The effect of structure and a secondary carbon source on the microbial degradation of chlorophenoxy acids

S. Evangelista, D.G. Cooper, V. Yargeau *

Department of Chemical Engineering, McGill University, Montreal, Quebec, Canada

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ABSTRACT

Pseudomonas putida, *Aspergillus niger*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Sphingomonas herbicidovorans* and *Rhodococcus rhodochrous* growing on glucose in a medium containing one of three chlorophenoxy acids at a concentration of 0.1 g L^{-1} (clofibric acid, (*R*)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop or MCP) and 4-chloro-2-methylphenoxyacetic acid (MCPA)) degraded these compounds to varying degrees; from nonmeasurable to almost complete removal. These results with the addition of glucose (2.5 g L^{-1}) as an easy to use carbon source indicated the formation of metabolites different from results reported in the literature for growth studies in which the chlorophenoxy acid was the sole carbon source. The metabolite, 4-chloro-2-methylphenol, which had been reported previously, was only observed in trace amounts for MCP and MCPA in the presence of *S. herbicidovorans* and glucose. In addition, three other compounds (M1, M3 and M4) were observed. It is suggested that these unidentified metabolites resulted from ring opening of the metabolite 4-chloro-2-methylphenol (M2).

The rate of biodegradation of the chlorophenoxy acids was influenced by the degree of steric hindrance adjacent to the internal oxygen bond common to all three compounds. The most hindered compound, clofibric acid, was converted to ethyl clofibrate by *R. rhodochrous* but was not degraded by any microorganisms studied. The more accessible internal oxygen bonds of the other two chlorophenoxy acids, MCP and MCPA, were readily broken by *S. herbicidovorans*.

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1. Introduction

Organic contaminants in the aquatic environment have long been issues of environmental concern. While some of the first pollutants to be observed included pesticides and herbicides, recently pharmaceuticals have also become important (Kümmerer, 2001; Emblidge and DeLorenzo, 2006; Kuster et al., 2008). This is due in part to advances in trace contaminant detection and the sensitivity of analytical instrumentation which aids in compound identification (la Farré et al., 2008). Environmental concerns stem from the capacity of these pollutants to affect the aquatic wildlife and surrounding ecosystems, as well as drinking water resources (Joss et al., 2006).

Pharmaceuticals are excreted into the aquatic environment in the $\text{ng-}\mu\text{g L}^{-1}$ range (Joss et al., 2006) in either the original form or as metabolites (Debska et al., 2004). Even after passage through sewage treatment plants they are often only partially eliminated (Winkler et al., 2001). Pharmaceuticals are highly problematic for a number of reasons. Not only are they ubiquitous and globally dis-

tributed (Enick and Moore, 2007), but they are designed to stimulate specific biological responses at very low concentrations (Fent et al., 2006). They are intended to be effective against bacteria and fungi and thereby resistant to biological degradation. Furthermore, the high polarity and low volatility of many pharmaceuticals ensures that they are likely to remain in the water systems, which they enter.

Microbial degradation of contaminants in the environment is a phenomenon which strongly influences the fate and persistence of such xenobiotics as pharmaceuticals and herbicides. For example, the decomposition of many herbicides is a result of aerobic metabolism occurring in top soils (Tett et al., 1997). Furthermore, small changes in the chemical structure of many xenobiotics can strongly influence their degradability (Hammond and Alexander, 1972). There has been research that has demonstrated that some xenobiotic contaminants, including some herbicides, could be degraded fortuitously, as a result of co-metabolism by microbial populations consuming other more readily-degradable carbon sources (Horvath, 1972; Janke and Fritsche, 1985). Some of these early studies and more recent examples with the co-metabolism of plasticizers (Gartshore et al., 2003; Nalli et al., 2006a,b) have also demonstrated that this mechanism often leads to the accumulation of stable metabolites.

* Corresponding author.

E-mail address: viviane.yargeau@mcgill.ca (V. Yargeau).

