30.0 m × 0.25 mm i.d. × 0.25 m thickness) (Supelco, USA). The oven temperature was set at 35°C for 1 min and was subsequently ramped to 100°C at 5 °C/min and finally ramped to 300°C at 25 °C/min and held for 10 min at 300°C. The total duration of the analytical run was 32 min.

When the alkylate concentrations were below 0.5 mg/L, the samples were reanalyzed using a GC/FID coupled to a Purge and Trap apparatus (Tekmar Dohrmann 3100 sample concentrator and a Tekmar Dohrmann AquaTek 70 Liquid Autosampler, Tekmar Dohrmann, USA). Purging was for

11 min at 40°C followed by a 4 min desorbing phase at 225°C. The GC/FID connected to a heated Purge and Trap was equipped with DB-1 column (30.0 m × 0.53 mm × 3 μm film thickness) and the oven temperature was set at 35°C for 6 min followed by ramping to 190°C at 12 °C/min and with a hold time of 6 min. The method detection limit of this procedure was approximately 0.5 μg/L for each alkylate.

A standard stock solution containing approximately 1000 mg/L of each alkylate was diluted with DCM to prepare standard solutions for alkylates. A total of ten standard solutions were prepared from the stock solution with DCM in 2 mL ABC auto sampler vials. The final volume per vial was 1 mL. The surrogate, 2,2,3-trimethylbutane was prepared in DCM and 2,2-dimethylhexane. It was used as an internal standard. The internal standard method published by US EPA (1996) was used for this study.

To determine if the decline in the alkylate concentrations in the columns was mainly due to biodegradation, dissolved oxygen (DO) and inorganic carbon (IC) in both the influent and effluent were analyzed. Micro electrodes from an OM-4 oxygen meter equipped with flow-through oxygen microelectrodes were used to measure DO and Shimadzu TOC-V_{CSH} total organic carbon (TOC) analyzer was used to measure IC. To extract the studied compounds from the columns, the extraction method previously described was followed (Cho et al., 2007).

1.3 Column experiments

1.3.1 Alkylates as sole sources of carbon under less agitated aquifer conditions

Glass columns, 25-mm in internal diameter, were operated to demonstrate that biodegradation, observed in well-agitated batch serum bottles, can also occur under less agitated aquifer conditions. Three sets of column experiments were prepared with 2,3-DMP, 2,4-DMP, and 2,2,4-TMP separately. Three glass columns were prepared for each experiment; 2 columns (one biologically active and one abiotic control) were continuously operated throughout the experiment, and another abiotic column was extracted at the beginning of the experiment to quantify the initial concentrations of the studied compounds in the column. Each column was charged with 90 g of aquifer materials and 6 mL of biomass. All columns were continuously supplemented with nutrient and buffer solutions containing salts and vitamins needed to support biological growth and maintain the pH in an acceptable range. The flow rate to each column was maintained at 0.25 L/day. Sodium azide was added at 2 g per column to inhibit biological activity in the killed controls. Each column was spiked with approximately 306 mg/kg of each alkylate compound. DO for the biologically active and abiotic control columns was measured to monitor changes in oxygen composition as biodegradation proceeded throughout the experiment. IC analysis was also performed to monitor the concentration changes of inorganic carbon in the aqueous effluent. The effluent samples were analysed by using Purge and Trap to monitor any compounds dissolved in the effluent samples. When the cumulative oxygen consumption reached the theoretical chemical oxygen demand of each alkylate compound in the biologically active columns, and the compounds of interest were not detected in the effluent, the contents of each tube were extracted and analysed by GC/FID and Purge and Trap to monitor if any alkylates remaining in the column.

1.3.2 Alkylates in the presence of ethanol under less agitated aquifer conditions

The biodegradability of 2,3-DMP, 2,4-DMP, and 2,2,4-TMP was investigated in the presence of ethanol under less agitated aquifer conditions. Similar to the experiments described earlier, 3 sets of column experiments were prepared and each set consisted of 3 glass columns (one biologically active column and two abiotic controls). Each glass column was spiked with approximately 306 mg/kg of each alkylate compound and 516 mg/kg of ethanol. Sodium azide was also used to inhibit biological activity in the control columns. DO and IC analyses were also performed to monitor changes in oxygen composition and inorganic carbon in the aqueous samples. Effluent samples were analyzed by Purge and Trap to monitor the compounds of interest, especially ethanol, dissolved in effluent samples. As in the batch experiments discussed earlier, at the end of the experiment the entire contents of each column were extracted and analyzed by GC/FID and Purge and Trap to measure any residual compounds in the column.

