A comparison of the ability of forest and agricultural soils to mineralize chlorinated aromatic compounds

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Abstract
Soils were sampled from two agricultural fields, two relatively pristine forests, and one suburban forest in Ontario, Canada. The ability of these soils to mineralize 2,4-dichlorophenoxyacetate, 3-chlorobenzoate, 4-chlorophenol, 2,4-dichlorophenol, pentachlorophenol, and atrazine was determined using 14C-labeled substrates. Direct pre-exposure was necessary before atrazine mineralization could be detected; however, it was not necessary for degradation of any of the other chemicals. 2,4-dichlorophenoxyacetate and pentachlorophenol mineralization was much higher in the agricultural soils relative to the pristine forest soils, but 3-chlorobenzoate and 2,4-dichlorophenol mineralization rates showed the opposite trend. Mineralization of 4-chlorophenol was about equivalent in all soils. Suburban forests soils were indistinguishable from agricultural soils with respect to their degradation of 2,4-dichlorophenoxyacetate and chlorobenzoate. Additionally, they were better able than any of the soils to withstand the toxic effects of pentachlorophenol. Pentachlorophenol mineralization was highly variable in the pristine forest soils, ranging from about 6 to 50%. Abiotic factors such as pH, soil type, and organic and moisture content did not account for these significant site differences. The selective forces responsible for these differences, and the possible differences in microbial populations are discussed.

Abbreviations: 3CBA = 3-chlorobenzoate; 2,4-D = 2,4-dichlorophenoxyacetate; PCP = pentachlorophenol; 4CP = 4 chlorophenol; DCP = 2,4-dichlorophenol.

Introduction
The ability of microorganisms to break down xenobiotics such as chlorinated aromatic compounds has long been assumed to be a recently evolved trait, selected for by the presence of pesticides and their residues in the environment. However, it is now clear that the ability to mineralize chlorinated aromatics such as 3-chlorobenzoate (3CBA) and 2,4-dichlorophenoxyacetate (2,4-D) is present in pristine soils that have not been directly exposed to xenobiotics (Fullilove et al. 1996). In addition, a high diversity of 3CBA mineralizers have been isolated from such soils (Fullilove et al. 1998). These findings are not surprising in the light of the discovery of high levels of natural chloroaromatic compounds produced by a variety of wood-rot fungi (de Jong et al. 1994). However, they do appear to conflict with the findings of others that 3CBA degrades in particular are rare (Beutelsbuech and Reinke 1993; deMeandres et al. 1995; Hickey and Focht 1990; Hickey et al. 1993). We were interested in finding the reason for these conflicting results and hypothesized that they might be due to the use of different methodologies or the study of soils harboring dissimilar microbial communities. Accordingly, the purpose of this study was to apply the same methods to study the mineralization abilities of soils from different ecosystems.

It is reasonable to assume that organic substrates that leach into soils will have a major impact on any species composition and capabilities of the bacterial community. Typically microbiologists have sampled
with corn, wheat, beans, barley, soy, and hay seed had a documented history of pesticide exposure, includ-
ing atrazine, 2,4-D amine and esters, bromoxynil, and monocrotophophosxycetate. The sites were mapped onto a grid, and random numbers were used to deter-
mine sampling locations within each site. Samples were taken using a flame-sterilized metal sampling device and kept in styrofoam boxes at 4 °C in the dark until use. Samples were composite samples of material from 5 to 20 cm below the surface of the mineral soil, and twelve samples were taken from each site. The sampling sites were determined by using a random number table to choose coordinates from a grid map of each of the

The water content of the soil was determined by drying overnight at 105 °C. For pH determinations 1 g of soil was mixed with 5 ml of distilled water and vortexed for 1 minute (pH meter: Orion model 420A). Soil organic carbon contents were determined by ashing dry soils at 550 °C for 1 hour.

Chemicals
Pentachlorophenol (PCP), UC-14CPCP (>98%), 4-
chlorophenol (4CP), 2,4-dichlorophenol (2,4DCP), UL-
14C2,4DCP (97%), 3-chlorobenzene (3CB) 2,4-
dichlorophenoxycetate (2,4D), UL-14C2,4D (>98%), UL-14C3CB (95%) and sodium bicar-
obonate-14C were obtained from Sigma Chemical Co. UL-14C2,4CB (98.1%) and UL-14C3CB (98%), from California Bionuclear Corp. (Los Angeles, Calif.) and atrazine (98.7%) from Ciba-Geigy Corp.

