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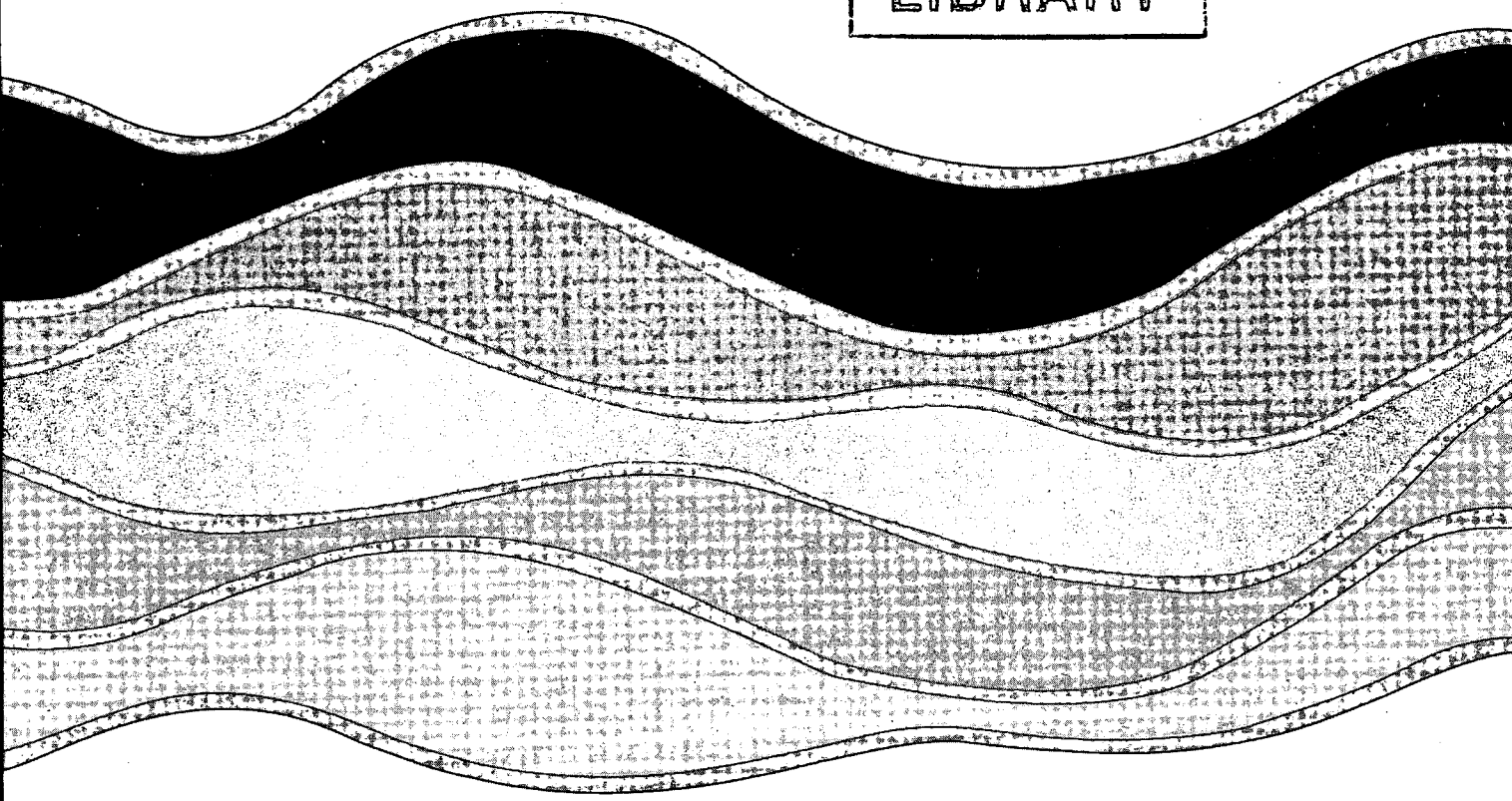

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NWRI Contribution No. 94-77

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MANAGEMENT PERSPECTIVE

Metolachlor is an important selective herbicide used for the control of several annual grassy weeds and certain broad-leafed weeds in corn, soybean, peanut, and other crops. It is the most heavily used agricultural pesticide in Ontario. An estimated 7.2 x 10^3 metric tons of agricultural pesticides (active ingredients) of all types were used in Ontario in 1988. Twenty-four percent of this total was metolachlor. The use of metolachlor in Ontario more than doubled from 1983 to 1988, during which time the related herbicide alachlor was withdrawn from the Canadian market. In order that an assessment can be made of the hazards of metolachlor use to aquatic ecosystems, more information is required on its occurrence in water, its toxicity to aquatic organisms, and its persistence and fate. There is virtually no information in the open literature on the aquatic fate and persistence of metolachlor. This work addresses research needs identified by the Ecosystem Interpretation Branch of Environment Canada in the development of Canadian water quality guidelines for metolachlor, and by the U.S. EPA during its re-registration of metolachlor in the late 1980s.

This study has shown that metolachlor is very stable in natural waters. No biodegradation or biotransformation of metolachlor was observed in the water of three lakes of different trophic status, even after a 170-day incubation in a cyclone fermentor system. Using a polynuclear aromatic hydrocarbon (PAH)-degrading bacterial culture as the test organism, metolachlor was estimated to have much greater environmental persistence than medium molecular weight PAHs. The white rot fungus *Phanerochaete chrysosporium* was able to biotransform metolachlor. Based on the three identified metabolites, a tentative metabolic pathway of metolachlor biotransformation by *P. chrysosporium* was proposed. The pathway involved demethylation, hydroxylation, and hydrolytic dechlorination. In summary, there is potential for the herbicide metolachlor to have a long-lasting impact on Canadian aquatic ecosystems.

SOMMAIRE À L'INTENTION DE LA DIRECTION

Le métolachlor est un important herbicide sélectif utilisé pour la lutte contre plusieurs mauvaises herbes graminées et certaines mauvaises herbes à feuilles larges dans des cultures comme celle du maïs, du soja, de l'arachide et quelques autres. C'est le pesticide agricole le plus intensément utilisé en Ontario. On estime que $7{,}2 \times 10^3$ tonnes métriques d'ingrédients actifs de pesticides agricoles de tous types ont été utilisées en Ontario en 1988. Le métolachor représente 24 % de ce total. L'utilisation de métolachlor en Ontario a plus que doublé de 1983 à 1988, période au cours de laquelle l'herbicide apparenté alachlor a été retiré du marché canadien. Afin de pouvoir évaluer les dangers de l'utilisation du métolachor pour les écosystèmes aquatiques, plus d'information est requise sur sa présence dans l'eau, sa toxicité pour les organismes aquatiques, et sur sa persistance et son devenir. Il n'existe à peu près pas d'information dans la littérature disponible sur le devenir et la persistance du métolachlor en milieu aquatique. Ce document répond à des besoins en recherche signalés par la Direction de l'interprétation de l'écosystème d'Environnement Canada pour l'élaboration de lignes directrices canadiennes de qualité des eaux pour le métolachlor, ainsi que par l'EPA (É.-U.) pour la réhomologation du métolachlor à la fin des années 1980.

L'étude a montré que le métolachlor est très stable dans les eaux naturelles. Aucune biodégradation ou biotransformation du métolachlor n'a été observée dans l'eau de trois lacs d'états trophiques différents, même après une période d'incubation de 170 jours dans un fermenteur à cyclone. À l'aide d'une culture bactérienne dégradant les hydrocarbures aromatiques polycycliques (HAP), on a estimé que la persistance du métolachlor dans l'environnement était très supérieure à celle des HAP de poids moléculaire moyen. Le champignon de la pourriture blanche *Phanerochaete chrysosporium* pouvait biotransformer le métolachlor. En se basant sur la caractérisation de trois métabolites, on a proposé un mécanisme métabolique provisoire de

biotransformation du métolachlor par *P. chrysosporium*. Ce mécanisme fait appel à la déméthylation, à l'hydroxylation et à la déchloration hydrolytique. En bref, il est possible que l'herbicide métolachlor ait des incidences durables sur les écosystèmes aquatiques canadiens.

ABSTRACT

Metolachlor is an important selective herbicide used for the control of several annual grassy weeds and certain broad-leafed weeds in corn, soybean, peanut, and other crops. It is the most heavily used agricultural pesticide in Ontario. There is, however, very little information in the open literature on the aquatic fate and persistence of metolachlor, a fact that hinders the assessment of its ultimate impact on the aquatic ecosystem. This study showed that metolachlor was very stable in natural water systems. No apparent biodegradation or biotransformation of metolachlor was observed in three test lake waters after an incubation period of 170 days. With a polynuclear aromatic hydrocarbon (PAH)-degrading bacterial culture as the test organism, metolachlor was estimated to have an environmental persistence much greater than medium molecular weight PAHs. Thus the extensive herbicidal use of metolachlor may have a long-lasting impact on Canadian aquatic ecosystems. The white rot fungus *Phanerochaete chrysosporium* was able to biotransform metolachlor. Based on the three identified metabolites, a tentative metabolic pathway of metolachlor biotransformation by *P. chrysosporium* was proposed, involving demethylation, hydroxylation, and hydrolytic dechlorination.

RÉSUMÉ

Le métolachlor est un important herbicide sélectif utilisé pour la lutte contre plusieurs mauvaises herbes annuelles graminées et certaines mauvaises herbes à feuilles larges dans des cultures comme celle du maïs, du soja, de l'arachide et quelques autres. C'est le pesticide agricole le plus intensément utilisé en Ontario. Toutefois, on ne trouve que très peu d'information dans la littérature disponsible sur le devenir et la persistance en milieu aquatique du métolachlor, et ceci gêne l'évaluation des incidences à long terme sur les écosystèmes aquatiques. Cette étude a montré que le métolachlor était très stable dans les systèmes aquatiques naturels. On n'a observé aucune biodégradation ou biotransformation visibles du métolachlor dans les eaux de trois lacs expérimentaux, après une période d'incubation de 170 jours. En utilisant une culture bactérienne dégradant les hydrocarbures aromatiques polycycliques (HAP) comme organisme d'essai, on a pu estimer que le métolachlor avait une persistance environnementale très supérieure à celle des HAP de poids moléculaire moyen. Par conséquent, l'utilisation intensive du métolachlor comme herbicide peut avoir des incidences à long terme sur les écosystèmes aquatiques canadiens. Le champignon de la pourriture blanche *Phanerochaete chrysosporium* pouvait biotransformer le métolachlor. En se basant sur les trois métabolites qui ont été caractérisés, on a proposé un mécanisme métabolique provisoire pour la biotransformation du métolachlor par *P. chrysosporium*, utilisant des étapes de déméthylation, d'hydroxylation et de déchloration hydrolytique.