1.3.3 Alkylates in the presence of BTEX under less agitated aquifer conditions

The biodegradability of each alkylate in the presence of BTEX was investigated. This experiment was performed similarly to the column experiments described earlier. In addition to 306 mg/kg of each alkylate compound, 332 mg/kg of a BTEX mixture (volume ratio, 1:1:1:1) was spiked in each glass tube. DO and IC in both the influent and effluent were monitored throughout the experiments. The effluent samples were also analyzed by Purge and Trap to monitor for any compounds dissolved in the effluent.

2 Results and discussion

Since the glass columns were continuously operated throughout the experiments without sacrificing columns for extraction (except for the one abiotic control column per each experiment extracted at the beginning of the experiment to verify the initial concentrations of spiked compounds), DO and IC in both influent and effluent of the columns were measured to monitor for oxygen consumption and inorganic carbon production in the aqueous phase as biodegradation progressed. The time-varying

concentrations of alkylates were calculated based on the cumulative oxygen consumption. The theoretical mass of oxygen required to mineralize each alkylate compound in this study was 96.7 mg for 2,3-DMP and 2,4-DMP and 96.4 mg for 2,2,4-TMP.

Figure 1a presents the cumulative oxygen consumption for the three biologically active columns fed 2,3-DMP, 2,4-DMP and 2,2,4-TMP, respectively. When alkylates were present as the sole carbon and energy sources, the cumulative oxygen consumption in all three columns increased gradually exceeding 100 mg. The time-varying concentrations of each alkylate were also calculated from the cumulative oxygen consumption data (**Fig. 2a**). As shown in **Fig. 2a**, the initial 306 mg/kg concentration of each alkylate was gradually decreased in the biologically active columns. In this experiment, all three compounds needed approximately 120 days or more to be mineralized completely. At the end of the experiment, the biologically

