PERGAMON
www.elsevier.com/locate/watres

Wat. Res. Vol. 34, No. 11, pp. 3014–3018, 2000

Printed in Great Britain
PII: S0043-1354(00)00035-X
0043-1354/00/$ - see front matter

EXPRESSION AND LONGEVITY OF TOLUENE DIOXYGENASE IN *PSEUDOMONAS PUTIDA* F1 INDUCED AT DIFFERENT DISSOLVED OXYGEN CONCENTRATIONS

REBECCA K. COSTURA and PEDRO J. J. ALVAREZ*

The University of Iowa, Department of Civil and Environmental Engineering, Iowa City, IA 52242-1527, USA

(First received 1 May 1999; accepted in revised form 1 August 1999)

Abstract—Toluene dioxygenase (TDO) in *Pseudomonas putida* F1 (PpF1) was used as a model to determine if enzymes that initiate aerobic BTEX degradation can be expressed at very low dissolved oxygen (DO) concentrations. Emphasis was placed on characterizing how the DO concentration during induction affects the level of enzyme expression. TDO was expressed at similar levels in PpF1 cells induced on toluene at different DO concentrations (ca. 1, 4, and 8 mg L^{-1}). TDO activity decayed exponentially with cell age at 0.25–0.48 day^{-1}. Nevertheless, the DO concentration during induction did not significantly affect TDO expression or longevity. PpF1 also grew on toluene at 0.1 mg L^{-1} DO, showing that TDO can be expressed at very low DO levels. This suggests that aerobic biotransformations are likely to contribute to BTX bioremediation even when an aerotolerant anaerobic respiration mode (e.g., denitrification) is prevalent. © 2000 Elsevier Science Ltd. All rights reserved

Key words—biodegradation, BTEX, enzyme decay, hypoxic, oxygen, toluene dioxygenase

INTRODUCTION

Priority pollutants such as benzene, toluene, and xylenes (BTX) are often found in oxygen-limited aquifers. Yet, the effect of dissolved oxygen (DO) concentration on the expression, activity, and longevity of oxygenase enzymes (that initiate aerobic BTX catabolism) is poorly understood. Based on a statistical analysis of DO and BTX concentrations at a contaminated site, Chiang *et al.* (1989) suggested that a minimum DO concentration threshold (ca. 2 mg L^{-1}) might exist for aerobic BTX degradation in aquifers. Nevertheless, laboratory studies have shown that some pseudomonads can degrade BTX under hypoxic conditions (DO < 2 mg L^{-1}) using nitrate as an electron acceptor (Olsen *et al.*, 1994, 1995; Kukor and Olsen, 1996). It is not clear if such nitrate-respiring strains used oxygen only as a co-substrate in initial BTX biotransformations, or if oxygen also served as electron acceptor.

There are reports that the DO concentration can affect the synthesis of enzymes for which oxygen is a substrate, although it is difficult to uncouple the effect that oxygen may have on enzyme induction versus enzyme activity or substrate uptake. Several studies have reported slower induction and lower BTX degradation rates for *Pseudomonas putida* in oxygen-limited media (Brazier *et al.*, 1990; Hack *et al.*, 1994; Viliesid and Lilly, 1992). Other researchers, however, have concluded that the rate of catechol 1,2-dioxygenase synthesis by *P. putida* does not change as the DO decreases (Mason, 1994). The interpretation of the effect of DO concentration on BTX degradation activity is confounded by the effect that oxygen has on respiration and growth rates, and on the regulation of gene expression. Hypoxic conditions could reduce oxygen diffusion into the cell and adversely affect aerobic metabolic activity, including the ability to incorporate essential components of the dioxygenase system such as Fe(II). It is plausible, for example, that iron-binding proteins that participate in iron uptake could be coded in an oxygen-sensitive operon whose expression is hindered under hypoxic conditions, as reported for *Escherichia coli* (Kammler *et al.*, 1993).

