APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1985, p. 429–433 Vol. 49, No. 2
0099-2240/85/020429-05$02.00/0
Copyright © 1985, American Society for Microbiology

Degradation of the Herbicide Mecoprop [2-(2-Methyl-4-Chlorophenoxy)Propionic Acid] by a Synergistic Microbial Community

HILARY M. LAPPIN,[1†] MICHAEL P. GREAVES,[2] AND J. HOWARD SLATER[1†*]

Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, West Midlands,[1] and Agricultural Research Council, Weed Research Organisation, Yarnton OX1 1PF, Oxford,[2] United Kingdom

Received 9 April 1984/Accepted 6 November 1984

A microbial community isolated from wheat root systems was capable of growth on mecoprop as the sole carbon and energy source. When exposed to fresh herbicide additions, the community was able to shorten the lag phase from 30 days to less than 24 h. The community comprised two *Pseudomonas* species, an *Alcaligenes* species, a *Flavobacterium* species, and *Acinetobacter calcoaceticus*. None of the pure cultures was capable of growing on mecoprop. Certain combinations of two or more community constituents were required before growth commenced. The mecoprop-degrading community could also degrade 2,4-dichlorophenoxyacetic acid and 2-methyl-4-chlorophenoxyacetic acid but not 2,4,5-trichlorophenoxyacetic acid.

Modern agricultural methods include the extensive application of a wide range of different herbicides which, in addition to their attack on the designated target organisms, also appear in the soil and within other regions associated with nontarget organisms. The application of some herbicides may affect crop growth and directly or indirectly influence the activity of rhizosphere microorganisms (7). The rhizosphere (10) is the zone of microbial activity associated with plant roots and is stimulated by the exudates of the root system. Although much is now known about the degradation of herbicides by the microflora generally (9, 19), much less is understood about the capacity of the rhizosphere microorganisms to degrade herbicides. This paper is concerned with the herbicide mecoprop [2-(2-methyl 4-chlorophenoxy)propionic acid], which is used extensively to control broad-leaved weeds in cereal crops. It is structurally related to the other widely used herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). A number of previous studies have emphasized the importance of interacting microbial communities in the degradation of halogenated compounds (2, 17, 18) and other xenobiotic compounds (3, 5, 8). Moreover, the importance of interacting microorganisms as starting points in the evolution of novel catabolic sequences is now well established (16–19).

This paper describes a microbial community, isolated from the rhizosphere of wheat, which was capable of utilizing mecoprop as a sole carbon and energy source. However, the detailed mechanism of mecoprop degradation remains to be elucidated.

MATERIALS AND METHODS

Herbicides. Mecoprop, 2,4-D, 2,4,5-T, and MCPA were all obtained as free acids from Fluka AG Chemische, Fabrik, Bucks, United Kingdom. Mecoprop, as a technical formulation, was donated by May and Baker Ltd., Ongar, Essex, United Kingdom.

Isolation and growth of microorganisms. Wheat (cv. Maris Dove) was grown in pots in sandy loam soil (15% clay, 13% silt, 72% sand [pH 5.9]) from a field at Weed Research Organisation for 25 days at 15°C with a 14- to 10-h light-to-dark regime. The seedlings were carefully removed, and loosely adhering soil was shaken off. The roots with adherent rhizosphere soil were then washed by hand shaking for 2 min in 25 ml of growth medium with the following composition (grams per liter of glass-distilled water): $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; NaEDTA, 0.12; NaOH, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.004; $CuSO_4 \cdot 5H_2O$, 0.001; Na_2SO_4, 0.0001; $Na_2MoO_4 \cdot H_2O$, 0.001; $CoCl_2 \cdot 6H_2O$, 0.0001; $MnSO_4 \cdot 4H_2O$, 0.0004. The medium had a pH of 7.0.