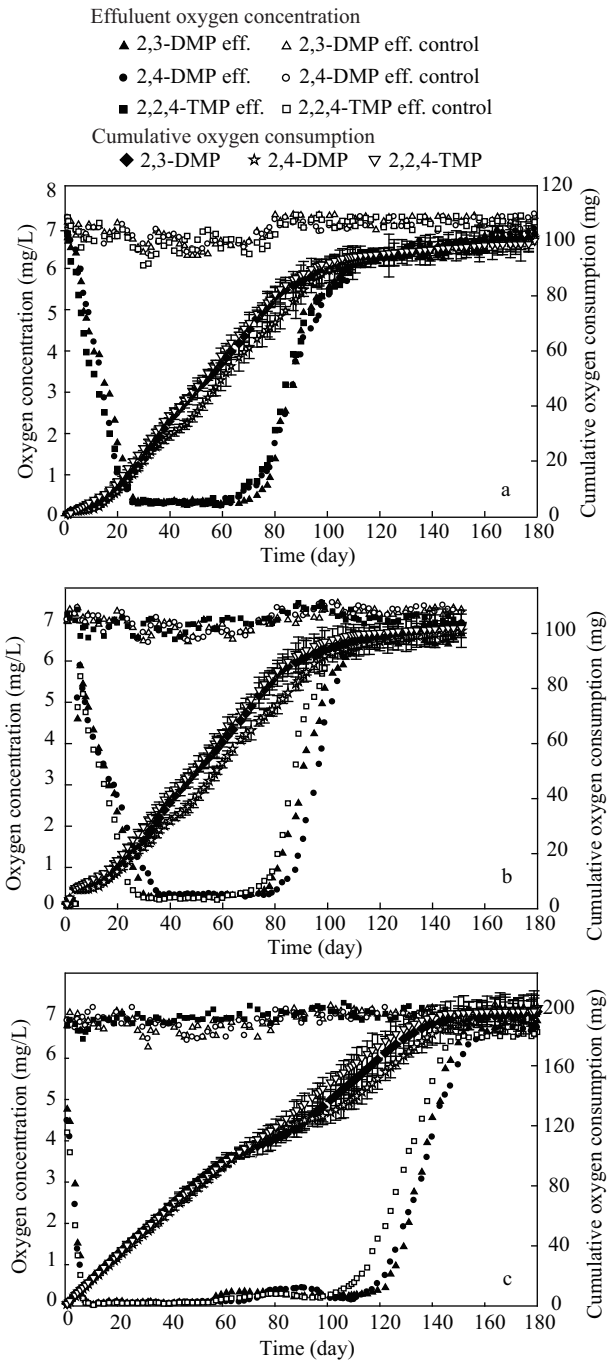

Fig. 1 Effluent oxygen concentration and cumulative oxygen consumption of alkylates degradation when present alone (a), with ethanol (b) and with BTEX (c). Errorbars represent the standard deviation from the mean of 3 indepentdent effluent samples

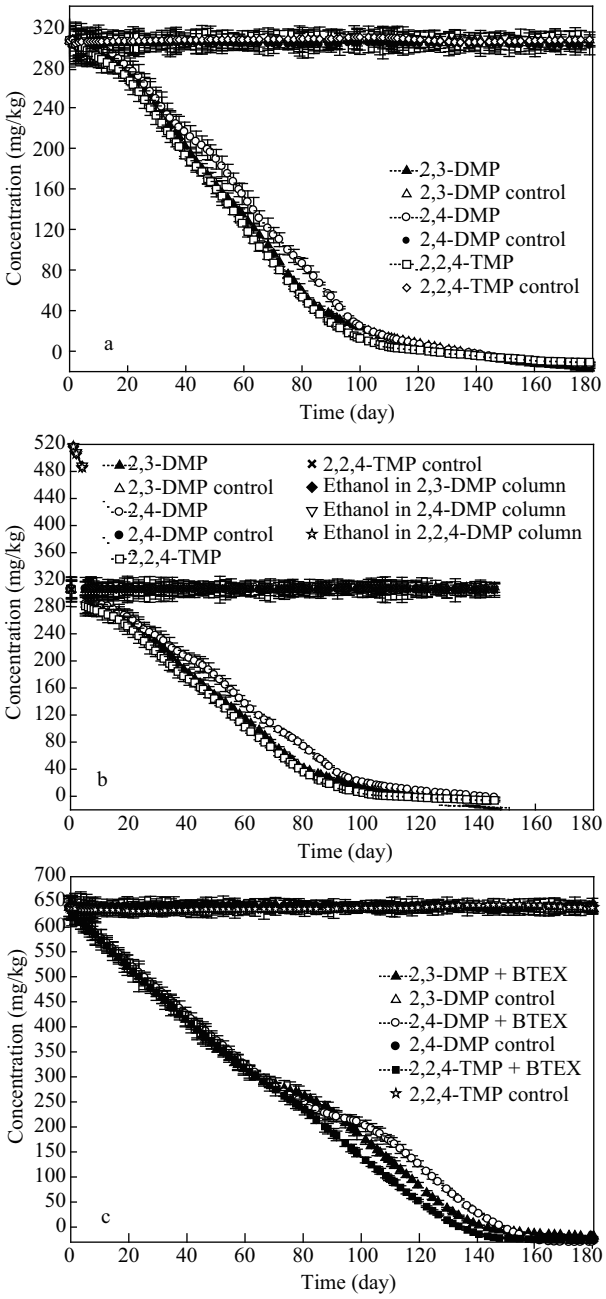

Fig. 2 Calculated time-varying concentrations of alkylates as a sole carbon source (a), in the presence of ethanol (b) and BTEX (c) under less agitated conditions. Error bars represent the standard deviation from the mean of 3 independent effluent samples.

active columns were extracted, and the concentrations of all the studied chemicals were below the detection limits. However, no changes were observed in the concentrations of the three alkylates in the abiotic control columns throughout the experiments (the entire column was extracted at the end of the experiment and more than 90% of each alkylate was recovered from the column). This confirms that the degradation of alkylates was mainly occurring by microbial activity and not by physical loss such as volatilization or dissolution. In the biologically active columns, an increase in IC concentrations was also observed in the effluent samples confirming partial dissolution of carbon dioxide as mineralization proceeded (**Fig. 3a**).

