



# Kinetics of toluene degradation by toluene-oxidizing bacteria as a function of oxygen concentration, and the effect of nitrate

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## Abstract

The kinetics of toluene degradation as a function of oxygen concentration were compared for six strains of toluene-oxidizing bacteria using initial rate assays. The effect of nitrate was also examined. Rates of degradation and the relative effect of oxygen on the degradation rate were correlated with the pathway for toluene oxidation. Strains which synthesize toluene dioxygenases, *Pseudomonas putida* F1, *P. fluorescens* CFS215, and *Pseudomonas* sp. strain W31, degraded toluene at significantly higher rates (151–166 nmol/mg per min) than strains synthesizing toluene monooxygenases, *Burkholderia cepacia* G4 (23 nmol/mg per min) and *B. pickettii* PKO1 (14 nmol/mg per min), or a methylmonooxygenase, *P. putida* PaW1 (12 nmol/mg per min). Rates declined 30–48% for the dioxygenase strains and 25% for PaW1 as the oxygen concentration was decreased from 240 to 50  $\mu$ M, but declined less than 10% for G4 and PKO1. Nitrate enhanced toluene degradation by the denitrifying strains PKO1 and W31 at oxygen concentrations below 30  $\mu$ M, but had no significant effect on any of the other strains. Biphasic kinetics were observed for all of the strains, with double-reciprocal plots of the data exhibiting an inflection point at a ‘critical oxygen concentration’ between 20 and 30  $\mu$ M. Below this concentration, the rate of toluene degradation was inhibited to a greater extent than predicted by the kinetic data for higher oxygen concentrations. For the denitrifying strains PKO1 and W31, however, monophasic kinetics were observed when nitrate was provided as an alternative electron acceptor. These observations suggest that biphasic kinetics result when rates of toluene degradation are limited by the availability of electron acceptor at the critical oxygen concentration, and that this limitation is overcome by denitrifying strains able to respire nitrate. Taken together, our findings suggest that the synthesis of monooxygenases and the ability to denitrify represent independent adaptations for toluene utilization in low oxygen environments. Moreover, these data support the use of nitrate in mixed electron acceptor strategies for the bioremediation of contaminated aquifers, as well as the targeted use of monooxygenase and dioxygenase strains in settings in which their physiological traits can be best exploited.

**Keywords:** *Pseudomonas*; *Burkholderia*; Kinetics; Toluene biodegradation; Oxygenase; Denitrification

## 1. Introduction

Benzene, toluene, ethylbenzene, and xylene

(BTEX) are hazardous aromatic compounds contained in gasoline and other petroleum products [1]. Collectively they are among the most commonly reported contaminants of ground water in the United States [2]. BTEX compounds can be degraded aerobically by indigenous subsurface micro-

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organisms by means of oxygenase-catalyzed reactions which require molecular oxygen for the hydroxylation of the aromatic ring or an alkyl substituent [3–5]. The biodegradation of hydrocarbons in contaminated aquifers is often limited by the availability of oxygen, which serves both as a co-substrate for oxygenase enzymes and as a terminal electron acceptor for cellular respiration [6].

Approaches to in situ bioremediation of ground water often include the injection of oxygen in the form of air, pure oxygen, or hydrogen peroxide. Problems associated with each of these methods have been reviewed [7]. The use of nitrate as an electron acceptor is attractive because of the higher solubility of nitrate than oxygen [8]. Mixed electron acceptor regimes, involving the addition of both nitrate and oxygen to contaminated aquifers, rely on the utilization of nitrate as a supplementary electron acceptor by denitrifying bacteria [5]. Evidence for the participation of denitrifying bacteria in the degradation of hydrocarbons under hypoxic (i.e. low oxygen) conditions has been previously reported [9–14]. In a previous study [15], we showed that the denitrifying, toluene-oxidizing bacteria *Burkholderia* (*Pseudomonas*) *pickettii* PKO1, *Pseudomonas* sp. strain W31, and *P. fluorescens* CFS215 were able to degrade 2–7 times more toluene in the presence of oxygen and nitrate than the non-denitrifiers *P. putida* F1, *P. putida* PaW1, and *Burkholderia* (*Pseudomonas*) *cepacia* G4. Similarly, we have recently reported that trichloroethylene was co-oxidized to a greater extent by PKO1 than by G4 under conditions of oxygen limitation imposed by the introduction of lactate, but only when nitrate was available as an alternative electron acceptor [16].