The root washings were made up to 100 ml with basal medium, and mecoprop was added to give a concentration of either 0.25 g of carbon per liter (0.53 g of mecoprop per liter; 2.1 mM) or 0.50 g of carbon per liter (1.06 g of mecoprop per liter; 4.2 mM). These cultures, in 250-ml Ehrlenmeyer flasks, were incubated at 25°C with shaking at 140 rpm on an orbital shaker. Growth was measured by determining the A_{600} of the culture with a 1-cm cell (Pye Unicam spectrophotometer SP1700). For batch culture enrichments, subcultures were made when released chloride ion concentration showed that at least 50% of the available mecoprop had been degraded.

An enriched mecoprop-degrading community was obtained after 4 months (30 subcultures). The constituent members were examined as follows. Appropriate dilutions of the culture were spread onto plates of nutrient agar (Oxoid Ltd., London), King's B agar (14), and malt agar (Oxoid) and incubated at 25°C for 2 to 3 days. Single colonies were isolated and purified by repeated streaking on the three media. Initially, the constituent members of the enriched communities were recognized on the basis of comparative colony morphology. Subsequently, the different isolates were identified by the Torry Research Station, Aberdeen, United Kingdom.

For growth on other halogenated phenoxyalkanoic acids, a concentration of 0.25 g of carbon per liter was selected; this was 0.46, 0.57, and 0.67 g/liter, or 2.3, 2.6, and 2.6 mM,

* Corresponding author.

† Present address: Department of Applied Biology, University of Wales Institute of Science and Technology, Cardiff CF1 3XF, Wales, United Kingdom.

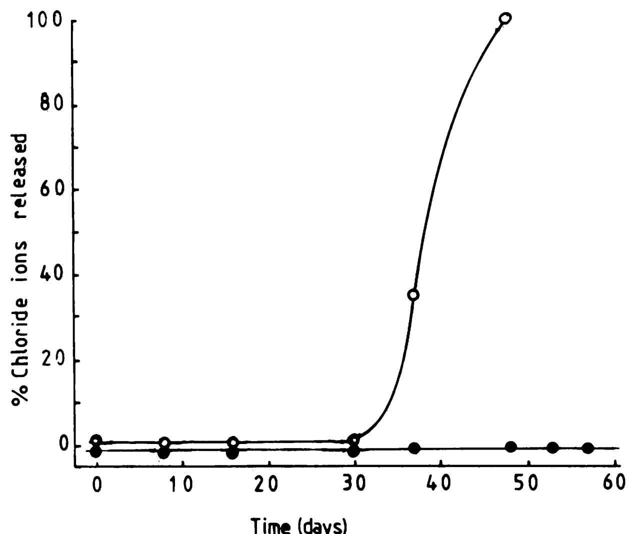

FIG. 1. Rate of chloride ion release with time with two concentrations of mecoprop. Symbols: ○, 0.25 g of carbon per liter (2.1 mM); ●, 0.5 g of carbon per liter (4.2 mM).

for MCPA, 2,4-D, and 2,4,5,-T, respectively. The cultures were incubated at 25°C, and growth was monitored by absorbance and chloride ion release at regular intervals over an 8-week period.

For growth of the community constituents on mixed substrates, 0.25 g of carbon per liter from both mecoprop and sodium succinate (0.84 g/liter; 3.12 mM) was added to the growth medium. Cultures were inoculated at 25°C, and the absorbance of the culture and the chloride ion release were monitored over a 7-day period.

Reconstitution and various combinations of community constituents. Pure cultures of the bacterial isolates from the mecoprop-degrading community were grown overnight in nutrient broth and then inoculated (1 ml) into mecoprop growth medium to give all possible permutations of the five community constituents.

Mecoprop breakdown. The disappearance of mecoprop was measured directly by monitoring the change in A_{279} in a UV spectrophotometer. Culture supernatants were prepared by centrifuging off the cells. A decrease in A_{279} indicated breakdown of mecoprop.

Chloride ion determination. Samples (1.0 ml) of culture were analyzed for chloride ion release (21) with a Marius Chlor-O-Counter (F. T. Scientific, Tewkesbury, Gloucester, United Kingdom).