The data observed when the alkylate compounds were present with ethanol were similar to those observed when they were present as the only carbon source. DO readings on both the influent and effluent of the columns were also monitored and the cumulative oxygen consumption computed (**Fig. 1b**). Ethanol was detected in the effluent during the first 4 days of the experiment and dropped to below its detection limit after day 4 in both the biologically active and abiotic control columns (**Fig. 2b**). Unlike ethanol, the alkylate compounds were below their detection limit in the effluent samples throughout the experiment due to their very low water solubility and high octanol-water partition coefficient (data not shown). For the first 4 days of the experiment, approximately 79% of the spiked ethanol was recovered in the effluent samples for the biologically active column compared to 87% for the abiotic control column (the remaining 13% of spiked ethanol was lost due to analytical limitations and/or volatilization). The theoretical mass of oxygen needed to completely degrade ethanol (516 mg/kg of spiked concentration) in this experiment was 97.1 mg. However, only approximately 7.3, 7.2, and 7.5 mg was consumed in the degradation of ethanol in 2,3-DMP, 2,4-DMP, and 2,2,4-TMP columns, respectively, during first 4 days. This suggests an average of only 7.4% of the spiked ethanol biodegraded in the biologically active column during the first 4 days (approximately 3.5 mg of ethanol biodegraded for 2,3-DMP and 2,4-DMP columns and 3.6 mg for 2,2,4-TMP column). This may have been due to oxygen limitations in the columns (**Fig. 2a**). The DO concentration in the influent varied between 6.0 and 7.4 mg/L, while in the effluent it was between 0.08 and 0.12 mg/L during the first 4 days of operation. Since the flow rate was 0.25 L/day, theoretically a maximum of 0.88 mg of ethanol can be biodegraded daily.

Figure 1b shows the cumulative oxygen consumption in the ethanol and 2,3-DMP, 2,4-DMP, and 2,2,4-TMP charged columns. Complete mineralization of 2,3-DMP, 2,4-DMP, and 2,2,4-TMP was also calculated when ethanol was present (**Fig. 2b**). In this case, alkylates biodegradation was likely to be delayed until day 4 based on the findings by Cho et al. (2007). As observed in