The purpose of the present work is to compare rates of toluene degradation by the toluene-oxidizing bacteria as a function of dissolved oxygen concentration, and to assess the effect of nitrate on degradation rates under these conditions. Chiang et al. [17] have shown that rates of degradation of BTEX compounds in groundwater microcosms are reduced at oxygen concentrations of 2 mg/l or less. However, there are no published reports, to our knowledge, of kinetic studies relating oxygen concentration to the degradation of toluene (or other BTEX compounds) by pure cultures of bacteria. As a consequence, there exists only limited information [18] on the effect of

oxygen limitation on the relative ability of toluene-oxidizing bacteria to utilize toluene. It is therefore unclear to what extent the specific type of toluene oxygenase (i.e. dioxygenase, monooxygenase, or methylmonooxygenase) influences degradation rates or their dependency on oxygen concentration. Moreover, the role of nitrate in enhancing oxygenase-mediated toluene degradation by aerobic denitrifying bacteria warrants further analysis, specifically in terms of determining whether the rate or merely the extent of degradation is increased, and defining the range of oxygen concentrations in which nitrate exerts this effect.

## 2. Materials and methods

### 2.1. Bacterial strains and media

Table 1 lists strains used in this study and relevant strain characteristics. All strains were grown in a basal salts medium (BM [16]).

### 2.2. Toluene degradation assays

The degradation of toluene was measured with resting cells in 160-ml serum bottles containing 150 ml buffer (0.2% NaCl, 40 mM Na/K phosphate buffer, pH 7.1). Potassium nitrate was added to a concentration of 1% (9.9 mM) where appropriate. All experiments were performed at 23°C. Initial oxygen concentrations, varying from approx. 6% (15 µM) to 100% (240 µM) dissolved oxygen saturation, were established by sparging for 15 min with defined proportions of air and N<sub>2</sub> using a gas mixing tube (Gilmont Instruments, Barrington, IL). Serum bottles were crimp-sealed with Teflon-faced butyl stoppers after sparging. A 16 gauge needle which was inserted through the stopper into solution served as a sampling port both for determination of dissolved oxygen concentration, measured with a polarographic needle electrode (Diamond General Corp., Ann Arbor, MI), and for withdrawal of samples for toluene determinations. Abiotic controls showed no net loss of toluene, and no measurable leakage of gas, as evidenced by the constancy of dissolved oxygen concentrations over a period of 20 min, the maximum incubation period used for these assays.

Toluene degradation assays were conducted using a previously described method [16]. This method has been demonstrated to be suitable for the measurement of initial rates of degradation in that mass transfer limitations are not encountered. For the present study, cells were grown aerobically (30°C, 250 rpm) for 18 h in BM with toluene (2.5 mM) supplied in the vapor phase, then washed in potassium phosphate buffer (40 mM, pH 6.8). Washed cells were resuspended in serum bottles to a final  $OD_{425nm}$  of 0.05 for dioxygenase strains, or 0.2 for monooxygenase strains to compensate for their low specific rates of degradation. Experiments were initiated by the addition of toluene (in *N,N'*-dimethylformamide) to yield a final concentration of 200  $\mu$ M for dioxygenase strains, or 100  $\mu$ M for monooxygenase strains to avoid problems of toxicity that were observed for these strains at higher concentrations (data not shown). Serum bottles were immediately resealed, and cell suspensions mixed for 30 s. Samples (1 ml) for toluene determinations were removed using a hypodermic syringe at 1 or 2 min intervals for a time period of 10–20 min. Although dissolved oxygen concentrations were not continuously monitored, oxygen consumption was concluded to have no significant effect on the initial rates of degradation since the progress curves were linear within the periods in which rate determinations were carried out.

### 2.3. Analytical methods

Toluene was quantitated by HPLC using methods described previously [5]. Cellular protein was measured using the Sigma Protein Assay Kit (Sigma Chemical Co., St. Louis, MO) following alkaline digestion of cell pellets at 90°C [27]. The production of nitrite was detected visually using the method of Smibert and Krieg [28].