Continuous-flow culture (chemostat) growth. An inoculum (10 ml) of a batch enrichment culture was grown in chemostat culture. The chemostat culture volume was 800 ml and was agitated at 1,000 rpm and aerated at the rate of 800 ml of air per min. The temperature was controlled at 25 ± 0.5°C, and fresh media were transferred to the culture vessel with a peristaltic glow inducer (Watson Marlow, Falmouth, Cornwall, United Kingdom) to give a dilution rate of 0.024/h. The basic defined growth medium (see above) was supplemented with 0.25 g of carbon per liter (mecoprop).

Materials. All chemicals were of the highest purity commercially available.

RESULTS

Batch culture enrichments with an inoculum taken from the rhizosphere of pot-grown wheat typically resulted in the onset of chloride ion release after prolonged lag phases on the order of 30 days (Fig. 1). Degradation, monitored by the release of chloride ions, was only obtained at starting concentrations of 0.25 g of carbon per liter (2.1 mM) or less and took ca. 20 days to complete (Fig. 1). In subsequent subcultures, enriched microbial communities shortened the lag phase to less than 24 h (Fig. 2), to give a specific growth rate of 0.087/h. As with all enrichment cultures, the maximum population phase was reached at the time chloride ion release was completed. In all cases, the final chloride ion concentration equaled the maximum calculated value for the

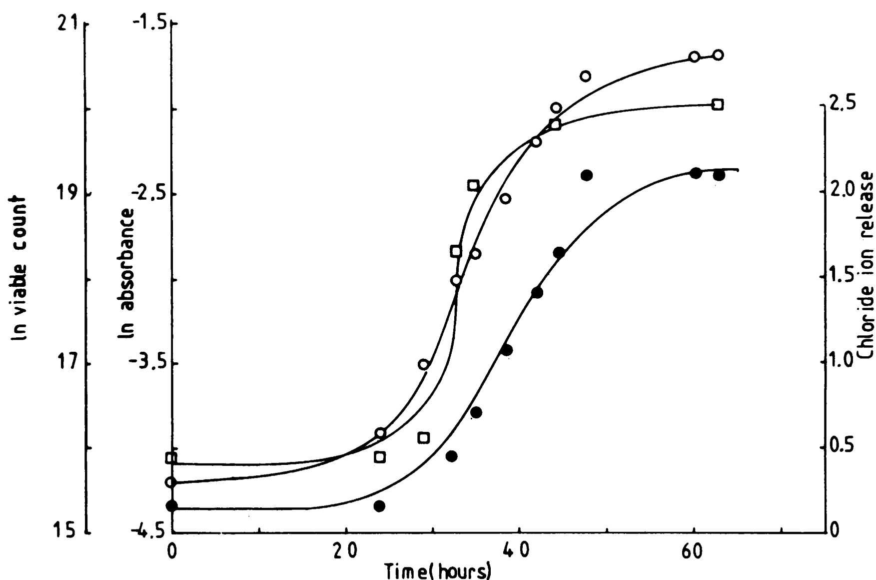

FIG. 2. Growth of a selected five-member microbial community on 0.25 g of carbon per liter (mecoprop). Symbols: ○, absorbance of culture (E_{600}); □, number of viable organisms per milliliter; ●, chloride ion release (micromoles of chloride per milliliter).

complete degradation of the supplied mecoprop. A linear relationship existed between absorbance and the concentration of chloride ion released, suggesting that the rate of breakdown of mecoprop was the rate-limiting step in the growth of the microbial community (Fig. 3).

Spectrophotometric scans of culture supernatants over the range of 240 to 340 nm revealed a substantial peak at 279 nm. During growth and chloride ion release, there was a concomitant decrease in the size of the 279-nm peak, demonstrating the disappearance of the primary mecoprop structure.

Prolonged incubation of more than 100 days of the primary enrichment cultures at higher substrate concentrations, such as 0.5 g of carbon per liter (Fig. 1), failed to demonstrate any degradation, and no organisms or communities were isolated which could degrade mecoprop. However, the enriched microbial community obtained at an initial concentration of 0.25 g of carbon per liter was able to grow in batch culture with starting concentrations up to 8.0 g of carbon per liter (67.2 mM) (Table 1). With a standard 10% (vol/vol) inoculum size, subcultures differing only in the initial mecoprop concentration illustrated a reduction in growth with increasing concentration (Table 1). The lag phase increased from 18 h at 1.0 g of carbon per liter to over 500 h at 8.0 g of carbon per liter. The specific growth rate decreased nearly 10-fold over the same concentration range (Table 1). There was no growth at mecoprop concentrations above 8.0 g of carbon per liter.