INTRODUCTION

Metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide] is an important selective herbicide used for the control of several annual grassy weeds, yellow nutsedge, and certain broad-leafed weeds in corn, soybean, peanut, and other crops (Krause *et al.*, 1985). Because of its effectiveness, metolachlor has been rapidly replacing other related chloroacetanilide herbicides (*e.g.*, alachlor and propachlor) (Chesters *et al.*, 1989), and global demand for it has risen. In Canada, metolachlor is now the most heavily used herbicide in the Province of Ontario. It was estimated that approximately 1,724,700 kg of active ingredient of this chemical were applied in Ontario in 1988 to control weeds in a variety of field crops (Moxley, 1989).

Metolachlor and alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide] are two structurally related chloroacetanilide herbicides. Their major difference in molecular structure is the methoxyalkyl chain attached to the N atom of the basic structure. This relatively small difference in molecular structure is apparently sufficient to impart some desirable properties to metolachlor as a herbicide. Some of these properties, among others, are higher lipid and water solubilities for metolachlor compared to alachlor (Chesters *et al.*, 1989). Lipophilicity facilitates the penetration of cell membranes and is therefore critical to herbicidal activity. Since lipophilicity is often associated with a chemical's persistence, the greater lipophilicity (in terms of octanol-water partition coefficient) of metolachlor compared to alachlor suggests greater persistence. Recent investigations have shown that metolachlor could be more persistent in soil and aquatic environments than other related chloroacetanilide herbicides (Chesters *et al.*, 1989).

Biodegradation and biotransformation are crucial in the detoxification and removal of toxic substances from the environment. Biotransformation of metolachlor by

microorganisms and aquatic animals has been reported (McGahen and Tiedje, 1978; Krause *et al.*, 1985; Saxena *et al.*, 1987; Liu *et al.*, 1991). However, relatively little is known about the fate and persistence of metolachlor in the natural aquatic environment. Such information is needed to assess and predict the ultimate impact of metolachlor on the environment. The objective of this study was to assess the resistance of metolachlor to degradation by naturally-occurring aquatic microorganisms and laboratory-cultured microorganisms, with emphasis on the isolation and identification of metabolites during biotransformation.

MATERIALS AND METHODS

Chemicals

Metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide] of reagent grade (97%) was obtained from Chromatographic Specialties Inc., Brockville, Ont. Technical grade metolachlor (93.5% active ingredient, 3% "safener" [Benoxacor] and approx. 3.5% emulsifier) and analytical standards of the following metabolites of metolachlor were obtained from Ciba-Geigy Canada Ltd. (Mississauga, Ont.) and Ciba-Geigy Corp., Agricultural Division (Greensboro, NC): CGA-40172, 2-hydroxy-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide; CGA-40919, 4-(2-ethyl-6-methylphenyl)-5-methyl-3-morpholinone; CGA-37735, 2-hydroxy-N-(2-ethyl-6-methylphenyl)acetamide; CGA-50720, N-(2-ethyl-6-methylphenyl)oxalamide; CGA-51202, N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)oxalamide; and CGA-41638,2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide. An analytical standard of the Benoxacor safener was also obtained (CGA-154281, 4-dichloroacetyl-3,4-dihydro-3-methyl-2H-1,4-benzoxazine). The molecular structures of these chemicals are shown in Figure 1. Pesticide grade organic solvents were obtained from Caledon Laboratories, Georgetown, Ont. The sodium sulfate used for drying organic extracts was heated to 500 °C for 24 hours before use. All other chemicals used in the

experiments were reagent grade or better. All glassware was rinsed with pesticide grade solvents before use.

Microorganisms and Culture Conditions

The resistance of metolachlor to biological degradation was tested with a stable mixed bacterial culture, with natural aquatic organisms from three lakes, and with a white rot fungus.

The stable mixed bacterial culture had previously been shown to rapidly degrade low and medium molecular weight polynuclear aromatic hydrocarbons (PAHs) and polynuclear aromatic nitrogen heterocycles (Liu *et al.*, 1992). The experiments were conducted using mineral salt medium in glass-Teflon cyclone fermentors with the technical grade metolachlor at a final concentration of 10 mg/L (Liu *et al.*, 1992). At various times, aliquots were withdrawn from the fermentors for the analysis of metolachlor and its metabolites. An abiotic control experiment (using 100 mg/L of $HgCl_2$) was also performed.

The resistance of metolachlor to biological degradation was also assessed with natural aquatic microorganisms using both the cyclone fermentor and shaker flask techniques. Three types of lake water (oligotrophic, mesotrophic and eutrophic) were collected from the Dorset region of Ontario and were used in the fermentors and shaker flasks without any addition of nutrient or inoculum. In the cyclone fermentor experiments, 1.5 L of the spiked (with the technical grade metolachlor at 5 mg/L) oligotrophic, mesotrophic, and eutrophic lake waters were placed in the fermentors and the disappearance of metolachlor from the test system was determined and compared to a control containing 100 mg/L of $HgCl_2$. In the shaker flask experiments, 1-L Erlenmeyer flasks, each containing 0.5 L of spiked lake waters (with the technical grade metolachlor at 5 mg/L), were incubated at room temperature (21 °C) on a rotary shaker (140 rpm). Aliquots were withdrawn from the flasks at various times for the analysis of metolachlor

and its metabolites. An abiotic control experiment (using 100 mg/L of $HgCl_2$) was also performed.

The resistance of metolachlor to fungal degradation was assessed in both liquid and solid growth media using the white rot fungus *Phanerochaete chrysosporium* (ATCC 20696). The growth medium was a modified malt extract medium (MME) of composition 6.0 g of malt extract base, 1.8 g of maltose, 6.0 g of glucose, and 120 mg of yeast extract per litre of distilled water. An appropriate amount of the technical grade metolachlor (in acetone) was added directly to this growth medium to achieve a final metolachlor concentration of 50 mg/L. The pH of the medium was then adjusted to 4.7 before sterilization at 120 °C for 15 minutes. A solid growth medium was also used which had essentially the same composition as the above liquid fungal growth medium, except that it was supplemented with 15 g of pure agar per litre.

The heterogeneous nature of the *P. chrysosporium* cultures (*e.g.*, a tangled mycelium in liquid media, or a radial mat on solid media) made it difficult to have each individual flask or agar plate inoculated with the same amount of fungal biomass. It was found that a reproducible amount of fungal inoculum could be obtained by using fungal plugs taken from a growing fungal culture on an agar plate. To obtain the fungal plugs the bulb end of sterilized Pasteur pipets was used to cut out the plugs from the plate. Since the plugs (containing fungal biomass) were uniform in size, they could be used as a convenient source of a similarly-sized fungal inoculum.

To assess the resistance of metolachlor to fungal degradation on solid growth medium, agar plates with or without metolachlor (50 mg/L) were inoculated with fungal plugs, and were incubated together with control agar plates (*i.e.*, plates without inoculum) at room temperature (21 °C) for various times. Sampling times for metolachlor and its metabolites were guided by the observation of the fungal growth radiating from the plug on the inoculated agar plates. In experiments with the liquid growth medium, inoculated

and control 1-L Erlenmeyer flasks, each containing 500 mL of MME with or without the addition of the technical grade metolachlor (final concentration at 50 mg/L), were incubated at room temperature on a rotary shaker (120 rpm). At appropriate times, 10 mL aliquots were withdrawn from the flasks aseptically for the analysis of metolachlor and its degradation products.

Sample Preparation and Chemical Analysis

For the analysis of metolachlor in PAH-degrading bacterial suspensions and in the natural waters, a 10-mL aliquot was withdrawn from the cyclone fermentors or shake flasks at various times. The samples were extracted with dichloromethane (1 x 10 mL and 2 x 5 mL), and the combined extracts were dried by passage through an anhydrous sodium sulfate column. A toluene "keeper" was added to the extracts, which were then reduced in volume to 5 mL on a rotary evaporator. Further concentration and solvent exchange into toluene was performed under a stream of nitrogen to achieve a final volume of 0.5 mL.

To assess the biodegradation of metolachlor by *P. chrysosporium* on solid agar growth medium, the inoculated agar plates were analyzed at appropriate times. The agar in each plate was cut into small pieces with a stainless steel knife and placed in a 125-mL Erlenmeyer flask containing 50 mL of dichloromethane. The flask was placed on a rotary shaker (220 rpm) for 1 hour and the dichloromethane was decanted from the flasks and dried by passage through anhydrous sodium sulfate. A toluene keeper was added to the resulting extracts were then concentrated to 5 mL on a rotary evaporator. Further concentration and solvent exchange into toluene was performed under a stream of nitrogen to obtain a final volume of 0.5 mL. To assess the degradation of metolachlor by *P. chrysosporium* in liquid growth medium, a 10-mL aliquot was withdrawn from the Erlenmeyer flask and extracted with dichloromethane (1 x 10 mL and 2 x 5 mL). Further

concentration of the extract was the same as for the bacterial degradation experiments described above.

The toluene extracts were analyzed on a Hewlett Packard 5890-II gas chromatograph with a single splitless injector - dual column - nitrogen-phosphorus/flame ionization detector (NPD/FID) technique. Both columns were DB-5 [polymethyl(5% phenyl)siloxane] (J & W Scientific - Chromatographic Specialties Inc., Brockville, Ont.), 0.25 mm i.d. x 30 m length, with 0.25 µm film thickness. Injector and detector temperatures were 200 °C and 300 °C, respectively. The initial column temperature was 90 °C for 2 minutes, and the program rate was 10 °/minute to 150 °C, then 4 °/minute to 280 °C, and then 8 °/minute to 300 °C with no final hold. The constant helium carrier gas flow rate was 0.8 mL/min. The gas flow rate for air and hydrogen was set up based on the type of detector used (FID, air - 400 mL/min, hydrogen - 30 mL/min ; NPD, air - 120 mL/min, hydrogen - 4 mL/min).

