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# Microbiological degradation of the herbicide dicamba

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## SUMMARY

*Pseudomonas paucimobilis* was isolated from a consortium which was capable of degrading dicamba (3,6-dichloro-2-methoxybenzoic acid) as the sole source of carbon. The degradation of dicamba by *P. paucimobilis* and the consortium was examined over a range of substrate concentration, temperature, and pH. In the concentration range of 100–2000 mg dicamba $L^{-1}$ (0.5–9.0 mM), the degradation was accompanied by a stoichiometric release of 2 mol of $Cl^-$ per mol of dicamba degraded. The cultures had an optimum pH 6.5–7.0 for dicamba degradation. Growth studies at 10 °C, 20 °C, and 30 °C yielded activation energy values in the range of 19–36 kcal $mol^{-1}$ and an average $Q_{10}$ value of 4.0. Compared with the pure culture *P. paucimobilis*, the consortium was more active at the lower temperature.

## INTRODUCTION

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is commonly used as a pre-emergence, post-emergence or pre-plant herbicide to control broadleaf weeds. It can be applied to leaves or soil, and registered uses include small grains, corn, sorghum, asparagus, perennial seed grasses, turf, grassland pasture, rangeland and non-croplands. Dicamba is one of the major herbicides used in the lawn care industry and functions as an auxin-like growth regulator. Dicamba (vapor pressure $3.4 \times 10^{-5}$ mm Hg) has a half-life of 1–6 weeks in soil, but may persist longer under adverse conditions such as low moisture. Dicamba ($pK_a$ 1.94) is a problematic herbicide because it has the potential to be highly mobile in soils, especially in limed agricultural soil, owing to its dissociated anionic character. It is often found in surface and ground water [1,5]. Although dicamba is transformed to 3,6-dichlorosalicylate which is much more hydrophobic than the parent molecule and tends to sorb in soil matrix, all available evidence to date indicates that this herbicide has the potential to migrate to subsurface aquifers.

Previous studies [10,12,15,16] have demonstrated that dicamba is aerobically mineralized to $CO_2$. To date, only three pure cultures (two *Pseudomonas* spp. and a *Moraxella* sp.) have been isolated which are capable of degrading dicamba [2,3,8,9]. Smith [15] and Krueger et al. [11] identified 3,6-dichlorosalicylate as the major metabolite in the aerobic pathway. A complete pathway for the aerobic degradation of dicamba has not been reported to date. Attempts to isolate other metabolites have been unsuccessful, suggesting that they may be short-lived or cannot be detected by the analytical methodology employed in previous studies. A partial degradative pathway was proposed by Krueger [9] who also demonstrated a stoichiometric release of inorganic chloride. Dicamba and its intermediate 3,6-dichlorosalicylate have been resolved and quantified by UV spectrometry, GC, and HPLC. Recently, Yang et al. [19] verified the formation of 3,6-dichlorosalicylate as the product of dicamba demethylase in *Pseudomonas maltophilia*. The formation of [$^{14}$C]-3,6-dichlorosalicylate and [$^{14}$C]-2,5-dihydroxy-3,6-dichlorobenzoate from [U-phenyl-$^{14}$C]-dicamba has also been demonstrated [9]. Furthermore, the evolution of $^{14}CO_2$ from [U-phenyl-$^{14}$C]-dicamba suggested that dicamba metabolites are channeled into central catabolic pathways. A pathway for the anaerobic degradation of dicamba has been proposed to involve the formation of 3,6-dichlorosalicylate and 6-chlorosalicylate preceding reductive dechlorination by an anaerobic consortium [18].

The environmental fate of this compound is dependent on a complexity of biotic and abiotic factors which have yet to be fully elucidated. The present study was undertaken to investigate the biodegradation of dicamba by a pure and mixed bacterial culture under aerobic conditions, with emphasis on the effects of temperature, pH, and substrate concentration. An attempt was also made to enrich for dicamba-degrading bacteria from freshly collected environmental samples that had a previous history of dicamba treatment. Dicamba biodegradation rates were determined in the temperature range of 10–30 °C in order to estimate temperature coefficient ($Q_{10}$) and activation energy values.