The microbial community which was enriched by batch culture procedures contained five bacteria. The organisms were closely associated and, because of their similar nutritional characteristics, were difficult to separate. Also, the possibility exists that other unidentified organisms are present within the community. In closed culture, the proportion of the community formed by each member varied with the age of the culture. Typically, a culture had the following composition after the completion of mecoprop degradation: a *Pseudomonas* species (60%), *Pseudomonas maltophilia* (25%), an *Alcaligenes* species (12%), *Acinetobacter calcoacetisus* (3%), and a *Flavobacterium* species (1%). An important finding was that none of the purified organisms

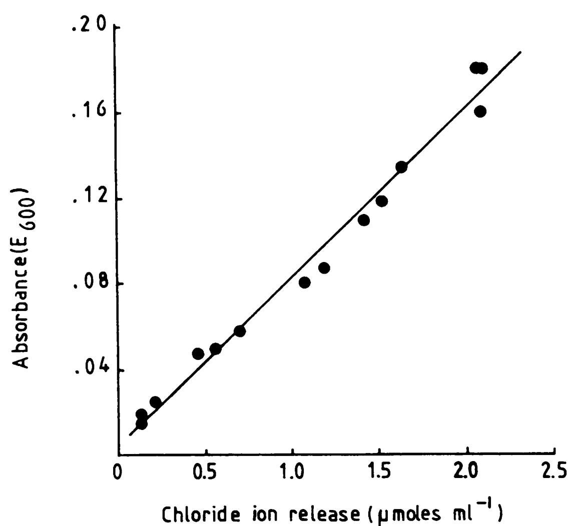

FIG. 3. Plot of increase in absorbance of culture (E_{600}) against released chloride ion concentration for the selected five-member microbial community growing on 0.25 g of carbon per liter (2.1 mM) (mecoprop).

TABLE 1. Influence of initial mecoprop concentration on the batch culture growth characteristics for a five-member mecoprop-utilizing community

Mecoprop concn (g of C per liter [mM])	Length of lag phase (h)	Specific growth rate (per h)	% Mecoprop used after growth ceased
1.0 (8.33)	18	0.09	67
2.0 (16.7)	31	0.08	58
3.0 (25.0)	47	0.06	51
4.0 (33.3)	61	0.08	32
5.0 (41.67)	144	0.02	39
6.0 (50.0)	240	0.02	30
7.0 (5.83)	288	0.02	27
8.0 (66.7)	528	0.01	14

was able to grow on mecoprop as the sole carbon and energy source (Table 2).

In chemostat culture with mecoprop at a growth-limiting concentration of 0.25 g of carbon per liter and an initial dilution rate of 0.02/h, the five-member community was maintained for nearly 1,600 h of continuous growth. The absorbance of the culture was 0.2, a value expected from batch culture with 100% breakdown of the herbicide and full chloride ion release. Again, even after a period of growth in the chemostat, none of the isolates was able to grow on mecoprop as the sole carbon and energy source.

Reconstituted experiments with all combinations of the five organisms in two- or three-member communities showed, with one exception, that mixed cultures containing the *Alcaligenes* species, the *Pseudomonas* species, or both were able to grow on mecoprop, as determined by an increase in the absorbance of the culture and chloride ion release. Equivocal results were obtained with the two-member mixture of the *Alcaligenes* species and *P. maltophilia*. With this exception, if growth did occur it was rapid, and degradation was completed within 48 h. Interestingly, the one three-member mixed culture which did not contain either the *Alcaligenes* species or the *Pseudomonas* species (which from the two-member studies appeared to have a significant role in mecoprop degradation) was able to degrade the herbicide just as rapidly as other combinations. Even in the presence of an easily utilizable substrate, none of the five cultures alone was able to grow on mecoprop.

