Journal of Industrial Microbiology, 7 (1991) 137–146
Elsevier

SIM 00319

Dechlorination and spectral changes associated with bacterial degradation of 2-(2-methyl-4-chlorophenoxy)propionic acid

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(Received 19 September 1989; revision received 6 September 1990; accepted 13 September 1990)

Key words: Phenoxyherbicide; Mecoprop degradation; Biodegradation; Mixed cultures

SUMMARY

The bacterial degradation of mecoprop (2-(2-methyl-4-chlorophenoxy)propionic acid) was studied using a mixed culture under aerobic conditions. The release of chlorine from mecoprop indicated incomplete degradation (75%), which did not proceed to completion upon extended incubation. The UV absorbance initially increased and this was associated with spectral distortion of the shoulder and trough regions and a slight shift in the maximum wavelength of absorption. GC-MS analysis indicated that 4-chloro-2-methylphenol was an intermediate in the degradative pathway of mecoprop. The GC-MS data also suggested the formation of other phenolic compounds with repositioned chloro- and methylgroups.

INTRODUCTION

The phenoxyalkanoic herbicide (±) 2-(2-methyl-4-chlorophenoxy)propionic acid (mecoprop) is often used in combination with other weed control agents to extend their herbicidal properties. Only few studies have been reported that deal with the microbiological degradation of this compound [3–5]. Moreover, previously published studies have largely focused on the biological degradation of mecoprop under field conditions [6,8,9].

For the present work, the microbiological degradation of mecoprop was assessed with a mixed culture under laboratory conditions by means of a mass balance. The mass balance was based on the disappearance of mecoprop and release of substituent chloride from the ring structure. The key parameters monitored for this work were the base consumption to maintain a constant pH, optical density, substrate concentration determined via HPLC, and spectral properties of samples of culture media. Spent culture media were also analysed by gas chromatography-mass spectrometry (GC-MS) in an attempt to identify soluble intermediates and chlorinated biotransformation products.

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MATERIALS AND METHODS

A mixed culture was enriched with mecoprop from a soil sample collected from a fertilizer manufacturing plant site. This culture, designated as SM, was maintained with mecoprop as the sole source of carbon and energy in a mineral salts medium, and used as the standard inoculum in all experiments. This culture was a mixture of Gram-negative bacteria but no attempt was made to identify any of the constituents. There was no evidence for the presence of Gram-positive bacteria or eukaryotic organisms. The stock culture was maintained in 100 ml cultures in 250-ml shake flasks (150 rev/min) containing (per liter) 1.0 g mecoprop, 0.5 g K_2HPO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeCl_3 \cdot 6H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, and 0.1 mg $ZnSO_4 \cdot 7H_2O$. The mineral salts and mecoprop stock solutions were autoclaved separately. All incubations were carried out at room temperature (22 ± 1 °C).

In experiment I (mass balance), the bacteria were grown in batch culture in a 2-l fermentor. The inoculum was first grown in shake flasks, centrifuged at 3000 rev/min for 10 min, and resuspended in sterile medium before inoculation. The culture vessel was fitted with a water condenser (5 °C) to minimize the evaporation of mecoprop and of volatile biotransformation products. The aeration rate of the fermentor was 1 l/min · l with a constant stirring at 150 rev/min. The pH was maintained at 7.4 by addition of NaOH via a pH-stat unit.

Samples of culture media were removed at 24-h intervals. Growth was monitored by measuring the optical density at 550 nm. The concentration of mecoprop was measured by UV absorbance at 279 nm which is the A_{max} characteristic to this compound. Spectral scans were recorded from 240 to 340 nm for centrifuged samples of culture media. The spectrophotometer used for UV scans was Varian 2200 with an accuracy of ± 0.5 nm. The instrument was equipped with a PC interface for data storage and for detailed analysis of the scans including peak ratios and shifts of as small as 1 nm in wavelengths of absorption maxima.

The concentration of mecoprop was also monitored with high pressure liquid chromatography (HPLC), consisting of a model 100A Altex pump operating in the isocratic mode with a flow rate of 2 ml/min, and a Hitachi 100-40 UV fitted with an Altex spectrophotometer flow cell set at 229 nm (range, 2.0; time constant, 0.3). The HPLC conditions were essentially the same as those previously developed for the analysis of 2,4-dichlorophenoxyacetic acid (2,4-D) [7]. A Hewlett Packard H3396 A integrator was set to peak width 0.2, threshold 1.0, area rejection 100, and a chart speed 0.5 cm/min. The HPLC column was a 4.6 mm × 150 mm Phenomenex reverse phase ODS1 with Spherisorb packing and 5 μm pore size. The mobile phase for HPLC consisted of 400 ml of acetonitrile, 6 g of K_2HPO_4 in 600 ml of distilled water, and 3.0 ml of concentrated phosphoric acid. The mobile phase was filtered using a 0.45-μm membrane filter followed by degassing with helium before use. The HPLC system had a detection limit of 1 mg mecoprop/l under these conditions.