## MATERIALS AND METHODS

### *Bacterial cultures and growth conditions*

The initial consortium was provided through the courtesy of M. Speed and D.J. Cork, Illinois Institute of Technology,

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Chicago, IL, USA. The consortium, designated as IIT for this study, was originally enriched from a storm water retention pond. The bacteria were maintained in a mineral-salts medium (pH 7.0) which contained (per liter) 1.3 g $K_2HPO_4$, 0.87 g $KH_2PO_4$, 0.66 g $(NH_4)_2SO_4$, 0.097 g $MgSO_4$, 0.025 g $MnSO_4 \cdot H_2O$, 5 mg $FeSO_4 \cdot 7H_2O$, 1.26 mg $CaSO_4 \cdot 6H_2O$, and 1000 mg dicamba. The media were filter-sterilized. Dicamba-containing solid media were prepared with (i) 0.5% (wt/vol) GelRite (Scott Laboratories, Carson, CA, USA), using 0.075% (wt/vol) $MgCl_2$ for gel formation, and (ii) 1.5% (wt/vol) Bacto agar. Analytical-grade dicamba (>99.9% purity) and technical-grade (86.8% purity) dicamba were obtained from Sandoz Crop Protection (Des Plaines, IL, USA).

The cultures were grown at pH 7 in shake flasks at 150 rev $min^{-1}$ and at 30 °C unless otherwise indicated. The cultures were subcultured twice a week using 10% (vol $vol^{-1}$) inocula. Log-phase bacteria were concentrated ten-fold, centrifuging suspensions at 3500 × *g* for 10 min. Aliquots of 0.1 ml of the concentrated IIT suspension were aseptically spread plated onto selective dicamba solid medium and non-selective trypticase soy agar (TSA). The plates were incubated at 30 °C until distinct colonies appeared. The isolates were checked for purity by Gram staining and microscopic examination.

Attempts were also made to obtain enrichment cultures from dicamba-treated environmental samples (soil, grass, compost), using the mineral salts medium amended with 100 mg dicamba $L^{-1}$.

*Bacterial identification*

Identification of dicamba-degrading pure cultures was based on Rapid NFT-API System (Analytical Products, Ayerst Laboratories, Plainview, NY, USA) and Flow N/F test system (Flow Laboratories, McLean, VA, USA). Biochemical identifications were confirmed by analysis of fatty acid composition using a Hewlett-Packard HP5898A Microbial Identification System (MIS, Palo Alto, CA, USA) developed by Miller and Berger [14].

*Analytical methods*

Bacterial growth was monitored by optical density at 660 nm and by colorimetric determination of protein using Folin reaction [13], with bovine serum albumin (Sigma Chemical Co., St Louis, MO, USA) as the standard. The $OD_{660}$ readings were converted to bacterial cell counts (per ml) based on calibration curves prepared separately for *P. paucimobilis* and consortium IIT. These calibration curves are defined by regression equations $y = 709\,x - 4.94$ ($r = 0.995$, *P. paucimobilis*) and $y = 3060\,x - 26.3$ ($r = 0.985$, culture IIT), where $y$ is bacterial cells × $10^6$ $ml^{-1}$ and $x$ is $A_{660}$.

The concentration of dicamba in aqueous solutions was determined by UV-spectroscopy. The analytical-grade and technical-grade dicamba had absorbance maxima ($A_{max}$) at 274 nm and 276.5 nm, respectively. The UV-absorbance ($A_{274}$) was linear at the range of 1–1000 mg dicamba $L^{-1}$. For culture studies, samples were centrifuged at 3500 × *g* to remove the cells. The absorption spectra of the supernatant media were recorded from 240 to 340 nm.

Reverse-phase HPLC methodology was based on using 40% acetonitrile–60% phosphate buffer (6.0 g $K_2HPO_4$ and 3.0 ml conc. $H_3PO_4$ $L^{-1}$) as a mobile phase [4]. The HPLC system comprised an Alltech (Deerfield, IL, USA) Econosphere $C_{18}$ column (220 mm × 4.6 mm; 5.0-μm pore size), an Applied Biosystems (Foster City, CA, USA) 400 solvent delivery pump (isocratic mode at 1 ml $min^{-1}$), an Applied Biosystems 783A programmable absorbance detector set at 229 nm, and a Spectra-Physics SP4290 integrator (San Jose, CA, USA). Inorganic chloride was determined by coulometric titration with a Haake Buchler chloridometer (Saddle Brook, NJ, USA).

