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Transformation of metolachlor in soil inoculated with a *Streptomyces* sp.

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Abstract

Microbial detoxication of pesticides may offer a promising alternative to existing physical-chemical treatment methods. We investigated a strain of *Streptomyces* sp. which can transform metolachlor in a liquid medium for its ability to decontaminate herbicide-treated soil. A cell suspension of *Streptomyces* sp. was added to a silt loam soil (Hagerstown, pH 6.1) which was amended with 10 μg of metolachlor containing 5 nCi ring-UL-^{14}C metolachlor per gram of soil, and the mixture was incubated at 28° C. Inoculation of the sterile soil resulted in the rapid transformation of metolachlor. Analyses of one-week-old samples indicated that approximately 70% of the added radioactivity was recovered in the ethyl acetate and water fractions as products from the inoculated reaction mixture, whereas in the uninoculated control less than 8% of the ^{14}C was found as products and about 80% was recovered in the form of unchanged metolachlor. In native soil, however, the rate of metolachlor disappearance was not enhanced by *Streptomyces* inoculation. In inoculated sterile soil the yields of products were affected by inoculum size, inoculation temperature and substrate concentration, but these variables had no effect on product formation in the inoculated native soil. Addition of Na_2CO_3 (200 μg/g soil) into native soil significantly promoted growth of *Streptomyces* due to the higher pH (7.8) and also stimulated transformation of metolachlor by 30%. Our results suggest that proliferation of the inoculated organisms under favorable conditions is essential for their function as metolachlor degraders in native soil.

Introduction

Metolachlor is one of the most extensively used chloroacetamide herbicides. Its chemical stability and its biological activity provide good control of most annual grasses and many broadleaf weeds, including late germinating grasses. Studies on metolachlor metabolism, degradation and dissipation in soils are described in a recent review article by LeBaron et al. (1988).

Our previous studies have demonstrated microbial cometabolism of metolachlor in growth media (Krause et al. 1985; Saxena et al. 1987). We have also reported catabolic metabolism of metolachlor by indigenous microbial populations in a soil perfusion system (Liu et al. 1988). Microorganisms isolated from the herbicide-perfused soil include species of bacteria, a *Streptomyces* sp., an *Actinomyces* sp., and a *Mucor*. Pure cultures of these microorganisms are highly active in transforming metolachlor in the growth medium, but none can mineralize the herbicide.

Various researchers have shown that microorganisms able to degrade organic pollutants in cul-

ture media or sterile soils sometimes fail to function when inoculated into natural environments (MacRae & Alexander 1965; Anderson et al. 1970; Lehtomaki & Niemela 1975). Reasons for the inability of microorganisms to enhance biodegradation in soil were discussed by Goldstein et al. (1985). Nevertheless, there are reports of biodegradation as a result of soil inoculation. Edgehill & Finn (1983), Pignatello et al. (1983) and Crawford & Mohn (1985) all demonstrated considerable removal of pentachlorophenol (PCP) from contamined soils and water following inoculation with a specific bacterial strain. Enhancement of biodegradation by soil inoculation has also been demonstrated with DDT (Kearney et al. 1969), isopropyl *N*-phenylcarbamate (IPC) (Clark & Wright 1970), parathion (Barles et al. 1979; Daughton & Hsieh 1977), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (Kilbane et al. 1983), polychlorinated biphenyls (Brunner et al. 1985) and oil hydrocarbons (Ismailov 1985). Therefore, it is of interest to investigate the metolachlor-transforming capability of the isolated microorganisms after their re-introduction to soil environments.

In the present study experiments on the ability of a *Streptomyces* sp. to transform metolachlor in sterile and native soils were performed and the disappearance of metolachlor was assessed after various incubation periods. Environmental factors favorable for the proliferation and expression of the degradative potential of the *Streptomyces* were also determined.

