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Metabolism of 2,4-dinitrotoluene (2,4-DNT) by *Alcaligenes* sp. JS867 under oxygen limited conditions

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Abstract

Transformation of 2,4-dinitrotoluene (2,4-DNT) by *Alcaligenes* JS867 under varying degrees of oxygen limitation was examined. Complete 2,4-DNT removal was observed under oxygen excess with near stoichiometric release (83%) of nitrite. Average kinetic parameters were estimated based on a dual-Monod biokinetic model with 2,4-DNT and O_2 as growth limiting substrates. The negative impact of nitrite accumulation on the reaction rate was adequately described by inclusion of a noncompetitive inhibition term for NO_2^-. Under aerobic conditions, μ_{max}, K_{sDNT}, and K_{iNO} were 0.058(0.004) hr^{-1}, 3.3($\pm$1.3) mg 2,4-DNT/L, and 1.2($\pm$0.2) hr^{-1}, respectively. At increasing oxygen limitation, rates of 2,4-DNT disappearance and nitrite production decreased and incomplete removal of 2,4-DNT commenced. JS867 was able to use NO_2^- as a terminal electron acceptor when grown on glucose or succinate under anaerobic conditions. However, during growth on 2,4-DNT and under O_2-limited conditions, JS867 did not use released nitrite as electron acceptor. The nearly constant molar ratios of DNT removed over NO_2^- released under various degrees of oxygen limitation suggested that oxygenolytic denitration pathways continued. No evidence of nitroreduction was obtained under the examined oligotrophic conditions. JS867 displayed a high affinity for oxygen consumption with K_{SO_2} value of 0.285($\pm$0.198) mg O_2/L. Our results indicate that under oligotrophic conditions with 2,4-DNT as dominant carbon source, oxygen availability and nitrite accumulation may limit 2,4-DNT biomineralization, but the accumulation of reduced 2,4-DNT transformation products will be small.

Introduction

Dinitrotoluenes (DNTs) are anthropogenic compounds synthesized as precursors in the manufacturing of commodity chemicals such as diisocyanate and the explosive trinitrotoluene (TNT). Contamination of soil and groundwaters with DNTs near TNT manufacturing plants, has prompted an interest in their cleanup, because dinitrotoluenes are toxic, mutagenic and potentially carcinogenic (Rickert et al. 1984). DNTs occur as isomeric mixtures of 2,4-DNT and 2,6-DNT in a ratio of approximately 4 : 1 (Popp and Leonard 1985).

Under aerobic conditions, 2,4-DNT and 2,6-DNT are mineralized by specific bacterial cultures as sole carbon and nitrogen source, and both the biochemistry and molecular biology has been studied in fair detail (Spanggord et al. 1991; Haigler et al. 1994; Spain 1995). Key in the mineralization pathway is the presence of several oxygenases that are involved in denitration and ring cleavage (Spain 1995). Hence, molecular oxygen is required as a reactant for mineralization. Three moles of oxygen are needed as oxygenase substrates for NO_2^- elimination and ring cleavage; additional oxygen may be required as a terminal electron acceptor (TEA) for the complete mineralization of 2,4-DNT. Therefore, large amounts of oxygen are needed to completely mineralize 2,4-DNT. The ultimate products of aerobic mineralization of DNT are carbon dioxide, water, and nitrite. In natural settings, nitrite is biochemically oxidized to nitrate by ubiquitous nitrifying bacteria, and the end products of aerobic biodegradation of DNT are therefore completely innocuous to human and ecological receptors. Using isolates and enrichments capable of 2,4-DNT and 2,6-DNT mineralization, successful treatment of DNT-laden waters and soils has thus been attained (Lendenmann et al. 1998; Nishino et al. 1999).

Under anaerobic conditions, mineralization of DNTs has not been demonstrated to date; however biotransformations readily occur that involve unspecific reduction of the nitro groups to nitroso, hydroxylamino, and eventually amine groups (McCormick et al. 1978; Liu et al. 1984; Cheng et al. 1996; Noguera and Freedman 1996; Lotrario and Woods 1997; Noguera and Freedman 1997). The potential accumulation of the highly reactive nitroso and hydroxylamino intermediates, which are more toxic than the parent compounds, plagues anaerobic processes for DNT biotransformation. Interestingly, such nitro-reduction has also been observed under aerobic conditions, although this seems contingent on the presence of abundant supply of primary carbon and energy sources (Noguera and Freedman 1996).