The five-member community was also able to grow on MCPA and 2,4-D as sole carbon and energy sources, but not

TABLE 2. Growth characteristics of the five bacteria isolated from a mecoprop-degrading community[a]

Carbon and energy sources	*A. calcoaceticus*	*P. maltophilia*	*Alcaligenes* sp.	*Pseudomonas* sp.	*Flavobacterium* sp.
Mecoprop	−	−	−	−	−
2,4-D	−	+	−	+	−
MCPA	−	−	−	−	−
2,4,5-T	−	−	−	−	−
Glucose	+	+	+	+	+
Succinate	+	+	+	+	+
Benzoate	+	+	+	+	+
Acetate	−	−	+	+	−
Lactate	+	−	−	+	+
Phenoxyacetate	−	−	−	−	−

[a] Pure cultures were grown overnight on nutrient broth and inoculated to give a 2% (vol/vol) inoculum with the carbon compounds at 0.25 g of carbon per liter.

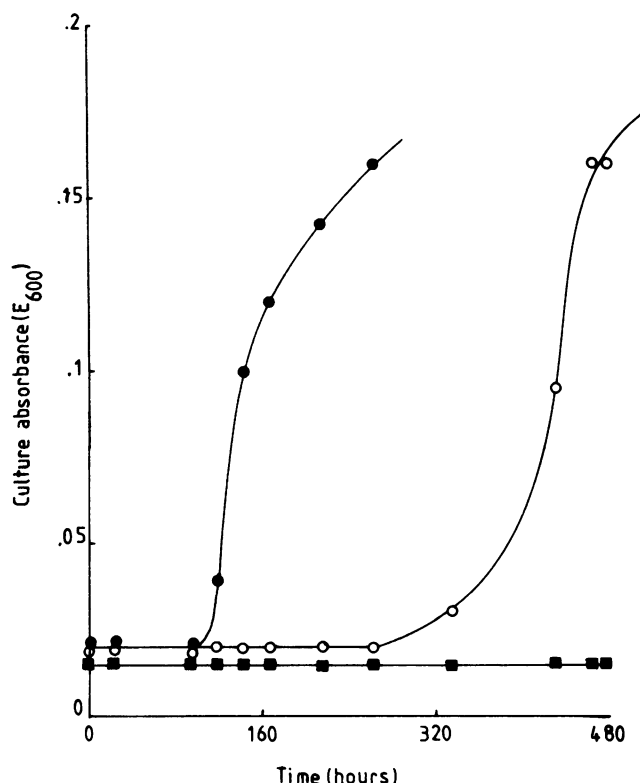

FIG. 4. Growth of the five-member microbial community on 2,4-D (○), MCPA (●), and 2,4,5-T (■), each at 0.25 g of carbon per liter.

on 2,4,5-T (Fig. 4). There were typical lag phases, but the cultures did not need prolonged adaptation times.

DISCUSSION

Since the initial use of chlorinated phenoxyalkanoic compounds as selective herbicides in the mid-1940s, there has been extensive interest in their environmental persistence and biodegradability. The pathways for 2,4-D and MCPA have been largely elucidated (1, 4, 6, 11, 12, 15, 24), but little has been published about the route of catabolism of mecoprop. Kilpi (13) isolated a microbial community from soil which had been exposed to mecoprop, but breakdown of the herbicide only occurred when benzoic acid was present as a cometabolic compound. Furthermore, the ability was lost on prolonged subculturing. Mecoprop is known to be degraded in natural environments, as disappearance rates ranging between 5 and 11 days have been reported (23).