Materials and methods

Materials

Metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide] of technical grade (95.4% purity) and ring-UL-^{14}C-metolachlor with a specific activity of 26.1 μCi/mg were provided by Ciba-Geigy Corp., Agricultural Division, Greensboro, NC, USA. The purity of the radiolabelled metolachlor was determined to be 97% by high-performance liquid chromatography.

A Hagerstown silt loam obtained from a field plot (3–7 cm below the soil surface) at University Park, Pennsylvania, was used. The properties of the soil are described in Table 1, and its moisture content at the time of use was 10% (W/W). The soil was stored in a covered plastic container at 20° C and was passed through a 2 mm sieve immediately before use. Soil sterilization was achieved by autoclaving at 121° C for 1 h on each of 3 consecutive days or by exposing to 3 Mrad γ-irradiation.

Preparation of inoculum

A strain of *Streptomyces* sp. isolated from metolachlor-perfused soil (Liu et al. 1988) was used. The culture was maintained on nutrient agar. After incubation at 28° C for 48 hr, the surface of the slant was scraped and the cells were inoculated into a 250 ml Erlenmeyer flask containing 100 ml of the liquid medium (Krause et al. 1985). After a 4-day incubation at 28° C on a rotary shaker (130 oscillations/min), the flask was allowed to stand for 30 min to permit settling of the cells. The supernatant was then carefully decanted, and the cells were washed aseptically with 100 ml of 0.01 *M* phosphate buffer, pH 6.5. This process was repeated twice. After the final washing, an aliquot of the suspension was diluted with the same buffer to a cell density of 24 mg/ml (based on cell wet weight).

Inoculation of metolachlor-amended soil with a Streptomyces sp.

Ten grams of soil were placed in a sterile 70 ml-glass bottle fitted with a teflon-lined screw cap (32 mm diameter). Metolachlor and ring-UL-^{14}C metolachlor were dissolved in a nutrient solution

Table 1. Properties of the soil used.

Soil series texture	Hagerstown silty loam
pH (1 : 1 H_2O)	6.1
Sand (%)	24.0
Silt (%)	54.0
Clay (%)	22.0
Organic matter (%)	2.8
Exch. P (kg/ha)	72.8
CEC (meq/100 g)	12.5
Exch. Ca (Cmol/kg)	4.4
Exch. K (Cmol/kg)	0.30
Exch. Mg (Cmol/kg)	0.70

containing 40 mg of yeast extract and 400 mg of sucrose per 100 ml of distilled water. Three milliliters of this herbicide solution were added to each bottle to yield a final concentration of 10 μg and 5 nCi (approximately 9000 dpm) per gram of soil. A cell suspension (0.5 ml equivalent to 12 mg wet weight) prepared as above was then added; and after thorough mixing of the soil, the inoculated samples were incubated in the dark at 28° C. The final moisture content of the inoculated soil sample was 45% (w/w), and was maintained at that level during prolonged incubation by the addition of sterile distilled water. Every second day the bottle was opened under a sterile hood to permit air exchange. Contaminated soils which were not inoculated with the *Streptomyces* sp. served as controls. Each treatment was performed in duplicate and experiments were repeated at least once.

Extraction and determination of radioactivity and metolachlor

At different time intervals, 10 g soil samples were extracted with 30 ml of methanol. After centrifugation (6000 g for 20 min) the methanol extract was decanted and saved, and the soil was re-extracted with another 30 ml of methanol. Radioactivity in the pooled methanol extract was determined by a scintillation counter. After evaporation of the combined methanol extract on a rotary evaporator, residues were dissolved in 10 ml of distilled water and extracted with an equal volume of hexane. Subsequently, the aqueous phase (8.0 ml) was re-extracted with ethyl acetate and the ^{14}C in 0.5 ml samples of both the ethyl acetate extract and aqueous solution were determined. The hexane extract (8 ml) was subsequently evaporated to dryness with a stream of nitrogen. Residues were dissolved in 1.0 ml hexane and injected into a gas chromatograph for analysis of metolachlor.