Because the O_2 flux into DNT laden aquifer settings may be low, it is imperative to study how O_2 limitation impacts the fate of DNT. On the one hand, the limit of oxygen presents a stoichiometric limitation to DNT transformation because of the need for O_2 as an oxygenase reactant. On the other hand, it is possible that bacterial strains may optimize their use of O_2. Recognizing that NO_2^- can serve as a TEA, if DNT mineralizing strains are facultative denitrifiers, they may preferentially use NO_2^- as TEA and thus maximize their use of O_2 for DNT mineralization. Such phenomenon has been observed in the case of BTEX mineralization under nitrate reducing conditions (Kukor and Olsen 1996). A more subtle result of O_2 limitation is the fact that the rates of NO_2^- to NO_3^- oxidation may be reduced, and the accumulation of NO_2^-, a known microbial inhibitor (Almeida et al. 1995; Sijbesma et al. 1996; Stein and Arp 1998), may impair DNT mineralization. Finally, it is plausible that under O_2 limiting conditions, the lack of terminal electron acceptors may result in the aspecific reduction of DNT, as occurs under anaerobic conditions.

The goal of this research was, then, to evaluate the fate of 2,4-DNT when an aerobic 2,4-DNT mineralizing strain, *Alcaligenes* JS867, was cultivated under oxygen limited conditions. Three specific objectives were: (1) evaluate the denitrification potential of JS867, (2) examine the effects of limiting dissolved oxygen conditions on kinetics and stoichiometry of 2,4-DNT transformation, (3) examine and quantify the inhibitory effects of nitrite on 2,4-DNT transformation.

Materials and methods

Bacterial inoculum

Strain JS867 was obtained from Shirley Nishino at AFRL/MLQR(Tyndall AFB, FL) and was closely related to *Alcaligenes xylosoxidans* (via 16S rDNA ribotyping) and *A. xylosoxidans* subsp. *denitrificans* (via Biolog ID)(Nishino, personal communication). Cultivation was at 30 °C on solid or in liquid defined media free of inorganic nitrogen, termed BLKN (Bruhn et al. 1987) supplemented with 2,4-DNT (3 mM) as sole carbon and nitrogen source. Maintenance media contained sorptive XAD-7 beads (10 g/L; Sigma, St. Louis, MO) to maintain a sub-toxic, long-term aqueous supply of DNT.

HPLC analysis

2,4-DNT concentrations were measured via isocratic high performance liquid chromatography (Varian Instruments, Palo Alto, CA) separation on a C6-hexyl column (Spherisorb, Alltech, Deerfield, IL) and UV-detection at 254 nm. Eluent was diH_2O : methanol (50 : 50) delivered at 1 ml/min and 10 μL sample volumes were injected; retention were recorded for 2,4-DNT (10.3 min), 2-amino-4-nitrotoluene (7.3 min) and 2-nitro-4-aminotoluene (6.4 min). The method's linear dynamic range was from 0.001 to 0.3 mM 2,4-DNT.

Nitrite analysis

Nitrite analysis was performed by a modification of the sulfanilamide standard method (Gerhardt et al. 1994): 0.4 ml of aqueous sample was mixed with 0.4 ml of sulfanilamide (1% (w/v) 10% HCl (v/v)) and 4 ml of diH_2O. After 5 min 0.4 ml of N-(napthyl)diethylenediamine (0.04% (w/v)) was added and incubated for 15 min. more before A_{543} was recorded. The method's linear dynamic range was from 0.01 to 0.5 mM.

Denitrification potential

Duplicate experiments were performed to examine nitrate and nitrite reduction. Test tubes containing 10 ml of $\frac{1}{4}$ × TSB (Tryptic Soy Broth; Difco, Detroit, MI) supplemented with either 0.01% $NaNO_3$ or 0.01% $NaNO_2$ and 0.17% bacto-agar were incubated under gentle agitation and inoculated with 0.1 ml of cell cultures grown overnight aerobically in TSB. Aerobic control growth tubes without $NaNO_3$ or $NaNO_2$ or agar were included to verify inoculum viability and negative control tubes were without nitrogen oxides. The test tubes were incubated at 25 °C and examined periodically (2, 4, 8, 12, and 24 hrs) for gas (N_2) formation and NO_2^- concentration.

Batch 2,4-DNT removal experiments

Serum bottles (125 ml) containing 75 ml of BLKN with 2,4-DNT (approx. 30 mg/L) were inoculated with a known cell mass of JS867 pregrown aerobically in BLKN containing 3 mM 2,4-DNT. Bottles were closed with rubber septa and aluminum crimp seals and incubated on a shaker table (150-rpm) at 30 °C. To attain oxygen limited conditions, sealed bottles were purged with a stream of N_2 gas before injecting a microfiltered quantity (5, 2, or 1 ml) of air directly into the liquid phase. Actual oxygen concentrations were measured in replicate bottles, by removing 50 μl of the head space with a gas tight syringe (Hamilton (CR-700-50) Reno, Nevada) and analysis for PO_2 via GC/TCD (HP 5890 Series II).

