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Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) in soil inoculated with *Pseudomonas cepacia* DBO1(pRO101), *Alcaligenes eutrophus* AEO106(pRO101) and *Alcaligenes eutrophus* JMP134(pJP4): effects of inoculation level and substrate concentration

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Abstract

Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) by two *Alcaligenes eutrophus* strains and one *Pseudomonas cepacia* strain containing the 2,4-D degrading plasmids pJP4 or pRO101 (= pJP4::Tn1721) was tested in 50 g (wet wt) samples of non-sterile soil. Mineralization was measured as ^{14}C-CO_2evolved during degradation of uniformly-ring-labelled ^{14}C-2,4-D. When the strains were inoculated to a level of approximately 10^8 CFU/g soil, between 20 and 45% of the added 2,4-D (0.05 ppm, 10 ppm or 500 ppm) was mineralized within 72 h. Mineralization of 0.05 ppm and 10 ppm 2,4-D by the two *A. eutrophus* strains was identical and rapid whereas mineralization by *P. cepacia* DBO1(pRO101) occurred more slowly. In contrast, mineralization of 500 ppm 2,4-D by the two *A. eutrophus* strains was very slow whereas mineralization by *P. cepacia* DBO1 was more rapid. Comparison of 2,4-D mineralization at different levels of inoculation with *P. cepacia* DBO1(pRO101) (6×10^4, 6×10^6 and 1×10^8 CFU/g soil) revealed that the maximum mineralization rate was reached earlier with the high inoculation levels than with the low level. The kinetics of mineralization were evaluated by nonlinear regression analysis using five different models. The linear or the logarithmic form of a three-half-order model were found to be the most appropriate models for describing 2,4-D mineralization in soil. In the cases in which the logarithmic form of the three-half-order model was the most appropriate model we found, in accordance with the assumptions of the model, a significant growth of the inoculated strains.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, CFU – colony forming units, PTYG – peptone, tryptone, yeast & glucose, DPM – disintegrations per minute

Introduction

As microbial degradation plays a major role in the elimination of xenobiotics in the environment (Alexander 1981), there is growing interest in the possibility of using introduced strains of xenobiot-

ic-degrading organisms to eliminate specific compounds. Although several bacteria and micro fungi able to degrade halogenated organic compounds under laboratory conditions have been isolated during the last decade (Ghosal et al. 1985; Chaudhry & Chapalamadugu 1991), few strains have been tested for their ability to degrade these compounds in natural soils. Short et al. (1990) found that inoculation of soil contaminated with 500 ppm 2,4-D with 10^9 colony forming units (CFU) per g soil *Pseudomonas putida* PPO301(pRO101), *P. putida* PPO301(pRO103) and *Alcaligenes eutrophus* JMP134(pJP4) enabled radish seeds to germinate in the soil three weeks later. Kilbane et al. (1983) found that inoculation of soil containing 500–20,000 ppm 2,4,5-trichlorophenoxyacetic acid with 5×10^7 CFU/g soil of *Pseudomonas cepacia* AC1100 resulted in 90% degradation of the compound within six weeks, whereas 2,4,5-trichlorophenoxyacetic acid persisted in non-inoculated soil. Most studies of xenobiotic-degrading organisms have been undertaken using either only one inoculation level or only one concentration of contaminant, even though the possible success or failure of laboratory-cultivated strains in contaminated soils depends heavily on both parameters (Goldstein et al. 1985; Ramadan et al. 1990).

In the present study we examined the effect of substrate concentration and inoculation level on the kinetics of 2,4-D mineralization by *A. eutrophus* AEO106(pRO101) (Harker et al. 1989), *A. eutrophus* JMP134(pJP4) (Don & Pemberton 1981) and *Pseudomonas cepacia* DBO1(pRO101) (Harker et al. 1989). All three strains are related in that they all contain the pJP4 plasmid or a derivative of it that enables them to mineralize 2,4-D in batch cultures. Although they use the same pathway to degrade 2,4-D, the strains differ by insertion of a transposon at a locus that is not essential for the expression of the tfd genes (i.e. 2,4-D genes) (Harker et al. 1989) or by the host environment of the plasmid.

The mathematical models most frequently used in studies of xenobiotic mineralization kinetics are derived from the Monod equation (Simkins & Alexander 1984, 1985). However, Scow et al. (1986) found that none of these models adequately describe mineralization kinetics in soil. In the present study we compared the three-half-order model (Brunner & Focht 1984), which was delevoped for soil, with other models applied to the study of mineralization kinetics in soil (Scow et al. 1986). The results obtained by model fitting are discussed in relation to growth of the strains.