In this work, we investigated the expression of a widely studied microbial enzyme system, toluene dioxygenase (TDO) of *P. putida* F1 (PpF1), as a model to evaluate aerobic biotransformation potential under DO concentration gradients found in BTX plumes. This enzyme was selected because of its ubiquity in aquifers and its broad substrate range (Gibson *et al.*, 1990). Emphasis was placed

*Author to whom all correspondence should be addressed. Tel.: +1-319-335-5065; fax: +1-319-335-5660; e-mail: pedro-alvarez@uiowa.edu

on determining if TDO can be expressed at low DO concentrations, and on characterizing how the DO concentration during induction affects the level of TDO expression. Thus, TDO activity was measured in whole cells that were induced at different DO concentrations. Because data on enzyme decay rates are extremely scarce (Tros *et al*., 1996), cells were also aged without a carbon source for different periods to characterize the decay of TDO activity. Degradation assays were conducted with whole cells to incorporate potential limitations related to substrate transport into the cell. *In vitro* TDO activity assays were also conducted with cell-free extracts to discern the effect of DO during growth on TDO expression.

MATERIALS AND METHODS

PpF1 cells were induced and grown on toluene vapors in a 5-L BioStat B fermentor (B. Braun, Allentown, PA) equipped with a DO meter and an airflow regulator that controlled the DO concentration in the growth medium. Three different DO concentrations (ca. 1, 4, and 8 mg L^{-1}) were used to grow PpF1. Cells were grown for 8–14 h to a final OD_{600} of 0.5 at 30°C and pH of 6.5–6.9, with longer growth times corresponding to lower DO concentrations. Cells were harvested during exponential growth phase using a J2-MC centrifuge (Beckman, Palo Alto, CA). Pellets were resuspended in modified Hutner's mineral medium (Cohen-Bazire *et al*., 1957) and resting cells were stored at 25°C for different intervals prior to conducting enzyme activity assays.

TDO activity was analyzed in cell-free extracts as described by Jenkins and Dalton (1985). In this assay, cells are broken by ultrasonic treatment and TDO activity is measured as the rate of increase in absorbance at 400 nm (A_{400}) when the cell-free extract reacts with indole. This rate was normalized to the protein concentration in the cell-free extract, which was measured with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) using the Bradford method (Bradford, 1976). These assays were conducted within 24 h of harvesting the cells. Longer resting periods with the tested cell densities decreased TDO activity below detection limits.

In vitro TDO activity measurements ignore potential limitations to BTX degradation associated with mass transport into the cell. Therefore, enzyme activity assays were also conducted with whole cells. Because TDO is the only enzyme capable of initiating toluene degradation in PpF1 (Finette *et al*., 1984), *in vivo* TDO activity was determined as the rate of toluene disappearance normalized to the dry cell weight (dcw) concentration. Toluene degradation assays were conducted in 250 mL bottles containing 50 mL of cell suspension (170 mg L^{-1} dcw), 100 mg L^{-1} of toluene, and 350 mg L^{-1} of chloramphenicol to inhibit *de novo* enzyme synthesis (Cundliffe and McQuillen, 1967). Chloramphenicol prevents confounding effects associated with cell growth, and ensures a constant enzyme concentration during the assay. Toluene was injected through Mininert valves caps (Supelco Inc., Bellefonte, PA) with a 10-μL gas-tight syringe 30 min after chloramphenicol, and the bottles were placed on a rotary shaker at 150 rpm. Aqueous samples were collected with a 500-μL gas-tight syringe, and toluene concentrations were monitored over time by gas chromatography using a 5890 Hewlett Packard apparatus (Palo Alto, CA) equipped with a DB-Wax capillary column (J&W Scientific, Folsom, CA) and a FID detector. Toluene losses from sterile (autoclaved) controls were negligible during the duration of the assay.

Toluene degradation activity assays were thus conducted with previously induced cells, grown under different DO concentrations and stored (i.e., aged) without a carbon source for different periods. DO concentration was only a variable during growth, as *in vivo* assays were conducted under air-saturated conditions (at least 8 mg L^{-1} DO). This eliminated confounding effects associated with differences in O_2 diffusion rates during the assay. Conducting degradation assays at different DO concentrations may have simulated *in situ* conditions better, but it would have complicated discerning the effect of DO on TDO expression. Adding chloramphenicol to induced cells allowed us to isolate this effect because chloramphenicol fixed the TDO concentration within a cell at the level it was induced at a given DO concentration. Thus, the level of TDO expression was not affected when cells were exposed to a higher DO concentration during the assay.