Mass spectral analyses were done with a Hewlett Packard 5890-II gas chromatograph, 7673 autosampler (1 µL injections), 5971A mass selective detector, MS Chem Station, and the same column and temperature program. The MSD was operated in electron impact mode with an ionization potential of 70 eV and a source temperature of 190 °C. The scan range was 50-500 amu.

RESULTS AND DISCUSSION

Mixed bacterial culture

The inability of the native microorganisms in the three test lake waters (see text below) to biodegrade or biotransform metolachlor over a very long contact period prompted a study of the persistence of metolachlor using a PAH-degrading culture with a known biodegradation potential (Liu *et al.*, 1992). After a six-month incubation, no

biodegradation or biotransformation of metolachlor was observed in cyclone fermentors or shaker flasks (data not shown). To check whether the PAH-degrading cultures still retained their degradation potential during this long incubation period, a mixture of naphthalene and phenanthrene was added to a test fermentor at the termination of the experiment. The immediate onset of the biodegradation of the two added PAHs verified the degradation potential of the culture and underscored the resistance of metolachlor to bacterial degradation, with a persistence greater than, or equal to, that of medium molecular weight PAHs. Since the occurrence of bacterial metabolism of metolachlor has been reported (Saxena et al., 1987; Liu et al., 1989), the role of bacteria in the biotransformation of metolachlor in the environment cannot be underestimated.

Natural water samples

Water quality can significantly affect the fate and persistence of a chemical in the aquatic environment. Contaminants are usually less persistent in eutrophic water, due to the higher level of nutrients and microorganisms. Consequently, it is essential to determine the persistence of chemicals in waters over a range of trophic status. Several parameters are commonly used in the classification of the trophic state of fresh-water lakes (Chapra and Dobson, 1981). In this study the trophic state of the three test lakes was determined on the level of total phosphorus and chlorophyll a in the water (see Table 1). Based on these criteria, the three Ontario lake waters (Blue Chalk Lake, Gull Feather Lake, and Moot Lake) used in our metolachlor persistence study would fall into the categories of oligotrophic (nutrient poor), mesotrophic (nutrient intermediate), and eutrophic (nutrient rich), respectively (see Table 2).

Metolachlor was quite persistent in all three natural waters, with no degradation found over 120 days when tested at 4, 15 and 40 mg/L in shaker flask experiments (see Figure 2). [In separate experiments the toxicity of metolachlor was assessed using two short-term bioassays (Thomson *et al.*, 1986; Kwan, 1993), and the results indicated that

metolachlor was not toxic to the test microorganisms at concentrations up to 500 mg/L (data not shown).] GC-NPD/FID and GC/MS analyses of extracts of the test solutions at the end of the experiments showed no degradation or transformation products. Metolachlor was also shown to be persistent in the cyclone fermentor experiments over a period of 170 days. The experimental results were complicated by losses of metolachlor from both control and test fermentors. Because there was generally as much, or more, loss of metolachlor from the control solutions as from the test solutions, however, the losses were attributed to volatilization from the vigorously mixed cyclone fermentor broth.

The persistence of metolachlor in aquatic systems is supported by results from other media. For example, Walker and Brown (1985) studied the relative persistence of five acetanilide herbicides in soil and concluded that metolachlor was the most persistent, with a half-life of more than 70 days. The measurement of degradation rates for herbicides in the field is often complicated by the fact that many factors could simultaneously affect the determination. Factors known to influence the persistence of metolachlor in soil systems include soil type and moisture (Zimdahl and Clark, 1982), soil temperature and depth (Bouchard *et al.*, 1982; Rao *et al.*, 1986), and the pre-treatment of soils with herbicides (Bailey and Coffey, 1986). Because of these variables, the values of the reported half-lives for metolachlor in soils ranged from 52 to 277 days (Bouchard *et al.*, 1982). There has been very little information in the open literature on the aquatic fate and persistence of metolachlor. LeBaron *et al.* (1988) calculated the chemical hydrolysis half-life of metolachlor at 20°C to be greater than 200 days at pH 1-9. Kochany and Maguire (1994) concluded that metolachlor was fairly stable to chemical degradation in natural lake water in the dark, with less than 4% loss after 100 days. Our biological degradation results are in agreement with the above results, and indicate that metolachlor is persistent in aquatic environments.

White rot fungus

In recent years the use of fungi to study the biodegradation of recalcitrant chemicals has generated a considerable amount of interest in the area of bioremediation research and applications. The interest in this subject arises from the ability of white rot fungi to degrade a diverse range of very persistent and/or toxic environmental contaminants (Barr and Aust, 1994). This ability appears to be a result of the non-specific substrate requirements of extracellular enzymes from such fungi. The failure of our PAH-degrading bacterial culture and the native microorganisms in the three test lake waters to biodegrade metolachlor prompted an investigation of the feasibility of using the white rot fungus *P. chrysosporium* to degrade metolachlor.

Unlike bacterial metabolism of organic chemicals, fungal biodegradation is often characterized by the accumulation of many degradation products. Figure 3 shows the production of normal growth metabolites by a culture of *P. chrysosporium* on the regular growth medium (MME) over a period of 48 days. The production of these normal metabolites was time-related, *i.e.*, the profile of metabolite production changed with the time of incubation. Because of this potential complication, several controls were used, *e.g.*, a growth control (fungal growth on MME medium without metolachlor), a medium control (sterile MME medium), and a chemical control (sterile MME medium plus metolachlor) in the fungal biodegradation experiments. Without such controls, it was difficult to differentiate metolachlor metabolites from chemicals derived from fungal growth on normal growth medium (for example, see Figure 4). As a further illustration, the peak at 30.77 min in the total ion chromatogram of the extract from the test growth plate (agar growth plate with metolachlor) (see Figure 5), was a metabolite resulting from normal fungal growth on the modified malt extract medium and those peaks occurring between 32.21 to 38.82 min were the actual metabolites from the fungal biotransformation of metolachlor.

From the numerous metolachlor metabolites produced by the white rot fungus *P. chrysosporium* (see Figure 5), only three could be tentatively identified by retention time and/or mass spectral matching with known standards or comparison with published mass spectra. Metabolite M-4 was tentatively identified as 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-hydroxy-1-methylethyl)acetamide by GC-NPD/FID, and GC-MSD retention time matching with a known standard (CGA-41638). The identification was confirmed by matching the electron impact GC-MSD spectrum for metabolite M-4 with CGA-41638. As shown in Figure 6, the EI/MS spectra were identical, with a molecular ion at m/z 269 and major fragment ions at m/z 238, 211, 162, and 146. The spectra also showed that the parent mass had a chlorine moiety. This metabolite occurs commonly in the metabolism of metolachlor by various organisms, including bacteria, actinomycete, fungi, and fish (McGahen and Tiedje, 1978; Krause *et al.*, 1985; Saxena *et al.*, 1987; Cruz *et al.*, 1993). Interestingly, the *in vitro* oxidation of metolachlor by rat liver cytosolic and microsomal enzymes also yielded M-4 (Feng and Wratten, 1989), thus suggesting that 2-chloro-N-(2-ethyl-6-methylphenyl)-N-2-hydroxy-1-methylethyl)acetamide is probably a major and/or universal metabolite during the biological degradation of metolachlor.

Without an available chemical standard, metabolite M-5 could be only tentatively identified by comparing its EI/MS spectrum with published data (Cruz *et al.*, 1993). As can be seen in Figure 7, M-5 had major fragment ions at m/z 254, 227, 210 (base peak), and 160 and the rest of the spectrum was consistent with the mass spectrum of the hydroxylated metabolite O-7 as reported by Cruz *et al.* (1993). On the basis of these GC/MS data, the M-5 was tentatively identified as 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(2-methoxy-1-methylethyl)acetamide. This compound was also identified as the metabolite F during the biotransformation of metolachlor by a soil actinomycete (Saxena et al., 1987).

Metabolite M-1 was also only tentatively identified, due to the lack of a chemical standard. GC/MS analyses indicated that M-1 had a molecular ion at m/z 263 and major

fragment ions at m/z 218, 191, 176, and 160 (see Figure 8). The spectral data also revealed the lack of a chlorine isotopic pattern, indicating that M-1 was a dechlorinated metabolite of metolachlor. As can be seen in Figure 8 the mass spectrum of M-1 matched fairly well that of bacterial metabolite S-1 of Liu *et al.* (1989), which was tentatively identified as 2-hydroxy-N-(2-methyl-6-vinylphenyl)-N-(2-methoxy-1-methylethyl)acetamide. The mass spectrum of M-1 was also similar to that of fungal metabolite IV of McGahen and Tiedje (1978), which was also tentatively identified as 2-hydroxy-N-(2-methyl-6-vinylphenyl)-N-(2-methoxy-1-methylethyl)acetamide. Consequently, metabolite M-1 is tentatively identified as that compound.

The results from the present study showed that the metabolic pathways of biotransformation of metolachlor by the white rot fungus *P. chrysosporium* were somewhat similar to those observed in fish, bacteria, and *Actinomycetes*. The metabolites M-4, M-5, and M-1 were also metabolites of metolachlor biotransformation by a soil fungus (McGahen and Tiedje, 1978), bacteria (Liu *et al.*, 1989), soil actinomycetes (Krause *et al.*, 1985; Saxena *et al.*, 1987), rat liver microsomal enzymes (Feng and Wratten, 1989), and by bluegill sunfish (Cruz *et al.*, 1993). The proposed metabolic pathway for metolachlor (see Figure 9) by *P. chrysosporium* includes O-demethylation of the N-alkyl substituent of metolachlor to yield metabolite M-4, and hydroxylation of the benzylmethyl group to form metabolite M-5. A subsequent hydrolytic dechlorination of metabolite M-5 may lead to the formation of the hypothetical intermediate 2-hydroxy-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(2-methoxy-1-methylethyl)acetamide, whose subsequent dehydrogenation yields the vinylphenyl metabolite M-1. Metabolite M-4 appears to be a very stable compound and would not undergo any further biotransformaation. When M-4 was incorporated into the MME agar plate as the test compound, no sign of biotransformation by P. chrysosporium was observed over an incubation period of of 2 months (data not shown).