Materials and methods

Soil system

A sandy loam which had not been sprayed with pesticides for at least 25 years was collected from an experimental farm in Roskilde, Denmark. The soil characteristics were: pH-H_2O: 6.5, cation exchange capacity: 8.4 meq/100 g dry wt, organic matter: 2.4%, clay 10.9%, silt: 30.7%, fine sand: 31.9%, coarse sand: 24.1%. The soil was passed through a 4 mm sieve on the day of collection and thereafter stored refrigerated in the dark at its natural moisture content for a maximum of 90 days. Three days prior to use, 50 g wet wt aliquots of soil were transferred to 300 ml Erlenmeyer flasks which were then sealed with a rubber stopper in order to prevent soil desiccation, and stored at 22 °C in the dark. After addition of bacteria and 2,4-D the soil in each system was adjusted to a final moisture content of 20.4% of dry wt, corresponding to a water potential, ψ, of -1.1 kPa.

Bacterial strains

The following three 2,4-D-degrading strains were used: *Alcaligenes eutrophus* JMP134(pJP4), a naturally-occurring soil bacteria containing a 2,4-D degrading plasmid (Don & Pemberton 1981), *Alcaligenes eutrophus* AEO106(pRO101) a cured derivative of JMP134 transformed with pRO101, a plasmid constructed by inserting the transposon Tn1721 on pJP4 at a place not disturbing the expression of the tfd genes (Harker et al. 1989), and *Pseudomonas cepacia* DBO1(pRO101), which was

constructed by transforming the plasmid-free strain DBO1 with plasmid pRO101 (Harker et al. 1989). The pJP4-carrying strain was obtained from D. Dwyer, GBF, Braunschweig, Germany, and the two pRO101-carrying strains from R.H. Olsen, University of Michigan, USA.

Soil inoculation

One ml aliquots of stock cultures kept at −80 °C were thawed at room temperature and transferred to flasks containing 20 ml of Luria Bertani (LB) medium (Maniatis et al. 1982). The flasks were shaken for 18 h at 150 rpm at 30 °C. The late log phase culture was harvested, washed twice in phosphate buffer (0.015 M pH = 7.4), and resuspended in buffer at a final concentration of approximately 5×10^9 CFU/ml. One ml of inoculum, or buffer in the case of controls, was carefully mixed with the soil using a sterile rod and the flasks kept overnight prior to amendment with 2,4-D. The population in each system was determined on agar plates immediately prior to amendment with 2,4-D and at intervals during mineralization as described in the accompanying article (Jacobsen & Pedersen 1992): briefly the two strains containing pRO101 were plated on agarplates containing tetracycline to control the indigenous flora, while the strain containing pJP4 was plated on unselective agar plates and detected by colony morphology. Each assay was conducted using triplicate soil systems.

Amendment of soil with 2,4-D

Uniformly (^{14}C)-ring-labeled 2,4-D (sp. act. 46 mCi/mmol; purity of 99%, as determined by TLC) (Amersham, Buckinghamshire, UK) was mixed with unlabeled 2,4-D (Sigma Chem. Co., St. Louis, MO, USA) in 0.1 M Na_2HPO_4. Although several 2,4-D concentrations were used, the same amount of labeled 2,4-D (28,000 dpm/g wet wt soil, 33,700 dpm/g dry wt soil), was added to each soil system, the 2,4-D or buffer being carefully mixed with the soil using a sterile pipette. The final 2,4-D concentrations used were 0, 0.06, 12 and 602 mg/kg dry soil (0, 0.05, 10 and 500 ppm wet wt) in the experiments comparing mineralization in the 3 strains, and 0, 0.07, 14 and 723 mg/kg soil (0, 0.06, 12 and 600 ppm wet wt) in the experiment comparing different inoculation levels of *P. cepacia* DBO1(pRO101).

Measurement of mineralization

The $^{14}CO_2$ evolved during mineralization of ^{14}C-2,4-D was trapped in KOH-containing glassfiber filters (Whatman GF/C) suspended in the flasks by a hook in the rubber stopper. The filters contained 0.2 ml 1N KOH, this being enough to absorb all the $^{14}CO_2$ released from the soil in a 20 h period (NB Hendriksen, personal communication). The filters were initially changed every hour, subsequently every 8 h. The efficacy of the system to trap the evolved $^{14}CO_2$ was tested by comparing results obtained when the filters were changed every hour or every 6 h as the possibility exists that some $^{14}CO_2$ might be lost by air exhange during the approximately 30 sec. that the flasks are open during the changing. This was tested in a *P. cepacia* DBO1(pRO101) inoculated soil system containing 10^6 CFU/g of soil and 10 ppm 2,4-D; no significant difference was seen at 5% level. Immediately after removal the filters were tranferred to a 20 ml scintillation vial containing 5 ml of Ready Protein® scintillation fluid (Beckman, Fullerton, USA). The vials were shaken at 30 rpm for at least 4 h to suspend the filters, allowed to stand for at least 4 h and then counted using a Packard TRI-CARB 460 CD Liquid Scintillation System (Packard, USA). In order to verify that the counting efficiency of the Ready Protein scintillation mixture was not influenced by the 0.2 ml 1 N KOH present in the filters, a quench correction curve was prepared using 50 µl, 100 µl, 200 µl, 300 µl, 400 µl and 500 µl of 1 N KOH in vials containing dry filters, an equal amount of ^{14}C, and 5 ml of scintillation fluid. Counting efficiency was not influenced by the presence of 200 µl 1 N KOH, and was only significantly decreased in the presence of 500 µl 1 N KOH.