In vivo TDO activity was analyzed statistically as a function of cell age and DO concentration during growth. Analysis of variance was conducted with the statistical software package, SPSS 7.5® for Windows (SPSS Inc., Chicago, IL) to determine if the effects of these two variables were statistically significant. In addition to running assays in triplicate, the experiment was repeated once to evaluate reproducibility.

RESULTS

Toluene-fed PpF1 cells grew under hypoxic conditions (DO < 2 mg L^{-1}) although at slower rates than when oxygen was not limiting. For example, the specific growth rate was 12 day^{-1} at 8 mg L^{-1} DO and 7 day^{-1} at 0.7 mg L^{-1} DO. The growth rate decreased sharply at lower DO concentrations (e.g., 2 day^{-1} at 0.1 mg L^{-1} DO) and no growth was observed at DO < 0.1 mg L^{-1}. Interestingly, extracts from 1-day-old cells grown at 0.7, 3.6, or 6.8 mg L^{-1} DO exhibited similar *in vitro* TDO activities, on the order of 10^{-3} A_{400} min^{-1} $(mg\text{-}protein)^{-1}$ (Table 1). Therefore, although hypoxic conditions resulted in slower growth, this did not hinder the level of TDO expression.

TDO activity was also measured in whole cells grown and induced at different DO concentrations.

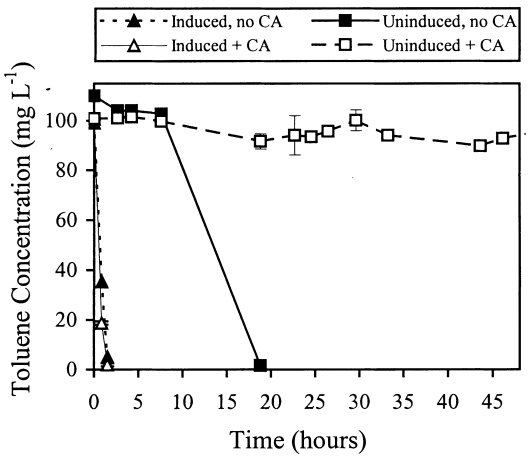

Fig. 1. Inhibition of *de novo* TDO synthesis by chloramphenicol.

Table 1. *In vitro* TDO activity of extracts from PpF1 cells grown on toluene at different dissolved oxygen concentrations. This assay used indole as enzyme substrate (Jenkins and Dalton, 1985). Results depict the mean ± SD from triplicate samples

DO Concentration during growth (mg L^{-1})	Specific TDO activity A_{400} min^{-1} (mg-protein)$^{-1}$ $\times 10^{-3}$
0.7	1.1 ± 0.6
3.6	0.6 ± 0.4
6.8	0.7 ± 0.5

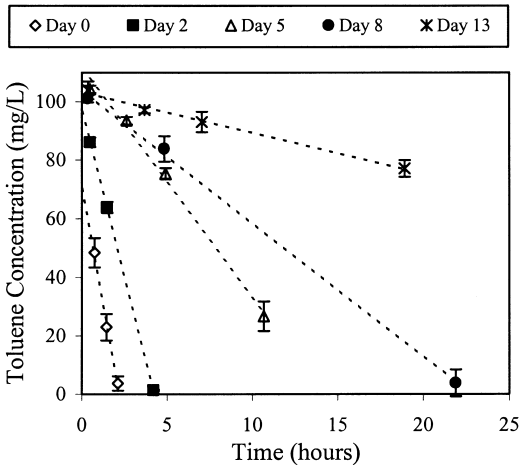

Fig. 2. Decrease in zero-order toluene degradation rates with increasing age of resting PpF1 cells. Data corresponds to cells (170 mg L^{-1} dcw) grown at 1 mg L^{-1} DO, Run A. Error bars represent one SD from triplicate reactors.

