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Effect of ethanol on BTEX biodegradation kinetics: aerobic continuous culture experiments

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Abstract

The use of ethanol as an automotive fuel oxygenate represents potential economic and air-quality benefits. However, little is known about how ethanol may affect the natural attenuation of petroleum product releases. Chemostat experiments were conducted with four pure cultures (representing archetypes of the known aerobic toluene degradation pathways) to determine how ethanol affects benzene, toluene, ethylbenzene, and xylene (BTEX) biodegradation kinetics. In all cases, the presence of ethanol decreased the metabolic flux of toluene (measured as the rate of toluene degradation per cell). This negative effect was counteracted by an ethanol-supported increase in biomass, which is conducive to faster degradation rates. When the influent total organic carbon (TOC) of the toluene–ethanol mixture was kept constant, the metabolic flux of toluene was proportional to its relative contribution to the influent TOC. This empirical relationship was used to derive a mathematical model that simulated effluent benzene concentrations as a function of the influent mixed-substrate composition, the dilution rate, and Monod kinetic coefficients. Under carbon-limiting conditions (1 mg/L influent benzene), the data and model simulations showed an increase in benzene removal efficiency when ethanol was fed at low concentrations (ca. 1 mg/L) because its positive effect on cell growth outweighed its negative effect on the metabolic flux of benzene. High ethanol concentrations, however, had a negative effect, causing oxygen limitation and increasing effluent benzene concentrations to higher levels than when benzene was fed alone. The slower BTEX degradation rates expected at sites with high ethanol concentrations (e.g., at gasohol-contaminated sites) could result in longer BTEX plumes and a greater risk of exposure.

Keywords: Benzene; Chemostats; Degradation; Ethanol; Kinetics; Substrate interactions; Toluene

1. Introduction

Benzene, toluene, ethylbenzene, and the three isomers of xylene (BTEX) are common groundwater pollutants generally associated with gasoline releases. All six BTEX compounds can depress the central nervous system, and chronic benzene exposure can cause leukemia [1]. Although considerable progress has been made towards understanding many hydrogeochemical factors that affect BTEX migration and biodegradation in aquifers, little attention has been given to how differences in gasoline formulation affect natural attenuation processes. In this regard, there is a recent effort to increase the use of ethanol as a gasoline oxygenate to reduce air pollution, and as a supplemental renewable fuel to reduce dependence on imported oil [2]. Consequently, the probability of encountering ethanol at BTEX-contaminated sites is increasing, and a better understanding of the effects of ethanol on BTEX biodegradation is needed to enhance our risk assessment and remediation capabilities.

The need to understand substrate interactions between BTEX and ethanol is very recent, and little research has been conducted on the potential effects of

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ethanol on BTEX biodegradation. Often, BTEX compounds are degraded by inducible enzymes that can be repressed when easily degradable substrates are present at high concentrations [3–5]. This may explain previous reports that ethanol was preferentially degraded over benzene when these compounds were fed concurrently to aquifer microcosms [6] and aquifer columns [7]. However, no preferential substrate utilization was observed in experiments with continuous cultures exposed to multiple substrates at low concentrations [8]. Apparently, preferential substrate degradation is a concentration-dependent phenomenon related to catabolite repression, and such regulatory mechanisms do not seem to occur under carbon-limiting conditions that are conducive to simultaneous utilization of multiple substrates [9]. Currently, the conditions leading to sequential (diauxic) versus simultaneous degradation of BTEX in the presence of ethanol are not well understood, and there are no comprehensive mathematical models that consider such substrate interactions.

This paper addresses the effect of ethanol on the metabolic flux of BTEX compounds when degraded by different reference strains. Emphasis was placed on developing an analytical model that incorporates the effect of such substrate interactions on metabolic flux repression and microbial growth. The implications of this dual-substrate utilization model on natural attenuation of gasohol releases are also discussed.