*Growth studies*

The effects of substrate concentration, temperature, and pH on growth of two dicamba-degrading cultures were evaluated. For inoculation, the consortium IIT and a pure culture derived therefrom were initially grown with 500 mg dicamba $L^{-1}$ for 48 h. Inocula (20 ml) were added to a final volume of 200 ml of dicamba minimal medium containing either 100, 250, 500, 1000, or 2000 mg dicamba $L^{-1}$ as the sole source of carbon. For temperature experiments, the test cultures were grown at 10 °C, 20 °C, and 30 ± 1 °C in mineral salts media supplemented with 500 mg dicamba $L^{-1}$ as the substrate. For pH experiments, the mineral salts medium was supplemented with 500 mg dicamba $L^{-1}$ and adjusted to initial pH values of 6.0, 6.5, 7.0, 7.5, and 8.0 with either NaOH or $H_2SO_4$. The medium contained 13.9 mM phosphate and 2.3 mM dicamba. No effort was made to increase the buffering capacity of the medium as the calculated maximum release of chloride would yield 4.6 mM HCl. Cultures were sampled at 0, 5, 10, 19, 25, 35, 48, 72 and 104 h. Growth was monitored by determining (i) the residual dicamba concentration with UV-spectroscopy and HPLC, (ii) inorganic chloride concentration by coulometric titration, (iii) optical density at 660 nm, and (iv) increase in protein concentration. Samples for HPLC and inorganic chloride analysis were centrifuged at 3500 × *g* for 10 min to remove the cells and frozen until analyzed. Two controls were incorporated into each experiment. The first control contained all the components in the dicamba minimal medium without an inoculum. The second control consisted of dicamba minimal medium without dicamba but was inoculated with a 10% inoculum.

The growth rate constant, $\mu$ ($h^{-1}$), was calculated from the linear portion of the growth curves using the formula:

$$\mu = 2.303\ (\log_{10}X - \log_{10}X_0)/t$$

where $X$ = total cell count at end of elapsed time, $X_0$ = total cell count at the start of elapsed time, $t$ = elapsed time (h). In the present work, optical density readings were used as a measure of bacterial cell counts.

Arrhenius plots were calculated based on growth rate, dicamba degradation (HPLC analysis), and chloride release using the equation:

$$k = Ae^{-E_a/RT}$$

where k = specific reaction rate constant, $E_a$ = activation

energy, R = universal gas constant, $T$ = temperature (°K), A = special constant for the particular reaction. The equation was linearized to:

$$\log k = -E_a/2.303\ R \times 1/T + \log A$$

The slope ($m$) of the linear portion of a plot of log k vs $1/T$ was used to estimate the activation energy:

$$m = E_a/2.303\ R$$

## RESULTS AND DISCUSSION

*Enrichment studies*

Initially, attempts were made to enrich for cultures capable of degrading dicamba, using source samples from various locations which had a history of repeated exposure to dicamba. These sources were clippings from dicamba-treated grass, composted grass clippings from dicamba-treated lawns, and composite turf-grass cores from test plots treated twice a year with dicamba. None of the samples examined yielded positive enrichments for dicamba-degraders.

Biodegradation is the main mechanism of dicamba removal in soils [16], and the apparent discrepancy between observed biodegradation in soil systems and the lack of successful enrichment cultures remains unclear at present. The only isolates of dicamba-degraders reported to date [8] originate from a storm water retention pond with a history of herbicide exposure for over three decades at a dicamba manufacturing facility. The mixed culture (consortium IIT) derived from the storm water consortium was used as the source inoculum in the present work.

*Isolation and identification of dicamba-degrading bacteria from consortium IIT*

Consortium IIT yielded three distinct isolates upon streaking to TSA. Only one of these isolates (strain 1) grew on dicamba as the sole source of carbon. The other two isolates grew on dicamba only in the presence of strain 1, suggesting that the two were capable of using dicamba metabolites. The interaction between the consortium members was not further explored.

Krueger [9] noted that a *Pseudomonas* sp. lost the ability to degrade dicamba when the culture was cured of plasmids using mitomycin C. In the present study, growth of strain 1 on TSA resulted in an irreversible loss of the ability to degrade dicamba, providing circumstantial evidence for the possible role of plasmids in dicamba degradation. This is in agreement with the findings of Cork et al. [2] who demonstrated that growth with succinate and in complex media resulted in a loss of a plasmid and an irreversible loss of the ability of a *Pseudomonas* sp. to degrade dicamba. In the present study, the incorporation of 1000 mg dicamba $L^{-1}$ and the mineral salts in 1.5% (wt $vol^{-1}$) Bacto agar proved an unsuccessful formulation in maintaining the ability to degrade dicamba. The selective pressure for dicamba degradation was successfully maintained by using 0.5% Gelrite with 0.075% $MgCl_2$ amended with 1000 mg dicamba $L^{-1}$ and the minerals salts.