Analytical methods

Gas-liquid chromatographic (GLC) analysis was performed on a Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector and a Hewlett Packard 3392A integrator. A capillary column (RTX-5, 30 m × 0.32 mm I.D.) purchased from Restek Corp. (Port Matilda, PA) was used. Samples were injected in the split mode with a split ratio of 80 : 7. Helium was used as the carrier gas at a fixed pressure of 48 psi. Isotherm elution was made at injector, detector, and column temperatures of 250° C, 275° C, and 220° C, respectively.

Thin-layer chromatography (TLC) was carried out on 0.25 mm silica gel plates 60 F-254 (E. Merck Darmstadt, West Germany). The plates were developed in a solvent system of hexane: methylene chloride : ethyl acetate (6 : 1 : 4, v/v).

Radioactivity was measured in Scintiverse II scintillation cocktail (Fisher Scientific Co., Fair Lawn, NJ) using a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove Village, IL). Samples of soil residues or silica gel removed from TLC plates were mixed with 15 ml of Scintiverse II in a glass vial to which 3 ml of H_2O was added to make a homogeneous gel and subsequently counted.

Results

The effect of a Streptomyces sp. on the transformation of metolachlor in sterile and native soils

One week after inoculation of a *Streptomyces* sp. into metolachlor-amended autoclaved soil, the radioactivity extractable into the methanol fraction was approximately the same for inoculated and uninoculated samples. However, only 13% of the

Table 2. TLC analysis of ethyl acetate extractable radioactivity.[a]

Rf on TLC	% of applied ^{14}C[b]
0.00	21.0
0.14	23.7
0.25	41.5
0.41	13.6
0.70 (metolachlor)	0.0
	99.8

[a] Sample was obtained one week after inoculation of an autoclaved soil with a *Streptomyces*.

[b] Based on 5000 dpm of radioactivity applied.