In these assays, chloramphenicol (350 mg L^{-1}) prevented *de novo* TDO synthesis when toluene was added without adversely affecting the activity of TDO initially present (Fig. 1). Specifically, previously induced (toluene-grown) PpF1 cells degraded toluene at similar rates with or without chloramphenicol, showing that chloramphenicol did not adversely affect pre-existing TDO activity. In addition, uninduced (pyruvate-grown) cells degraded toluene (after an adaptation period of 8 h) in the absence of chloramphenicol. Uninduced cells, however, could not degrade toluene when chloramphenicol was added to inhibit *de novo* TDO synthesis. Therefore, chloramphenicol eliminated confounding effects associated with potential changes in TDO concentration or activity during the assay and ensured that the measured activity corresponded to the level of TDO expression for the DO concentration under which cells were induced. In addition, the initial toluene concentration was chosen to be high (100 mg L^{-1}) relative to reported values of the half-saturation coefficient for toluene degradation (0.04 to 20 mg L^{-1}) (Alvarez *et al.*, 1991; Button, 1985; Robertson and Button, 1987). This resulted in constant (zero-order) degradation rates (e.g., Fig. 2), which simplified data analysis.

In vivo TDO activity decreased exponentially with cell age, but the DO concentration during induction did not significantly affect TDO activity or longevity (Fig. 3). Table 2 summarizes a regression analysis of TDO decay kinetics for duplicate fermentation runs. The difference in toluene degradation rates between the two runs was statistically significant, despite efforts to reproduce reaction conditions and cell preparation procedures. It is unknown if this difference was due to small variations in the catabolic potential of the different start-up cells, as suggested by Sommer *et al.* (1998). Regardless of why initial conditions differed, two phenomena were reproducible: (1) the exponential decay in TDO activity; and (2) the lack of effect of DO concentration during growth on *in vivo* TDO activity and longevity (Fig. 3). The latter observation corroborates the novel finding shown by *in vitro* assays, that low DO levels during growth do not hinder TDO expression.

DISCUSSION

Although DO concentration gradients commonly develop in contaminated aquifers, little research has been conducted to evaluate aerobic BTX catabolism in these gradients. In this work, PpF1 grew on toluene at DO levels as low as 0.1 mg L^{-1}. Because PpF1 can grow on toluene only if TDO is active (Finette *et al.*, 1984), this constitutes evidence that TDO can be expressed at 0.1 mg L^{-1} DO. This supports the notion that some aerobic BTX biotransformations can occur in hypoxic environments in conjunction with aerotolerant anaerobic respiration

Table 2. Regression analyses of *in vivo* TDO activity assays depicted in Fig. 3. TDO activity decreased exponentially with cell age

Run	DO during growth (mg L^{-1})	Initial (maximum) TDO activity (g-toluene g-dcw^{-1} h^{-1})	Decay coefficient (day^{-1})	R^2
A	1	0.20 ± 0.01	0.25 ± 0.03	0.9845
A	4	0.19 ± 0.01	0.30 ± 0.02	0.9972
A	8	0.20 ± 0.01	0.30 ± 0.01	0.9985
B	1	0.37 ± 0.02	0.44 ± 0.05	0.9946
B	4	0.29 ± 0.01	0.46 ± 0.03	0.9974
B	8	0.33 ± 0.01	0.48 ± 0.01	0.9999

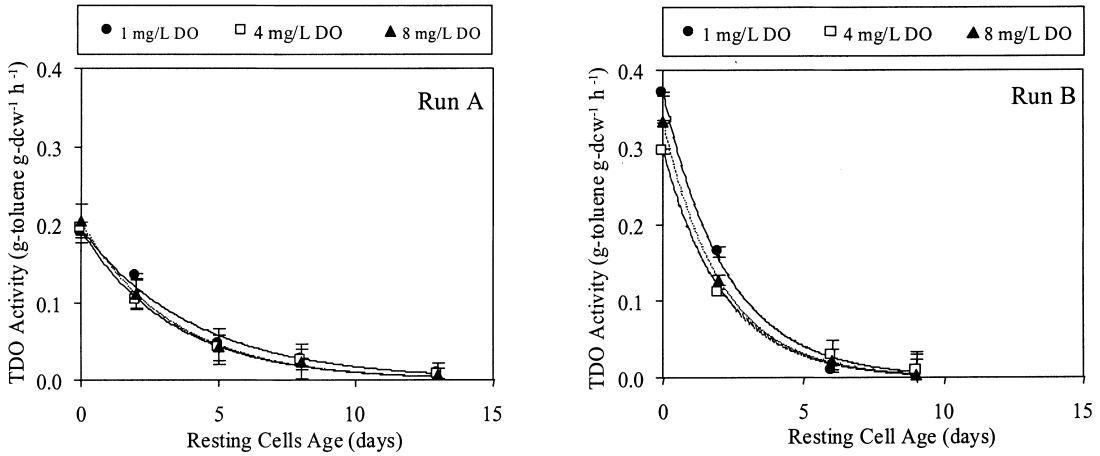

Fig. 3. Exponential decay in specific TDO activity for resting PpF1 cells grown at different DO concentrations. Results of duplicate fermentation runs are depicted. Lines depict non-linear regressions summarized in Table 2. Error bars depict one SE.