Analysis of data

Colony counts were averaged and converted to CFU/g and log CFU/g. The latter data were analyzed using routines in SAS/STAT version 6.03 (Statistical Analysis Systems, SAS Institute, Cary, NC, USA). The effects of 2,4-D amendment on bacterial survival was analysed by analysis of variance (proc. ANOVA).

Nonlinear regression analysis was performed on all $^{14}CO_2$ evolution data (after subtraction of background counts) by the curve fitting program in Fig.P. (Biosoft, Cambridge, UK). This program fits curves to data by minimizing the least squares of the differences between the data and the model curve by the Marquart algorithm.

The data were analysed using five different models chosen according to their appropriateness for describing mineralization processes in soil, or their simplicity (Robinson 1985; Simkins & Alexander 1984; Scow et al. 1986; Focht & Brunner 1985).

The models chosen were:

The first-order kinetic model:

$$P = So[1 - \exp(- k \times t)] \quad (1)$$

where P is product formation (i.e. CO_2 in the present study), and So is initial substrate concentration. This model is relevant in cases where the inoculated strain is not expected to grow.

The logarithmic kinetic model:

$$P = So + Xo[1 - \exp(k \times t)] \quad (2)$$

where Xo is equal to $Bo \times q$ as described by Simkins and Alexander (1984) where Bo is the initial cell density and q is the cell quota or inverse yield.

The two compartment model:

$$P = So_1[1 - \exp(k_1 \times t)] + So_2[1 - \exp(k_2 \times t)] \quad (3)$$

which was developed by Scow et al. (1986) to describe CO_2 evolution during mineralization of compounds in soil. This model is a 'double' first-order model where the variables and parameters So_1 and k_1 and So_2 and k_2 are for compartments 1 and 2, respectively. Compartment 1 and 2 could, for example, be bound and free compounds in the soil.

The three-half-order kinetic model:

$$P = So\{1 - \exp[(- k_1 \times t) - (k_2 \times t^2)/2]\} + k_0 \times t \quad (4)$$

which was developed (Brunner & Focht 1984) specifically to describe CO_2 evolution during mineralization in soil. k_1 is a proportionality constant per unit time, k_2 is the rate of increase of the first-order rate constant with time, and k_0 is a zero-order rate constant. The model is designated the linear form of the three-half-order model since it assumes either linear- or zero-growth of the investigated population. The v_m and t_m values can be calculated as follows:

$$t_m = (-k_1 + \sqrt{k_2})/k_2 \quad (4a)$$

$$v_m = S_0\sqrt{k_2}(\exp(k_1^2/2 \times k_2 - 1) + k_0 \quad (4b)$$

Focht and Brunner (1985) simplified the logarithmic form of the three-half-order model by eliminating the k_1 term since they found it was insignificant in all cases where substantial growth of the investigated organism occurs. The truncated logarithmic form of the three-half-order model can thus be given as:

$$P = S_0(1 - \exp(\exp((-\mu \times t_m) \times (1 - \exp(\mu \times t))) + k_0 \quad (5)$$

The v_m term can be calculated as:

$$v_m = S_0 \times \mu \times \exp(\exp(-\mu \times t_m) - 1) + k_0 \quad (5a)$$

As outlined by Simkins et al. (1986), the three-half order models differ from models derived from Monod kinetics in that they allow growth of the investigated population on substrates other than the added xenobiotic.

In order to evaluate mineralization kinetics in the three strains, and the effects of inoculation level and substrate concentration, each of these