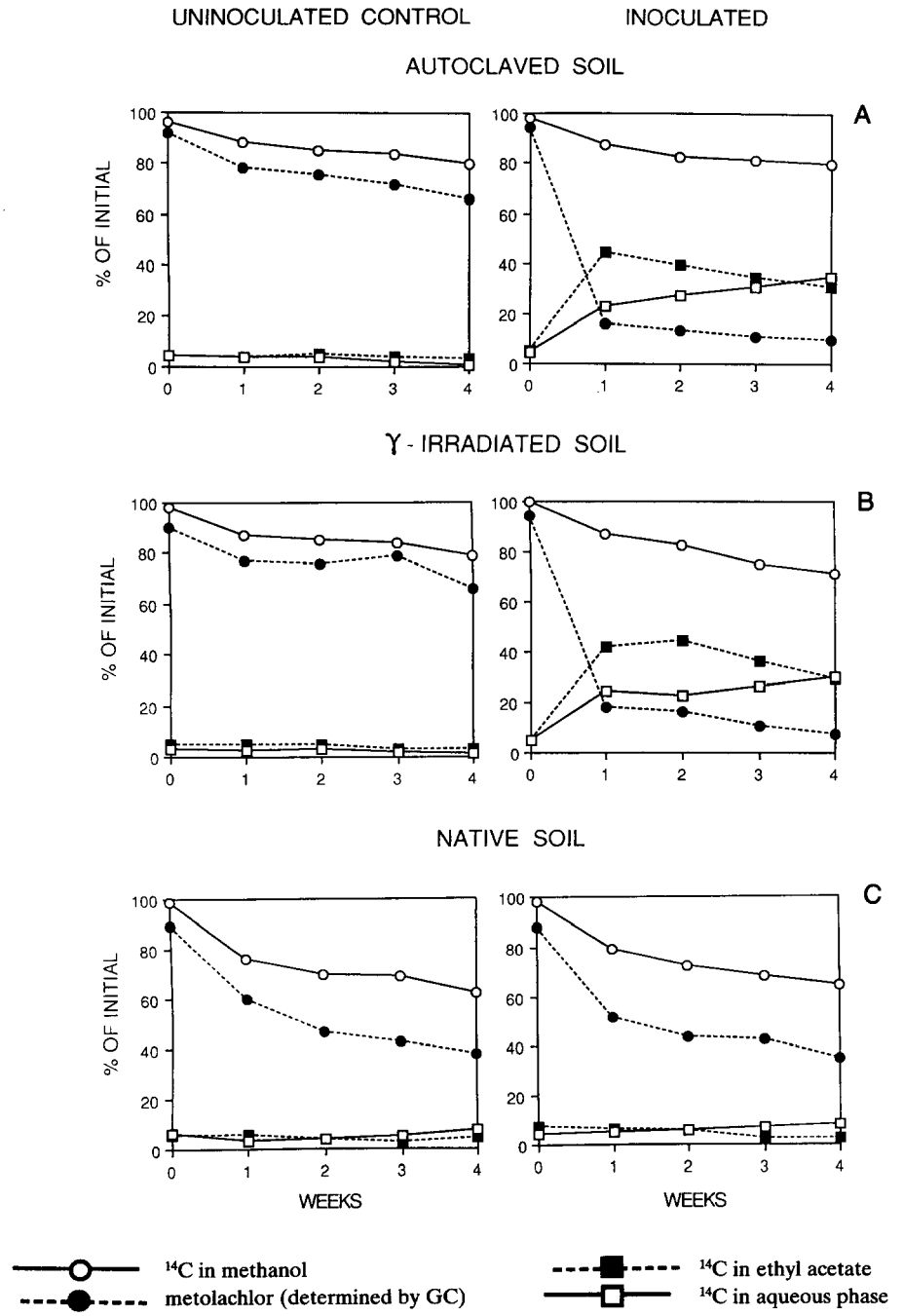
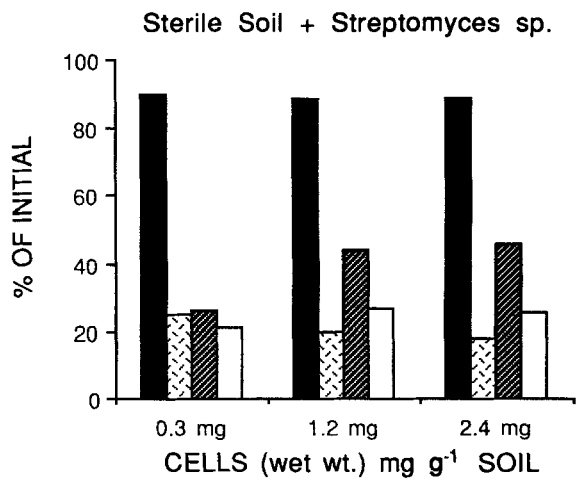
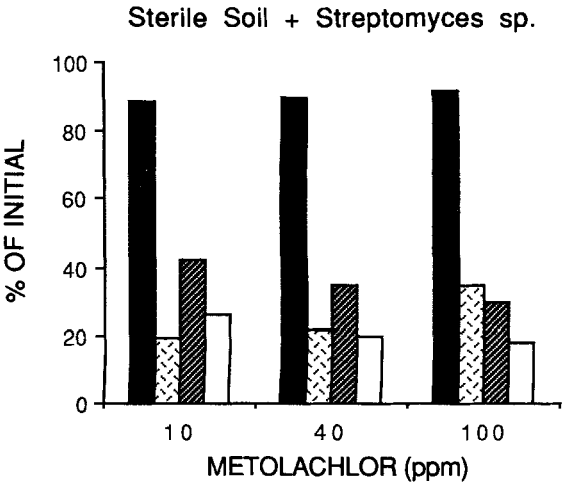
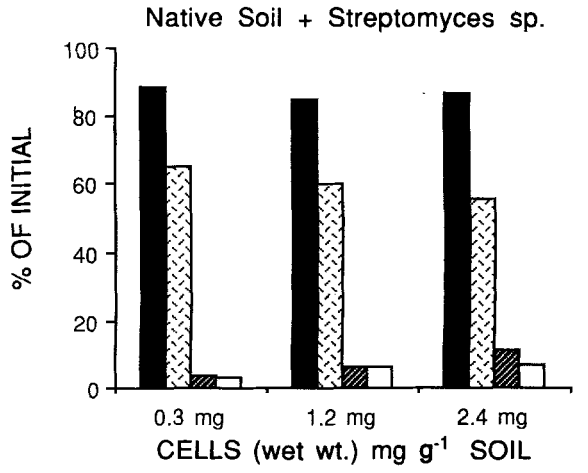