Aqueous samples (1 ml) were removed periodically with disposable syringes; a fraction of the sample was immediately mixed with MeOH (HPLC grade; Sample : MeOH 70 : 30); followed by 5 min centrifugation at 14,000 rpm (Eppendorf, 5415C) and used for HPLC analysis. Remaining aqueous sample was retained for nitrite analysis.

Use of nitrite released from transformation of 2,4-DNT as terminal electron acceptor

Serum bottles (2,4-DNT @13 mg/L) were inoculated with JS867 cells, incubated aerobically and monitored until removal of 2,4-DNT was complete. Upon DNT depletion, glucose or succinate was added at 10 mM and 6 mM from aseptic stock solutions. Bottles were purged with N_2 gas and samples periodically withdrawn for nitrite analysis.

Batch profile fitting

Under conditions of oxygen excess, experimental profiles were fit using a biokinetic model based on typical Monod growth kinetics assuming negligible cell decay and incorporating a non-competitive inhibition term describing the systemic effects of NO_2^- on microbial activity

$$\frac{\mathrm{d}S_{\mathrm{DNT}}}{\mathrm{d}t} = \frac{-\mu_{\max}}{Y\left(1+\frac{S_{\mathrm{NO}}}{K_{\mathrm{iNO}}}\right)}\left(\frac{S_{\mathrm{DNT}}}{K_{S_{\mathrm{DNT}}}+S_{\mathrm{DNT}}}\right)X, \quad (1)$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = -Y\frac{\mathrm{d}S_{\mathrm{DNT}}}{\mathrm{d}t}, \quad (2)$$

$$\frac{\mathrm{d}S_{\mathrm{NO}}}{\mathrm{d}t} = \left(-0.154\frac{\mathrm{d}S_{\mathrm{DNT}}}{\mathrm{d}t}\right) - \left(0.123\frac{\mathrm{d}X}{\mathrm{d}T}\right), \quad (3)$$

with S_{DNT} = 2,4-DNT concentration (mg 2,4-DNT/L), S_{NO} = nitrite concentration (mg NO_2^--N/L), $\mu_{\max}$ = maximum specific growth rate coefficient (hr^{-1}), K_{SDNT} = half-saturation coefficient for 2,4-DNT (mg 2,4-DNT/L), K_{iNO} = nitrite inhibition coefficient (mg NO_2^--N/L), X = biomass concentration (mg DW/L), Y = growth yield coefficient (mg DW/mg 2,4-DNT), t = time (hrs).

The nitrite mass balance (Eq. 3) is the net effect of NO_2^- release due to 2,4-DNT transformation (0.154 mg N/mg DNT) and the assimilation of nitrogen in new biomass (0.123 mg N/mg DW based on an average biomass formula of $C_5H_7O_2N$ (Rittmann and McCarty 2000). Because the biomass concentrations obtained in batch cultures were small (between 1 and 10 mg DW/L) it was not possible to determine them with good precision. Therefore, the biomass concentrations were calculated employing growth yield coefficients that were estimated from the difference between the nitrite available in the removed DNT and the residual nitrite measured at the end of a batch assay.

Under conditions of oxygen limitation, the biokinetic model was expanded to include a Monod type dependency of growth on both the oxygen concentration (Eq. 6) as well as the DNT concentration and an explicit mass balance on oxygen in the system (Eq. 7). Dual Monod expressions have been used successfully to describe growth under dual-substrate limited conditions (Haas 1994). To facilitate oxygen tracking, a mass balance on the total oxygen concentration in the reaction vessel was developed, although the Monod dependency was written in terms of the aqueous phase oxygen concentration. A new term, Ω, measures the total mass of oxygen present in the system, expressed per unit volume of aqueous phase (Naziruddin et al. 1995):

$$\Omega V_L = S_{O_2,aq} V_L + S_{O_2,g} V_G. \tag{4}$$

It can then easily be shown that

$$S_{O_2,aq} = \frac{\Omega}{\left(1 + K_H \frac{V_G}{V_L}\right)}. \tag{5}$$

The governing equation on DNT (Eq. 1) is subsequently modified to include an additional Monod term for the aqueous phase oxygen concentration, which is written in terms of Ω (Eq. 6). The equations for biomass growth (Eq. 2), and NO_2^- evolution remain as before (Eq. 3) under the assumption that NO_2^- is not utilized as a terminal electron acceptor (see Results & Discussion).