2. Materials and methods

2.1. Experimental approach and rationale

Continuous culture techniques such as chemostats are well suited to study the effects of alternative substrates on the degradation of target pollutants. This is so because the specific utilization rate of a given compound in a mixture can be controlled (and held constant) by varying its percentage in the feed solution [8], or by adjusting the dilution rate of the chemostat [10]. Note that chemostats do not mimic the mass transfer limitations and other sub-optimal conditions encountered in aquifers, which invalidates extrapolating biodegradation rate coefficients from such experimental systems to the field. Nevertheless, the mechanistic understanding and rate laws obtained with chemostats could be applied to contaminated sites provided that appropriate site-specific parameters are used.

Pure cultures were used in these experiments to preclude confounding effects from microbial population shifts and to isolate the effect of ethanol on BTEX degradation kinetics. Four reference strains that harbor different (aerobic) toluene degradation pathways were used to investigate response variability. The tested strains were *Pseudomonas putida* mt-2, which has the *tol* plasmid and attacks toluene at the methyl group [11]; *Pseudomonas putida* F1, which has the *tod* gene and attacks the ring using toluene dioxygenase [12]; *Burkholderia pickettii* PKO1, with the *tbu* gene coding for toluene *meta*-monooxygenase [13]; and *Pseudomonas mendocina* KR1, with the *tmo* gene expressing toluene *para*-monooxygenase [14].

2.2. Chemostats

Commercially available chemostats are not compatible for precise work with volatile organic compounds. System requirements include that BTEX contact only glass and Teflon to reduce sorption losses, little or no headspace to eliminate partitioning considerations, and a constant feed of BTEX at concentrations near 1 mg/L or less to be representative of field conditions. This was achieved with a dual pump system and fittings typically used with liquid chromatography instruments (Fig. 1). Five 300 mL Pyrex bottles with inlet and outlet ports were used as chemostats. The inlets were connected to a 10-liter mineral medium reservoir. This medium was pumped to the chemostats with a peristaltic pump. The inlet tubings were also connected to a gas-tight syringe pump where BTEX and ethanol were added. A heated water bath (70°C) was used to prevent microbial contamination of the medium reservoir. Before each run, the chemostats and the medium reservoir were autoclaved and purged with nitric acid solution (20%) for at least 24 h to ensure sterility.

A modified Hutner's mineral salts basal medium (MSB) was used to provide trace metals and nutrients to support microbial growth. The recipe for MSB was

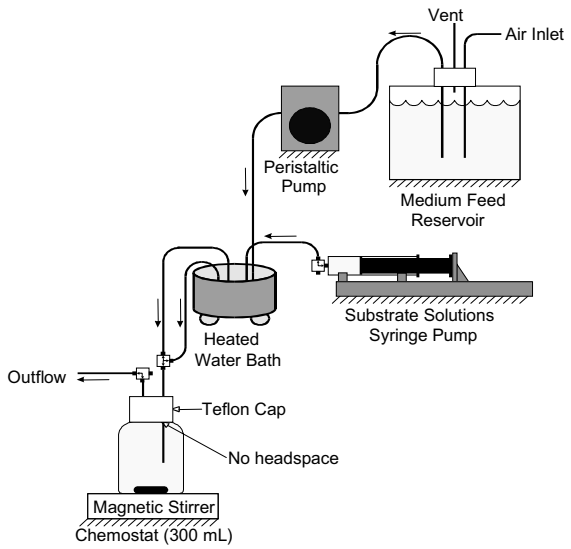

Fig. 1. Chemostat schematic.

obtained from the laboratory of David T. Gibson, and was first described by Cohen-Bazire et al. [15]. Toluene (or benzene) was added to a sterile gas-tight syringe (Hamilton, Reno, Nev.) containing sterile water to obtain a concentration of 100 mg/L. The syringe pump flow was diluted 1:100 by the peristaltic pump flow delivering the MSB, and the influent BTEX concentration was approximately 1 mg/L. Ethanol was also added through the syringe pump. The outlet flow rate was kept at 75 mL/h so that the dilution rate (D) was 0.25 h^{-1}. This dilution rate represents 55–65% of the maximum specific growth rate (μ_{max}) for the reference strains, which were reported to be 0.38 h^{-1} for F1, 0.40 h^{-1} for mt-2, and 0.45 h^{-1} for KR1 when grown on toluene at 28°C [16]. Thus, D was sufficiently low to prevent washout and sufficiently high to ensure relatively short cell residence times to avoid significant cell decay and attachment within the reactors.