A standard mecoprop stock solution for HPLC was prepared by adding 100 mg of mecoprop (98% pure) to a 100-ml volumetric flask containing 10 ml of 0.5 N NaOH and made up to volume with double distilled and deionized water. The standards were prepared by adding graded amounts of the mecoprop stock solution to 100-ml volumetric flasks containing 25 ml of 0.5 N NaOH and 4.0 ml of glacial acetic acid. A similar protocol was used in preparing a 4-chloro-2-methylphenol (4,2-CMP) standard.

Samples of culture media were prepared for HPLC by adding 1.0-ml aliquots to 4.0 ml of 0.25 N NaOH followed by filtration through a 0.45 μm Gelman Acro LC25 disposable syringe filter. An equivalent volume of 1 N acetic acid was added to this volume. 20-μl standards and samples were loaded onto the HPLC system.

Inorganic chloride was measured with a coulometric titrator (Haake Buchler). The instrument displayed linearity in the range of 1.8 to 1065 mg Cl^-/l with an accuracy of $\pm 0.5\%$.

For experiment II, a 10-l batch of the test culture was grown in a fermentor with 1 g/l mecoprop. The culture vessel was stirred at 150 rev/min and aerated at 1 l/min · l. The medium was inoculated (2% v/v) with the test culture followed by incubation for 8 days at 22 °C. The initial pH 7.4 decreased to pH 6.4 during the incubation and no attempt was made to maintain a constant pH. At termination, the biomass was removed by filtration (Millipore Pellicon cassette system) and the clear culture solution was extracted with 8 l of ethylacetate, followed by drying with anhydrous sodium sulfate and concentration in a rotary evaporator to approx. 20 ml.

Gas chromatograph-mass spectrometer (GC-MS) analysis of extracts was carried out with a Hewlett-Packard 5890 gas chromatograph-5970B mass spectrometer, equipped with an RTE VI data system. GC separations for total ion chromatogram (TIC) were performed using a 30 meter × 0.25 mm Supelco SPB-5 capillary column. Injections (1 μl) were made and the temperature was programmed as follows: 40 °C for 6 min, ramp 10 °C/min to 300 °C, held at 300 °C for 5 min. The MS was tuned to meet standard U.S. EPA criteria for decafluorotriphenyl phosphine. The MS was operated in full scan mode over a mass range of 40 to 450 Da. Identification of peaks was carried out using a standard reverse search program of the on-line NBS/Wiley mass spectral library.

For HPLC, a 10-ml sample of the ethylacetate-extract of spent culture media was concentrated to a 5-ml volume in a rotary evaporator and then extracted with 20 ml of 0.1 N NaOH. The resulting precipitation, presumed to be protein, was removed by centrifugation and the supernatant was diluted 10-fold with distilled water and then prepared for HPLC as described for experiment I. In order to differentiate between impurities and biotransformation products of the substrate, mecoprop standards were analysed by HPLC at concentrations similar to those present in the sample.

Mecoprop (98% purity) and 4,2-CMP were obtained from Aldrich Chemical Corp.

RESULTS AND DISCUSSION

In experiment I, the SM culture removed 75% of the mecoprop in 7 days of incubation (Fig. 1). The residual of 25% remained constant up to 13 days. An inverse relationship was established between the A_{550} and the residual concentration of mecoprop as monitored by HPLC.

The degradation of mecoprop was an acid-producing reaction (Fig. 1). The rate of base consumption was similar to the rate of chloride formation, suggesting that hydrochloric acid was produced as a product of dechlorination of the parent molecule.