Benzylic hydroxylation of the aralkyl side chains and O-demethylation at the N-alkyl substituent have been demonstrated to be major pathways in the biological transformation of metolachlor (Krause *et al.*, 1985; Feng and Wratten, 1989; Cruz *et al.*, 1993). The process of hydrolytic dechlorination appears to occur less frequently in the metabolism of metolachlor. Only one report (LeBaron *et al.*, 1988) was found in the literature that described hydrolytic dechlorination and O-demethylation as being major metabolic pathways for metolachlor in the rat. Metabolism studies with the white rot fungus may also be complicated because of the multiplicity of products produced through the action of non-specific lignin peroxidases and manganese-dependent peroxidases. Evidence for the multiplicity of products is given in Figure 10, which shows mass spectra of three metabolites (M-1, M-2 and M-3) of metolachlor produced by *P. chrysosporium*. These closely-eluting metabolites (see Figure 5) had very similar mass spectra, with parent ions at m/z 160 and major ions at m/z 218, 191, 174/176, and 145. Some of these metabolites may be diastereoisomeric isomers, as has been suggested for other microbial metabolites of metolachlor (Krause *et al.*, 1985).

The inability of the *P. chrysosporium* culture to mineralize metolachlor is consistent with the results of previous studies. Krause *et al.* (1985) used uniformly ring-labelled ^{14}C-metolachlor to study microbial transformation by a soil actinomycete and concluded that degradation of the aromatic moiety of metolachlor did not occur during the biotransformation process. A similar conclusion was also reached by Saxena *et al.* (1987) in their screening study for metolachlor-degrading microorganisms. It thus appears that metolachlor is only transformed, and not mineralized, by common microorganisms. If the major metabolites are biologically active, aquatic organisms may thus be still at risk. However, it is known that some stable metabolites derived from pesticides possess more active sites or functional groups than do the parent compounds. Consequently, the metabolites may bind more easily to organic matter (including humic substances) in soil or sediment, a phenomenon that is expected to increase their persistence (Kozak *et al.*,

1983), but perhaps also to reduce their biological availability and toxicity to aquatic organisms.

In summary, this study has shown that the herbicide metolachlor is very stable to biological degradation in natural waters, and persists much longer than medium molecular weight PAHs in bacterial cultures. This work, together with recent work indicating that metolachlor is both chemically and photochemically stable in natural lake water (Kochany and Maguire, 1994), indicates that metolachlor is likely fairly persistent in natural waters. Further work is planned on the nature of metolachlor interactions with suspended solids and sediment, and the environmental behaviour of some of the major metabolites arising from biological transformation.

ACKNOWLEDGEMENT

We thank J.R. Purdy and D. Simmons of Ciba-Geigy for the gift of metolachlor and some of its degradation products. Part of this work was done while D. Liu was a visiting professor at the Research Institute for Bioresources, Okayama University, Kurashiki, Japan. This work was supported in part by PESTMYOP funding from Environment Canada.

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Table 1. Classification of the trophic state of lakes*.

Parameters	Trophic State		
	Oligotrophic	Mesotrophic	Eutrophic
total phosphorus (µg P/L)	< 9.9	9.9 - 18.5	> 18.5
chlorophyll a (µg/L)	< 2.0	2.0 - 5.0	> 5.0

* Based on the trophic classification by Dillon and Rigler (1975).

Table 2. Water quality data for Blue Chalk Lake, Gull Feather Lake and Moot Lake.

Parameters	Blue Chalk Lake (oligotrophic)	Gull Feather Lake (mesotrophic)	Moot Lake (eutrophic)
alkalinity (mg/L)	4.5	1.7	0.8
Al (µg/L)	10.0	72.3	106.7
Ca (mg/L)	2.6	2.5	2.1
chlorophyll a (µg/L)	1.4	3.8	6.9
Cl (mg/L)	0.4	0.4	0.4
conductivity (µS/cm)	27.9	27.9	22.9
DIC (mg/L)	1.4	1.3	0.6
DOC (mg/L)	1.8	5.4	6.5
Fe (µg/L)	60.5	747.9	518.2
K (mg/L)	0.4	0.4	0.4
Mg (mg/L)	0.8	0.7	0.5
Mn (µg/L)	29.6	74.1	43.5
Na (mg/L)	0.8	0.7	0.5
NH_4 (µg/L)	17.7	53.8	21.2
NO_3 (µg/L)	15.4	36.1	20.9
TKN (µg/L)	175.4	350.3	565.7
pH	6.7	5.8	5.6
Si (mg/L)	0.2	1.5	0.8
SO_4 (mg/L)	6.3	7.8	6.1
Total P (µg/L)	5.53	11.54	14.53

FIGURE CAPTIONS

Fig. 1. Chemical structure of metolachlor and some metabolites.

Fig. 2. Shake flask study on metolachlor persistence in lake waters.

Fig. 3. Extracts of fungal growth on MME agar plates.

Fig. 4. Comparison of total ion chromatograms of extracts from a fungal growth plate (*i.e.*, MME plate without metolachlor) and a metolachlor degradation plate (*i.e.*, MME plate with metolachlor).

Fig. 5. Total ion chromatogram of the extract from the metolachlor degradation plate.

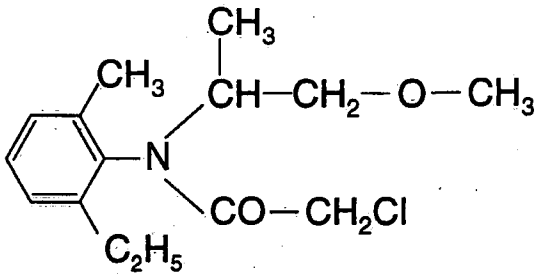
Fig. 6. Mass spectra of (A) CGA-41638, and (B) metabolite M-4 from the metolachlor degradation plate.

Fig. 7. Mass spectra of (A) metabolite O-7 of Cruz *et al.* (1993), and (B) metabolite M-5 from the metolachlor degradation plate.

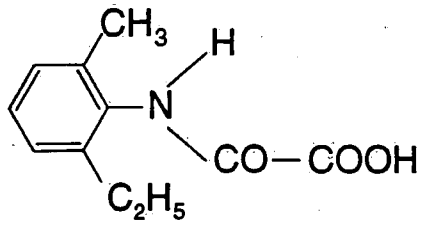
Fig. 8. Mass spectra of (A) metabolite S-1 of Liu *et al.* (1989), and (B) metabolite M-1 from the metolachlor degradation plate.

Fig. 9. Proposed metabolic pathway of metolachlor by the white rot fungus *P. chrysosporium*.

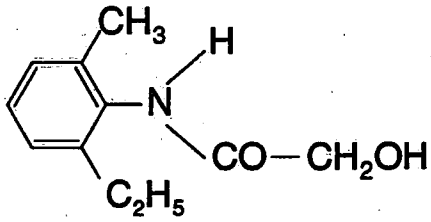
Fig. 10. Comparison of mass spectra of metolachlor metabolites M-1, M-2 and M-3.



Metolachlor



CGA-50720 (Metabolite)



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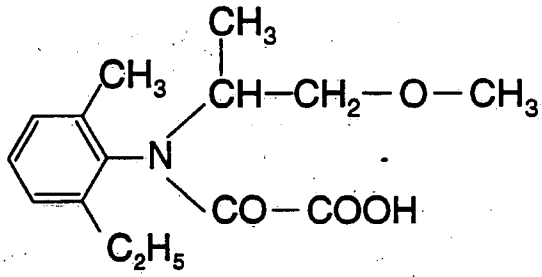

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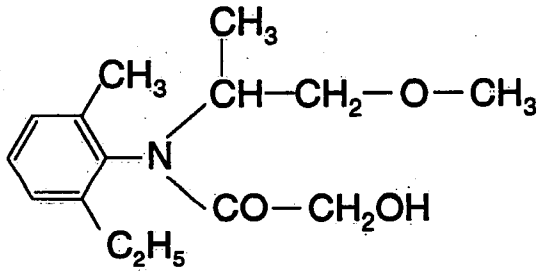

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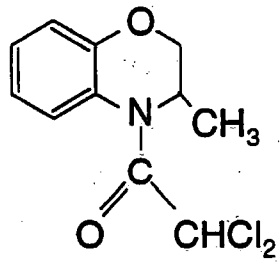

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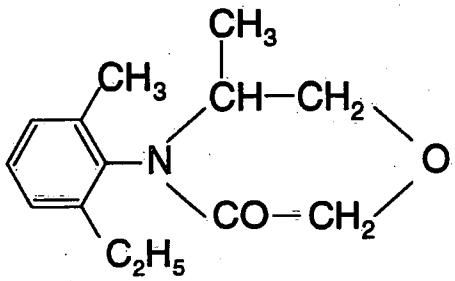