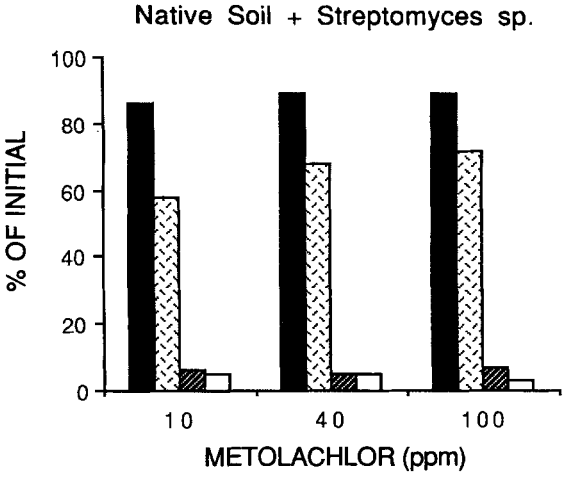
Fig. 1. The effect of a *Streptomyces* sp. on the transformation of metolachlor in sterile and native soils.

^{14}C in this fraction was determined to be metolachlor in the inoculated sample, while in the uninoculated control about 80% of the added chemical remained in the form of metolachlor. The ^{14}C in the ethyl acetate and water fractions constituted 45% to 25% of the applied chemical, respectively, in the inoculated sample, whereas a very low percentage of ^{14}C (2–4%) was found in these two fractions from









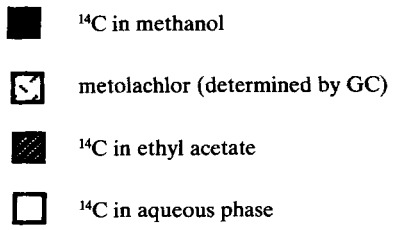


Fig. 2. The effect of inoculum size on the transformation of metolachlor. (Incubation time: 1 week.)

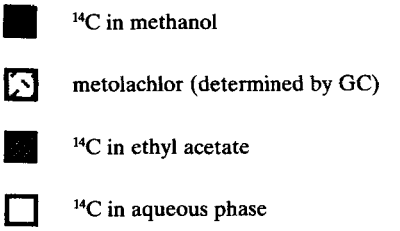


Fig. 3. The effect of metolachlor concentration on the transformation of metolachlor by a *Streptomyces* sp. (Incubation time: 1 week.)

the uninoculated control (Fig. 1A). TLC analysis of the ethyl acetate extract from inoculated samples (one week old) revealed the presence of four radioactive spots, but no metolachlor was detected in this fraction (Table 2). The major spot at Rf 0.25 contained 41% of the applied radioactivity.

In the inoculated autoclaved soil, the amount of ^{14}C extracted into the ethyl acetate fraction declined gradually after the first week, and at the same time, more radioactivity was recovered in the water fraction. A similar trend in metolachlor transformation was observed for γ-irradiated soil samples (Fig. 1B). In strong contrast, there was essentially no difference in the rates of transformation of metolachlor between the inoculated and uninoculated native soil (Fig. 1C). More metolachlor disappeared from the uninoculated native soil than from the sterile soil without inoculation. With or without inoculation, the radioactivity remaining in soil residues after 4 weeks of incubation accounted for approximately 15% (data not shown). There were no evident changes in the transformation of the herbicide in the inoculated sterile soil when incubation was extended to 4 weeks. Because there was a strong decrease in metolachlor concentration during the first week, we repeated the experiment in autoclaved soil with daily, rather than weekly, sampling. Transformation of metolachlor occurred as early as 2 days after inoculation, with metolachlor disappearing linearly until the third day, after which the rate of metolachlor disappearance gradually leveled off to reach a steady state (data not shown). Based upon these results, all subsequent experiments were incubated only for one week.