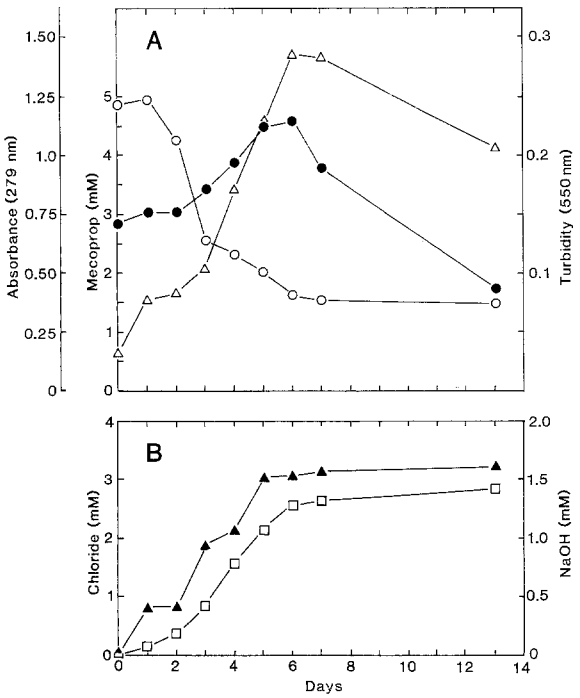


Fig. 1. Microbiological degradation of mecoprop in experiment I. **A.** Concentration of mecoprop measured by HPLC (○); UV absorbance at 279 nm (●); and the turbidity (△). **B.** Base consumption (□) and the concentration of inorganic chloride (▲) in the culture media.

TABLE 1

Characterization of spectral changes of culture media in Experiment I

Day	A_{279}/A_{248}	A_{279}/A_{296}	UV peak structure[a]	
			Mecoprop shoulder peak (A_{286})	Transient shoulder peak (A_{282})
0	5.7	42.5	+ + +	ND
1	5.5	13.4	+ +	+ + +
2	4.8	11.9	+ +	+
3	3.5	2.9	+ +	+
4	2.9	3.3	+	+
5	2.5	3.0	ND	+
6	2.3	2.4	ND	+
7	2.5	2.5	ND	+
13	3.4	7.8	+ + +	ND

[a] +, relative intensity; ND, not detected.

Both HPLC and A_{279} were used to analyse mecoprop. The mecoprop concentration determined by A_{279} resulted in significantly higher values than those derived via HPLC. This discrepancy may be attributed in part by the accumulation of biotransformation products in the culture medium. Fig. 2 shows that the decrease in the concentration of mecoprop is not coupled with the dechlorination reaction, again implying the formation of chlorinated biotransformation products. The accumulation of

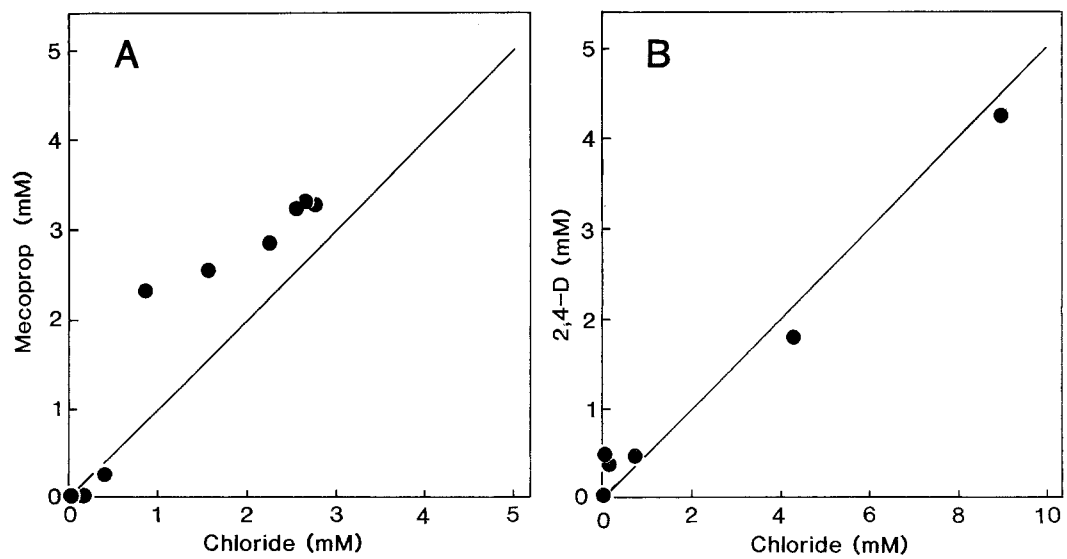


Fig. 2. Release of inorganic chloride from mecoprop (**A**) and 2,4-D (**B**). The line depicts the theoretical release of chloride based on 100% dechlorination on successive days of incubation. The sample points for mecoprop are from experiment I: the sample points for 2,4-D are based on a previously published study [2].

the intermediate was greatest on day 3, when 65% of the mecoprop was still chlorinated and thus partially metabolized (Fig. 2A). Further dechlorination took place during the subsequent days of incubation, but the mass balance calculations on day 13 still indicated that 20% of the degraded mecoprop persisted in a chlorinated form (Fig. 2A). In contrast, in a similar experiment where 2,4-D was the sole carbon source [2], the disappearance of 2,4-D was coupled to dechlorination throughout the time course (Fig. 2B) and the respective UV spectra of centrifuged culture samples displayed 2,4-D as the only pre-