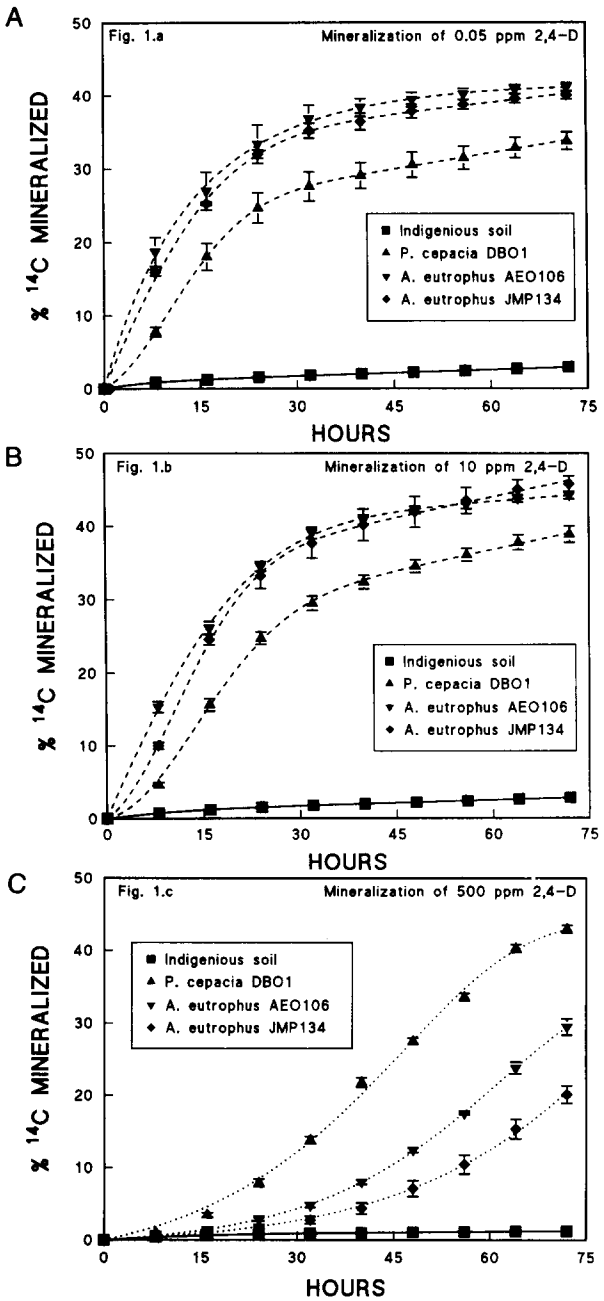


Fig. 1. Mineralization of 0.05, 10 and 500 ppm 2,4-D in soil inoculated with approx. 1×10^8 CFU/g soil *P. cepacia* DBO1(pRO101), *A. eutrophus* AEO106(pRO101) or *A. eutrophus* JMP134(pJP4). The mineralization curves were generated by either the linear form (broken lines) or the truncated logarithmic form (dotted lines) of the three-half-order model. Mineralization by the indigenous soil micro flora could not be fitted to any model (solid lines). Error bars indicate SEM of triplicate experiments. The corresponding data of survival of the strains are presented in Fig. 2.

nonlinear kinetic models was fitted to the mineralization curves.

F-tests performed on the Chi^2 values generated by each model were used to make a primary selection of the models, as suggested by Robinson (1985). Further selection was based on the degree of correlation between parameters, the standard deviation of parameter estimates and how realistic the parameter estimates were.

Results

Comparison of 2,4-D Mineralization by A. eutrophus *JMP134(pJP4),* A. eutrophus *AEO106 (pRO101) and* P. cepacia *DBO1(pRO101)*

Inoculation with all three bacterial strains greatly enhanced 2,4-D mineralization in the soil, in contrast to negligible mineralization by the indigenous flora (Figs. 1a-c). As the linear or logarithmic forms of the three-half-order model generally gave the best fits and the lowest standard deviation on the parameter estimates, it was decided to fit the mineralization data to them. The curves shown in Figs. 1a-c are thus the fits of either the linear or logarithmic forms of the three-half-order model. In general, the logarithmic form gave the lowest Chi^2 values and standard deviation on parameter estimates in the case of amendment with 500 ppm 2,4-D, whereas the linear form was more appropriate for soils amended with 10 and 0.05 ppm 2,4-D. Mineralization in non-amended soil could not be fitted to any of the models.

From Figs. 1a and 1b it can be seen that the magnitude and time course of the mineralization of 0.05 and 10 ppm 2,4-D by *A. eutrophus* AEO106 (pR0101) and *A. eutrophus* JMP134(pJP4), although slightly greater and faster in the AEO106 strain, were almost identical: mineralization was initially rapid, subsequently slowing down markedly, and total mineralization in 72 h was about 40%. In contrast, mineralization of 0.05 and 10 ppm 2,4-D by *P. cepacia* DB01(pR0101) occurred in a more sigmoidal manner and was slower than that in the two *Alcaligenes* strains, total mineralization in 72 h being only about 35%.