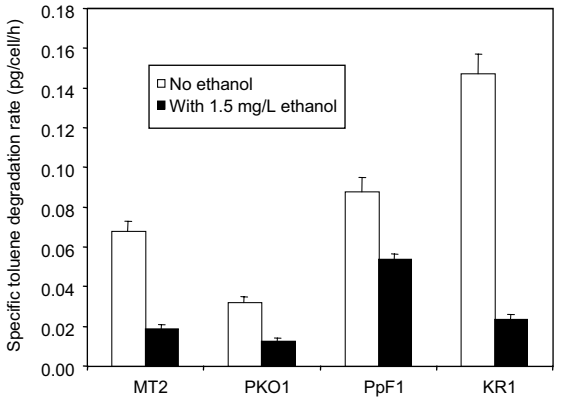

Fig. 2. Effect of ethanol on toluene metabolic flux for different reference strains fed toluene (1 mg/L) at $D = 0.25\,h^{-1}$, 17°C. PKO1=*B. Pickettii* PKO1, KR1=*P. mendocina* KR1, PpF1=*P. putida* F1, and MT2=*P. putida* mt-2.

2.3. Analytical methods

The biomass concentration was measured using the most probable number (MPN) technique. The medium used for the MPN method was MSB with 2 g/L sodium succinate and 10 mg/L resazurin. The MPN was performed using a 96-well microplate and an eight-channel multipipettor with a 150 μL test volume, eight replicates per dilution, and 12 dilution steps with a dilution factor of three [17]. The presence of bacteria was indicated by a color change in resazurin from blue to pink due to a decrease in redox potential caused by microbial respiration. The initial concentration in the chemostats was about 10^9 cells/L.

Samples taken from the chemostats (1 mL) were filtered through a 0.22 μm-pore-size bacterial filter prior to BTEX and ethanol analyses. BTEX and ethanol were analyzed by gas chromatography with a Hewlett-Packard 5890 instrument equipped with an HP 19395A automatic headspace sampler and a flame ionization detector. Separation was achieved with a 30 m, 0.53 mm diameter DB-wax column. Detection limits were approximately 1 μg/L for BTEX and 50 μg/L for ethanol. Analytical details are described elsewhere [18,6].

Control runs were conducted without bacteria to discern BTEX biodegradation from volatilization losses. Such losses were minor (<5%) indicating that BTEX removal was due to biodegradation.

3. Results and discussion

3.1. Effect of ethanol on the metabolic flux of toluene

Chemostats were run with different pure cultures under aerobic, carbon-limited conditions reminiscent of the edge of a plume. Even a low influent ethanol concentration (e.g., 1.5 mg/L) decreased the metabolic flux of toluene, measured as the toluene degradation rate per unit cell (Fig. 2). A similar inhibitory response was observed when all six BTEX compounds were fed concurrently with ethanol (data not shown). Apparently, ethanol diffuses freely through the cell membrane and is readily metabolized using constitutive enzymes [19]. The metabolic flux of ethanol hinders that of toluene and other BTEX compounds, possibly because their degradation pathways converge into central metabolic processes that become saturated (e.g., Kreb's cycle).

It should be noted that relatively high ethanol concentrations might also be inhibitory by repressing the induction of oxygenase enzymes that are needed to initiate aerobic BTEX catabolism. This phenomenon, known as catabolite repression, saves cellular energy that would be used for synthesis of enzymes that are not needed when ethanol is abundant. Such regulatory mechanisms are unlikely to be expressed under carbon-limiting conditions [9] and were not studied in this work. Nevertheless, previous chemostat studies have shown that substrates that are degraded by constitutive enzymes (e.g., succinate) can exert catabolite repression of BTEX-degrading enzymes, and that this effect is more pronounced at relatively high growth rates and under nutrient-limiting conditions [3,4,20].