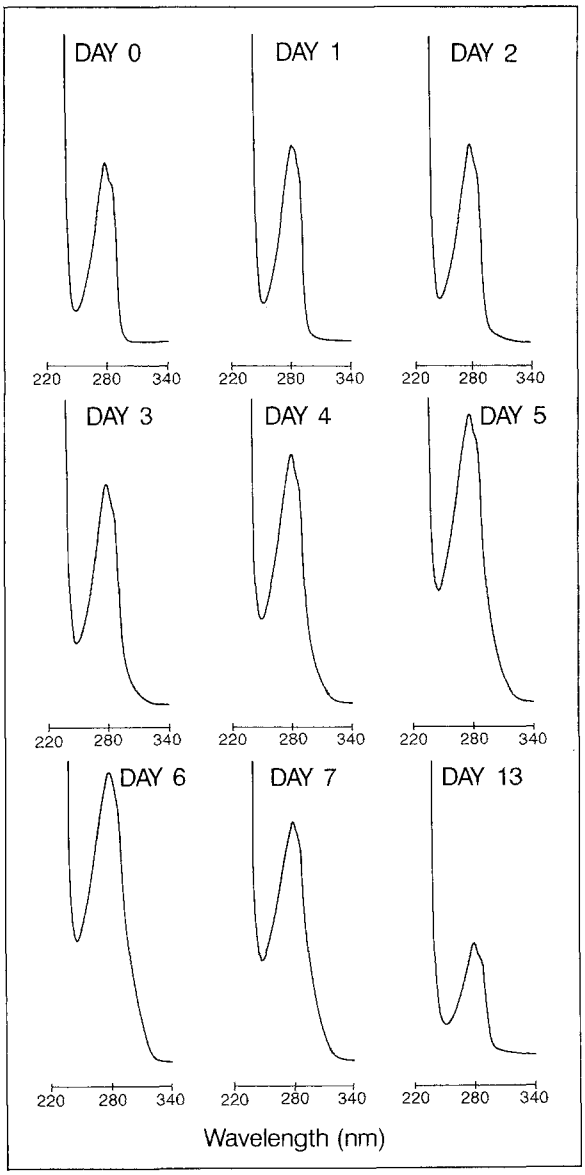


Fig. 3. Spectral scans of samples of culture media in experiment I.

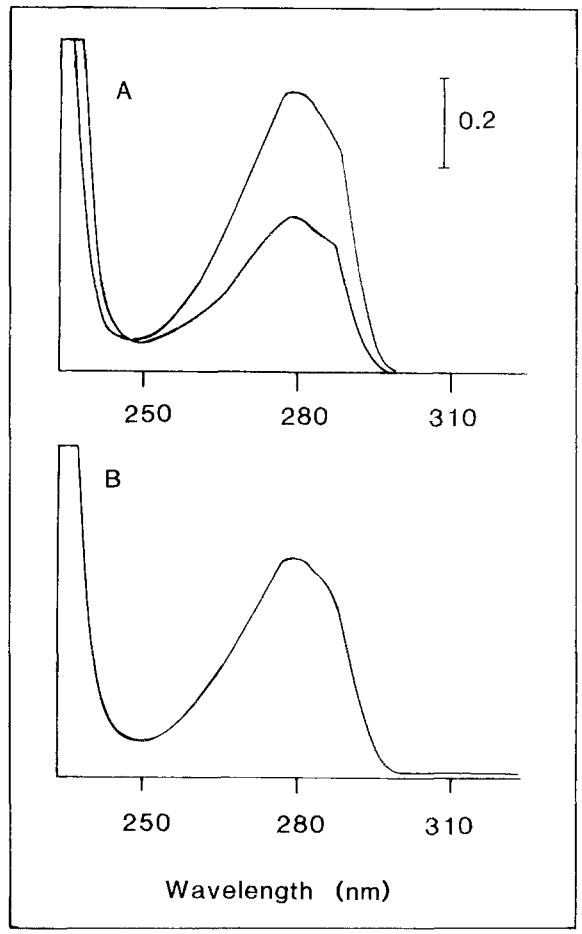


Fig. 4. Spectral scans of 4,2-CMP and mecoprop standards. **A.** 50 ppm of 4,2-CMP (upper scan) and 50 ppm of mecoprop (lower scan). **B.** 25 ppm each of 4,2-CMP and mecoprop.