Clofibric acid, (R)-2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop or MCPP) and 4-chloro-2-methylphenoxyacetic acid (MCPA) are structurally-related compounds that are common environmental contaminants belonging to the class of chlorophenoxy acids. Clofibric acid is a pharmaceutical and MCPP and MCPA are herbicides. Clofibric acid is the active metabolite of blood lipid regulators, which act by lowering serum triglyceride production (Daughton and Jones-Lepp, 2001). In 1976, clofibric acid became the first pharmaceutical contaminant to be detected in the environment (Hignite and Azarnoff, 1977), owing to its structural resemblance to the herbicide mecoprop (Kümmerer, 2001). While largely being replaced by newer drugs, it remains an environmental contaminant of concern due to its widespread occurrence and persistence, with a half-life estimated to be greater than 21 years (Emblidge and DeLorenzo, 2006). It is still frequently detected in ground, surface and drinking waters world-wide (Dordio et al., 2009). A recent paper has reported that biodegradation of this compound is possible by an activated sludge that had been acclimated for three years and this degradation yielded chlorophenol as a metabolite (Kosjek et al., 2009).

Zipper et al. (1996) showed that *Sphingomonas herbicidovorans* could metabolize MCPP as the sole carbon and energy source and that it is degraded via cleavage of the ether bond, resulting in the formation of the metabolites 4-chloro-2-methylphenol and pyruvate (Nickel et al., 1997). The metabolite 4-chloro-2-methylphenol was also detected during degradation studies of MCPA by a microbial consortium (Oh et al., 1995). The goal of the work presented here was to reconsider the biodegradation of the chlorophenoxy acids in a medium that contains a more easily degraded carbon source in addition to the target pollutant. It was expected that different patterns of degradation would be seen as observed in the earlier work with plasticizers (Gartshore et al., 2003; Nalli et al., 2006a,b).

2. Material and methods

2.1. Reagents

Clofibric acid (2-(4-chlorophenoxy)-2-methylpropanoic acid, CAS 882-09-7), (R)-mecoprop ((R)-2-(4-chloro-2-methylphenoxy)propionic acid, MCPP, CAS 16484-77-8), 4-chloro-2-methylphenoxyacetic acid (MCPA, CAS 94-74-6) and ethyl clofibrate (CAS 637-07-0) were obtained from Sigma–Aldrich Canada Ltd., as were 4-chloro-2-methylphenol (CAS 1570-64-5) and pyruvic acid (CAS 127-17-3). All other solvents, chemicals and nutrient media were purchased from Fisher Scientific Canada.

2.2. Microorganisms and degradation experiments

Microorganisms used in this study were purchased from the American Type Culture Collection (ATCC). They include *Pseudomonas putida* ATCC 12633, *Aspergillus niger* ATCC 16888, *Bacillus subtilis* ATCC 6051, *Pseudomonas fluorescens* ATCC 13525, *Sphingomonas herbicidovorans* ATCC 700291 and *Rhodococcus rhodochrous* ATCC 13808. These microorganisms were selected for their known or expected ability to degrade chlorophenoxy acids or similar compounds. While each strain was tested for its ability to degrade clofibric acid, only *S. herbicidovorans* and *R. rhodochrous* were selected for studies with MCPA and MCPP. Between experiments the microorganisms were kept on nutrient agar plates at 4 °C.

All experiments were performed in 500-mL Erlenmeyer flasks. Microorganisms were first cultured in 100 mL of Difco Nutrient Broth (8 g L⁻¹ in distilled water). Difco Brain Heart Infusion (32 g L⁻¹ in distilled water) was used to grow *R. rhodochrous*. Flasks were capped with foam plugs and autoclaved (Model 3021-S, AMS-

CO) at 121 °C and 220 kPa for 30 min prior to inoculation. Once stationary phase was achieved, a 1-mL broth sample was transferred into 100 mL of the glucose-containing media used for biodegradation. Inoculation was normally done using 1 mL aliquots but in a few cases 50-mL inocula were used.