All three isolates are gram-negative rods. The N/F Flow system identified two of these isolates as *Pseudomonas paucimobilis* (strain 1) and an *Achromobacter* sp., both with excellent discrimination. The *P. paucimobilis* isolate produced a brown pigment which resembled nostoxanthin on the basis of the spectral properties of a crude extract in methanol, ethanol, and acetone, in close agreement with previous description of carotenoids from *P. paucimobilis* [6,7]. The Rapid NFT-API system yielded similar identification, again with excellent match, and identified the third isolate as a *Flavobacterium* sp. but with low match. The MIS by fatty acid analysis yielded poor matches (similarity index <0.06) of all three isolates with the database. While the identification of *P. paucimobilis* as a dicamba-degrading species is the first report to our knowledge, members of the *Pseudomonas* genus, in general, possess a wide spectrum of degradative pathways. Krueger [9] reported that a pseudomonad, subsequently identified as *Pseudomonas fluorescens* [17], completely mineralized dicamba.

*Growth studies*

The growth of the test cultures with 500 mg dicamba $L^{-1}$ is presented in Figs 1 and 2. Growth measurement by cell counts (based on $A_{660}$) and by protein assay were in general agreement for both cultures. Utilization of dicamba was accompanied by concurrent release of $Cl^-$ during growth. The data are in excellent agreement with the theoretical stoichiometry of 2 mol of $Cl^-$ released per mol of dicamba degraded (Fig. 3). Abiotic degradation of dicamba and chloride release were negligible in all experiments.

*Effect of dicamba concentration*

*P. paucimobilis* and consortium IIT completely degraded dicamba at concentrations up to 2000 mg $L^{-1}$ within the experimental time course of 104 h. The degradation proceeded to the complete depletion of the parent compound, as measured by reverse-phase HPLC. The concomitant release of $Cl^-$ was stoichiometric and in excellent agreement with the disappearance of dicamba. UV-spectral analysis of dicamba could only be used for cultures with low cell densities. At concentrations greater than 250 mg dicamba $L^{-1}$, spectral interference caused by nostoxanthin pigment in the test cultures precluded the use of UV-spectrometry for dicamba analysis.

Figure 4 shows growth rate constants of both test cultures at different substrate concentrations. The growth rate constants ($\mu$) leveled off between 500 and 1000 mg dicamba $L^{-1}$. *P. paucimobilis* was more sensitive than the consortium to higher substrate concentrations. The maximum growth rate constant was 0.175 $h^{-1}$ for *P. paucimobilis* and 0.179 $h^{-1}$ for the consortium. Krueger [9] and Speed [17] reported $\mu$ values in the range of 0.190–0.133 $h^{-1}$ for dicamba-degrading *Pseudomonas* cultures.

*Effect of pH*

Growth of the test cultures with dicamba was investigated at initial pH range of 6.0, 6.5, 7.0, 7.5, and 8.0. Apparent lag phases were not observed at these pH values. The rate of