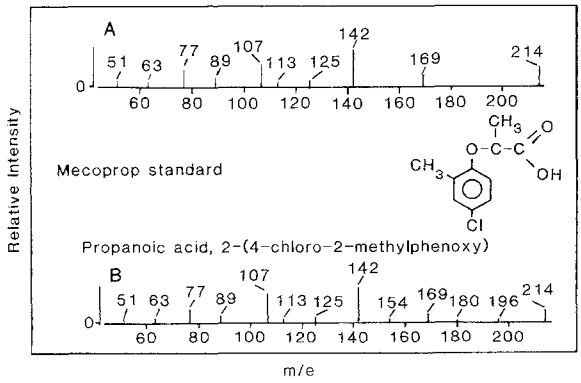


Fig. 5. Mass spectral data for (**A**) mecoprop standard and (**B**) the respective library scan.

dominant UV-absorbing species under otherwise comparable experimental conditions.

The spectral scans of mecoprop cultures incubated for up to 13 days displayed several changes in UV absorbance after day 1. These changes involved spectral distortions and a transient shoulder peak (Table 1) as well as a transient increase in UV absorption over the 240–280 nm range (Fig. 3). The characteristic mecoprop spectrum can be seen in the UV data for day 0. The major spectral features of this compound include a strong absorption region in the 260–320 nm range which can be attributed to the aromatic $\pi \rightarrow \pi^*$ electronic transitions [1]. This