modes, such as nitrate-based respiration (Kukor and Olsen, 1996).

The DO concentration during growth did not significantly affect TDO activity or longevity (Fig. 3). Apparently, hypoxic conditions did not hinder the ability of PpF1 to incorporate essential components of the dioxygenase system such as Fe(II). Nevertheless, the fact that higher DO concentrations had no apparent benefit on the level of catabolic enzyme expression does not necessarily mean that attempts to saturate BTX contaminated aquifers with oxygen have limited benefits. The DO concentration can influence other processes that affect contaminant degradation kinetics. For example, other researchers have found that PpF1 exhibits slower trichloroethylene degradation rates under hypoxic conditions (Leahy *et al.*, 1996). In such cases, it seems that slower oxygen diffusion into the cell can limit metabolic processes that affect degradation rates.

Data on enzyme decay rates are extremely scarce. This motivated us to study TDO longevity for resting PpF1 cells grown (and induced) on toluene. The decay coefficients estimated in this work (0.25–0.48 day^{-1}) are larger than typical coefficients for aerobic cell decay (0.06–0.2 day^{-1}) (Corseuil and Weber, 1994; Metcalf & Eddy Inc., 1991). This decrease in activity, however, is not necessarily due to enzyme turnover. TDO is an NADH-dependent enzyme, and NADH depletion by other metabolic processes would hinder its activity. Thus, unlike resting cells that are not fed during storage for bioaugmentation applications, cells that are feeding and regenerating NADH might experience a slower decay in TDO activity. Interestingly, the observed decay in TDO activity is much slower than that reported for other genera expressing methane monooxygenase (Henry and Grbič-Galic, 1991) or biphenyl dioxygenase (Kohler *et al.*, 1988). These activities also decayed exponentially in resting cells, but with coefficients on the order of 2 day^{-1}. Although these other oxygenases also require NADH, it is unknown whether their faster decay in activity was due to differences in cell content of endogenous substrates that might be used to regenerate NADH (e.g., lipids or poly-β-hydroxybutyrate) (Henry and Grbič-Galic, 1991), or to other physiological differences affecting enzyme longevity.

In conclusion, the finding that PpF1 cells grown at about 1 mg L^{-1} DO have comparable TDO expression levels as cells grown in air-saturated medium, show that TDO synthesis is not hindered by hypoxic conditions. These results also suggest that PpF1 is able to incorporate essential components of the TDO system (e.g., Fe(II)) at DO levels as low as 0.1 mg L. Thus, aerobic biotransformations are likely to contribute to BTX bioremediation through a wide range of DO concentrations, even when an anaerobic aerotolerant respiration mode (e.g., denitrification) is prevalent.

Acknowledgements—We thank David T. Gibson who provided *P. putida* F1, Milind Deshpande for his assistance in fermentation runs at the Center for Biocatalysis and Bioprocessing of the University of Iowa, and Mike Vermace, Gene Parkin, Sol Resnick and Peter Adriaens for valuable discussions. This work was funded by the National Science Foundation and the USEPA Office of Exploratory Research.

REFERENCES

Alvarez P. J. J., Anid P. J. and Vogel T. M. (1991) Kinetics of aerobic biodegradation of benzene and toluene in sandy aquifer material. *Biodegradation* **2**, 43–51.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Brazier A. J., Lilly M. D. and Herbert A. B. (1990) Toluene *cis*-glycol synthesis by *Pseudomonas putida*: kinetic

data for reactor evaluation. *Enzyme Microb. Technol.* **12**, 89–94.

Button D. K. (1985) Kinetics of nutrient-limited transport and microbial growth. *Microbiol. Rev.* **49**, 270–297.