All reference strains degraded ethanol faster than toluene, with specific degradation rates ranging from 0.25 to 0.64 pg/cell/h for strains *P. mendocina* KR1 and *B. pickettii* KRO1, respectively (Table 1). This suggests that ethanol can be preferentially metabolized over BTEX compounds, and corroborates previous studies reporting faster ethanol degradation than other gasoline

Table 1
Specific degradation rates (U) for different strains when toluene or ethanol was fed as the sole substrate at a dilution rate of $0.25\,h^{-1}$ at 17°C

Strain	Toluene degradation rate (pg/cell/h)	Ethanol degradation rate (pg/cell/h)
B. pickettii PK01	0.032	0.64
P. mendocina KR1	0.15	0.25
P. putida F1	0.088	0.28
P. putida mt-2	0.068	0.48

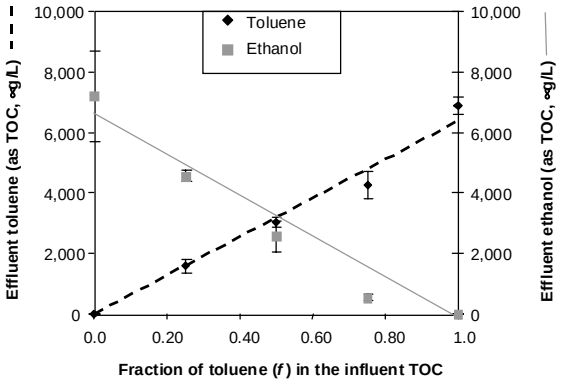

Fig. 3. Simultaneous degradation of toluene and ethanol by *P. putida* F1 at 22°C. When the influent TOC (from toluene plus ethanol) was constant (ca. 10 mg/L), the effluent toluene concentration was proportional to its influent TOC contribution.

constituents under both aerobic and anaerobic conditions [6,7,21].

Further studies were conducted to quantify the concentration-dependent effect of ethanol on toluene degradation. Chemostats were run by keeping the influent total organic carbon (TOC) constant, at about 10 mg/L, and varying the percentage of ethanol and toluene that contributed to the influent TOC. Ethanol and toluene were utilized simultaneously, and their effluent concentrations were proportional to their relative contribution to the TOC in the feed (Fig. 3). These observations were in good agreement with the pioneering experiments conducted by Egli et al. [8] with continuous cultures of *E. coli* growing on sugar mixtures. The effluent toluene concentration when ethanol was present in the mixture (S_{mix}) could thus be described as

$$S_{mix} = fS_{alone}, \quad (1)$$

where f is the fraction of BTEX (toluene in this case) in the influent TOC, and S_{alone} is the effluent toluene concentration when fed alone. This relationship is represented as the black dotted line in Fig. 3.

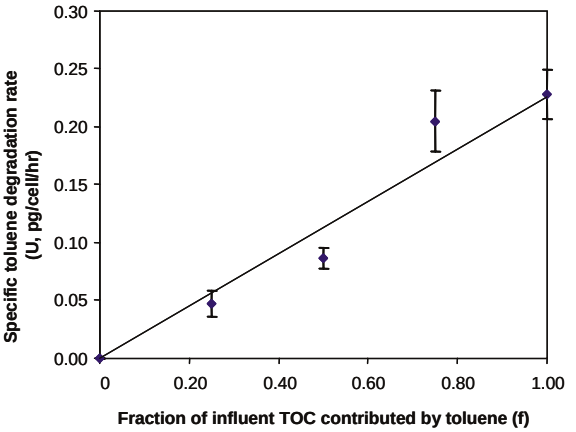

Fig. 4. The specific degradation rate of toluene (U) for *P. putida* F1 fed a toluene–ethanol mixture at 22°C. The influent TOC (from toluene plus ethanol) was constant ($\sim$10 mg/L).