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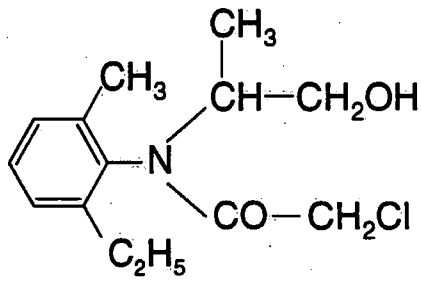
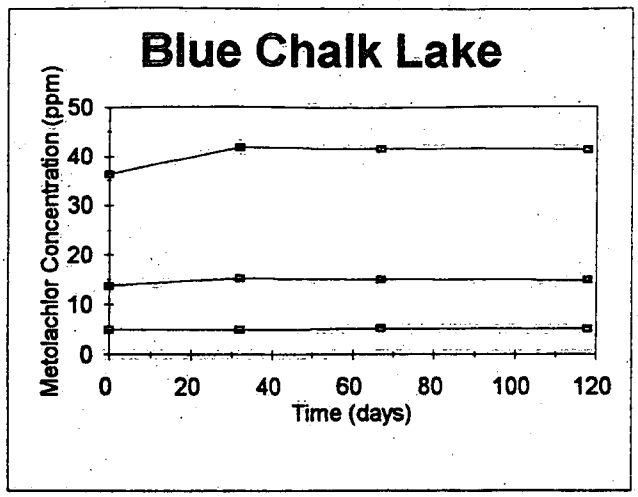
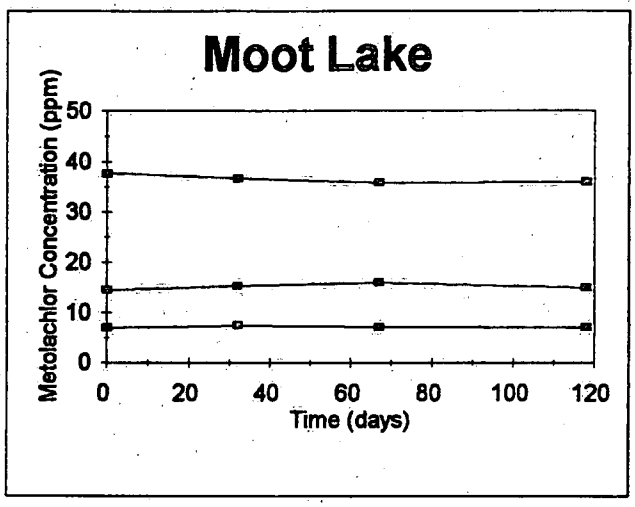
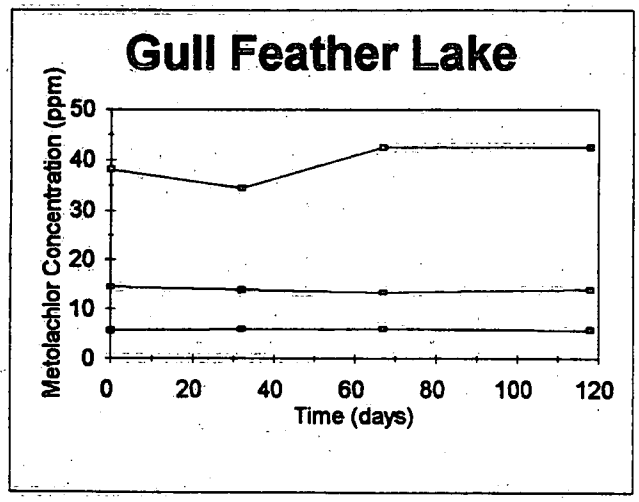
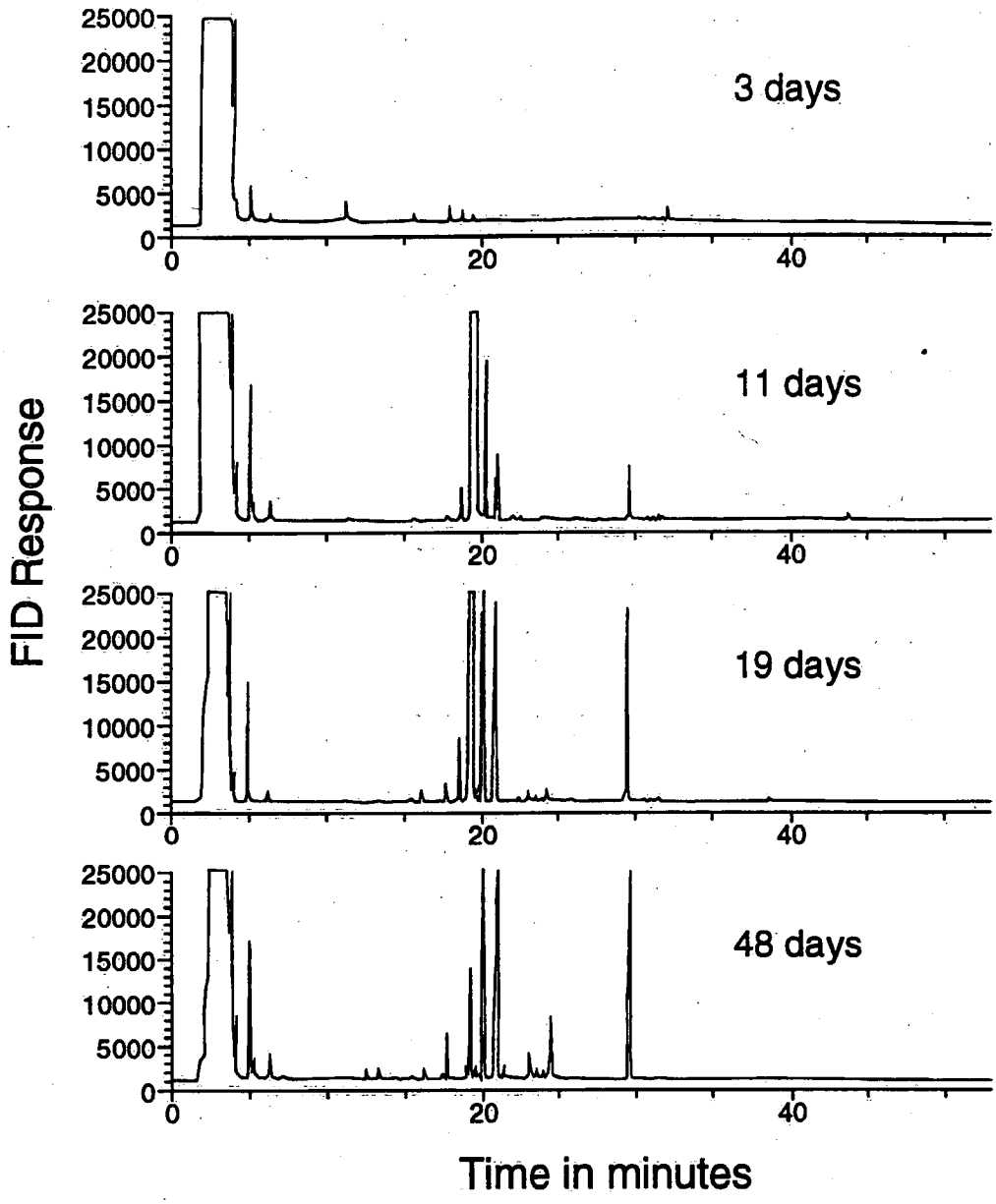


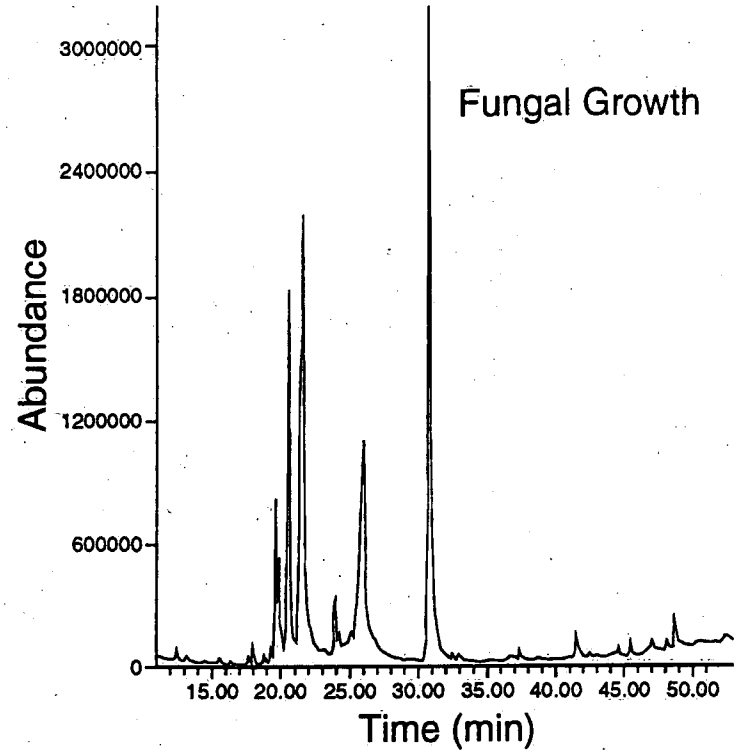
CGA-41638 (Metabolite)

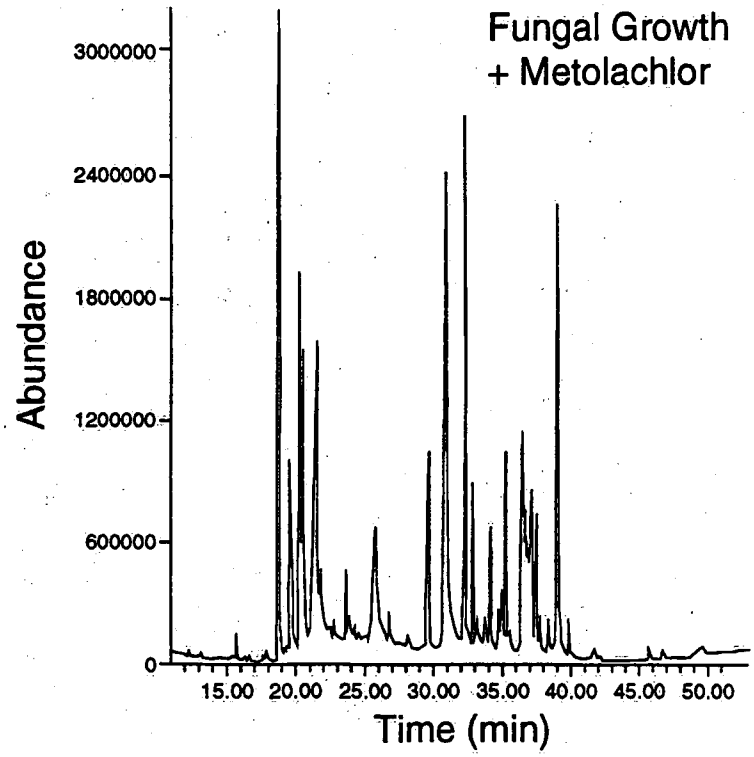

Blue Chalk Lake
Metolachlor Concentration (ppm)
0
10
20
30
40
50
0
20
40
60
80
100
120
Time (days)