The biodegradation media contained NH₄NO₃ (4 g L⁻¹), KH₂PO₄ (4 g L⁻¹), Na₂HPO₄ (6 g L⁻¹), MgSO₄·7H₂O (0.2 g L⁻¹), CaCl₂·2H₂O (0.01 g L⁻¹), FeSO₄·7H₂O (0.01 g L⁻¹), Na₂EDTA (0.014 g L⁻¹), glucose (2.5 g L⁻¹), yeast extract (0.01 g L⁻¹) and either clofibric acid, MCPP or MCPA (0.1 g L⁻¹). All components were added to the flasks prior to autoclaving, as it was determined that none of the compounds were heat labile.

All manipulations with the microorganisms were performed in a laminar flow hood (Model VBM600, The Baker Company). The cultures were grown at 26 °C in an incubator shaker (Innova 44, New Brunswick Scientific Co., Inc.) at 150 rpm. Optical density measurements at 600 nm were used to confirm that growth occurred in all conditions tested.

2.3. Sample preparation and analysis

Samples for liquid chromatography (HPLC) analysis, 1.5-mL, were taken periodically from the shake flasks, were placed in 2-mL plastic micro-centrifuge tubes and were centrifuged at 21 000g for 10 min using an IEC MicroMax centrifuge. The supernatant was then filter-sterilized using 0.25 µm pore-size syringe filters and stored in HPLC vials (Fischer Scientific Canada) at 4 °C until analyzed. An Agilent 1200 HPLC equipped with a diode array detector (DAD) and an Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 µm) were used. Conditions were as follows: the column was eluted with a mobile phase consisting of 70% NaH₂PO₄ (20 mM, pH 2.8) and 30% acetonitrile. The mobile phase flow rate was 0.7 mL min⁻¹ and the column temperature was 40 °C. The detector was set to 273 and 230 nm with a stop time of 20 min and a post time of 10 min and the volume injected was 5 µL. The error of replicates was on average 10%.

Samples for gas chromatography (GC) analysis, 2-mL, were taken from the shake flasks using 2-mL sterile pipettes and placed in 3.5-mL glass vials. The pH was adjusted to between 1.0 and 2.0 by adding concentrated hydrochloric acid (Fisher Scientific). The samples were then extracted using chloroform containing benzoic acid (1 g L⁻¹) as internal standard, in a 1:2 (solvent:broth) volume ratio. Recoveries of 80% were obtained on average. The organic phase (extract) was collected and stored in amber vials at 4 °C for quantitative analysis by GC. A Varian CP 3800 (Varian, St-Laurent, QC, Canada) gas chromatograph equipped with a split/splitless injector 1177 and a flame ionization detector (FID) was used with a Varian Chrompack Capillary Column, CP SIL 8CB, 30 m × 0.32 mm × 0.25 µm. The operating conditions were as follows: the carrier gas flow rate was 1.5 mL min⁻¹ with an initial hold time of 2 min. The injection temperature was 250 °C, with an initial column temperature at 40 °C, a temperature ramp of 10 °C min⁻¹ and a detector temperature 300 °C. The split ratio was 10 and the injection volume was 3 µL.

The gas chromatography–mass spectrometry (GC–MS) analysis was done with a ThermoQuest (Trace GC 2000) gas chromatograph equipped with a RTX-5 MS column (length: 30 m, film thickness: 0.25 µm, I.D.: 0.25 mm) and a ThermoQuest (GCQ/Polaris MS) mass spectrometer which used the Xcalibur 1.2 Polaris Q software. Conditions were as follows: column flow rate 1.5 mL min⁻¹, initial column temperature 125 °C with hold time 1 min, a temperature ramp of 10 °C min⁻¹–225 °C with hold time 1.5 min, temperature ramp of 7 °C min⁻¹–300 °C with hold for 1 min. The split ratio was 100. MS conditions were as follows: full scan mode (20–650), positive ionization, source temperature 200 °C, transfer line temperature 275 °C, maximum ion time 25 µs.