In aquifers, natural attenuation of BTEX compounds often follows first-order kinetics (i.e., the degradation rate is proportional to the BTEX concentration) [22]. In completely mixed systems such as chemostats, the effluent concentration is the same as the concentration to which cells are exposed. Since this is the concentration that controls the rate, and this concentration is a function of the mixture composition (defined by f, Eq. (1)) it follows that that the BTEX degradation rate is also a function of f. This observation can be used to postulate an empirical equation for the effect of ethanol on the rate of BTEX utilization by continuous cultures

$$U_{mix} = fU_{alone}, \quad (2)$$

where U_{mix} is the specific BTEX utilization rate when ethanol is present (i.e., the degradation rate per cell, which is a measure of metabolic flux), U_{alone} is the corresponding rate without ethanol, and f was previously defined as the fraction of BTEX in the feed mixture (as TOC). This equation fits the data reasonably well (solid line, Fig. 4), and suggests that the metabolic flux of a substrate in a mixture can be proportional to its relative availability.

Note that both the rate of toluene degradation (Table 1) and the level of impact of ethanol (Fig. 2) are variable from strain to strain. Nevertheless, the proposed direct proportionality between the specific toluene degradation rate and the fraction of toluene contributing to the total available TOC was observed for all four strains used in this work (data not shown). This suggests that Eq. (2) could be applicable to field situations, provided that mass transfer phenomena are not rate limiting.

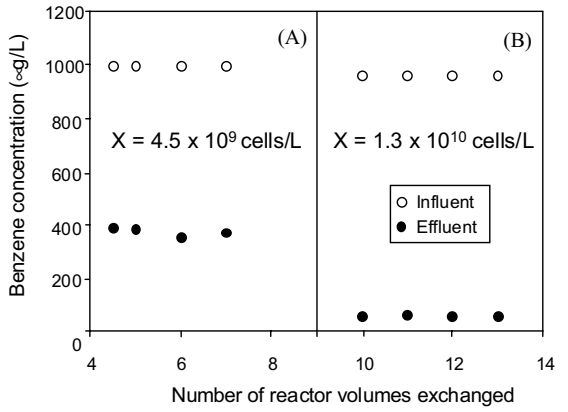

Fig. 5. Benzene degradation by *P. putida* F1 at 17°C (A). The addition of ethanol at 1.5 mg/L (B) enhanced the degradation of benzene due to an increase in microbial concentration (X).

3.2. Effect of ethanol on microbial growth

The negative effect of ethanol on the metabolic flux of BTEX can be counteracted to some degree by its positive effect on microbial growth. When mass transfer is not rate limiting, the degradation rate is proportional to the microbial concentration. Thus, an increase in biomass concentration is conducive to faster biodegradation rates. Fig. 5 shows the results of a chemostat experiment with *Pseudomonas putida* F1 (PpF1) degrading benzene in the absence and presence of ethanol. This experiment was run with relatively low influent concentrations of benzene (1 mg/L) and ethanol (1.5 mg/L), which represents about 3 mg/L (each) of chemical oxygen demand (COD). Therefore, the oxygen demand exerted by the substrates was less than the available dissolved oxygen (8–9 mg/L) and carbon-limiting conditions prevailed. Adding ethanol to the influent decreased the steady-state effluent benzene concentrations from 378 ± 15 to 62 ± 3 μg/L. This effect was attributed to an ethanol-supported increase in the biomass concentration, which increased from 4.5×10^9 to 1.3×10^{10} cells/L. In this case, enhanced growth did offset the negative effect that ethanol had on the specific toluene degradation activity (Fig. 2). Similar results were also observed for the other three archetype strains when benzene or toluene was fed with ethanol under carbon-limiting conditions. Nevertheless, this beneficial effect is not likely to be common in contaminated sites, where the high oxygen demand exerted by ethanol could drive the system anaerobic and rapidly consume any oxygen that diffuses in, thus hindering BTEX bioremediation [2].

The fact that ethanol can have both a positive and a negative effect suggests the need for a mathematical model that integrates these effects to predict the overall rate of BTEX degradation. Such a model would be useful to delineate the range of ethanol concentrations that enhance or hinder BTEX degradation. As a first step towards incorporating the effects of ethanol on fate-and-transport models, we combined the empirical relationship describing a decrease in the specific BTEX degradation rate when ethanol is present (Eq. (2)) with mass balance equations for ethanol, BTEX, and biomass. This yielded a generic dual-substrate utilization model that predicted the overall effect of ethanol on BTEX degradation by continuous cultures.