Chiang C. Y., Salanitro J. P., Chai E. Y., Colthart J. D. and Klein C. L. (1989) Aerobic biodegradation of benzene, toluene, and xylene in a sandy aquifer-data analysis and computer modeling. *Ground Water* **27**, 823–834.

Cohen-Bazire G., Sistrom W. R. and Stanier R. Y. (1957) Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *Journal of Cellular and Comparative Physiology* **49**, 25–68.

Corseuil H. X. and Weber Jr W. J. (1994) Potential biomass limitations on rates of degradation of monoaromatic hydrocarbons by indigenous microbes in subsurface soils. *Water Research* **28**, 1415–1423.

Cundliffe E. and McQuillen K. (1967) Bacterial protein synthesis: the effects of antibiotics. *J. Mol. Biol.* **30**, 137–146.

Finette B. A., Subramanian V. and Gibson D. T. (1984) Isolation and characterization of *Pseudomonas putida* PpF1 mutants defective in the toluene dioxygenase enzyme system. *J. Bact.* **160**, 1003–1009.

Gibson D., Zylstra G. J. and Chauhan S. (1990) Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1. In *Pseudomonas*: Biotransformations, Pathogenesis, and Evolving Biotechnology, ed. S. Silver, pp. 121–132. American Society for Microbiology, Washington, DC.

Hack C. J., Woodley J. M., Lilly M. D. and Liddell J. M. (1994) The production of *Pseudomonas putida* for the hydroxylation of toluene to its *cis*-glycol. *Appl. Microbiol. Biotechnol.* **41**, 495–499.

Henry S. and Grbič-Galic D. (1991) Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. *Appl. Environ. Microbiol.* **57**, 236–244.

Jenkins R. O. and Dalton H. (1985) The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. *FEMS Microbiology Letters* **30**, 227–231.

Kammler M., Schon C. and Hantke K. (1993) Characterization of the ferrous iron uptake system of *Escherichia coli*. *J. Bacteriol.* **175**, 6212–6219.

Kohler H.-P., Kohler-Staub D. and Focht D. D. (1988) Cometabolism of polychlorinated biphenyls: enhanced tranformation of aroclor 1254 by growing bacterial cells. *Appl. Environ. Microbiol.* **54**, 1940–1945.

Kukor J. J. and Olsen R. H. (1996) Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. *Appl. Environ. Microbiol.* **62**, 1728–1740.

Leahy J. G., Byrne A. M. and Olsen R. H. (1996) Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. *Appl. Environ. Microbiol.* **62**, 825–833.

Mason J. R. (1994) The induction and repression of benzene and catechol oxidizing capacity of *Pseudomonas putida* ML2 studied in perturbed chemostat culture. *Arch. Microbiol.* **162**, 57–62.

Metcalf and Eddy Inc (1991) *Wastewater Engineering Treatment, Disposal, and Reuse*. McGraw-Hill Inc, New York, NY.

Olsen R. H., Mikesell M. D. and Kukor J. J. (1994) Enumeration and characterization of BTEX-degrading bacteria from hypoxic environments functional with mixed electron acceptors. *Res. Microbiol.* **145**, 47–49.

Olsen R. H., Mikesell M. D., Kukor J. J. and Byrne A. M. (1995) Physiological attributes of microbial BTEX degradation in oxygen-limited environments. *Environmental Health Perspectives* **103**, 49–51.

Robertson B. R. and Button D. K. (1987) Toluene induction and uptake kinetics and their inclusion in the specific-affinity relationship for describing rates of hydrocarbon metabolism. *Appl. Environ. Microbiol.* **53**, 2193–2205.

Sommer H. M., Spliid H., Host H. and Arvin E. (1998) Examination of reproducibility in microbiological degradation experiments. *Biodegradation* **9**, 65–82.

Tros M. E., Schraa G. and Zehnder A. J. B. (1996) Transformation of low concentrations of 3-chlorobenzoate by *Pseudomonas* sp. strain B13: kinetics and residual concentrations. *Appl. Environ. Microbiol.* **62**, 437–442.

Viliesid F. and Lilly M. D. (1992) Influence of dissolved oxygen tension on the synthesis of catechol 1,2-dioxygenase by *Pseudomonas putida*. *Enzyme Microb. Technol.* **14**, 561–565.