The effect of inoculum size on the transformation of metolachlor by a Streptomyces sp.

The rate of metolachlor transformation was not entirely proportional to the amount of *Streptomyces* sp. initially added to the soils (Fig. 2). In sterile soil, reducing the initial cell density to 0.3 mg g^{-1} soil (i.e., one-fourth of the normal inoculum) decreased the formation of products in the ethyl acetate fraction by 41%. However, the amount of

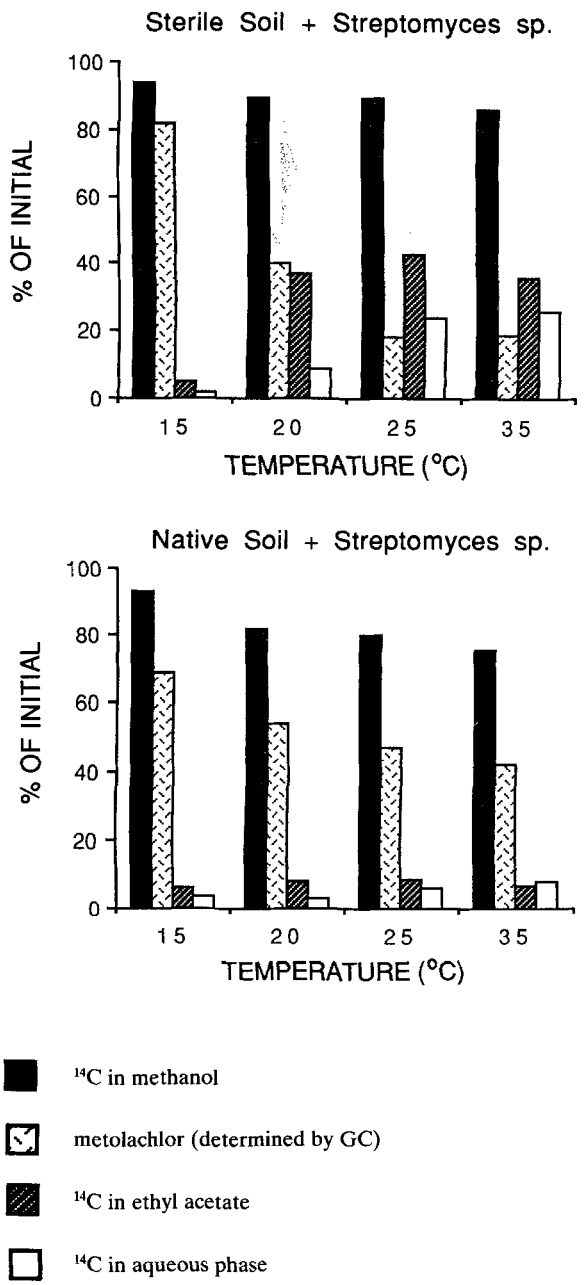


Fig. 4. The effect of incubation temperature on the transformation of metolachlor by a *Streptomyces* sp. (Incubation time: 1 week.)