$$\frac{dS_{DNT}}{dt} = \frac{-\mu_{max}}{Y\left(1+\frac{S_{NO}}{K_{iNO}}\right)} \left(\frac{S_{DNT}}{K_{S_{DNT}}+S_{DNT}}\right) \left(\frac{\Omega}{K_{SO_2}\cdot\left(1+K_H\frac{V_G}{V_L}\right)+\Omega}\right) X, \tag{6}$$

$$\frac{d\Omega}{dt} = Y^{O_2/\Delta DNT}\left(\frac{dS_{DNT}}{dt}\right), \tag{7}$$

with $S_O = S_{O,aq}$ = aqueous phase oxygen concentration (mg O_2/L), $S_{O,G}$ = gaseous phase oxygen concentration (mg O_2/L), K_{SO_2} = half-saturation coefficient for O_2 (mg O_2/L), K_H = dimensionless Henry's Law coefficient for O_2 (0.031 at 25 °C), V_G = volume of the headspace (0.05 L), V_L = volume of the liquid (0.075 L), Ω = aqueous plus gaseous oxygen mass expressed per unit aqueous volume (mg O_2/L), $Y^{O_2/\Delta DNT}$ = stoichiometric coefficient (mg O_2/mg DNT removed, calculated from mass balance after cessation of substrate removal).

Numerical profiles of 2,4-DNT and NO_2^--N, derived from simultaneous solutions to equations 1–3 or 2–5 were fit to experimental profiles by minimizing the residual sum of squared errors (RSSE) using the Solver® routine in MS EXCEL®.

Results

While assaying the use of nitrate as terminal electron acceptor in batch cultures, the reduction of nitrate typically results in a transient increase of nitrite and its subsequent disappearance in case of nitrite reduction. JS867 reduced nitrate to nitrite, and further reduced nitrite (Figure 1). In addition, nitrite supplied as sole nitrogen oxide was completely reduced by JS867.

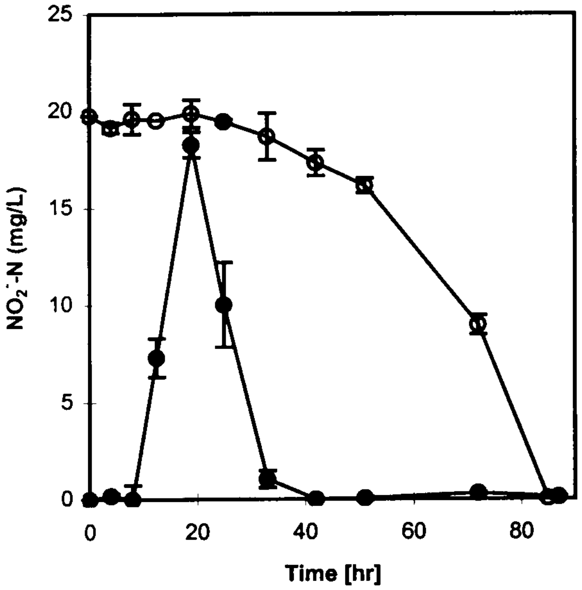

Figure 1. Nitrite profiles in test tubes containing $\frac{1}{4}$ × TSB and NO_3^- (filled circles), or NO_2^- (open circles) at approx. 20 mg N/L inoculated with *Alcaligenes* JS867. Mean and range of duplicate experiments are shown.

When oxygen was in excess, JS867 completely removed 2,4-DNT in approximately 140 hours. Nitrite accumulated to 0.276 mM when 0.165 mM of 2,4-DNT was completely removed (Figure 2). Because no soluble N-species other than NO_2^--N were expected under these conditions (no evidence of oxidation or reduction of NO_2^- to NO_3^- or NH_4^+, respectively and no evidence of accumulation of organic DNT intermediates, see below), a mass balance analysis indicates that approximately 18% of the stoichiometrically available NO_2^- in 2,4-DNT was incorporated in new biomass. Initial attempts to fit the chemical profiles using simple Monod kinetics failed: Such models consistently underpredicted rates at the onset and overpredicted rates near substrate exhaustion. These observations are consistent with product inhibition. Acceptable curve fits were attained using a modified biokinetic model that incorporated a non-competitive inhibition term for NO_2^-. A typical sensitivity analysis was conducted to assess the identifiability of the three kinetic parameters by inspecting the profiles of $\mu_{max}(dS/d\mu_{max})$, $K_S(dS/dK_S)$, and $K_{iNO}(dS/dK_{iNO})$ with time for the employed experimental conditions