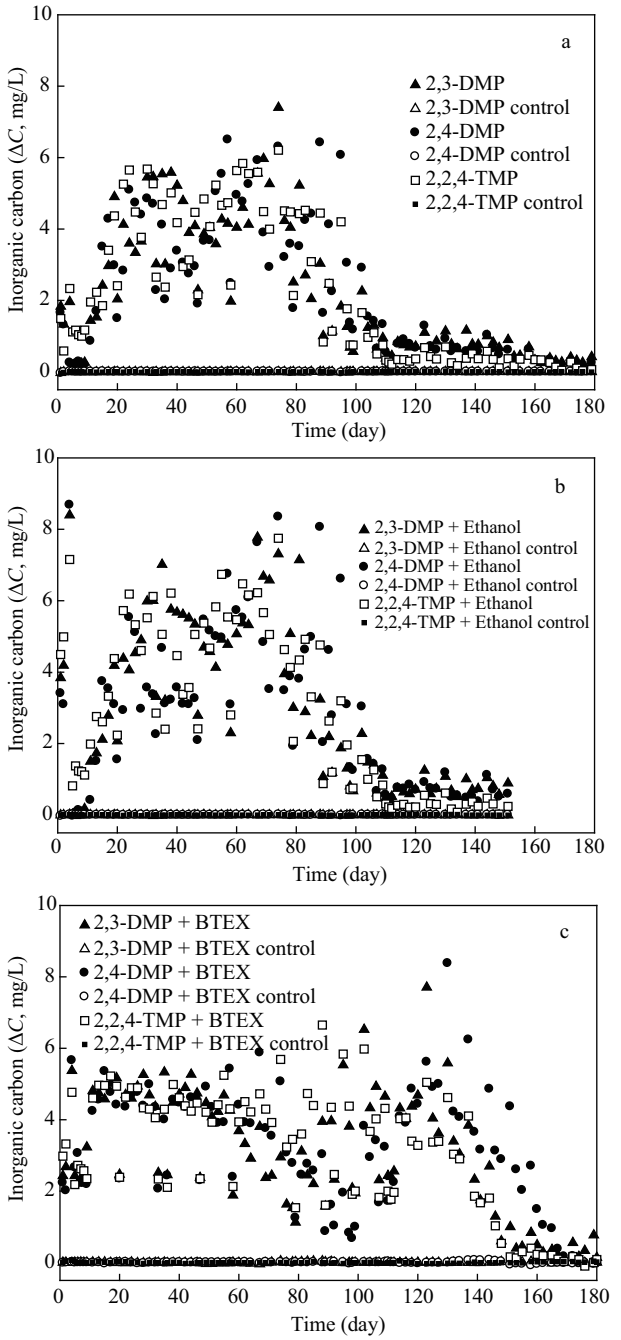

Fig. 3 Change of inorganic carbon (ΔC = effluent–influent) when alkylates were present alone (a), in the present of ethanol (b) or BTEX (c).

the first experiment, 2,3-DMP, 2,4-DMP and 2,2,4-TMP degraded completely after 120 days. Regardless of the presence or absence of ethanol, complete mineralization of these alkylates was observed within the same time period. This suggests that the concentration of ethanol used in this experiment did not inhibit the microbial utilization of 2,3-DMP, 2,4-DMP, or 2,2,4-TMP. At the end of the experiment, the abiotic control columns were extracted and the concentrations of alkylates remained unchanged (more than 90% of alkylates was recovered) confirming

the removal of alkylates was due to biological activity. However, the concentration of ethanol in the extract solution was below the detection limit since ethanol, being miscible with water, exited the columns with the effluent. The biologically active columns were also extracted at the end of the experiment and no ethanol or alkylates were detected. In the biologically active columns, an increase in the IC concentrations was observed in the effluent samples confirming partial dissolution of carbon dioxide as mineralization proceeded (**Fig. 3b**).

In regard to the biodegradation of alkylates in the presence of BTEX, as in the first two experiments, the DO in both the influent and effluent of the columns was monitored (**Fig. 1c**). Unlike the experiment where ethanol was present, time-varying concentrations of individual BTEX and alkylate cannot be estimated by DO and IC data since individual BTEX concentration cannot be calculated only with DO and IC data (unlike serum bottle experiment, the column was being operated for the entire duration of the experiment without sacrificing for extraction). Thus, the combined time-varying-concentrations of both alkylates and BTEX were calculated based on cumulative oxygen uptake data (**Fig. 2c**). The combined theoretical mass of oxygen to completely biodegrade both BTEX (94 mg of oxygen needed) and alkylates (97 mg of oxygen needed for each alkylate) in this study was approximately 191 mg. As shown in **Fig. 1c**, cumulative mass of oxygen consumed by alkylates and BTEX biodegradation gradually increased up to approximately 200 mg in the biologically active column. As in the experiment where ethanol was added, complete biodegradation of 2,3-DMP, 2,4-DMP, and 2,2,4-TMP was also calculated when they were present with BTEX (**Fig. 2c**). At a combined concentration of approximately 638 mg/kg of each alkylate and BTEX, all three alkylates required approximately 150 days to be biodegraded completely. Effluent samples from the biologically active columns were monitored for aqueous presence of alkylates or BTEX. BTEX concentrations in the effluent were consistently below their detection limits after day 60 for the 2,3-DMP and 2,2,4-TMP columns compared to day 80 for the 2,4-DMP column. From this result, it was assumed that the majority of BTEX were degraded by day 60 (2,3-DMP and 2,2,4-TMP column) or day 80 (2,4-DMP column) with only biodegradation of alkylates occurring thereafter. At the end of the experiment, the biologically active column was extracted, and none of the added compounds were detected in the extract. In addition, an increase in the IC concentrations was also observed in effluent samples, confirming that the decrease of alkylates was primarily due to biological activity and not to physical losses (**Fig. 3c**).

In the continuously operated abiotic columns, BTEX was detected in the effluent samples throughout the experiment during the 180 days of operation. However, the alkylate compounds in the effluent samples were below their detection limits from day 1 in both the biologically active and abiotic control columns. The two abiotic columns were extracted one at the beginning and the other at the end of the experiment. Compared to the concentrations extracted at the beginning of the experiment, more than 89% of alkylates were recovered at the end of the experiment. However, only 33% of BTEX was recovered at the end of the experiment since a portion of BTEX was dissolved in effluent water and volatilized.