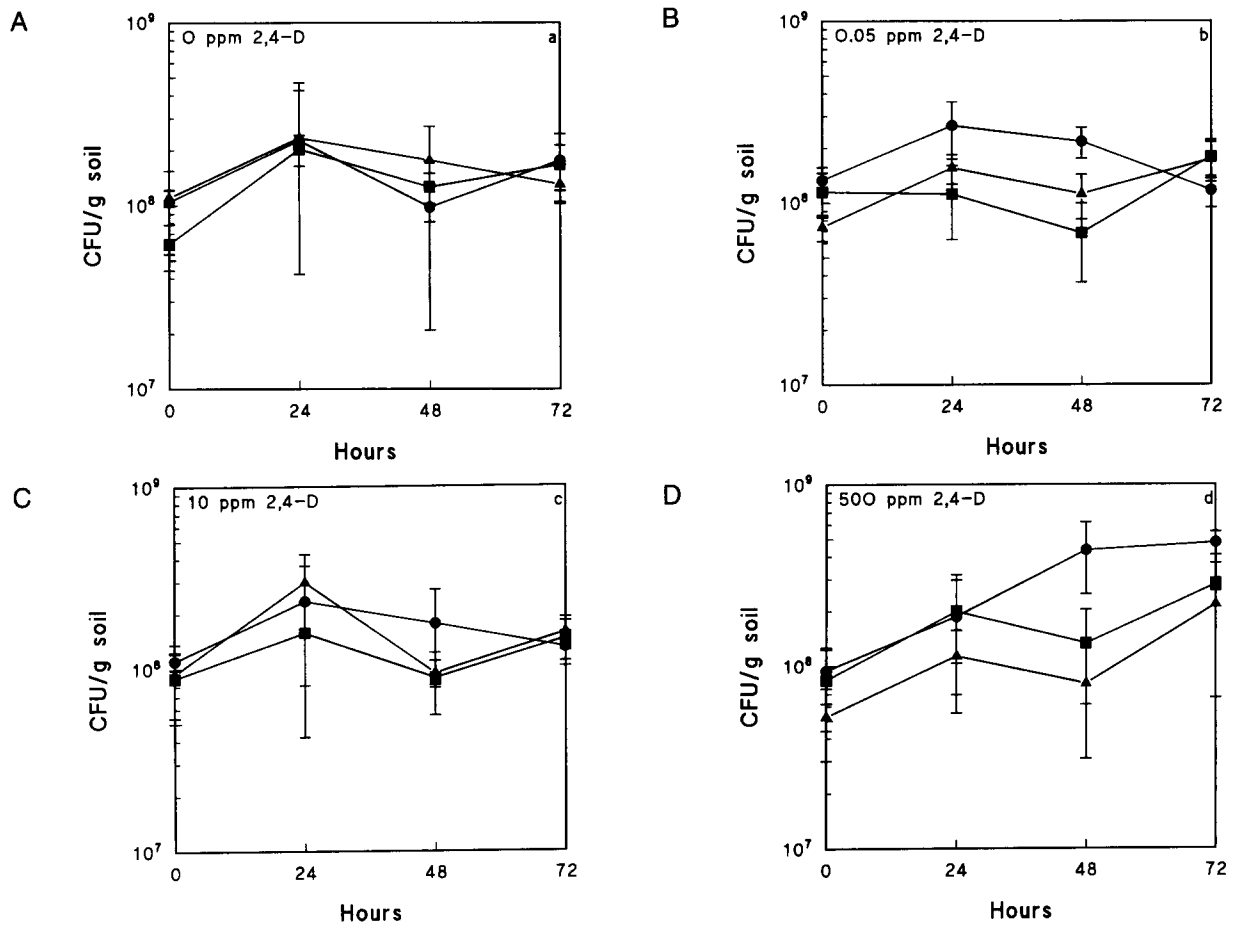


Fig. 2. Survival of *P. cepacia* DBO1(pRO101), *A. eutrophus* AEO106(pRO101) and *A. eutrophus* JMP134(pJP4) in soil amended with 0, 0.05, 10 and 500 ppm 2,4-D (Figs. a-d). The corresponding mineralization data are presented in Fig. 1a-c. Error bars represent SD of triplicate experiments.

With 500 ppm 2,4-D, the pattern of mineralization was markedly different, especially in the case of the two *Alcaligenes* strains. Mineralization by both *A. eutrophus* AEO106(pR0101) and *A. eutrophus* JMP134(pJP4), although faster in the former, occurred in a manner opposite to that at the low 2,4-D concentrations, initially being negligible and slowly becoming more and more rapid. By 32 h, only about 5% of the 2,4-D had been mineralized as compared with about 35% at the low 2,4-D concentrations. Total mineralization in 72 h was about 30% for *A. eutrophus* AEO106 (pR0101) and 20% for *A. eutrophus* JMP134 (pJP4), as compared with about 40% at the low 2,4-D concentrations. In contrast to the situation at low 2,4-D concentration, mineralization of 500 ppm by *P. cepacia* DB01(pR0101) occurred more rapidly than in the *Alcaligenes* strains. With *P.cepacia* DBO1(pRO101) mineralization of 500 ppm 2,4-D in 72 h was about 45%, which was slightly greater compared to the lower 2,4-D concentrations. As the curve was more sigmoidal and the lag phase much longer at high concentrations than at low concentrations, relative mineralization of the high concentrations of 2,4-D was less for the first 48 h than that with the low concentrations but greater thereafter.

Significant growth occurred in most soil systems, including the non-amended soil, during the mineralization experiment (Figs. 2a-d). The average inocula were 1.4×10^8, 6.4×10^7 and 1.0×10^8 CFU/g soil for *P. cepacia* DBO1(pRO101), *A. eu-*

trophus JMP134(pJP4) and *A. eutrophus* AEO106 (pRO101), respectively. The average concentration of *P. cepacia* DBO1(pRO101) was about 40% higher than that of the two *Alcaligenes* strains. Only 500 ppm 2,4-D had a significant effect on the populations of the three strains (Fig. 2d). The difference in population densities of the strains was due to the difference in inoculum. Thus when CFU's were corrected to the same inoculum level there was no significant difference in the effect of 500 ppm 2,4-D on population growth.