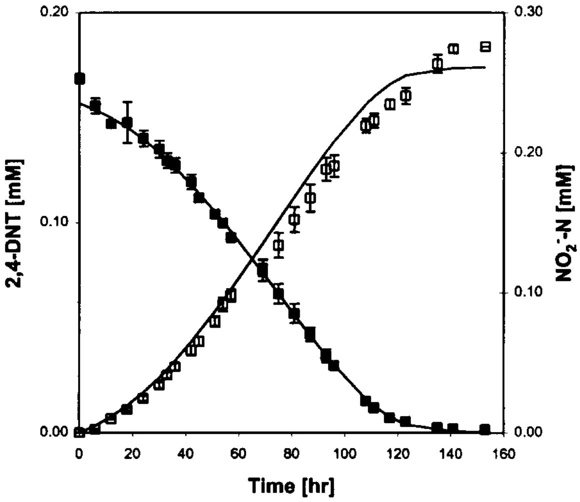

Figure 2. Experimental profiles of NO_2^--N (□) and 2,4-DNT (■) during aerobic batch transformation of 2,4-DNT by *Alcaligenes* JS867 (X_0 = 0.2 mg DW/L). Error bars represent one standard deviation (n = 3). Smooth lines are best-fit profiles.

(Robinson 1985). The sensitivity equations displayed little overlap (results not shown), indicating that the parameters μ_{max}, K_{SDNT} and K_{iNO} had only a small degree of correlation. Thus, the biokinetic parameters could be identified from the experimental profiles (Robinson 1985). Average best fit kinetic parameters were μ_{max} = 0.058(±0.004) hr^{-1}, K_{sDNT} = 3.3(±1.3) mg DNT/L and K_{iNO} = 1.2(±0.2) mg NO_2^- N/L.

To examine whether JS867 was capable of rapidly using nitrite released from 2,4-DNT denitration as a terminal electron acceptor, batch transformation of 2,4-DNT under aerobic conditions was followed with a N_2 purge and spike of either succinate or glucose. Rapid onset of nitrite removal occurred with either carbon source, while in the absence of exogenous carbon source, nitrite was not depleted (Figure 3).

Batch experiments under progressively limiting oxygen conditions were performed to test whether strain JS867 was capable of using nitrite as a terminal electron acceptor while mineralizing 2,4-DNT (Table 1). Nitrite production and 2,4-DNT disappearance occurred under all conditions. Complete removal of 30 mg/L of 2,4-DNT occurred in 62 hours under aerobic conditions (15 mg O_2), while approximately 87.5, 50, and 12.5% of the initial 2,4-DNT was mineralized in bottles that contained 1.6, 0.70, and 0.58 mg O_2, respectively (Figure 4). Inspection of chromatograms did not indicate new transformation products such as aminonitrotoluenes under oxygen limited conditions.

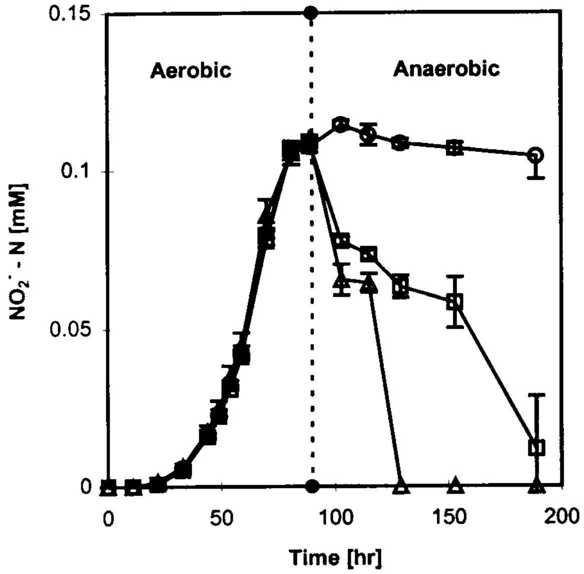

Figure 3. Nitrite profiles from batch experiments evaluating use of NO_2^- as terminal electron acceptor by *Alcaligenes* JS867. After complete removal of 2,4-DNT (≅80 hrs), flasks were purged with N_2 and supplemented with glucose (□, 10 mM) or succinate (△, 6 mM) or not supplemented (○). The maximum theoretical nitrite release was 0.145 mM.

The rates of 2,4-DNT removal and NO_2^- production decreased under increasing oxygen limitation. Approximately 73, 72, 42, and 17.5% of the total amount of NO_2^- stoichiometrically available in 2,4-DNT was released in vessels that were spiked with 15 (excess), 1.6, 0.70, and 0.58 mg O_2, respectively (Figure 4).