3. Results

Clofibric acid (structure shown in Fig. 1a) was resistant to microbial degradation when exposed to cultures of *P. putida*, *A. niger*, *B. subtilis*, *P. fluorescens* and *S. herbicidovorans* grown on glucose in the presence of clofibric acid. However, *R. rhodochrous* grown in this manner was able to transform clofibric acid even though it could not degrade it. A peak for clofibric acid was observed at 12.50 min in the GC and the peak of a new compound, which appeared in early the experiment, was observed at 14.10 min. This metabolite was identified as the ester, ethyl clofibrate (structure shown in Fig. 1b), which had both the same GC retention time and mass spectrum as an authentic standard (data not shown). As can be seen in Fig. 2, in the initial experiments, the changes due to the removal of clofibric acid and the appearance of the ester monitored by the GC peak area were small (solid symbols) but, after several transfers the culture became acclimated to clofibric acid and there was an almost complete removal of clofibric acid due to conversion to the ester (empty symbols).

Although *S. herbicidovorans* growing in the presence of glucose as a carbon source could not affect clofibric acid this approach did lead to degradation of both MCPA and MCPP (structures shown in Fig. 1c and d, respectively). There was no evidence for degradation or conversion of either compound by *R. rhodochrous*. The GC chromatogram for MCPP and MCPA did not show any new peaks near the peaks for the parent compounds in a manner analogous to the observations for clofibric acid, so it was concluded that ethyl esters were not being formed. HPLC analysis indicated the formation of metabolites by *S. herbicidovorans* and the HPLC peak area was used to monitor the formation of metabolites of the herbicides.

Fig. 3 demonstrates the degradation of MCPP using different growth conditions of *S. herbicidovorans*. The set of data presented using empty symbols and dotted lines, was from a degradation study started with an inoculum that was 50 times larger than that used for the experiment shown using solid symbols and lines. In both cases MCPP was completely degraded and metabolites were produced but the patterns of metabolite production were not identical. Four metabolites were detected, which were not observed in blanks obtained from *S. herbicidovorans* growing on glucose in the absence of the chlorophenoxy acids. A major metabolite M1 and three minor metabolites M2, M3 and M4, were observed at various elution times reported in Table 1. M2, with a retention time of 11.64 min, was not detected in experiments using the larger inoculum and concentrations of M2 and M4 were, in both cases, too small to be reported in Fig. 3. The faster degradation observed with the larger inoculum may explain why M2 was not detected. Similar removal and metabolite profiles were observed for MCPA but only one example is shown for this system using the smaller inoculum (Fig. 4).

Fig. 2. Degradation of clofibric acid (●) by *Rhodococcus rhodochrous* and appearance of a metabolite (◆). Repeat of the experiment after acclimation – clofibric acid (○) and metabolite (◇).

Fig. 3. Degradation of MCPP by *Sphingomonas herbicidovorans* started with an inoculum of 1 mL; MCPP (●), metabolite 1 (■) and metabolite 3 (▲) and an inoculum of 50 mL; MCPP (○), metabolite 1 (□) and metabolite 3 (△).

Table 1
HPLC retention times (min) of chlorophenoxy acids and their metabolites.

Parent compounds	Metabolites observed				
		Major metabolite M1	Minor metabolites		
			M2	M3	M4
MCPP	13.01	4.44	11.64 ^a	3.31	3.20 ^a
MCPA	10.71	4.44	11.64 ^a	3.31	3.11 ^a
4-Chloro-2-methylphenol	11.64	4.44	–	3.31	–
Pyruvic acid	2.17	–	–	–	–

^a Observed for some experiments in small quantities.

Fig. 1. Structures of: (a) clofibric acid, (b) ethyl clofibrate, (c) MCPA, (d) MCPP, and (e) 4-chloro-2-methylphenol.