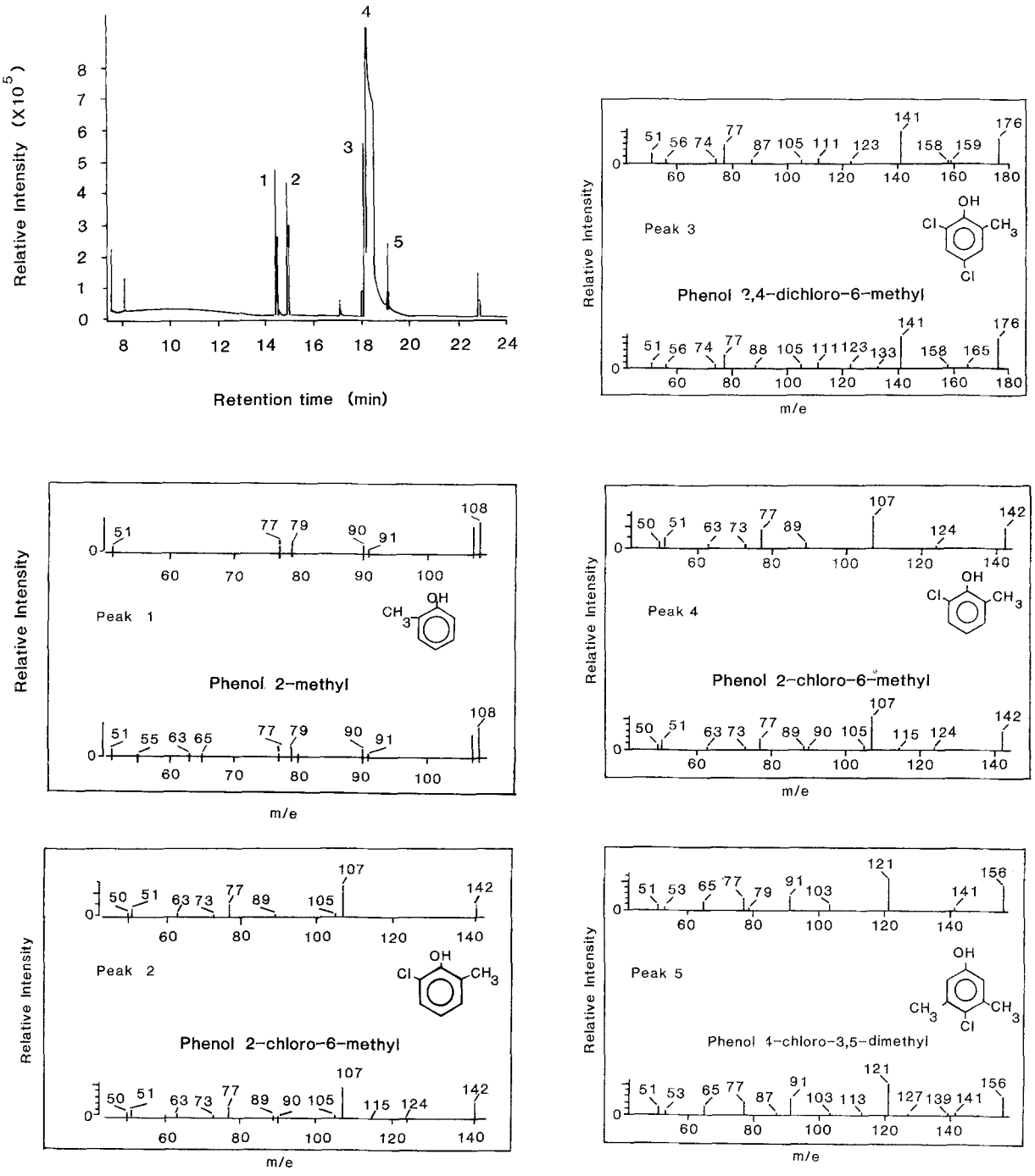


Fig. 6. TIC and mass spectral data for the 4,2-CMP standard. Mass fragmentation patterns (top m/e scale in each panel) and the respective best library match (bottom m/e scale) are shown for the five major peaks in the TIC.

TABLE 2

Compound identification based on mass fragmentation patterns of five major peaks in the total ion chromatogram of 4,2-CMP standard

Peak no.	Retention time (min)	Compound identification	Probability (%)	Abundance (%)
1	14.42	2-methyl-phenol	88	9.7
2	14.88	2-chloro-6-methyl-phenol	89	6.1
3	18.04	2,4-dichloro-6-methyl-phenol	99	10.9
4	18.22	2-chloro-6-methyl-phenol	86	68.7
5	19.06	4-chloro-3,5-dimethyl-phenol	79	4.6

absorption region sharply rises at 296 nm and reaches a maximum at 279 nm. A minor shoulder peak is present at 296 nm. Below 279 nm the absorption sharply decreases and reaches a trough region at 248 nm.

These wavelengths can be used to define the shape of the peak and by using the absorbance ratios at these wavelengths spectral distortions in the culture media become apparent. The A_{279}/A_{248} ratios decreased during the incubation (Table 1), indicating the presence of at least one additional UV absorbing species in this range. Similarly, the A_{279}/A_{296} ratios decreased, suggesting the formation of a metabolic product with a characteristic UV absorbance different from that of mecoprop. The typical mecoprop shoulder region at 296 nm gradually changed during incubation until it was again predominant on day 13. This loss of the shoulder region was accompanied with a transient shoulder at 282 nm (Fig. 3), which disappeared by day 13 when the spectral data again depicted a typical mecoprop spectrum.

One suspected partial degradation product was 4-chloro-2-methylphenol (4,2-CMP) which was reported by Smith [7] to be formed during the microbiological degradation of mecoprop. In the present work, it was found that the mecoprop standard used in these experiments contained a trace amount of 4,2-CMP as an impurity. However, the peak area of 4,2-CMP was only about 0.13% of that of mecoprop and therefore 4,2-CMP was usually not seen as a discreet peak in the HPLC chromatograms of mecoprop standards.

While the HPLC chromatograms obtained in the mass balance experiments indicated a gradual decrease in mecoprop concentration, they did not display peaks that would correspond to the retention time of the 4,2-CMP standard. Thus there was no evidence in the HPLC chromatograms to suggest the accumulation of 4,2-CMP as a major metabolite. Depending on the sample, three to five other minor peaks were also present but each had a relative abundance of $< 1.2\%$ of the mecoprop peak area. Control experiments revealed their presence also in the parent substrate.

UV scans determined during the time course displayed an initial increase in absorbance, characteristic of the formation of a compound with a higher absorption molar coefficient than the parent compound mecoprop. At the same time, there was a shift in the A_{max} from 279 nm to 280 nm and a spectral distortion in the shoulder region of the spectrum (Fig. 3), suggesting a change in the UV absorbing species. The molar absorption coefficients determined in this study were $1.4 \times 10^3\ M^{-1}\ cm^{-1}$ for mecoprop and $1.7 \times 10^3\ M^{-1}\ cm^{-1}$ for 4,2-CMP. UV scans for mecoprop and 4,2-CMP standards and their mixture are shown in Fig. 4. It should be noted that if the increase in UV absorbance was caused by a ring cleavage product, it would be a strongly polar compound and could not be resolved by the HPLC methodology employed in this study.