Ratios of 2,4-DNT removed to NO_2^- released (Δ2,4-DNT/ΔNO_2^-) under various degrees of oxygen limitation were calculated (Table 2). If denitrification were occurring, an increase in the Δ2,4-DNT/ΔNO_2^- ratio would be expected under oxygen limited conditions due to the use of released nitrite as a terminal electron acceptor. The constant values of the Δ2,4-DNT/ΔNO_2^- from 0.73–0.80 under various degrees of oxygen limitation suggested that denitrification did not occur.

The experimental profiles from the oxygen limited conditions were fit using kinetic parameters μ_{max}, K_S, K_{iNO} retrieved from the profiles obtained during oxygen excess. The half saturation coefficient for oxygen K_{SO_2} was the only additional parameter estimated in the various duplicate oxygen-limited treatments. Curve fits to the oxygen limited experiments explicitly considered oxygen depletion concomitant with DNT removal (Eq. 7). Adequate curve fits were obtained using the aerobic biokinetic parameters, although the

Table 1. Experimental oxygen concentrations under various degrees of oxygen limitation

V_{air} (ml)	$P_{O_2,expt}$ (%)	$[O_2]_g$ (M)	$[O_2]_{aq}$ (M)	Ω, mg O_2/L	Σ Mass O_2 (mg)
50	20.1(±0.2)	8.38×10^{-3}	2.52×10^{-4}	200(±2.6)	15.0(±0.2)
5	2.1(±0.2)	7.76×10^{-4}	2.33×10^{-5}	20.6(±0.15)	1.6(±0.2)
2	0.94(±0.07)	3.89×10^{-4}	1.17×10^{-5}	9.3(±0.05)	0.70(±0.05)
1	0.78(±0.05)	3.23×10^{-4}	9.69×10^{-6}	7.7(±0.04)	0.58(±0.04)

Table 2. Experimental stoichiometric ratios (Mass/Mass) and estimates of the oxygen half saturation coefficient, K_{SO_2}, obtained during oxygen-limited 2,4-DNT transformation by *Alcaligenes* JS867

V_{air} (ml)	ΔDNT/ΔNO_2^-	ΔDNT/$M_{O_2,initial}$	K_{SO_2} (mg/L)	K_{SO_2} (μM)
50	0.73(±0.00)	n/d[1]	n/d	n/d
5	0.76(±0.01)	1.22 (±0.01)	0.083 (±0.03)	2.6
2	0.79(±0.02)	1.68 (±0.10)	0.150(±0.002)	4.7
1	0.80(±0.08)	0.73(±0.02	0.591(±0.116)	18

[1] Not determined.

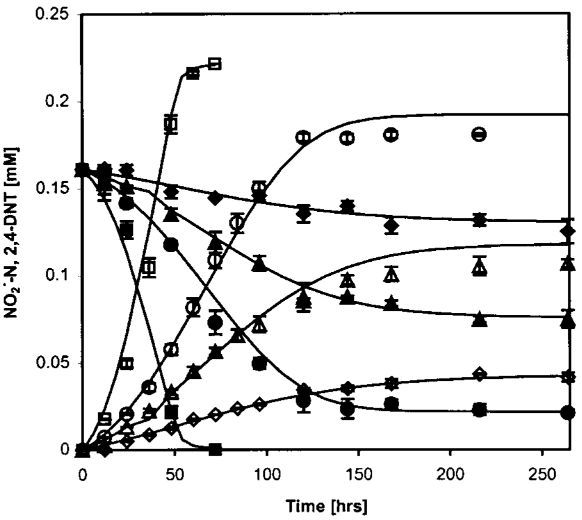

Figure 4. Experimental profiles of 2,4-DNT (closed symbols) and NO_2^--N (open symbols) during batch transformation of 2,4-DNT by *Alcaligenes* JS867 (X_0 = 0.2 mg DW/L) under oxygen limited conditions. Mean and range of duplicate experiments are shown. The volume of air supplied per vessel was in excess (□), 5(○), 2(△), or 1(◇) ml. Smooth lines are best-fit profiles.

K_{SO_2} estimate varied with the degree of oxygen limitation: K_{SO_2} estimates increased slightly with increased degree of oxygen limitation with an overall average K_{SO_2} value of 0.30(±0.23) mg O_2/L (Table 2); a simultaneous fit of all profiles at different oxygen limitations yielded a K_{SO_2} estimate of 0.134 (±0.010) mg/L, although this parameter resulted in very poor curve fits at highest degree of oxygen limitation.