Fig. 4. Degradation of MCPA (x) by *Sphingomonas herbicidovorans* started with a 1-mL inoculum; metabolite 1 (■) and metabolite 3 (▲).

Table 1 summarizes the HPLC data for the parent compounds, the metabolites observed and standard compounds that were tested (4-chloro-2-methylphenol (structure shown in Fig. 1e) and pyruvic acid) because these were reported in the literature as metabolites. The retention time for the standard 4-chloro-2-methylphenol (11.64) was very close to the elution time for another standard, 4-chlorophenol, which is also a possible metabolite as reported in Kosjek et al. (2009). The retention times of these two standards were both identical to the retention time of the minor metabolite M2. GC–MS analysis confirmed that metabolite M2, observed at a retention time of 11.64, was 4-chloro-2-methylphenol and there was no evidence for the production of 4-chlorophenol.

Experiments were done adding 4-chloro-2-methylphenol (M2) to the glucose containing growth medium to evaluate if the further degradation of this metabolite, reported in the literature, may lead to the formation of the other metabolites observed here. These experiments demonstrated the biodegradation of this compound and the formation of two metabolites (Fig. 5). The positions of the HPLC peaks for these two metabolites were identical to those of the major metabolite M1 observed from the degradation of MCPP and MCPA as well as one of the minor metabolites M3, which could not be identified by GC–MS.

Selected standard compounds including 4-chlorophenol, 2-methylphenol, pyruvate and phenol did not have the same elution times as any of the metabolites observed by HPLC analysis. Benzoic acid did have the same elution time as one minor metabolite (M4) that was sometimes observed but the amount of this metabolite was too small to either confirm or dismiss this possible identification.

Fig. 5. Degradation of 4-chloro-2-methylphenol (◆) by *Sphingomonas herbicidovorans*; metabolite 1 (■) and metabolite 3 (▲).

4. Discussion

When biodegradation was observed, there were two possible types of behaviour. Clofibric acid exhibited a high resistance to microbial attack and the only new compound observed was due to the conversion of clofibric acid to ethyl clofibrate. After several transfers to acclimate the culture to the compound, total esterification of the original amount of clofibric acid added was possible. This conversion could be a survival strategy by the organism; ethyl clofibrate, a more hydrophobic compound, would be less soluble in the aqueous phase. This has not been proven but it has been suggested that the conversion of butyric acid to the ethyl ester by *Zoogloea ramigera* could be a survival mechanism (Joyce and Dugan, 1972).

The two structurally very similar analogues, of the above compound, MCPA and MCPP (Fig. 1c and d) did not generate any noticeable amounts of esters in our experiments but this is not surprising as both were readily biodegraded to other metabolites by *S. herbicidovorans* and it may simply be a matter of faster alternative pathways. Two metabolites had been reported previously after biodegradation of MCPP by *S. herbicidovorans* when this herbicide was the only carbon source available (Nickel et al., 1997). These were pyruvic acid, which was never observed in any of our studies and 4-chloro-2-methylphenol, which was sometimes observed in small amounts during our experiments with either MCPP or MCPA.

The structures of MCPP and MCPA can both be seen to contain a common fragment, which is the structure of 4-chloro-2-methylphenol (Fig. 1e), reported in the literature as a metabolite of these compounds. Thus, the appearance of this compound as a fragment during the earlier biodegradation studies was not unexpected. However, only very small amounts of 4-chloro-2-methylphenol were detected and in only some experiments. When a sample of 4-chloro-2-methylphenol was itself subjected to biodegradation by *S. herbicidovorans* (Fig. 5) instead of the herbicides, a large quantity of the major metabolite M1, and the common minor metabolite M3 observed for the herbicides, were observed in the HPLC (i.e. identical elution times). It is reasonable to conclude that 4-chloro-2-methylphenol (M2) was a short-lived metabolite from the biodegradation of either MCPP or MCPA, which was quickly biodegraded leading to two other compounds (M1 and M3). Because the minor metabolite M3 only appeared after there was an appreciable quantity of the major metabolite M1, it is reasonable to suggest that this minor metabolite was a product of further biodegradation of the major metabolite. Neither of these compounds was identified but their elution times eliminated the possibilities of phenol, 2-methylphenol (o-cresol) or 4-chlorophenol (all possible fragments of the structures in Fig. 1). The most reasonable guess is that they are formed by opening the aromatic ring and other work has reported ring opening of (R)-mecoprop by *Alicyclospira denitrificans* (Tett et al., 1997) and other similar herbicides (Müller et al., 2004).