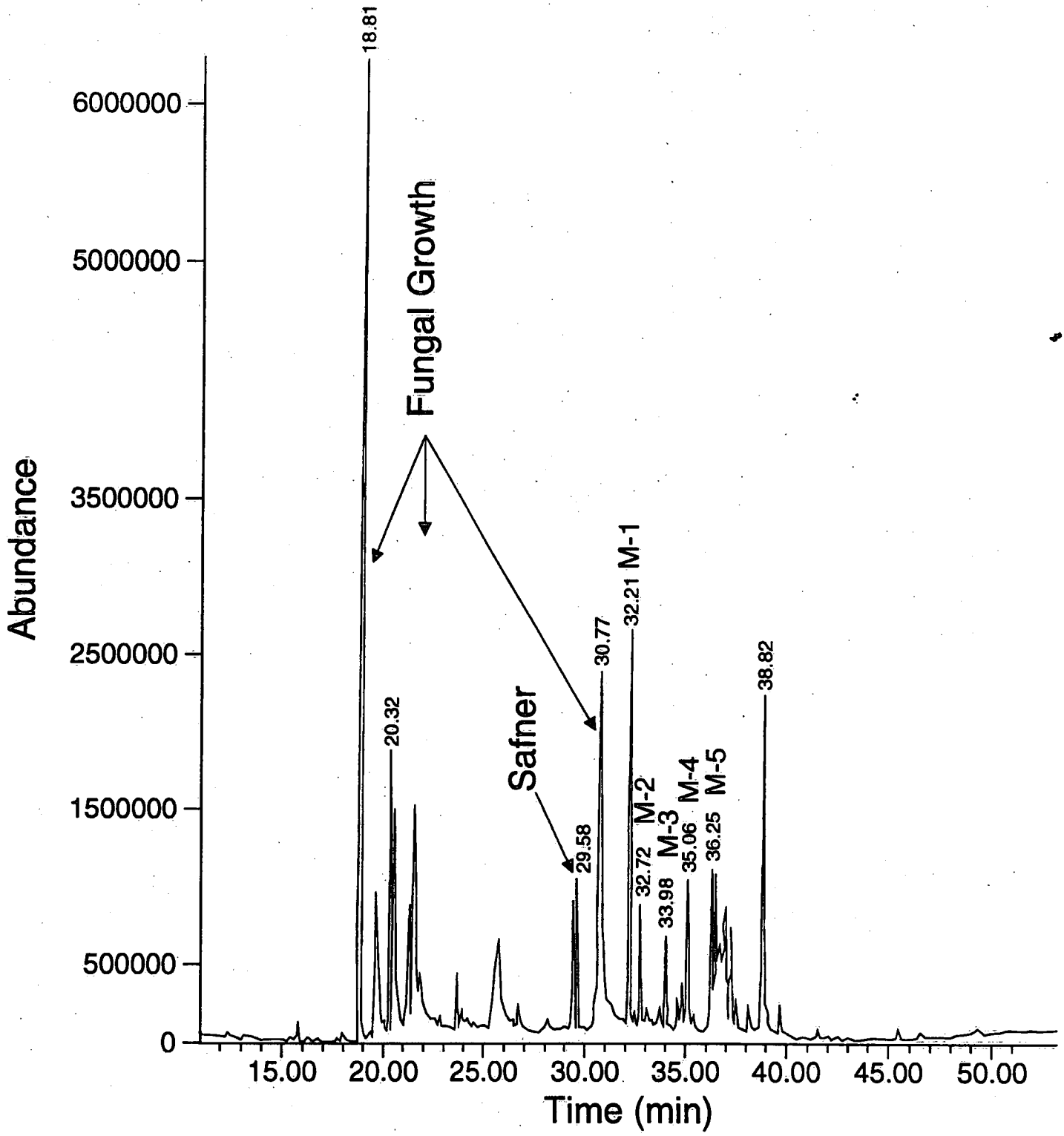

Moot Lake
Metolachlor Concentration (ppm)
0
10
20
30
40
50
0
20
40
60
80
100
120
Time (days)

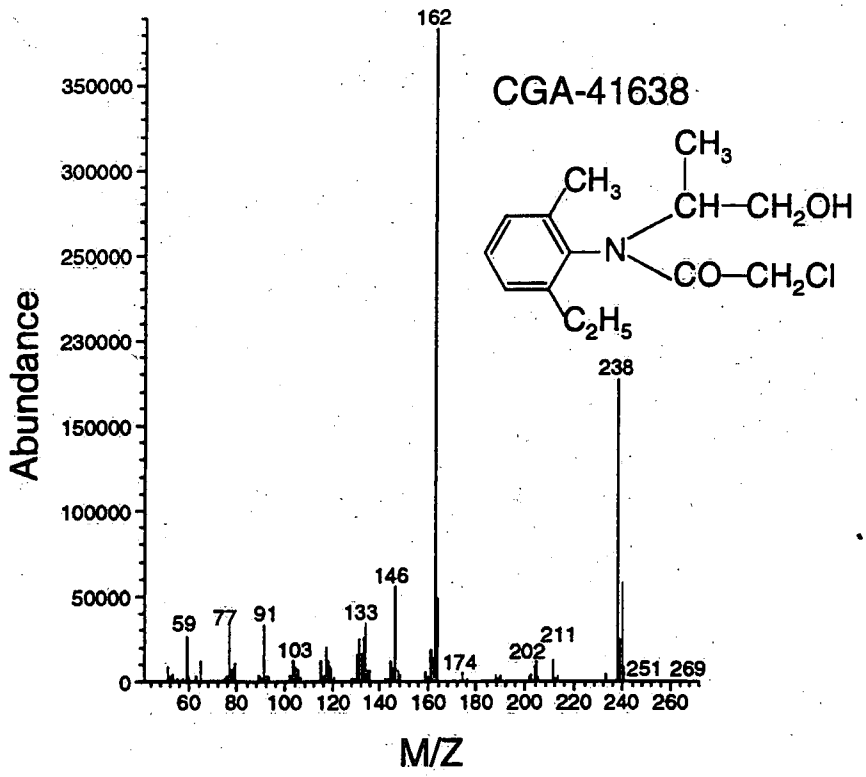

Gull Feather Lake
Metolachlor Concentration (ppm)
0
10
20
30
40
50
0
20
40
60
80
100
120
Time (days)

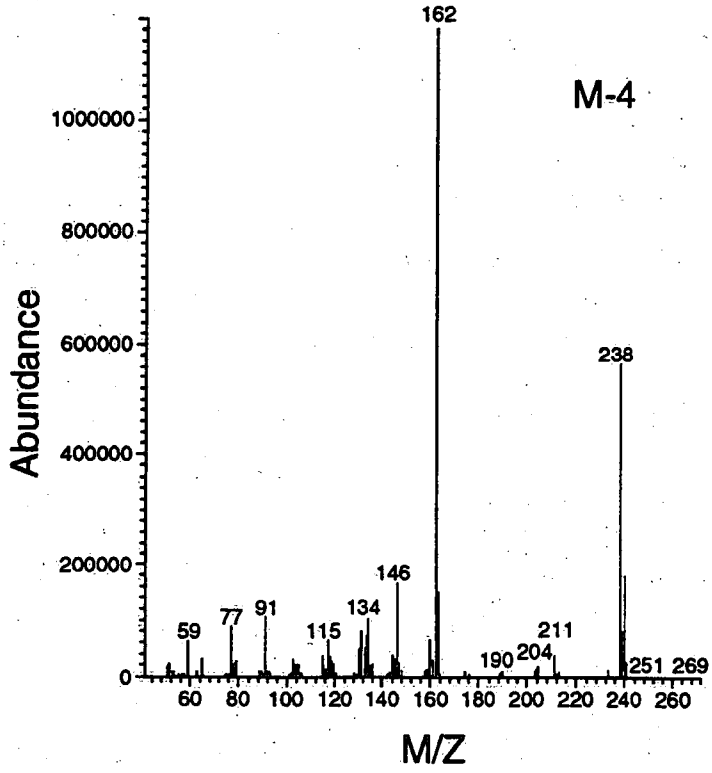

FID Response
25000
20000
15000
10000
5000
0
0
20
40
3 days
11 days
19 days
48 days
Time in minutes

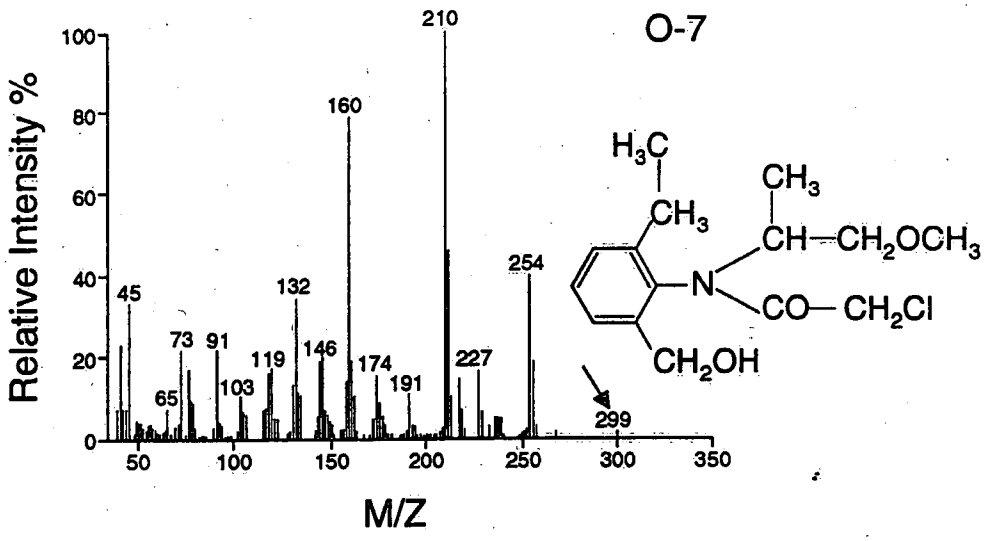

Fungal Growth
Abundance
3000000
2400000
1800000
1200000
600000
0
15.00 20.00 25.00 30.00 35.00 40.00 45.00 50.00
Time (min)