Discussion

Strain JS867 was capable of aerobic growth on 2,4-DNT as sole carbon and nitrogen source, resulting in the release of NO_2^- as sole water soluble transformation product because no aromatic transformation products were observed via HPLC analysis. Complete aerobic mineralization of 2,4-DNT has been demonstrated for an increasingly large set of bacterial isolates (Spanggord et al. 1991; Bausum et al. 1992; Spain 1995). Although 2,4-DNT transformation under aerobic conditions can lead to accumulation of reduced transformation products, such finding has only been demonstrated with strains that cannot mineralize 2,4-DNT and when an excess of readily assimilable primary carbon source is present (McCormick et al. 1978; Liu et al. 1984; Cheng et al. 1996; Noguera and Freedman 1996; Noguera and Freedman 1997). No transformation products were observed with JS867. Our findings indicate that under oligotrophic conditions, reductive transformations of nitro-substituents on nitroarenes may not be obligatory. The observation of reduced transformation products of 2,4-DNT in microcosms derived from DNT contaminated aquifers, therefore, suggest the presence of readily assimilable organic carbon sources (Bradley and Chappelle 1995; Bradley et al. 1997).

An approximate cell yield of 0.22 mg cell DW/mg 2,4-DNT was computed from the incorporation of approximately 18% of the stoichiometrically available NO_2^--N in biomass and a typical pseudo-chemical formula for biomass of $C_5H_7O_2N$. This value exceeds an earlier estimate of 0.07 mg cell VSS/mg 2,4-DNT reported for an aerobic 2,4-DNT mineralizing enrichment culture (Freedman et al. 1996), but is consistent with the yield value of 0.42 mg biomass COD/mg DNT ($\approx$0.3 mg biomass DW/mg DNT) we reported for a continuous fluidized bed biofilm reactor (FBBR) treating a mixture of 2,4-DNT and 2,6-DNT as sole substrates (Lendenmann et al. 1998).

Estimated biokinetic parameters were a $\mu_{\max}$ value of 0.058($\pm$0.004) hr^{-1}, a K_S value of 3.3 ($\pm$1.3) mg 2,4-DNT/L and a NO_2^- inhibition coefficient, K_{iNO}, of 1.2($\pm$0.2) mg NO_2^-/L. These values are consistent with reported values of 0.1 hr^{-1} and 1.82–5.46 mg DNT/L for $\mu_{\max}$ and K_S measured in batch assays for another 2,4-DNT mineralizing strain; although NO_2^- inhibition was not observed (Heinze et al. 1995). However, much lower K_s values were reported in the FBBR study, in the range of 0.029–0.36 mg 2,4-DNT/L depending on the operating conditions (Smets et al. 1999).

In earlier studies, no direct evidence of nitrite toxicity during 2,4-DNT transformation was obtained. The employed phosphate buffer (total analytical molarity of 27×10^{-3} M, pH of 7.2) provided a buffering capacity of 0.0155 mole/L (Stumm and Morgan 1996). This capacity far exceeded the maximum computed proton production of 0.32×10^{-3} mole H^+/L associated with complete transformation of DNT (30 mg/L DNT = 0.16×10^{-3} M DNT and assuming a 2 : 1 NO_2^-/DNT ratio). Thus pH changes during the batch assays could be ruled out. Further, oxygen limitations could be excluded as these experiments were conducted with excess headspace oxygen. Thus, in the current study, nitrite toxicity caused the reaction rates to decrease as the batch transformation of DNT progressed and nitrite accumulated. The use of a biokinetic model that exhibited sensitivity to the nitrite inhibition coefficient allowed us to quantify nitrite inhibition. It is often assumed that the true causative agent is free nitrous acid (FNA) by its action as a protonophore (Almeida et al. 1995). With the acid dissociation constant of nitrous acid (pK_a = 3.32 at 25 °C), and the measured maximum nitrite concentration of 3 to 4 mg/L, a FNA value of 0.5 μg N/L was computed at the medium pH of 7.2. This value is much smaller than other reports for the onset of nitric toxicity of approximately 60 μg N/L (Anthonissen et al. 1976; Almeida et al. 1995). Although this finding suggests a limitation to aerobic mineralization of 2,4-DNT by strains like JS867, it must be recognized that natural and engineered environments typically are not batch systems, and accumulation of nitrite may not be an issue. Furthermore, in natural systems, when O_2 is abundant, a ubiquitous microflora of nitrifying organism converts nitrite rapidly to nitrate as was observed in an earlier FBBR study (Lendenmann et al. 1998).