3.3. Model development

The overall specific growth rate of the culture (μ) is assumed to equal the sum of the contribution to the growth rate by each substrate, i (μ_i):

$$\mu = \sum_i \mu_i. \tag{3}$$

The mass balance for the microbial concentration (X) in the chemostat is

accumulation = growth − outflow:

$$\frac{dX}{dt} = \mu X - DX. \tag{4}$$

At steady state, Eq. (4) equals zero, and $\mu = D$ (i.e., the specific growth rate is controlled by the dilution rate of the culture, D, which is the flow rate divided by the chemostat volume).

The mass balance of substrate i in the chemostat system is given by

accumulation = (input–output) − amount degraded:

$$\frac{dS_i}{dt} = D(S_i^o - S_i) - X\frac{\mu_i}{Y_i}, \tag{5}$$

where Y_i is the yield coefficient for substrate i (mass of cells produced per mass of substrate degraded). Note that μ/Y represents the specific substrate utilization rate, U. At steady state, Eq. (5) equals zero. Rearranging terms

$$\mu_i X = D Y_i (S_i^o - S_i). \tag{6}$$

Substituting Eq. (6) into Eq. (3) for each substrate j, and replacing μ with D, we get

$$D = \sum_j \frac{D Y_j}{X}(S_j^o - S_j)$$

$$\Rightarrow X = \sum_j Y_j (S_j^o - S_j). \tag{7}$$

Substituting this value of X into the mass balance for the substrate of interest, i (Eq. (6)), we get

$$\mu_i \sum_j Y_j(S_j^o - S_j) = D Y_i(S_i^o - S_i)$$

$$\Rightarrow (D - \mu_i)(S_i^o - S_i) - \mu_i \sum_{j \neq i} \frac{Y_j}{Y_i}(S_j^o - S_j) = 0. \tag{8}$$

If only two substrates are present (e.g., S_1 = benzene and S_2 = ethanol), Eq. (8) yields a model that considers the effect of both substrates on microbial growth

$$(D-\mu_1)(S_1^o-S_1)-\mu_1\frac{Y_2}{Y_1}(S_2^o-S_2)=0$$

and

$$(D-\mu_2)(S_2^o-S_2)-\mu_2\frac{Y_1}{Y_2}(S_1^o-S_1)=0. \quad (9)$$

The next step is to account for the substrate interaction term, which should be a function of the substrate mixture composition (defined by f). Assuming that the biokinetic coefficients for one substrate do not change by the presence of the other substrate, and recognizing that the specific growth rate is proportional to the specific substrate utilization rate, we can postulate an empirical relationship for dual-substrate utilization based on Eq. (2) and Monod's equation:

$$\mu_1=f\mu_{\max_1}\left(\frac{S_1}{K_{s_1}+S_1}\right)$$

and

$$\mu_2=\mu_{\max_2}\left(\frac{S_2}{K_{s_2}+S_2}\right). \quad (10)$$

Thus, the interaction term (f) is only applied to benzene (S_1). Note that this is mathematically equivalent to assuming non-competitive inhibition by ethanol on benzene degradation [23]. Substituting (10) into (9) yields a dual-substrate utilization model that describes the effluent concentrations (S_1 and S_2) as an implicit function

$$\left(D-f\mu_{\max_1}\left(\frac{S_1}{K_{s_1}+S_1}\right)\right)(S_1^o-S_1)-f\mu_{\max_1}\left(\frac{S_1}{K_{s_1}+S_1}\right)\frac{Y_2}{Y_1}(S_2^o-S_2)=0$$

and

$$\left(D-\mu_{\max_2}\left(\frac{S_2}{K_{s_2}+S_2}\right)\right)(S_2^o-S_2)-\mu_{\max_2}\left(\frac{S_2}{K_{s_2}+S_2}\right)\frac{Y_1}{Y_2}(S_1^o-S_1)=0. \quad (11)$$