Contamination of soils and groundwater by gasoline compounds has been of great concern in recent years (Wang and Deshusses, 2007; Malandain et al., 2010). As a result, studies have been reported that focused on the bioremediation of gasoline-contaminated soils and groundwater, and the remediation of these media from contamination with fuel oxygenates, especially MTBE and ethanol (Kharoune et al., 2001; Magar et al., 2002; Zoeckler et al., 2003; Wang and Deshusses, 2007; Reinauer et al., 2008; Malandain et al., 2010). However, limited information is available on the biodegradation of alkylates, potential high-octane alternatives to MTBE (Auffret et al., 2009). Alkylate compounds are expected to be widely used in both non-oxygenated gasoline and some ethanol-containing gasoline (Rice, 1999; Machado et al., 2011). For example, 2,2,4-TMP has an octane rating of 100 and is attractive to the refining industry as an octane enhancer since it can be produced by former MTBE production plants (Auffret et al., 2009).

The biodegradation of monoaromatic compounds, such as BTEX, and *n*-alkanes has been evaluated in many studies, and they have been reported to be biodegradable under aerobic conditions (Atlas and Cerniglia, 1995; Solano-Serena et al., 1998; Auffret et al., 2009). However, highly branched alkanes such as alkylates have not been easily or rapidly biodegradable due to their molecular structure (Atlas and Cerniglia, 1995; Solano-Serena et al., 1998, 2000, 2004; Marchetti et al., 2003; Cho et al., 2007). Recent laboratory studies by Solano-Serena et al. (1998, 1999) showed the partial (less than 50%) degradation of alkylate compounds (2,2,4-TMP and 2,3,4-TMP) by microorganisms. However, these authors suggested that the resistance of alkylates to biodegradation is not due to the inhibitory capacity of isoalkanes or cycloalkanes, but rather to a population deficiency of the naturally occurring microorganisms degrading these hydrocarbons (Solano-Serena et al., 1998, 1999). Cho et al. (2007) found that 2,3-DMP, 2,4-DMP and 2,2,4-TMP were completely mineralized under aerobic condition in well-mixed serum bottle microcosms by a microbial culture that had been previously enriched on alkylates. Consequently, the biodegradation of all three alkylates (2,3-DMP, 2,4-DMP, and 2,2,4-TMP) in this study is not surprising since the columns were inoculated with a culture pre-acclimated to these compounds. When alkylates were present alone as a sole carbon and energy sources under less agitated aquifer

Table 2 Zero-order degradation constants for the alkylates in the presence and absence of ethanol or BTEX under less agitated column (unit: mg/(kg·day))

Alkylate	Alone	With ethanol	With BTEX
2,3-DMP	–3.65 ± 0.72	–3.71 ± 0.83	–4.92 ± 1.58
2,4-DMP	–3.25 ± 0.74	–3.20 ± 0.82	–4.70 ± 1.98
2,2,4-TMP	–3.56 ± 0.82	–3.57 ± 0.96	–4.55 ± 1.49

Data are expressed as mean ± SD. The values were deduced from the linear declining portions of the degradation curves in **Fig. 2**.

Table 3 Times to onset of alkylate degradation and total disappearance of cosubstrates under less agitated conditions (unit: day)

Alkylates	Alone	With ethanol		With BTEX	
	Lag in onset of alkylate degradation	Complete disappearance of ethanol*	Lag in onset of alkylate degradation	Complete disappearance of BTEX	Lag in onset of alkylate degradation
2,3-DMP	8.2	4.0	8.7	64	85
2,4-DMP	9.3	4.0	10.2	87	102
2,2,4-TMP	3.7	4.0	5.2	65	72

* Disappearance of ethanol was mostly due to dissolution in effluent.

condition, all three alkylates degraded completely after approximately 120 days.