The parameter estimates provided by the three-half-order models for the curves shown in Figs. 1a-c are summarized in Table 1. It can be seen that the time it takes (t_m) for maximal velocity (v_m) to be achieved increases with 2,4-D concentration. However t_m of *P.cepacia* DBO1(pRO101) did not increase as much as t_m of the two *Alcaligenes* strains.

As pointed out above, some of the curves were not sigmoidal. Consequently, as the used truncated version of the three-half-order model was not designed to fit relationship in which growth does not occur, an appropriately good fit to the data was not always obtained. The three cases in which other models were more appropriate are discussed below.

The first-order model was, according to F-test ($P < 0.05$), the best model to describe mineralization of 0.05 ppm 2,4-D by the two *A. eutrophus* strains. The rate constant, k, estimated from the first-order model was 0.070 and 0.062 for the AEO106 and JMP134 strains, respectively, values which are close to the k_1 rate constants obtained with the linear form of the three-half-order model.

The third case in which another model better described the data than the three-half-order model (F-test, $P < 0.05$) was the mineralization of 500 ppm 2,4-D by *A.eutrophus* JMP134(pJP4). The curve was logarithmic, a sigmoidal form not being attained within the duration of the experiment, and the logarithmic model best described the data.

The effect of inoculum level on mineralization by P.cepacia *DBO1(pRO101)*

In order to determine the influence of inoculum size on mineralization, additional studies were undertaken using *P. cepacia* DBO1(pRO101). The

Table 1. Three-half-order model kinetic constants for 2,4-D mineralization in soil inoculated with *P. cepacia* DBO1(pRO101), *A. eutrophus* AEO106(pRO101) and *A. eutrophus* JMP134(pJP4).

Treatment	Chi^2	S_0 % $^{14}CO_2h^{-1}$	K_1 $10^{-3}h^{-1}$	K_2 h^{-1}	μ h^{-1}	k_0	v_m % CO_2h^{-1}	t_m h
0.05 ppm 2,4-D:								
DBO1	0.071	23	0.013	7.1		0.15	1.4	10
AEO106[a]	1.3	43	0.073	−1.0[c]		0.022[c b]		
JMP134[a]	5.3	33	0.059	3.0[c]		0.103[c]	1.2	−1.4
10 ppm 2,4-D:								
DBO1	1.2	26	0.000	5.3		0.19	1.4	14
AEO106	0.60	40	0.048	2.1		0.065	1.8	−1.3
JMP134	0.89	33	0.013	6.6		0.19	2.0	10
500 ppm 2,4-D:								
DBO1	6.5	44			0.053	0.000[c]	0.9	47
AEO106	0.085	36			0.058	0.000[c]	0.8	62
JMP134[d]	0.68	59[c]			0.046	0.000[c]	1.0	89[c]

[a] The first-order kinetic model gives a higher Chi^2-value but describes the data better according to the F-test ($P < 0.05$).
[b] The mineralization pattern was not sigmoidal and the v_m and t_m could not be calculated.
[c] Parameter estimate not significant.
[d] The logarithmic kinetic model gives a higher Chi^2-value but describes the data better according to the F-test ($P < 0.05$).
The curves obtained by fitting the model are illustrated in Figs. 1a-c.

mineralization of 0.06 ppm, 12 ppm and 600 ppm 2,4-D in uninoculated soil and soil inoculated with 6×10^4, 6×10^6 and 1×10^8 CFU/g *P. cepacia* DBO1(pRO101) is illustrated by Figs. 2a-c. It can be seen that the mineralization pattern of 2,4-D in soil was strongly influenced by the inoculum density.

The linear and logarithmic forms of the three-half-order model gave the best fit (F-test; $P < 0.05$) in those cases in which model fitting was appropriate. Mineralization by the indigenous flora, mineralization of 0.06 ppm at low inoculation levels (6×10^4 and 6×10^6 CFU/g soil) and mineralization of 12 ppm 2,4-D by 1×10^4 CFU/g soil, could not be fitted appropriately to any model.

The constants estimated by fitting the data to the linear or logarithmic form of the three-half-order model are summarized in Table 2. t_m, the time taken to reach maximum mineralization rate, was inversely related to the initial inoculation levels. The estimates of S_o and t_m for the mineralization of 600 ppm 2,4-D by 6×10^4 CFU/g soil were not significant because of large variation due to the very low mineralization and the non-sigmoidal curve.

Discussion

The present study demonstrates that it is possible to enhance mineralization of 2,4-D in soil by inoculation with 2,4-D- degrading bacteria.

During the three day period that mineralization was followed, mineralization in the uninoculated soil was negligible. Other studies have shown that 2,4-D can be mineralized in natural soil, although with a lag period of between two and ten days (Ou et al. 1978; Ou 1984; Kunc et al. 1984, Parker & Doxtader 1982). If the duration of the present experiment had been longer, we might have found greater mineralization by the indigenous soil microbiota.