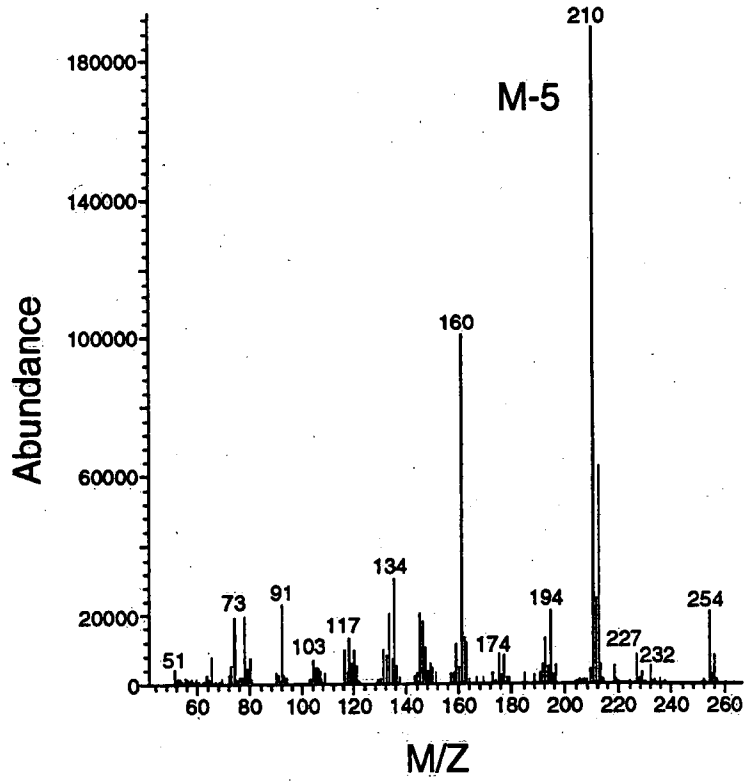

Fungal Growth
+ Metolachlor
Abundance
3000000
2400000
1800000
1200000
600000
0
15.00 20.00 25.00 30.00 35.00 40.00 45.00 50.00
Time (min)

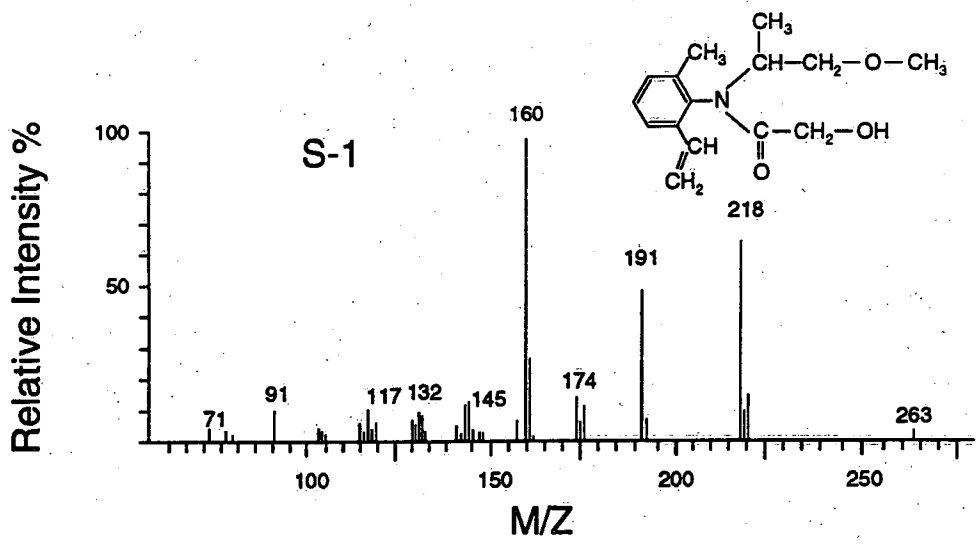

Abundance
6000000
5000000
3500000
2500000
1500000
500000
0
18.81
Fungal Growth
20.32
Safner
29.58
30.77
32.21
M-1
M-2
32.72
M-3
33.98
M-4
35.06
M-5
36.25
38.82
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
Time (min)

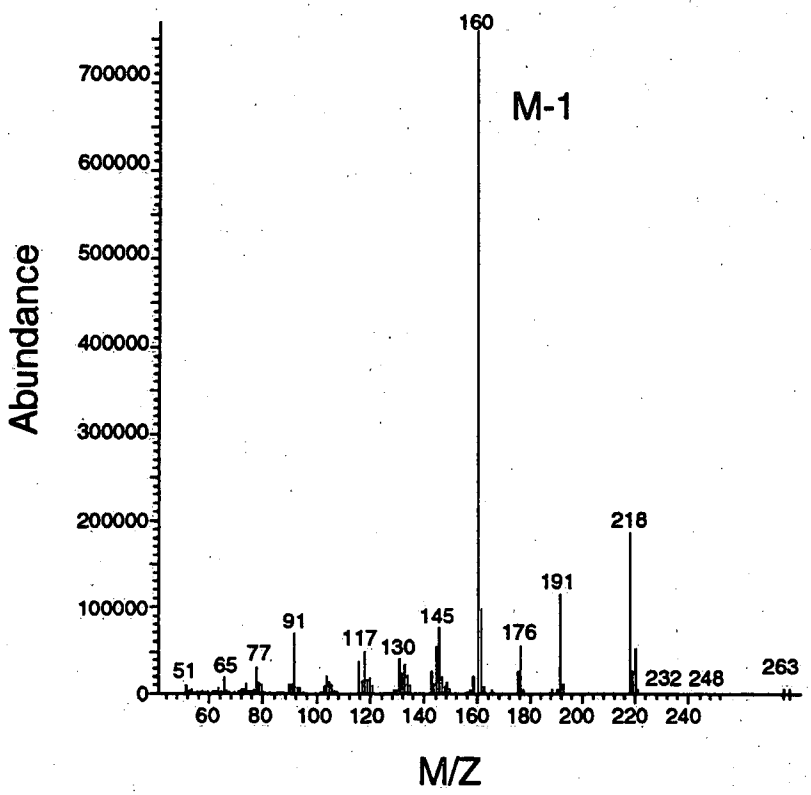

CGA-41638
Abundance
M/Z
162
238
146
59
77
91
133
103
174
202
211
251
269
350000
300000
250000
230000
150000
100000
50000
0
60
80
100
120
140
160
180
200
220
240
260
CH3
CH3
CH—CH2OH
N
CO—CH2Cl
C2H5

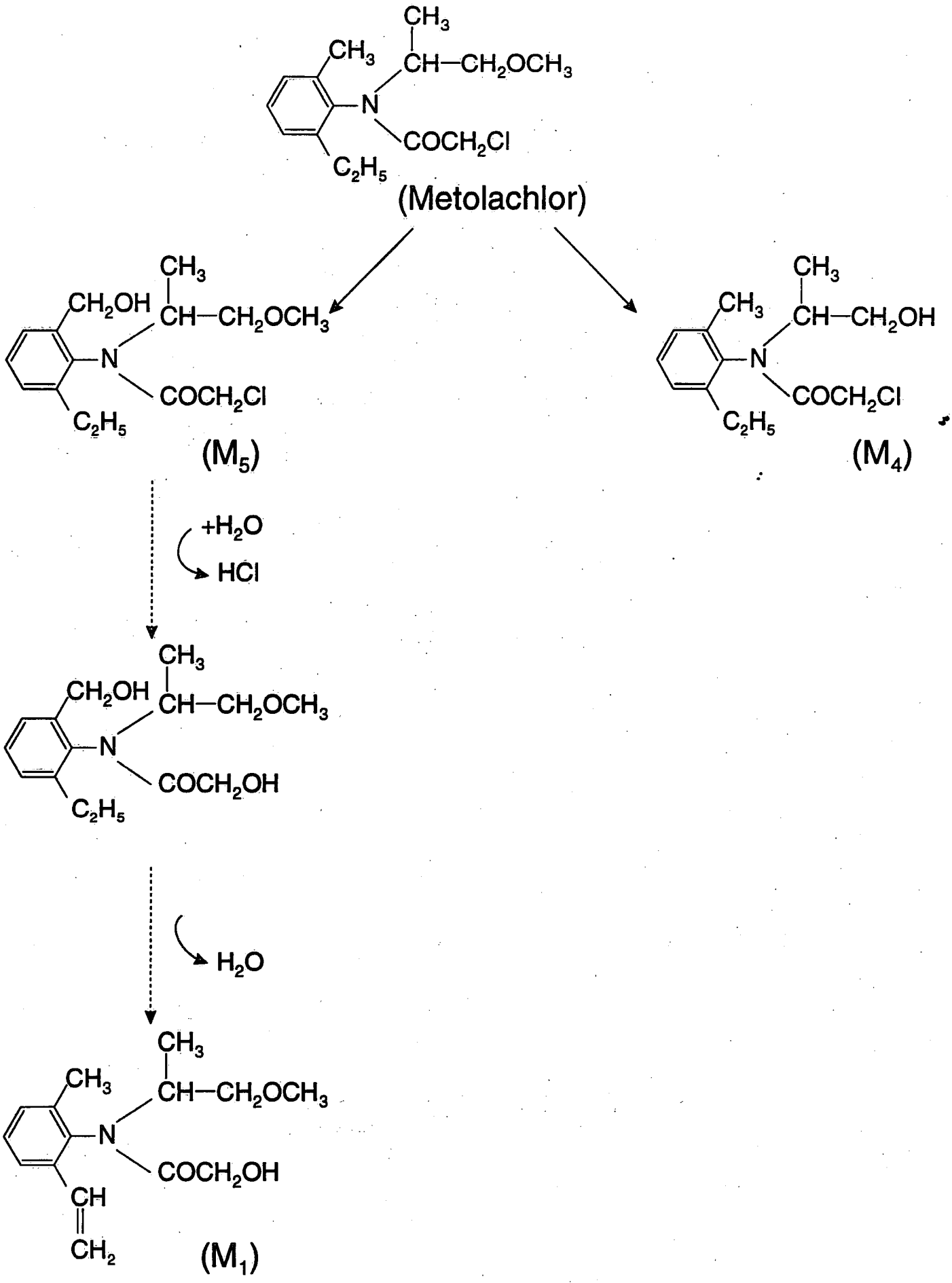

M-4
Abundance
M/Z
162
238
146
59
77
91
134
115
211
190
204
251
269
1000000
800000
600000
400000
200000
0
60
80
100
120
140
160
180
200
220
240
260