### 2.4. Treatment of data and statistical methods

Initial degradation rates were calculated by regression analysis of the linear portion of progress curves as described by Tros et al. [29]. A minimum of five data points were used for each rate calculation. Statistical analysis of data was performed using the SAS System for Personal Computers, Release 6.04 [30].

Analysis of variance was done by the PROC GLM procedure. Specific differences among the data were identified using the Student–Newman–Keuls test [31]. The level of significance was established at  $\alpha=0.05$ .

## 3. Results and discussion

The kinetics of toluene degradation as a function of dissolved oxygen concentration were studied in batch experiments with toluene-oxidizing bacteria which degrade toluene via dioxygenase, monooxygenase, or a methylmonooxygenase pathways. The effect of nitrate, an alternative electron acceptor for nitrate-respiring and denitrifying strains, was also examined. Our data show that the relationship between the concentration of oxygen and rates of toluene degradation was complex and highly strain-dependent.

In these experiments, both the initial rate of toluene degradation and the relative effect of oxygen on degradation rate were highly correlated with the type of oxygenase catalyzing the initial step in toluene oxidation. Examination of linear plots of kinetic data reveals that under fully aerobic conditions, i.e. a dissolved oxygen concentration of 240  $\mu$ M, strains which synthesize toluene dioxygenases, F1 (Fig. 1A), CFS215 (Fig. 1B), and W31 (Fig. 1C), degraded toluene at a significantly higher rate than those which synthesize a toluene 2-monooxygenase, G4 (Fig. 1D), a toluene 3-monooxygenase, PKO1 (Fig. 1E), or a xylene methylmonooxygenase, PaW1 (Fig. 1F). Rates were relatively uniform for the dioxygenase strains, ranging from 151 nmol/min per mg for W31 to 166 nmol/min per mg for F1. Corresponding values of 23, 14, and 12 nmol/min per mg were obtained for G4, PKO1, and PaW1, respectively. The degradation of toluene by dioxygenase strains, although more rapid than that of other strains under aerobic conditions, was more greatly affected by the availability of oxygen. As the oxygen concentration was decreased from 240 to 50  $\mu$ M, rates declined approx. 40, 48, and 30% for F1, CFS215, and W31, respectively, but were reduced by less than 10% for G4 and PKO1. An intermediate effect was observed for PaW1, for which the rate of degradation was reduced by 25%. Below 50  $\mu$ M dissolved oxygen, rates decreased dramatically for all strains,

although degradation by all strains was still measurable at the lowest dissolved oxygen concentration tested, approx. 15  $\mu\text{M}$ .

Although it is tempting to speculate that these results may be indicative of a generally high  $V_{\text{max}}$  and a high  $K_s$  for the oxygen-dependent utilization of toluene by dioxygenase systems, and a low  $V_{\text{max}}$  and low  $K_s$  for monooxygenase systems, such constants could not be calculated in view of the unusual kinetics that were observed, as described below. Nevertheless, these findings are consistent with those of Duetz et al. [18], who previously noted physiological differences in the effect of oxygen on toluene utilization by toluene-oxidizing bacteria. In their study, the ability of strains to compete in chemostats under various conditions was at least partially ascribed to differences in toluene degradation pathways. G4, which we have shown to degrade toluene at rates which are less affected by oxygen than F1 or PaW1, was found to outcompete these strains under conditions of oxygen limitation. Taken together, our observations suggest that strains synthesizing toluene dioxygenases and those synthesizing toluene monooxygenases may be adapted to different environments, with dioxygenase strains more suited to the utilization of toluene when oxygen is readily available, and the monooxygenase strains better adapted to conditions of low or fluctuating oxygen concentrations. PaW1, on the basis of its kinetic performance relative to the other strains, seems to be poorly adapted for toluene utilization under either set of conditions. This finding is in agreement with the evi-

dently inferior ability of this strain to compete with strains that oxidize the aromatic ring of toluene, as reported by Duetz et al., who postulated that the widespread occurrence of bacteria with TOL pathways was due to their ability to degrade di-substituted aromatic compounds, rather than to their ability to degrade toluene.