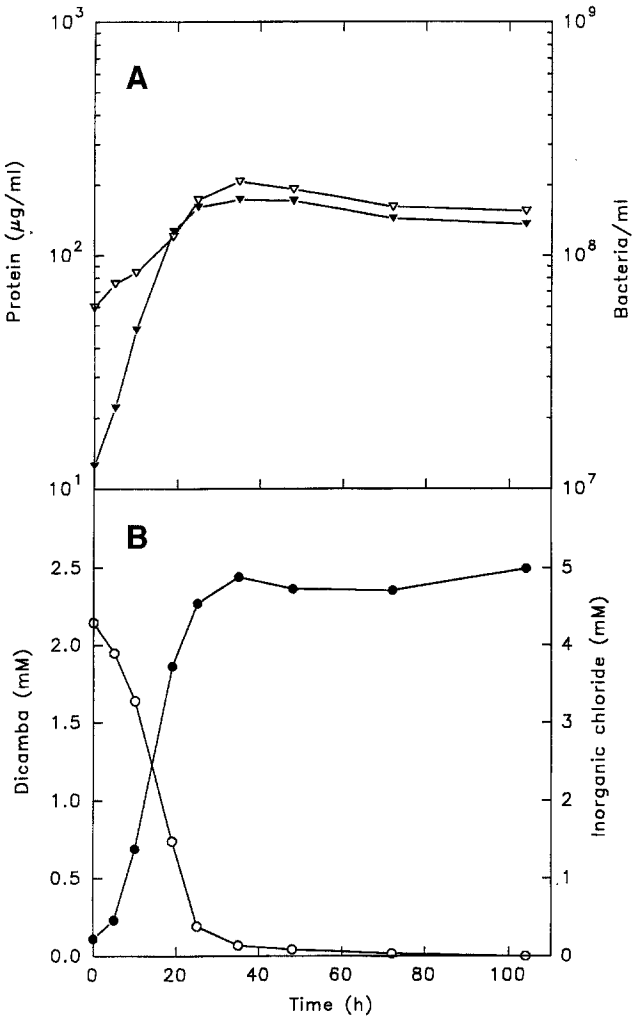


Fig. 1. Growth of *P. paucimobilis* on 500 mg dicamba $L^{-1}$ as determined by (A) $A_{660}$ (▼) and protein assay (▽); and (B) inorganic chloride release (●) and HPLC-analysis of residual dicamba concentration (○).

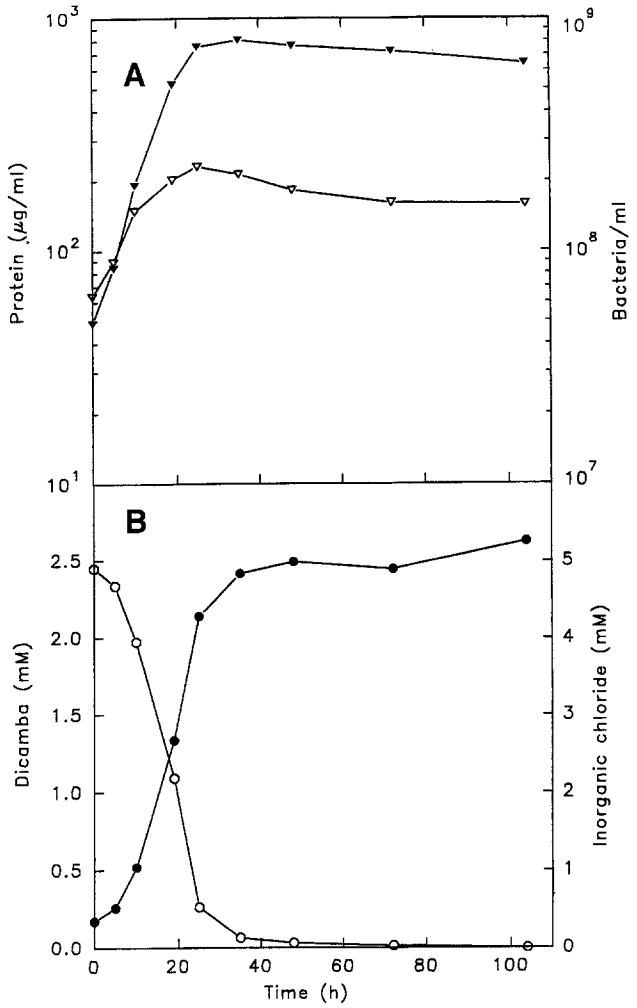


Fig. 2. Growth of consortium IIT on 500 mg dicamba $L^{-1}$ as determined by (A) $A_{660}$ (▼) and protein assay (▽); and (B) inorganic chloride release (●) and HPLC-analysis of residual dicamba concentration (○).

biodegradation of dicamba by the mixed culture at the lowest and highest pH was considerably faster than that observed for the pure culture, signifying differences in species and physiological diversity. The optimal for *P. paucimobilis* was pH 7.0 and for the consortium pH 6.5–7.0. These results were in keeping with Krueger's [9] data, showing dicamba degradation at pH range 7–8 and decrease at pH <6. Speed [17] reported an optimum pH 6.7 for dicamba degradation by *P. fluorescens*.