The work reported in this paper demonstrates a number of fundamentally important points concerned with biodegradation studies. It is obvious that prolonged enrichment times are required in laboratory cultures to achieve the selection of the appropriate catabolic capability. Moreover, a certain mecoprop concentration (2.1 mM) must not be exceeded if this selection is to occur. We do not know the nature of mecoprop toxicity at high concentrations, but as we have observed previously (19), the presence of toxic compounds, especially halogenated ones, can induce recalcitrance by killing those organisms which can initially degrade the compound in question (25, 26). We believe that many cases of apparent recalcitrance to degradation have been reported simply because inappropriate initial concentrations have been chosen in the attempts to find the appropriate catabolic potential. Thus, an enrichment strategy which starts with very low concentrations may, as in this instance, produce either a pure or a mixed culture capable of degrading much higher concentrations than were initially used for the primary enrichment. Thus, the five-member community was capable of growing at mecoprop concentrations which were 32 times greater than the initial enrichment conditions. It may be, therefore, that a much wider genetic potential for biodegradation does exist in nature but that this potential, either in the form of single organisms or synergistically acting mixed cultures, is never realized because it is actively selected against in laboratory cultures by the very conditions designed to produce the capability.

Furthermore, neither during the initial enrichment nor during the subsequent growth studies has it proved possible to obtain a single organism with the complete capacity to degrade the herbicide. Once again, as we have highlighted elsewhere (16, 18–20, 22), synergistic microbial communities can be important factors for biodegradation to occur, and undue adherence to pure culture techniques can severely jeopardize the changes of demonstrating biodegradation.

This particular community has, to our knowledge, some unusual, if not unique, features. The community cannot be divided into standard primary and secondary utilizers (see, for example, reference 18), nor does it contain a precise synergism between two or more members of the community (see, for example, references 3 and 5). The reconstituted experiments showed that various combinations of two or more organisms generated the full potential to degrade mecoprop. Following on from this observation it is, therefore, interesting to note that prolonged batch and continuous culture studies with the five-member community failed to reduce its complexity even though a two-member association of, say, the *Pseudomonas* species and the *Flavobacterium* species would have given the necessary catabolic potential. As yet we do not have a sufficiently complete understanding of the kinetic parameters involved with the pure or mixed cultures; what is fully apparent is that the five-member community is the preferred stable entity. This situation is analogous to the preferred stability of a three-member orcinol-degrading community selected in preference to an equally capable two-member derivative community (A. Osman, A. T. Bull, and J. H. Slater, Abstr. 5th Int. Ferment. Symp., p. 124). Clearly, therefore, in the five-member mecoprop community, there are several combinations of organisms with the capacity to degrade mecoprop coexisting within a single stable microbial community.

ACKNOWLEDGMENTS

H.M.L. thanks the Science and Engineering Research Council for a CASE studentship. We thank F. Taylor (W.R.O.) for helpful technical discussions.

LITERATURE CITED

1. **Bollag, J.-M., G. G. Briggs, J. E. Dawson and M. Alexander.** 1968. Enzymatic degradation of chlorocatechols. J. Agric. Food Chem. **16**:829–833.
2. **Bordeleau, L. M., and R. Bartha.** 1968. Ecology of a pesticide transformation: synergism of two soil fungi. Soil Biol. Biochem. **3**:281–284.
3. **Daughton, C. G., and D. P. H. Hsieh.** 1977. Parathion utilization by bacterial symbionts in a chemostat. Appl. Environ. Microbiol. **34**:175–184.
4. **Evans, W. C., and B. S. W. Smith.** 1954. The photochemical inactivation and microbial metabolism of the chlorophenoxyacetic acid herbicide. Biochem. J., vol. **57**.