The effect of nitrate on initial rates of toluene degradation was found to be variable and dependent on both the bacterial strain and the concentration of dissolved oxygen. No significant effect was observed for the obligately aerobic strains F1 (Fig. 1A) and PaW1 (Fig. 1F), or for the nitrate-respiring strain G4 (Fig. 1D). G4 did exhibit dissimilatory nitrate reduction, as evidenced by the production of high concentrations of nitrite, which was detected colorimetrically. Rates of toluene degradation by the denitrifying strains W31 (Fig. 1C) and PKO1 (Fig. 1E) were not affected under fully aerobic conditions, or with 82  $\mu\text{M}$  dissolved oxygen in the case of PKO1. However, rates were almost doubled in the presence of nitrate at oxygen concentrations of 9–24  $\mu\text{M}$  for W31, and 15–22  $\mu\text{M}$  for PKO1. Nitrite was detected in all experiments conducted with PKO1 at 82  $\mu\text{M}$  or less dissolved oxygen, while the accumulation of nitrite by W31 was observed only at 24  $\mu\text{M}$ . The results for the denitrifying strain CFS215 (Fig. 1F) differed from those of W31 and PKO1 in that nitrate had no effect on toluene degradation.

Based on these results, it can be concluded that the rate of degradation of toluene under these conditions is increased by the utilization of nitrate as an alter-

Table 1  
Bacterial strains used in this study

Strain	Source or reference	Toluene pathway
Strict aerobes		
<i>Pseudomonas putida</i> F1	D.T. Gibson	2,3-dioxygenase [19,20]
<i>P. putida</i> PaW1	ATCC 33015	xylene monooxygenase [21,22]
Nitrate-respirer <sup>a</sup>		
<i>Burkholderia cepacia</i> G4	P.H. Pritchard	2-monooxygenase [23]
Denitrifiers		
<i>B. pickettii</i> PKO1	[24]	3-monooxygenase [25]
<i>Pseudomonas</i> sp. strain W31	[26]	2,3-dioxygenase [26]
<i>P. fluorescens</i> CFS215	[5]	2,3-dioxygenase (Mikesell, M.D. and Olsen, R.H., unpublished data)

<sup>a</sup>Nitrate-respirers defined as those strains which reduce nitrate to nitrite but do not denitrify.