During oxygen limitation, the amount of removed 2,4-DNT and the amount of released NO_2^- progressively and proportionally decreased. The stoichiometric capability of 2,4-DNT to drive dissimilatory reduction of the released NO_2^--N can be computed as follows. Three moles of oxygen are required per mole of 2,4-DNT as reactant in the initial oxygenase-catalyzed metabolic transformations of 2,4-DNT (Spanggord et al. 1991; Haigler et al. 1994). The theoretical oxygen demand of 2,4-DNT (assuming complete oxidation) is 8 moles O_2 per mole DNT. Therefore, the oxygen needed as terminal electron acceptor solely is 5 mole/mole DNT. The removal of approximately 30 mg/L of 2,4-DNT (under oxygen limited conditions, 75 ml), would therefore require a minimum of 1.2 mg of O_2 as oxygenase reactant. Aromatic ring cleavage results in the formation of 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (Haigler and Spain 1991; Spain 1995). The theoretical complete oxidation of 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid to CO_2 with nitrite as an alternative terminal electron acceptor (i.e. oxidant) would require 8.67 moles of NO_2^- with N_2 as the dissimilatory end point. On the other hand, 2 moles of NO_2^- are produced during the initial oxygenolytic denitration reactions of 2,4-DNT. As a result, if dissimilatory nitrite respiration were established, nitrite reduction would be highly electron acceptor limited and complete reduction of the available nitrite expected. New biomass synthesis would, somewhat, reduce the total amount of nitrite required as an electron acceptor. In our experiments, however, nitrite accumulated and its accumulation was commensurate with the mass of 2,4-DNT removed; this was evidenced by the near constant value of the Δ2,4-DNT/ΔNO_2^- ratio under the various levels of oxygen limitation.

Because nitrite reduction was not observed, and oxygen the sole terminal electron acceptor used, the oxygen limited experiments allowed an estimation of the half-saturation coefficient for oxygen. This estimation was possible because we employed a comprehensive biokinetic model that explicitly considered oxygen depletion (Eq. 7) stoichiometrically to and concomitant with 2,4-DNT removal (Eq. 1, Eq. 6), nitrite production (Eq. 3), and biomass growth (Eq. 2). To our knowledge, this constitutes the first report on oxygen affinity coefficients for the biotransformation of a nitrotoluene. These values varied from 2.6–18 μM; the observed increase in the K_{SO_2} estimate with increased degree of oxygen limitation is surprising. The opposite trend would be expected if physiological adaptation to low oxygen concentrations had occurred. At this point, we cannot explain this observation, which may simply reflect experimental imprecision. The estimated values are in the low range of half-saturation coefficients reported for oxygenases involved in aromatic compound transformation measured with whole cells or isolated enzymes (Krooneman et al. 1996; Kukor and Olsen 1996). However, K_m values for oxygen consumption via respiratory chain are typically in the sub-micromolar range, especially during conditions of low oxygen concentration (Krooneman et al. 1996; Preisig et al. 1996; Jünemann 1997). Therefore, although the oxygenases in JS867 exhibit a high affinity for O_2, oxygenase activity rather than terminal respiration is probably impaired first under oxygen limited conditions.

The accumulation of NO_2^- during the oxygen limited experiments was not due to JS867's inability to use NO_2^- as terminal electron acceptor, because NO_2^- was readily reduced in a complex growth medium (Figure 1), and NO_2^- reduction was readily established after expression of 2,4-DNT transformation (Figure 3), consistent with the tentative identification of JS867 as *A. xylosoxidans* (Busse and Auling 1992). Although assimilative nitrite reduction contributed to the removal of NO_2^-, it must be emphasized that oxygen as terminal electron acceptor must have been absent or at very low concentrations (N_2 flushing in Figure 3 and limited aeration in Figure 1) in these experiments. Hence, any active metabolism was contingent on the use of other terminal electron acceptors, with NO_2^- the only terminal electron acceptor available, and dissimilatory NO_2^- reduction occurred.

Conclusion

In sum, our studies with *Alcaligenes* sp. JS867 demonstrated that aerobic 2,4-DNT mineralizing strains can successfully mineralize 2,4-DNT when O_2 is present in stoichiometric excess. Under hypoxic conditions, rates of 2,4-DNT and total amount of 2,4-DNT mineralization decreased. These trends were adequately described by expanding the biokinetic model to include an oxygen affinity term. J867 has fairly strong affinity for oxygen consumption (K_{SO_2} = 2.6-18 μM). JS867 can perform complete denitrification, but simultaneous 2,4-DNT transformation and NO_2^- reduction under hypoxic conditions, was not observed. Transformation products that result from nitroreduction of 2,4-DNT were not measured under hypoxic conditions, suggesting that such nitroreductase activity may not be prevalent under oligotrophic environments where 2,4-DNT presents the major carbon source. Finally, NO_2^- released from 2,4-DNT transformation has an inhibitory effect on 2,4-DNT removal and may impair 2,4-DNT transformation if persistent.

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