We used only one soil type in the present experiment, and did not investigate the effect of using the same soil previously amended with 2,4-D, because it has already been shown that 2,4-D is readily degraded in many soils, and that pre-amendment of a soil only slightly enhances mineralization of 2,4-D by the indigenous microflora (Smith et al. 1989).

The comparative study of the three 2,4-D degrading organisms revealed that the mineralization patterns differed. This could be due to several fac-

Table 2. Three-half-order model kinetic constants for 2,4-D mineralization in soil inoculated to different levels of *P. cepacia* DBO1(pRO101).

Treatment CFU/g soil	CHi^2	S_0 % $^{14}CO_2h^{-1}$	K_1 $10^{-3}h^{-1}$	K_2 h^{-1}	μ h^{-1}	k_0	v_m % CO_2h^{-1}	t_m h
0.06 ppm 2,4-D:								
6×10^4 [b]								
6×10^6 [b]								
1×10^8	0.63	19	0.016	2.9[a]		0.089	0.67	13
12 ppm 2,4-D:								
6×10^4 [b]								
6×10^6	1.3	24			0.089	0.000[a]	0.81	35
1×10^8	0.20	27	0.003	4.1		0.12	1.2	15
600 ppm 2,4-D:								
6×10^4	0.0009	8.5[a]			0.15	0.015	0.47	62[a]
6×10^6	0.34	21			0.12	0.000[a]	0.94	41
1×10^8	7.7	36			0.079	0.000[a]	1.1	36

[a] Parameter not significant.

[b] Data could not be fitted to any model.

The curves obtained by fitting the model are illustrated by Figs. 3a-c.

tors, e.g. a difference in adhesion or cell membrane properties. In a study by Kukor et al. (1989) with different strains bearing pRO103, a deletion mutant of pRO101 that constitutively expresses the 2,4-D degradation genes (Harker et al. 1989), it was found that in addition to the plasmid encoded enzymes, a chromosomally encoded maleyl-acetate (MAA) reductase was involved in the complete degradation of 2,4-D. MAA reductase activity in *A. eutrophus* AEO106(pRO103) was found to be higher than in *P. cepacia* DBO1(pRO103) (Kukor et al. 1989). If this is also the case for the pRO101 strains used in the present study, and if cromosomally encoded MAA reductase activity also influences the 2,4-D degradation rate in these strains, this might explain the more rapid mineralization (higher k_1 values) of the low concentrations of 2,4-D by the two *A. eutrophus* than by *P. cepacia* DBO1(pRO101).

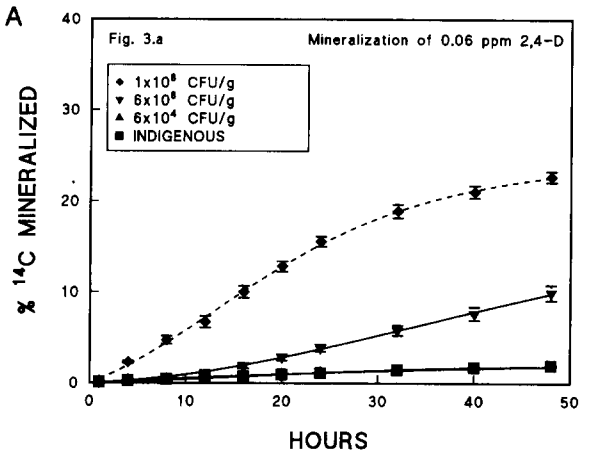
Because of the difference in the average inoculation levels used for *A. eutrophus* JMP134 < *A. eutrophus* AEO106(pRO101) < *P. cepacia* DBO1(pRO101) i.e. 4.6×10^7, 1.0×10^8 and 1.4×10^8 CFU/g respectively, one might have expected mineralization to have been slowest in the former and fastest in the latter. However, this was only observed with 500 ppm 2,4-D, for which the logarithmic version of the three-half-order model was the most appropriate. This indicates that the observed significant growth of the three strains influenced mineralization. The highest inoculation level with *P. cepacia* DBO1(pRO101) may account for its superior performance to the other two strains in mineralization of 500 ppm 2,4-D, since its maximum population was reached earliest. With the lower concentrations of 2,4-D, growth of all three strains was not significantly different from that in unamended soil, justifying the use of the linear form of the three-half-order model.