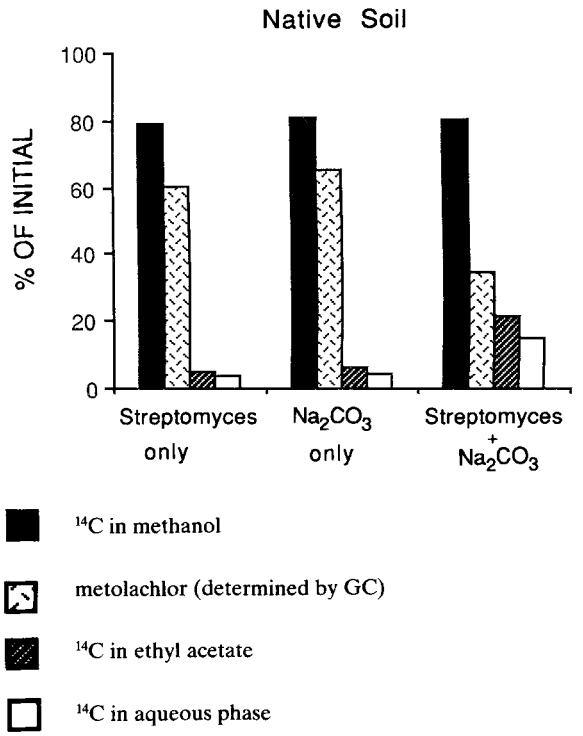


Fig. 5. The effect of soil amended with Na_2CO_3 on the transformation of metolachlor by a *Streptomyces* sp. (Incubation time: 1 week.)

radioactivity in the ethyl acetate fraction was not noticeably increased when the size of the inoculum was doubled. In native soil, increasing the size of the inoculum had no effect on the formation of transformation products.

The effect of substrate concentrations on the transformation of metolachlor by a Streptomyces sp.

As shown in Fig. 3, the amount of ^{14}C extracted into ethyl acetate and water was found to be higher at lower metolachlor concentrations. At initial metolachlor concentrations of 10, 40, and 100 $\mu g\ g^{-1}$ soil, the percentage of the metolachlor which remained unaltered after a one-week incubation was 19, 22 and 35%, respectively. In general, no difference was found in the amount of ^{14}C recovered in the ethyl acetate or water fraction if native soil supplemented with various metolachlor concentrations was inoculated with the *Streptomyces* sp.

The effect of incubation temperature on the transformation of metolachlor by a Streptomyces sp.

As illustrated in Fig. 4, it is evident that incubation temperature has an effect on the transformation of metolachlor. At 15° C no visible proliferation or sporulation of the inoculated *Streptomyces* sp. was observed and consequently there was almost no transformation of metolachlor. At higher incubation temperatures, a mycelial mat and heavy sporulation was seen, and the rate of metolachlor transformation was also increased. Indeed, metolachlor transformation was found to be closely related to the observed growth of the *Streptomyces* sp. At 25° C the rate of metolachlor transformation was similar to that at 28° C, which is the temperature used for all other experiments. Raising the incubation temperature to 35° C had essentially no effect on metolachlor transformation but a slightly smaller amount of ^{14}C was found in the ethyl acetate fraction under this condition. In the *Streptomyces*-inoculated native soil, the incubation temperature did not seem to affect metolachlor transformation.

The effect of soil amended with Na_2CO_3 on the transformation of metolachlor by a Streptomyces sp.

When native soil was amended with Na_2CO_3 at a level of 200 $\mu g\ g^{-1}$ soil, the growth of *Streptomyces* sp. was markedly enhanced as the rise in soil pH from 6.1 to 7.8 favored their proliferation. At the same time, the disappearance of metolachlor was accelerated with accompanying changes in the distribution of radioactivity in the various fractions. For instance, the recovery of ^{14}C in the ethyl acetate fraction of the inoculated, amended soil was approximately 16.5% greater than that of the inoculated control without amendment and about 15% greater than that of uninoculated, Na_2CO_3-amend-

ed soil (Fig. 5). In addition, the amount of radioactivity in the water fraction was also considerably enhanced in Na_2CO_3-amended soil inoculated with the *Streptomyces* sp.

Discussion

Over the past two decades, research into the use of microbial strains to enhance biodegradation of polluted soil has become an important area of study. However, to this end, few advancements were made with respect to the practical application of this technology in the natural soil environment. As Goldstein et al. (1985) have pointed out, for microbial inocula to bring about degradative reactions in a contaminated ecosystem, the inoculated strains must first overcome ecological constraints. Therefore, knowledge of in situ biochemical and ecological processes is essential for implementation of microbial decontamination measurements.