Wang and Deshusses (2007) reported that the presence of xylene completely inhibited MTBE degradation Deeb et al. (2001) also suggested that BTEX and MTBE degradation occurred primarily via two independent and inducible pathways. Similarly, Cho et al. (2007) reported that biodegradation of alkylates did not commence until BTEX were entirely degraded. In our experiments, two independent degradation pathways were observed in the presence of BTEX (**Fig. 1c**). The first degradation pathway ended between 58 and 68 days for 2,3-DMP, 2,4-DMP and 2,2,4-TMP (**Fig. 1c**). After approximately 20 days of lag period in all three experiments, the second degradation pathway started. Based on these observations, it is assumed that BTEX biodegradation was complete before the initiation of alkylates bioutilization.

Corseuil et al. (1998) reported that when BTEX was coming led with ethanol, the rate of aerobic biodegradation of BTEX decreased since microorganisms preferentially utilized ethanol. This resulted in oxygen depletion and the persistence of BTEX contamination (Corseuil et al., 1998). Cho et al. (2007) observed that the total biodegradation time for alkylates in serum bottles was the same regardless of whether these alkylates were present alone or with ethanol. In these continuous flow column experiments, ethanol did not inhibit the microbial utilization of alkylates since ethanol was either rapidly degraded or dissolved in the effluent.

As shown in **Fig. 1**, influent DO concentrations for both abiotic and biotic columns and effluent DO concentrations for abiotic columns in all experiments were between 6.0 and 7.4 mg/L throughout the experiments. However, effluent DO concentrations for biologically active columns were below 0.5 mg/L during the period when biodegradation was actively occurring. Shaler and Klecka (1986) reported that DO concentrations below 1 mg/L may be rate limiting for the biodegradation of chlorinated aromatic compounds, which have a requirement for molecular oxygen as a cosubstrate for metabolism. It was also reported that MTBE biodegradation is dependent on DO concentration with K_{O_2} of 0.7 mg O_2/L (Park, 1999). Similarly, limited degradation of ethanol and BTEX in our experiments was likely due to oxygen limiting conditions in the columns. Thus, if oxygen is not a limiting factor, ethanol and BTEX may be degraded earlier in the time series. Generally, the greater the oxygen supply, the faster biodegradation takes place (Zhou and Crawford, 1995). Also if oxygen is supplied to the soil microorganisms, the microbial community could adapt to the contaminated environment through selective enrichment and degrade the petroleum hydrocarbons at relatively fast rates (Zhou and Crawford, 1995). Based on these observations, it is likely that higher degradation rates of alkylates are possible if enough oxygen is available for aerobic degradation.

Zero-order biodegradation rate coefficients for each alkylate were calculated from the linear declining portions of the degradation curves (**Table 2**). Similar to the experiments performed by Cho et al. (2007), the rate coefficients were similar whether the alkylates were in the presence or absence of ethanol or BTEX. In addition to the zero-order rate coefficients, the lag periods before the onset of the declining period of the alkylates were estimated and compared to the length of time for complete disappearance of the co-substrates, ethanol or BTEX (Cho et al., 2007). As shown in **Table 3**, only BTEX significantly delayed the onset of biodegradation of alkylates. Ethanol did not inhibit the biodegradation rate or the lag period for the alkylates. However, the presence of BTEX significantly increased the lag periods to the onset of alkylate biodegradation especially under less agitated conditions.

3 Conclusions

The objective of this study was to investigate the biodegradability of three alkylates (2,3-DMP, 2,4-DMP

and 2,2,4-TMP) under less agitated conditions that might exist in groundwater aquifers. When alkylates were present alone, all three compounds degraded completely after approximately 120 days. To confirm the degradation of alkylates was mainly occurring by microbial activity, both the biologically active and abiotic columns were extracted at the end of the experiment. No studied chemicals were detected in the biologically active columns compared to more than 90% of alkylates were recovered from the abiotic control columns. When ethanol was present, alkylates biodegradation was likely to be delayed until day 4. However, complete mineralization of the alkylates was observed within the same time period regardless of the presence or absence of ethanol. This suggests that the concentration of ethanol used in this experiment did not inhibit the microbial utilization of alkylates. By contrast, the presence of BTEX significantly delayed the onset of biodegradation of alkylates especially under less agitated conditions.

Limited degradation of ethanol and BTEX was observed in this experiment due to oxygen limiting conditions in the columns. Thus, if enough oxygen is supplied for aerobic degradation, the degradation rates of alkylates are expected to be higher.

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