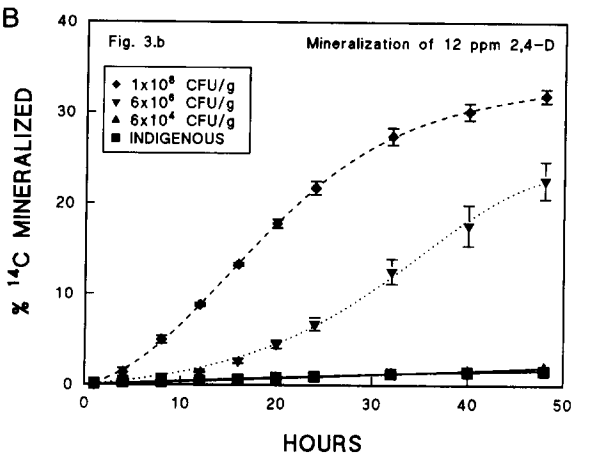
That initial inoculation level influenced mineralization kinetics (i.e. t_m was inversely related to inoculation levels), is in accordance with other reports. Focht & Brunner (1985) found that t_m was higher for the mineralization of 100 ppm biphenyl in indigenous soil than in soil inoculated with biphenyl-degrading organisms. Although t_m was not calculated, the same conclusion can be derived from several other studies (Ramadan et al. 1990; Simkins & Alexander 1984; Crawford & Mohn 1985; Greer et al. 1990).

As discussed elsewhere (Jacobsen & Pedersen 1992), survival and growth of the *P. cepacia* DBO1 (pRO101) in 2,4-D amended soil at different initial inoculation levels is influenced by the amount of 2,4-D present in the soil. Survival is the net-result of growth and death of the bacterial strain. The ability of *P. cepacia* DBO1(pRO101) to grow in the presence of high levels of 2,4-D indicates that the initial inoculation level will be of only minor influence in long term experiments. In the present experiment with 600 ppm and 6×10^4 CFU/g soil, t_m was calculated to be longer than the actual duration of the experiment. Although variation was high, t_m being 62 ± 50 h, the population actually grew to 5×10^6 CFU/g soil in 48 h (see Jacobsen & Pedersen 1992). An initial inoculum of roughly equivalent size i.e. 6×10^6 CFU/g resulted in a very significant mineralization (Fig. 3c).

Ramadan et al. (1990) found that at very low inoculation levels (330 CFU/ml), another strain of *P. cepacia* failed to mineralize 1 ppm *p*-nitrophenol in non-sterile lake-water, whereas it succeeded in sterile lake-water. The present study indicates that because of growth, the mineralization of high levels of 2,4-D can be achieved by inoculation with low levels of *P. cepacia* DBO1(pRO101). The inoculation level chosen to ensure the survival and efficiency of a particular strain should therefore be balanced against the soil concentration of the contaminant; if the contaminant supports growth of the introduced strain, it might be feasible to use a low inoculation level. If however the soil concentration of the substrate is low, we found that mineralization of the compound at low inoculation levels might be negligible, as was the case with 2,4-D in the present study. An explanation might be that at low soil concentrations a relative lower amount of a compound is avaiable for degradation since physical barriers (i.e. diffusion) would have a more significant influence. One way to overcome this problem might be to inoculate with high levels of the xenobiotic-degrading strain.

It can be concluded that the ability of the three 2,4-D-degrading strains tested, *P. cepacia* DBO1





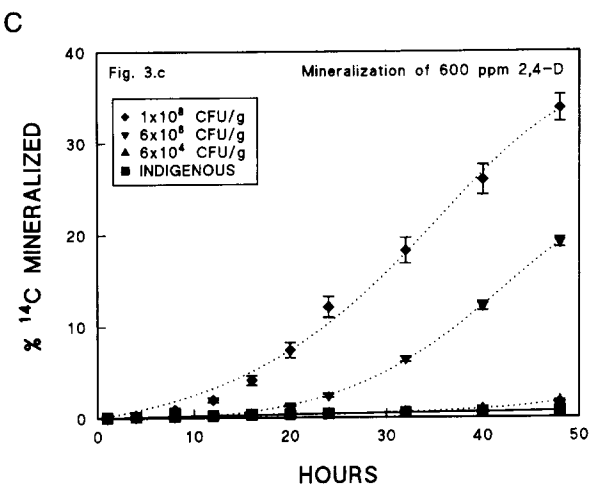


Fig. 3. Mineralization of 0.06, 12 and 600 ppm 2,4-D by indigenous soil or soil inoculated to a level of 6×10^4, 6×10^6 or 1×10^8 CFU/g soil of *P. cepacia* DBO1(pRO101). Treatments are indicated on the figures. The curves were generated by either the linear form (broken lines) or the truncated logarithmic form (dotted lines) of the three-half-order model. Curves which could not be fitted by the model are indicated by solid lines.

(pRO101), *A. eutrophus* AEO106(pRO101) and *A. eutrophus* JMP134(pJP4), to mineralize 2,4-D in soil varies depending on the soil concentration of 2,4-D.

Considering that the 2,4-D degradation pathway in the plasmids is identical, it is interesting that the strains did not perform equally well in soil. This indicates that the success of a strain for bioremediation depends not only on the ability to degrade a compound in batch culture, but also on its ability to function and survive in such a complex ecosystem as soil. The study lends further support to the recommendation that genetically engineered strains should be evaluated not only on their genetic construction but also on the basis of their phenotypic character (Tiedje et al. 1989).

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