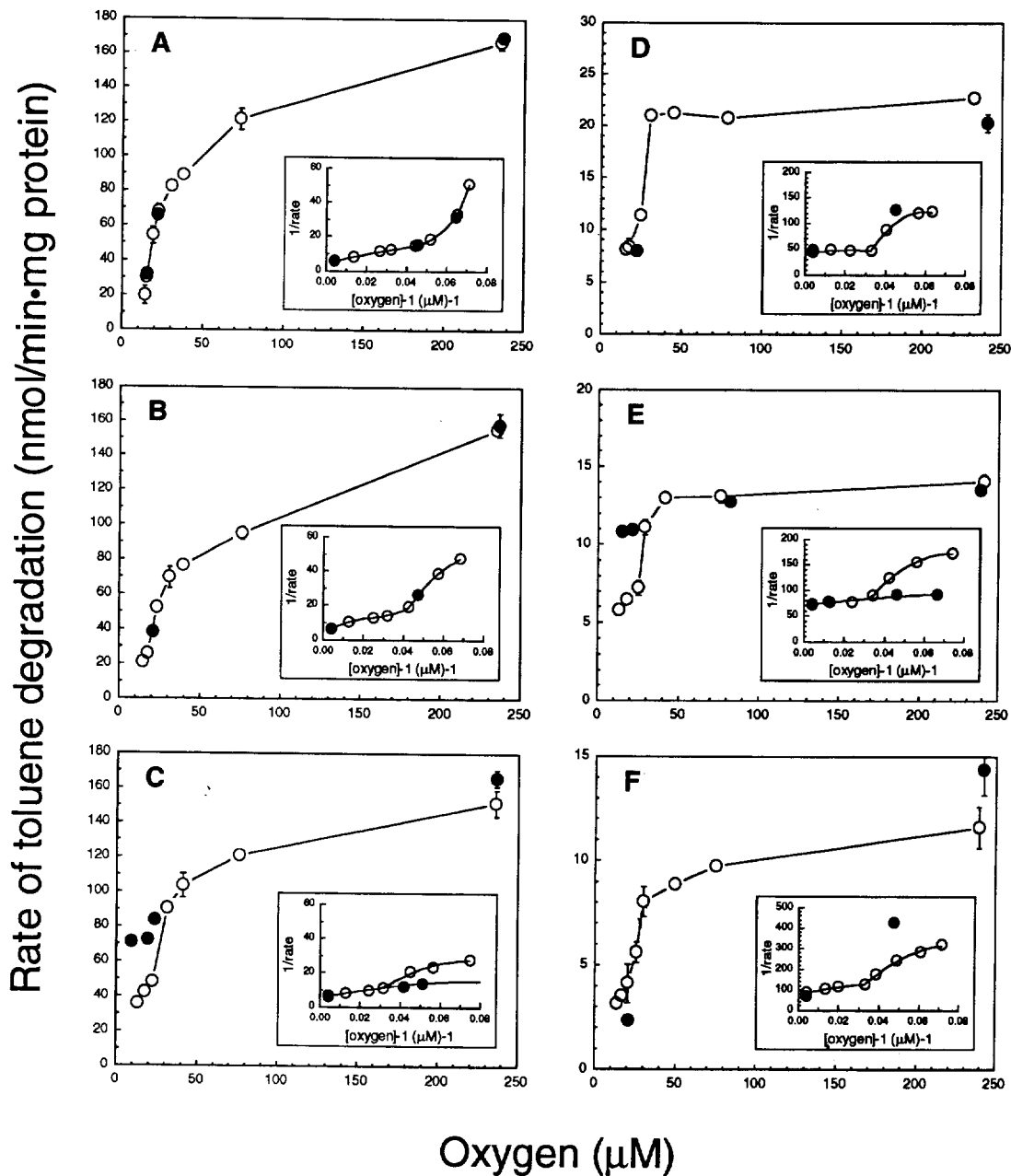


Fig. 1. Kinetics of the initial rate of toluene degradation by (A) *Pseudomonas putida* F1, (B) *Pseudomonas fluorescens* CFS215, (C) *Pseudomonas* sp. strain W31, (D) *Burkholderia cepacia* G4, (E) *Burkholderia pickettii* PKO1, and (F) *Pseudomonas putida* PaW1 as a function of oxygen concentration alone (○), and in the presence of nitrate (●). Error bars represent standard deviations for two independent experiments. Insets show double-reciprocal plots of the data. Note that one of the data points (●) in panel C inset is off-scale.

native electron acceptor by denitrifying, toluene-oxidizing bacteria. We therefore confirm and extend our previous finding that the extent of toluene degrada-

tion is increased by nitrate under the same conditions [15], and the results of other investigators who reported that the biodegradation of BTEX com-

pounds in ground water was improved by the addition of nitrate when oxygen is present [9,11–13]. Nitrate respiration may represent an inadequate alternative mechanism for the dissimilation of nitrate in this regard, since nitrate did not enhance the degradation of toluene by G4 under conditions in which the reduction of nitrate to nitrite was observed. Our results also demonstrate that conditions must be suitable for the induction and activity of denitrification enzymes in order for stimulation of toluene degradation to occur. In the case of PKO1 and W31, it is apparent that denitrification was induced under the aerobic conditions for growth that were employed in this study, and that denitrification occurred in the presence of oxygen. These observations are not without precedent, in that the expression of denitrification enzymes in the presence of oxygen has been previously demonstrated in a number of strains [32–36], and aerobic denitrification is now a well established phenomenon [37]. The failure of nitrate to stimulate rates of toluene degradation for CFS215 contrasts with the results of our previous study [15] showing that, under hypoxic conditions, nitrate increased the extent of toluene degradation by this strain after a period of 4 days, possibly indicating that the induction of denitrification by CFS215 requires a period of growth in a low oxygen environment.