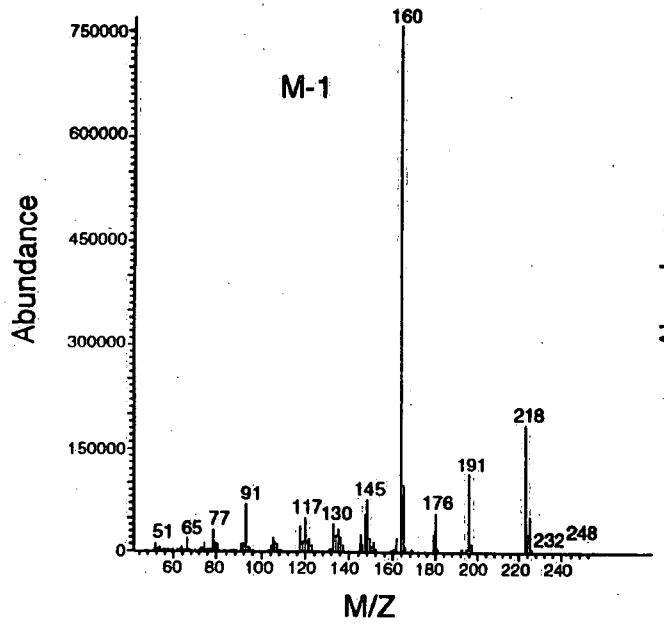

O-7
Relative Intensity %
M/Z
210
160
254
45
73
91
132
119
146
174
227
103
65
191
299
H_3C
CH_3
CH_3
CH—CH_2OCH_3
N
CO—CH_2Cl
CH_2OH

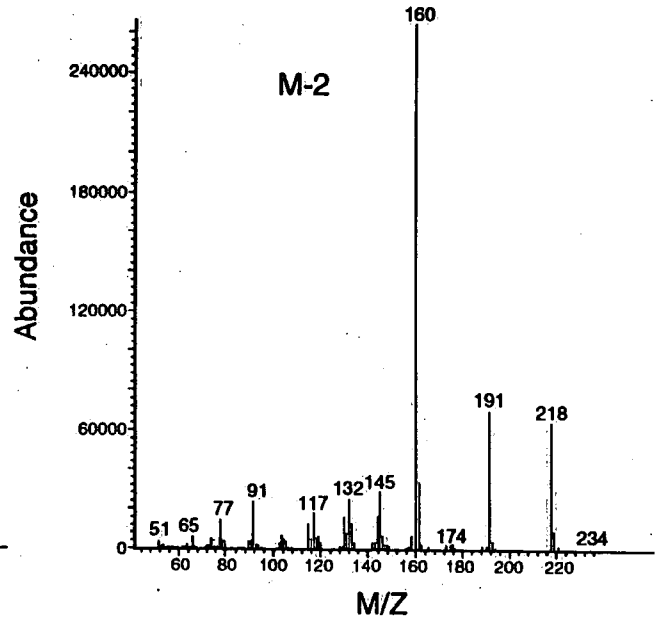

M-5
Abundance
M/Z
210
160
134
73
91
194
254
117
103
174
227
232
51

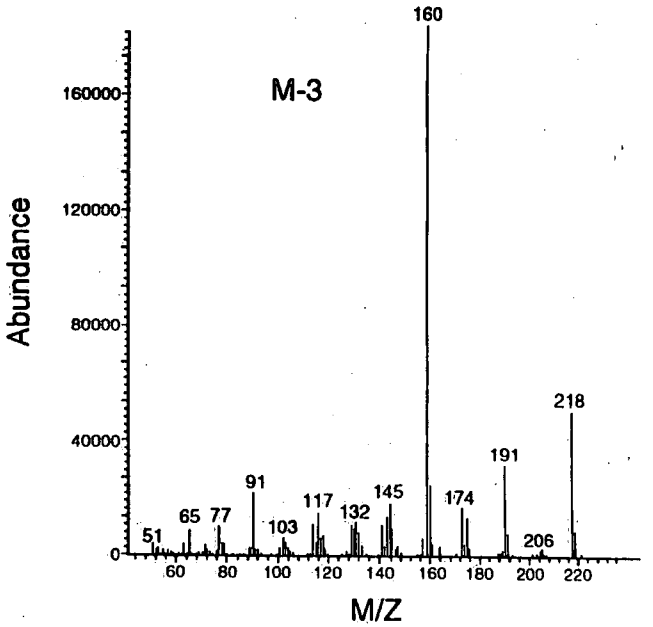

S-1
Relative Intensity %
100
50
160
218
191
174
91
117
132
145
71
263
100
150
200
250
M/Z
CH3
CH3
CH–CH2–O–CH3
N
C–CH2–OH
O
CH
CH2

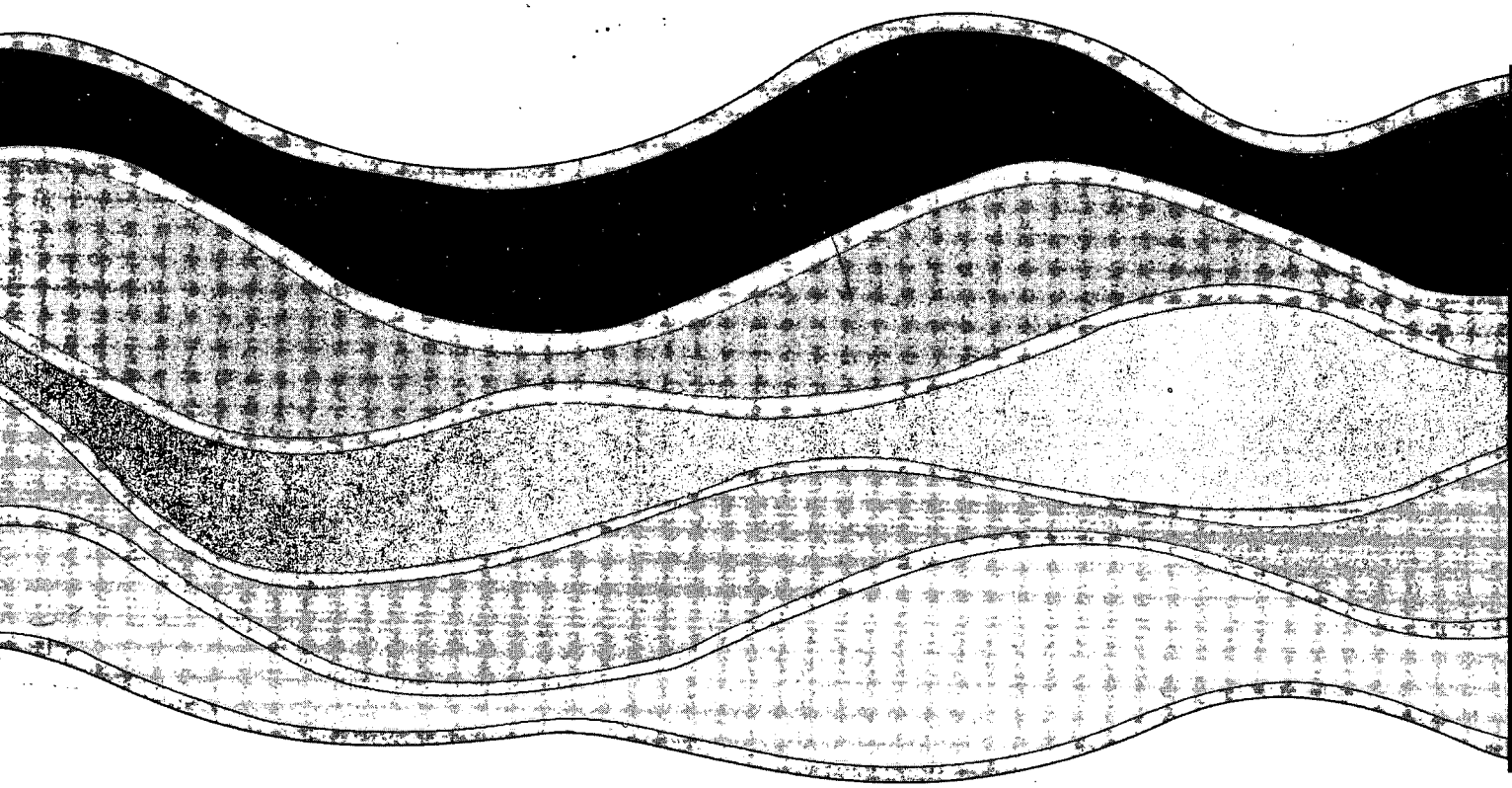

M-1
Abundance
700000
600000
500000
400000
300000
200000
100000
0
160
218
191
91
145
117
130
176
77
51
65
232
248
263
60
80
100
120
140
160
180
200
220
240
M/Z


(Metolachlor)
(M5)
(M4)
+H2O
HCl
H2O
(M1)


M-1
Abundance
M/Z
750000
600000
450000
300000
150000
0
160
218
191
91
145
117
130
176
51
65
77
232
248
60
80
100
120
140
160
180
200
220
240


M-2
Abundance
M/Z
240000
180000
120000
60000
0
160
191
218
91
117
132
145
77
51
65
174
234
60
80
100
120
140
160
180
200
220


M-3
Abundance
M/Z
160000
120000
80000
40000
0
160
218
191
91
145
117
132
174
65
77
103
51
206
60
80
100
120
140
160
180
200
220



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