GC-MS analysis of a mecoprop standard displayed one major peak in the total ion chromatogram (TIC). The mass spectrum of the major peak, which displayed a

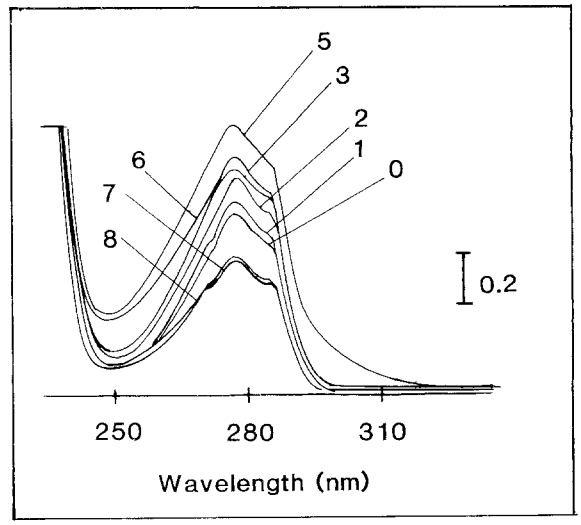
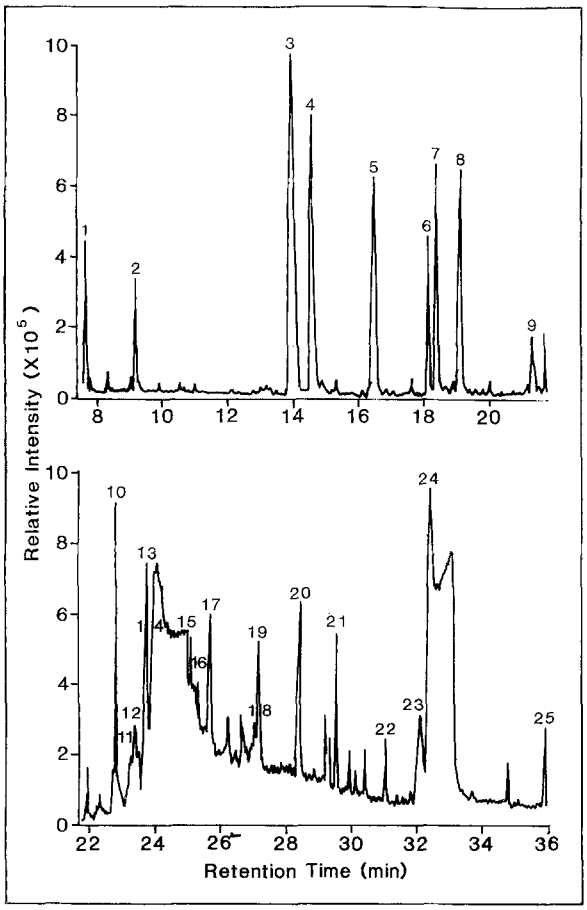
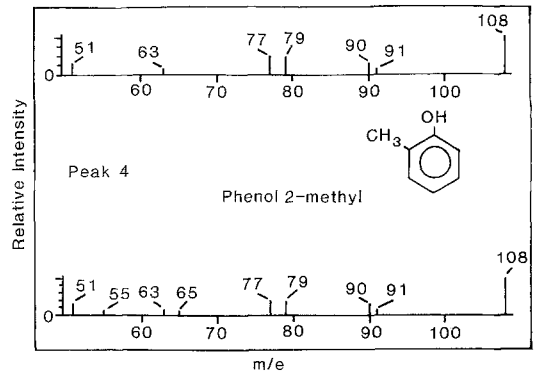


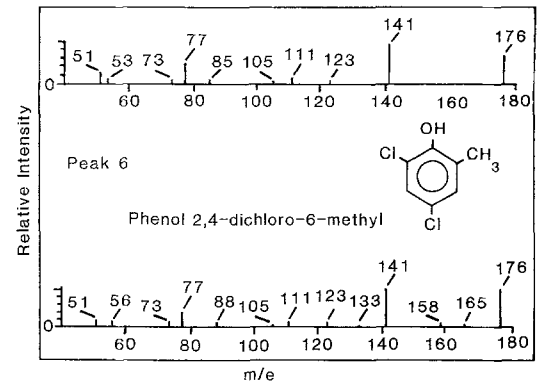
Fig. 7. UV spectral scans of the culture media on successive days of incubation (indicated by numbers) in experiment II.

retention time of 23.8 min, is shown in Fig. 5. The mass spectral library search correctly identified the peak as mecoprop, with a 96% match probability.