The only difference observed in the biodegradation of MCPP and MCPA is that sometimes the degradation resulted in an additional minor metabolite (M4) eluting at 3.20 for MCPP whereas it eluted at 3.11 min for MCPA. These metabolites must contain the portion of these compounds, which make their structures slightly different – the carbon atom adjacent to the central oxygen that has a methyl substituent for MCPP but not for MCPA.

The patterns of metabolite formation lead to some conclusions about the mechanisms of interaction of the three, structurally similar, organic acids with microorganisms. In the first place, the formation of an ethyl ester was a slow reaction and was not observed unless the acid remained in the system for a long enough period of time. This would explain why it was only observed for clofibric acid, which was resistant to biodegradation.

The recalcitrant nature of clofibric acid is consistent with the known persistence of this compound in the environment (Daughton and Ternes, 1999; Winkler et al., 2001; Schulman et al., 2002; Emblidge and DeLorenzo, 2006). It is acknowledged that molecular structure can play a significant role in the recalcitrance of xenobiotics (Alexander and Aleem, 1961; Hammond and Alexander, 1972; Karthikeyan et al., 1999). Chlorinated substitutions or structural characteristics such as ring substitutions and the nature of the aliphatic side chain of compounds influence the persistence of these compounds in the environment, by affecting their susceptibility to biological degradation. Clofibric acid does contain a chlorophenolic group but the most important feature of its resistance to degradation can be seen to be much simpler than this and is, in fact, due to steric hindrance from a single extra methyl group. MCPP has a single methyl group on the carbon atom adjacent to the ether function whereas clofibric acid has two methyl groups (Fig. 1a). It has been suggested previously that the mechanism by which *S. herbicidovorans* degrades MCPP (Nickel et al., 1997) is via cleavage of the ether bond and the appearance of 4-chloro-2-methylphenol, or its metabolitic products, is consistent with this mechanism. The recent report of biodegradation of clofibric acid by an activated sludge also implies that this is the active mechanism (Kosjek et al., 2009). Our results indicated that this pathway for the degradation of clofibric acid is severely hindered by the presence of a second methyl group on the carbon adjacent to the ether function. A similar phenomenon was observed, for a different type of enzymatic reaction (Hammond and Alexander, 1972). They reported that oxidation of small aliphatic acids was blocked by the presence of two methyl substituents on a carbon atom adjacent to the site of the enzymatic attack.

While the two methyl groups inhibited rupture at the ether bond, a single branch did not affect the degradability of MCPP by *S. herbicidovorans*. The compound with no methyl functions at this position, MCPA, was degraded at a similar rate to that observed for MCPP and both reactions generated comparable amounts of the major metabolite M1.

5. Conclusion

This work demonstrated that the particular resistance to microbial degradation of clofibric acid is due to the presence of two methyl groups adjacent to the expected point of enzymatic attack. This led to the accumulation of the ethyl ester of this compound instead of breakdown products. Only one of these methyl groups was necessary for this pattern of behaviour and the two herbicides differing only by the presence or absence of a single methyl group were both easily degraded. However, the pattern of biodegradation was influenced by the presence of a more easily degraded carbon source and the result was the accumulation of secondary metabolites created from biodegradation of the primary metabolite observed for both herbicides in earlier studies.

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