When rate data for toluene degradation as a function of dissolved oxygen concentration was examined using double-reciprocal plots, evidence for biphasic kinetics was obtained. In all cases, the plots are essentially linear at oxygen concentrations above 30  $\mu\text{M}$ . At an inflection point in the data between 20 and 30  $\mu\text{M}$ , however, each of the plots breaks sharply upward, indicating that there exists a 'critical' oxygen concentration below which rates of toluene degradation are inhibited to a greater extent than is predicted by the kinetics observed at higher oxygen concentrations. This region of the plot was generally curved in a concave downward direction, such that degradation rates decrease the most markedly at the inflection point and then begin to level off at the lowest oxygen concentrations tested. Double-reciprocal plots for rates of degradation in the presence of nitrate yielded points that generally fell on or near the plots established using data for rates of degradation in the absence of nitrate. Notably, though, the

rate data for toluene degradation by the denitrifying strains PKO1 and W31 gave rise to linear plots with no inflection, i.e. biphasic kinetics were not observed when nitrate was present. Moreover, these data points were co-linear with the linear plots of corresponding data collected under conditions in which nitrate was not present and the oxygen concentrations were greater than 30  $\mu\text{M}$ . Consequently, for the denitrifying strains which exhibited evidence of nitrate reduction, the inhibition of toluene degradation at oxygen concentrations less than 30  $\mu\text{M}$  was relieved by the presence of nitrate, and this effect resulted in the elimination of the second 'phase' of the biphasic plots such that monophasic kinetics were observed and linear plots obtained.

Given the role of nitrate as an alternative electron acceptor for denitrifying bacteria, these results suggest that the critical oxygen concentration corresponds to the onset of conditions which are electron acceptor limiting for toluene degradation, and that this limitation is removed for denitrifying strains able to reduce nitrate in the presence of oxygen. By inference, then, the activity of the toluene oxygenase must be linked in some way to the activity of the cytochrome oxidase(s), as well as to the activity of denitrification enzymes in denitrifying strains. Since NAD(P)H is a co-substrate for the oxygenase reaction, and its availability is controlled by the rate of respiration via the concerted action of the TCA cycle and the electron transport chain leading to the cytochrome oxidase(s), it can be surmised that when the concentration of oxygen is low, the decreased rate of respiration may mediate a net decrease in the availability of NAD(P)H for the oxygenase reaction, diminishing its rate. For denitrifying strains, the co-respiration of oxygen and nitrate, as previously documented [36,38,39], would be expected to effect concomitant increases in the rate of respiration and in the rate of NAD(P)H regeneration via the TCA cycle, thereby maintaining its availability for the toluene oxygenase reaction. These conclusions mirror those of our prior work [16], in which we showed that the co-oxidation of TCE was limited by the introduction of the electron donor lactate, and that, for the denitrifying strain PKO1, this effect was alleviated by the presence of nitrate. Our findings are also compatible with those of Krooneman et al. [40], who reported two  $K_m$  values for oxygen

uptake of an *Alcaligenes* strain grown on 3-chlorobenzoate. They concluded, on the basis of the KCN-sensitivity of uptake at low oxygen concentrations, that the low  $K_m$  represented oxygen consumption via respiration, while the high  $K_m$  reflected the use of oxygen by oxygenases. In contrast, other investigators have suggested that oxygen limitation of the utilization of aromatic compounds is primarily the consequence of the activity of oxygenases [41] or the cytochrome oxidase(s) [42]. In those studies, however, the conclusions were based solely on a comparison of kinetic constants with literature ' $K_{do}$ ' values, and double-reciprocal plots of the data were either not presented [41], or did provide some indication of biphasic kinetics as we have described [42].

In conclusion, the results of the present study suggest that the degradation of toluene via monooxygenase pathways and the ability to denitrify under aerobic conditions represent independent adaptations for the utilization of toluene by aerobic bacteria in low oxygen environments. These findings support the use of nitrate in mixed electron acceptor regimes as a feasible strategy for the in situ bioremediation of contaminated ground water, as discussed earlier. Moreover, the available evidence indicates that bioremediation efforts can be improved by more targeted approaches to exploit the physiological traits of the toluene-oxidizing bacteria to their best advantage. Such approaches may include, for example, the introduction of appropriate monooxygenase strains for in situ applications in which oxygen is limiting, and the use of dioxygenase strains for bioreactors, in which oxygen concentrations can be maintained at high levels to support the higher maximum rates of degradation exhibited by these strains. It should be emphasized that additional work is clearly necessary to confirm the relevancy of the present study to the biodegradation of toluene in these types of environments.

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