*Effect of temperature*

Growth of both test cultures was investigated at 10 °C, 20 °C, and 30 °C. The activity of the consortium was somewhat faster at 10 °C when compared with the pure culture of *P. paucimobilis*, suggesting greater microbial diversity in relation to temperature. Both cultures grew fastest at 30 °C. Speed [17] determined that the temperature range for biodegradation of dicamba by *P. fluorescens* was between 15 °C and 35 °C, with 30 °C being the optimal temperature. Arrhenius plots based on cell growth, substrate utilization, and chloride release in the present work are shown in Fig. 5. The respective activation energy values ($E_a$) were in the range of 19–36 kcal $mol^{-1}$ (Table 1). These values are in good agreement with an $E_a$ of 22 kcal $mol^{-1}$ derived from cell counts of *P. fluorescens* [17]. The $Q_{10}$ values based on cell counts, residual substrate concentration, and $Cl^-$ release are presented in Table 1. In the mesophilic range 20–30 °C the $Q_{10}$ values were virtually identical for the three parameters. The $Q_{10}$ values averaged 4.0 based on all data presented in Table 1.

In temperate climates with seasonal variations, temperature variations are a major determinant in influencing biodegradation rates in the environment. The effects of many other environmental and biological factors influencing the degradation of dicamba are still largely unknown. An understanding of the physical and chemical factors which govern the rate of degradation of dicamba is critical in regulation and environmental evaluation of this agrochemical. The disappearance of dicamba and the release of inorganic chloride, both coupled with growth of the bacteria, indicate dissimilatory substrate utilization. The lack of intermediates detectable by HPLC suggests ring-cleavage; however, mineralization based on $^{14}CO_2$-

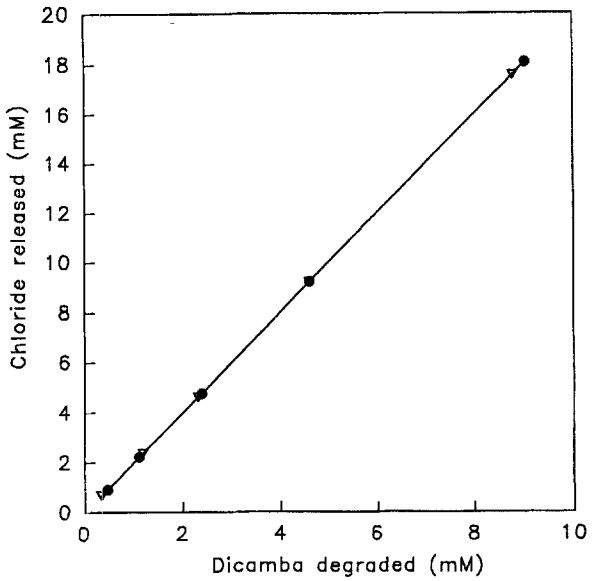


Fig. 3. The relationship between dicamba degradation and inorganic chloride release. Symbols: ●, *P. paucimobilis*; ▽, consortium IIT.

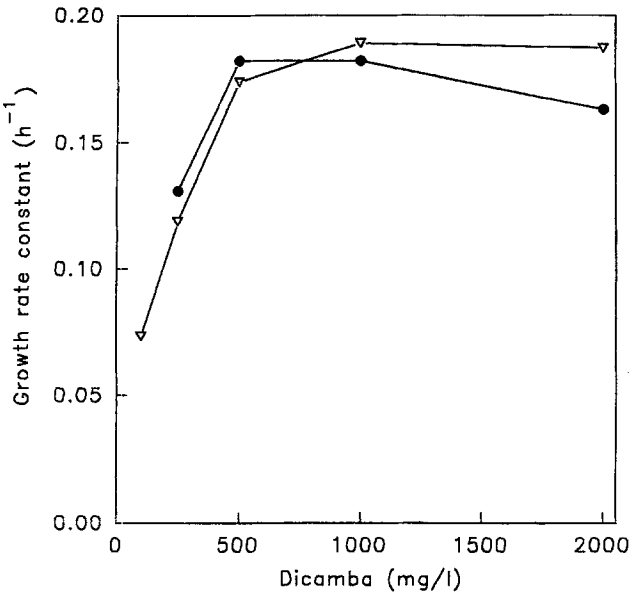


Fig. 4. Growth rate constants of *P. paucimobilis* and consortium IIT at various dicamba concentrations. Symbols: ●, *P. paucimobilis*; ▽, consortium IIT.

evolution from $^{14}C$-labelled dicamba was not performed in this study. Future work should also include the elucidation and molecular basis of the degradative pathway.