A 4,2-CMP standard sample resulted in five significant peaks in the TIC (Fig. 6). The respective MS fragmentation data indicated that the substituent Cl and CH_3 groups of 4,2-CMP were subject to repositioning and removal (Fig. 6). A partial listing of the best matches of the fragmentation patterns is shown in Table 2 for the five major peaks in the TIC. Peak 4 (retention time 18.2 min) was the largest peak in the TIC and was identified as 2-chloro-6-methylphenol. This peak is taken to represent the standard 4,2-CMP because it is recognized that MS does not readily differentiate between isomers on a library search. A minor TIC peak also produced a fragmentation pattern that was characteristic of 4,2-CMP (peak 2, Table 2), but it should be again recognized that the position of substituent groups cannot be completely verified







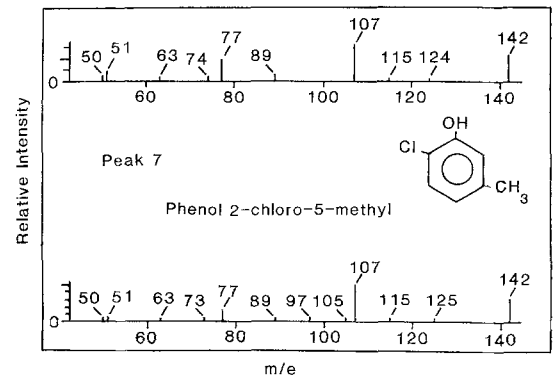


Fig. 8. TIC and mass spectral data for the spent culture media analysed after 8 days of incubation in experiment II. Mass fragmentation patterns (top m/e scale in each panel) and the respective library match (bottom m/e scale) are shown for peaks 4, 6, and 7. Identification of other peaks based on mass fragmentation patterns is summarized in Table 3.

TABLE 3

Compound identification based on mass fragmentation patterns of eight major peaks in the total ion chromatogram of spent culture media in experiment II. The remaining mass fragmentation patterns in other TIC peaks did not yield positive identification

Peak no.	Retention time (min)	Compound identification	Probability (%)	Abundance (%)
4	14.53	2-methyl-phenol	83	8.3
		3-methyl-phenol	79	
6	18.10	2,4-dichloro-6-methyl-phenol	99	3.0
7	18.35	2-chloro-5-methyl-phenol	79	4.8
		2-chloro-6-methyl-phenol	66	
8	19.08	phthalic anhydride	73	5.6
11	23.23	1,1-*bis*(dodecyloxy)-hexadecane	78	1.4
12	23.40	4-chloro-2-methyl-phenol	66	0.9
		2-(4-chloro-2-methyl-phenoxy)-propanoic acid	60	
13	23.77	5-octadecane	70	6.8
18	27.03	hexadecanoic acid	88	1.0

by MS. The presence of 4,2-CMP because of thermal degradation during the GC-MS analysis cannot be ruled out at the present time.

In experiment II, the UV absorbance again displayed an initial increase and spectral distortion of both shoulder regions (Fig. 7), again suggesting a transient accumulation of an intermediate. GC-MS analysis of spent culture media exhibited multiple peaks in the TIC (Fig. 8). Eight of these peaks could be identified by matching with the mass spectral library search (Table 3). Several phenolic compounds were identified with repositioned chloro or methyl groups (Fig. 8, Table 3). Although the MS fragmentation pattern does not differentiate between repositioned substitutions, an identification may also be based on the retention time of the peak in the TIC relative to that of the authentic compound. Peak 7 (retention time 18.4 min) produced identification which was comparable with that of the 4,2-CMP standard. Peak 12 (Table 3) can be attributed to the parent substrate, mecoprop.

CONCLUSIONS

In conclusion, a mixed bacterial culture could be maintained with mecoprop as the sole source of carbon and energy. However, mecoprop was only partially degraded and yielded biotransformation products as evidenced by spectral changes in the UV range characteristic to phenoxyherbicides. Changes in UV absorbance were transient and included spectral distortions and a shift in the wavelength of A_{max}. If the bacteria were capable of using mecoprop without the accumulation of aromatic intermediates, UV absorbance could be directly used as a measure of residual concentration. However, the absorbance first increased, again suggesting the formation of UV absorbing aromatic intermediate species in the culture medium. In the degradative pathway of mecoprop, one of the first intermediates is 4,2-CMP which is formed upon removal of propionate from mecoprop. GC-MS produced fragmentation patterns for authentic 4,2-CMP which were characteristic of repositioned substitutions in the ring structure. The presence of 4,2-CMP in samples of spent culture media was verified by GC-MS based on both peak retention and fragmentation pattern. Mecoprop degradation was consistently incomplete based on dechlorination and HPLC data. Several reasons may account for the incomplete degradation. The pathway of mecoprop degradation may be subject to regulation by downstream intermediates yet to be elucidated, and these may also be inhibitory directly to the bacteria, thereby accounting for the cessation of mecoprop degradation.

ACKNOWLEDGEMENTS

We thank Carla Gierke and Richard Driggs for technical assistance, Kevin B. Hallberg for stimulating discussions, and Laurie Haldeman for typing the manuscript. Partial financial support for this study was received from the Ohio EPA and O.M. Scott & Sons Company.

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