5. **Feinberg, E. L., P. I. N. Ramage, and P. W. Trudgill.** 1980. The degradation of n-alkylcycloalkanes by a mixed bacterial culture. J. Gen. Microbiol. **121:**507–511.
6. **Gaunt, J. K., and W. C. Evans.** 1971. Metabolism of 4-chloro 2-methyl phenoxyacetate by a soil pseudomonad. Ring fission, lactonizing and delactonizing enzymes. Biochem. J. **122:**533–542.
7. **Greaves, M. P.** 1980. Herbicide effects on the root microflora, p. 28–30. *In* Report of the Weed Research Organisation, 1978–1979. Agriculture Research Council, London, United Kingdom.
8. **Gunner, H. B., and B. M. Zuckerman.** 1968. Degradation of Diazon by synergistic microbial action. Nature (London) **217:**1183–1184.
9. **Hill, I. R., and S. J. L. Wright.** 1978. The behaviour and fate of pesticides in microbial environments, p. 79–136. *In* Pesticide microbiology. Academic Press, Inc., London.
10. **Hiltner, L.** 1904. Uber neuere Ersahrungen und Probleme aus den Gebiete der Bodenbakteriologie unter besouderer Berucksichtigung der Grundungung und Brache. Arb. Dtsch. Landwirtsch. Ges. **98:**59–78.
11. **Kaufman, D. D., and P. C. Kearney.** 1976. Microbial transformations in the soil, p. 29–64. *In* L. J. Audus (ed.), Herbicides, physiology, biochemistry and ecology. Academic Press, Inc., London.
12. **Kearney, P. C., and D. D. Kaufman.** 1972. Microbial degradation of some chlorinated pesticides, p. 166–189. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.
13. **Kilpi, S.** 1980. Degradation of some phenoxy acid herbicides by mixed cultures of bacteria from soil treated with 2-(2-methyl-4-chloro) phenoxypropionic acid. Microb. Ecol. **6:**261–270.
14. **King, O. E., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
15. **Loos, M. A., R. N. Roberts, and M. Alexander.** 1967. Phenols as intermediates in the decomposition of phenoxyacetates by an *Arthrobacter* species. Can. J. Microbiol. **13:**679–690.
16. **Reanney, D. C., P. C. Gowl, and J. H. Slater.** 1983. Genetic interactions among microbial communities, p. 379–421. *In* J. H. Slater, R. Whittenbury, and J. W. Wimpenny (ed.), Microbes in their natural environments. Society for General Microbiology Symposium 34. Cambridge University Press, Cambridge, United Kingdom.
17. **Reineke, W., and H.-J. Knackmuss.** 1979. Construction of haloaromatic utilizing bacteria. Nature (London) **277:**385–386.
18. **Senior, E., A. T. Bull, and J. H. Slater.** 1976. Enzyme evolution in a microbial community growing on the herbicide Dalapon. Nature (London) **263:**476–479.
19. **Slater, J. H., and A. T. Bull.** 1982. Environmental microbiology: biodegradation. Philos. Trans. R. Soc. London **297:**575–597.
20. **Slater, J. H., and D. Godwin.** 1980. Microbial adaptation and selection, p. 137–160. *In* D. C. Ellwood, J. N. Hedger, M. J. Latham, J. M. Lynch, and J. H. Slater (ed.), Contemporary microbial ecology, Academic Press, Inc., London.
21. **Slater, J. H., D. Lovatt, A. J. Weightman, E. Senior, and A. T. Bull.** 1979. The growth of *Pseudomonas putida* on chlorinated aliphatic acids and its dehalogenase activity. J. Gen. Microbiol. **114:**125–136.
22. **Slater, J. H., and H. J. Somnerville.** 1979. Microbial aspects of waste treatment with particular attention to the degradation of organic compounds, p. 221–261. *In* A. T. Bull, D. C. Ellwood, and C. R. Ratledge (ed.), Microbial technology: current status, future prospects. Cambridge University Press, Cambridge, United Kingdom.
23. **Smith, A. E., and B. J. Hayden.** 1981. Relative persistence of MCPA, MCPB and mecoprop in Saskatchewan soil and the identification of MCPA in MCPB-treated soils. Weed Res. **21:**179–183.
24. **Tiedje, J. M., J. M. Duxbury, M. Alexander, and J. E. Dawson.** 1969. Metabolism pathway of degradation of chlorocatechols by an Arthrobacter species. J. Agric. Food Chem. **17:**1021–1026.
25. **Wigmore, G. J., and D. W. Ribbons.** 1980. *p*-Cymene pathway in *Pseudomonas putida*: selective enrichment of defective mutants by using halogenated substrate analogs. J. Bacteriol. **143:**816–824.
26. **Wigmore, G. J., and D. W. Ribbons.** 1981. Selective enrichment of *Pseudomonas* spp. defective in catabolism after exposure to halogenated substrates. J. Bacteriol. **146:**920–927.