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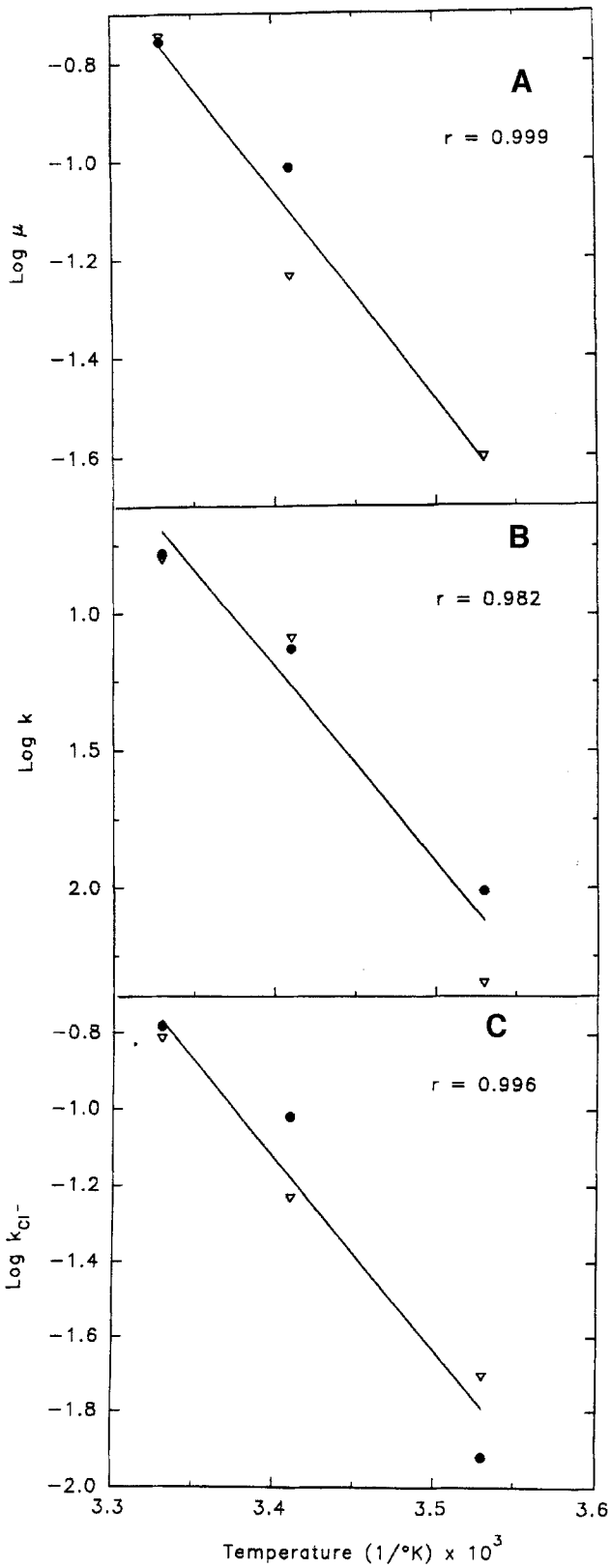


Fig. 5. Arrhenius plots of dicamba degradation based on (A) growth rate constants ($\mu$); (B) dicamba degradation rate constants (k); and (C) inorganic chloride release rate constants ($k_{Cl^-}$). Symbols: ●, *P. paucimobilis*; ▽, consortium IIT.

TABLE 1

Activation energy ($E_a$) and temperature coefficient ($Q_{10}$) values

| Parameter | $E_a$ (kcal mol$^{-1}$) | $Q_{10}$ 10–20 | 20–30 | 10–30 |
|---|---|---|---|---|
| *P. paucimobilis* | | | | |
| Cell growth | –[a] | – | 1.80 | – |
| Dicamba degradation | 28.4 | 7.49 | 2.28 | 4.13 |
| Chloride release | 26.0 | 8.02 | 1.72 | 3.72 |
| Consortium IIT | | | | |
| Cell growth | 19.6 | 2.32 | 3.08 | 2.67 |
| Dicamba degradation | 35.5 | 10.9 | 2.10 | 4.80 |
| Chloride release | 20.4 | 2.94 | 2.63 | 2.78 |

[a]–, Sufficient data not available for calculation.

HPLC systems, respectively, at our disposal, and Robert H. Edgerley Environmental Toxicology Fund for partial financial support (A.M.F.).

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