A thermodynamic model of microbial growth kinetics [24] was used to estimate the yield coefficients for benzene (Y_1) and ethanol (Y_2) using oxygen as an electron acceptor (Table 2). The maximum growth rate (μ_{max}) was determined from batch incubations with acclimated PpF1 cells (amended with 50 mg/L benzene) as the slope from the increase in $\ln(OD_{600})$ versus time during the exponential growth phase [16]. The value for K_S was measured as the chemostat effluent concentration (S) when the dilution rate (D) was set at one-half of μ_{max}. The estimated Monod coefficients (Table 2) are within the range of values reported in the literature [16,25,26]. The system of equations (11) yielded three roots, but only one represented a biologically plausible mathematical solution (i.e., the non-negative roots that have values below influent concentrations are the only "logical" or biologically plausible outputs) (Fig. 6).

Using independently obtained parameters, the model accurately predicted a decrease in effluent benzene concentrations when ethanol was fed at low levels (Fig. 6A). Enhanced benzene removal suggests that (under carbon-limiting conditions) the positive effect of ethanol on microbial growth is more dominant than its negative effect on the metabolic flux of benzene.

Table 2
Biokinetic parameters for aerobic biodegradation of benzene and ethanol by PpF1

Compound	K_S (μg/L)	Y (g-cell/g-compound)	μ_{max} (h^{-1})
Benzene	13	1.18	0.35
Ethanol	88	0.93	0.46

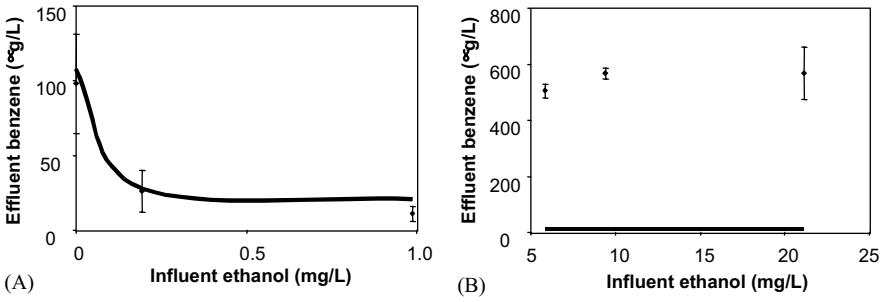

Fig. 6. Effect of ethanol on effluent benzene concentration for chemostats with PpF1 fed benzene (1 mg/L) at 22°C. (A) Carbon-limiting conditions and (B) oxygen-limiting conditions. The solid line is the model (Eq. (11)). Error bars depict standard deviations ($n=3$).

The model, however, does not account for O_2 limitation, and failed to predict that higher influent ethanol concentrations would have a negative effect on benzene removal by PpF1 (Fig. 6B). Adding ethanol at $\geqslant 5$ mg/L increased effluent benzene concentrations beyond the level when benzene was fed alone. Ethanol constitutes a significant additional electron acceptor demand compared to that exerted by other soluble components of gasoline, and its degradation is likely to deplete O_2 that could otherwise be available for BTEX degradation. A decrease in the extent of aerobic BTEX degradation in oxygen-limited aquifers is particularly important for the fate of benzene, which is the most toxic of the BTEX compounds and is relatively recalcitrant under anaerobic conditions [27–32].

4. Summary and conclusions

Dual-substrate utilization (e.g., benzene and ethanol) can be modeled satisfactorily under carbon-limiting conditions by assuming that the metabolic flux of a substrate is proportional to its availability, and by taking into account microbial growth. The inhibitory effect of ethanol on specific BTEX degradation activity can be offset by a fortuitous growth of specific degraders on ethanol, which is conducive to faster degradation rates. However, the high ethanol concentrations expected near the source of gasohol contaminated sites are expected to be very inhibitory due to an ethanol-driven consumption of nutrients and electron acceptors (e.g., O_2) that would otherwise be available for BTEX degradation. Thus, the presence of ethanol in gasoline is likely to hinder the natural attenuation of BTEX releases, which would contribute to longer BTEX plumes and a greater risk of exposure. The overall effect of ethanol is likely to be system specific, depending largely on the release scenario and the assimilative capacity of the aquifer (e.g., the electron acceptor pool).

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