This study was sought to explore the feasibility of transforming metolachlor by the seeding of soil with a *Streptomyces* sp. It is hoped that microbial decontamination of metolachlor and other pollutants may offer a promising alternative to the costly chemical or physical detoxication technologies currently being used. Although our previous studies have established that this microorganism can completely transform metolachlor in an enrichment culture (Liu et al. 1988), in situ experiments must be undertaken as to date there are no reports describing this type of research with chloroacetamide herbicides.

In our study, a one-time application of the *Streptomyces* sp. was used in most experiments, and the moisture content of all samples was held constant throughout the incubation period. Although additional inoculations of the *Streptomyces* sp. at days 3 and 6 were attempted in a preliminary experiment, no enhancement of metolachlor transformation was noted.

The data in Figs. 1 and 2 show that inoculation of sterile soils with the *Streptomyces* sp. resulted in a rapid transformation of metolachlor and a substantial formation of products in both the ethyl acetate and the aqueous phase, whereas only negligible amounts of radioactivity were found in the ethyl acetate or water fraction in the uninoculated sterile soils. Therefore, the enhanced transformation of metolachlor in sterile soil was evidently due to inoculation with the *Streptomyces* sp. Nearly 80% of metolachlor was transformed in the sterile soil after 1 week of inoculation. This relatively rapid metabolic function of the *Streptomyces* sp. correlated with its heavy sporulation and prolific growth at this stage. As the incubation period was extended to 4 weeks, the ^{14}C in the ethyl acetate fraction was reduced slightly, but its TLC profile was essentially unchanged among samples obtained at different time periods.

Our results with native soils, however, did not provide much encouragement. Essentially, no differences were observed between the *Streptomyces*-inoculated and uninoculated soils in terms of product formation. The inability of vegetative cells of the *Streptomyces* sp. to sporulate and multiply in the native soil was easily visualized by the observed lack of a thin white film which always formed on the surface of the inoculated sterile soils. Among the diverse microbial populations in native soil there may exist microorganisms which antagonize the sporulation and proliferation of the inoculated culture and impose a competitive barrier to the effectiveness of the potential degrader. It is apparent that adequate establishment of the inoculated culture is a prerequisite for its metabolic activity on metolachlor transformation.

At 15° C, because of poor sporulation and growth of the *Streptomyces* sp., the amount of products formed was much less than that at the higher temperatures. Temperature, however, did not have any effect on metolachlor transformation in native soil.

Increasing the pH of the native soil by the addition of Na_2CO_3 stimulated both sporulation and growth of *Streptomyces* sp., and enhanced the formation of transformation products in the inoculated samples by approximately 15% over that in the uninoculated control. Using the dilution plate method the population density of the *Streptomyces* sp. cultures grown for one week with added Na_2CO_3 was determined to be 6-fold higher than the control without Na_2CO_3 amendment (data not shown). Similarly, it has been reported that the severity of the common scab of potatoes, which is

caused by *Streptomyces scabies,* was enhanced greatly as the pH of the soil was changed from 5.2 to 8.0 (Agrius 1988). It is also possible that the suppression of competitive microbial populations due to change in soil pH may favor the successful establishment of the *Streptomyces* sp.

The inoculum of *Streptomyces* sp. used in this study was much larger than the possible occurrence of these organisms in nature; however, since the culture was originally isolated from soil, its reintroduction into the environment should not cause ecological problems. Since microbial populations appear more effective in the degradation of metolachlor (Liu et al. 1988), it is our intention to investigate the behavior of these mixed cultures towards the herbicide.

Conclusions

The present study has demonstrated the feasibility of transforming metolachlor through the inoculation of a *Streptomyces* sp. into sterile soil; however, with the native soil attempts were unsuccessful. Further investigations are needed to learn to manipulate environmental variables such that the application of the inoculated culture to a native soil also produces transformation of a particular pollutant.

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