Mineralization of labeled compounds
Mineralization studies were carried out by adding 2 g (dry weight equivalent) of soil to sterile 20-ml scin-
tillation vials, and amending them with 50 μCi of unlabelled substrate and 0.05 μCi of labeled substrate. Atrazine was dissolved in ether, which was left to evaporate after adding it to the soil. Water was then added to these soils to obtain final moisture contents of 25%. A sterile, 1-ml glass vial was filled with 0.5 ml of a 1 M NaOH solution and placed in the scintillation vial as a 14CO2 trap. For each chemical tested, exper-
iments were run in triplicate. All vials were kept in containers saturated with water to prevent desiccation. To monitor mineralization activity, NaOH solutions were read and replaced on a weekly basis. The NaOH
was mixed with 10 mL of scintillation cocktail (Beckman, Ready Safe) and 1.5 mL of methanol and vortexed for 10 seconds. The amount of radioactivity was counted for 1 minute in a scintillation counter (Beckman Instruments Inc., LS6000). To calibrate the amount of Na<sup>14</sup>CO<sub>3</sub> trapped in the solution, aliquots of a labeled NaHCO<sub>3</sub> stock solution were put into 1 mL of water in scintillation vials including a vial with 0.5 mL of 1 M NaOH and allowed to dissociate. After one week all the bicarbonates had dissociated to carbon dioxide and was captured in the NaOH solution. Counts of Na<sup>14</sup>CO<sub>3</sub> were regressed against μCi added to water.

To ensure that observed mineralization was due to biotic activity, a control experiment was performed with three replicates for each chemical at 50 μg/g dry soil in which soil was sterilized by autoclaving for 30 minutes. Because relatively high counts were observed from the sterile soil treated with DCP, another experiment at higher concentrations with autoclaved soil was performed. Four replicates were tested, one sample from Highland Creek, Graythorpe, and Springwater and one agricultural sample all at 100, 300, 500 and 750 μg/kg. To determine if mineralization was due to bacterial rather than fungal action, a set of experiments were carried out where fungicides were added to the soil. Nystatin (5 μg/g) was added by dissolving in ether, dispensing over soil surface, and waiting for 1 hour for the ether to evaporate before other amendments were carried out. Cycloheximide (100 μg/g) was added with the water and substrates.

**Response of soils to higher concentrations**

In order to determine the effects of substrate concentration on mineralization rates, we examined the soil response to 4 concentrations higher than 50 μg/g. In order to avoid complete inhibition of activity, the minimum inhibitory concentration was estimated by monitoring substrate degradation in liquid media inoculated with soil. Chloroaromatic substrate concentrations were determined using HPLC. These data were used to set up mineralization experiments with 3 replicates at four different concentrations for four compounds.

In order to examine the effect of higher concentrations of the same chemicals, three samples from each site were chosen. The samples that were best able to mineralize the compounds at 50 μg/g were chosen for this. The agricultural samples used for the concentration study were from Elora field 11 for CIBA, DCP, and PCB and field 30 for 2,4D, and Arkell field 3 for all compounds.

**Chemical analysis**

Liquid chloroaromatic concentrations were determined using a Waters HPLC system that included a NovaPak C18 reverse phase column and a Waters 9600 Photo Diode Array detector. The mobile phase consisted of equal parts of 1% (v/v) phosphoric acid and acetonitrile.

**Results**

**Soil characteristics**

The characteristics of the sampled soils are shown in Table 1. The soils had similar organic contents, ranging from 5.5 to 7.7%, and similar moisture contents. The agricultural sites and the Highland Creek forest site were slightly basic, and the two more rural forest sites were slightly acidic.

**Mineralization at 50 μg/g dry soil**

The correlation between the radioactivity trapped in the NaOH solutions and 14CO2 added to the water was significant. The regression line μCi = 1.325 ± 3.258 × 10<sup>-4</sup> + 3.258 × 10<sup>-4</sup> × CPM (r<sup>2</sup> = 0.983) was used to convert counts to μCi for determination of mineralization rates. Typical mineralization curves are shown for two of the chemicals in Figure 1. The graphs show some of the main findings of this study. First, there is a large difference in the ability of the soils to mineralize the test compounds. These differences are chemical specific, i.e., one cannot conclude that agricultural soils degrade all chloroaromatic compounds better than do forest soils or vice versa. Second, for all chemicals, average mineralization rates from the Graythorpe
and Springwater soils did not significantly differ from each other, nor did average rates from the agricultural soils, Arkell and Elora. Highland Creek forest soils, however, show more variability between samples and behaved like agricultural soils with respect to some chemicals, but like forest soils with respect to others.

The mean mineralization levels achieved after 60 days for all chemicals applied at 20 μg/g dry soil for all sites is given in Figure 2. The forest sites Springwater and Graythorpe mineralized significantly more 3CBA (p < 0.0001) and DCP (p < 0.0001) than did the agricultural soils, but significantly less PCP (p < 0.0001) and atrazine (p < 0.0001). The 60-day mineralization rates are comparable for 2,4-D and 4CP. The Highland Creek samples appear to behave like agricultural soils, mineralizing comparable amounts of 3CBA, 2,4-D, DCP and 4CP. However, there is no atrazine degradation in Highland Creek forest soil, and PCP is degradation midway between that of the agricultural soils and the other more remote forest sites. In all three forest sites PCP mineralization was patchy, ranging from 2.7 to 56% in Highland Creek, from 6.3 to 37% in Graythorpe, and 8.6 to 17.7% in Springwater. It is apparent that bacteria capable of mineralizing PCP can be found in forested systems, but their distribution, or their activity is limited.

Graythorpe and Springwater have lower pHs than Highland Creek forest soils and this might have contributed to differences in mineralization rates (Table 1). Both PCP and 4CP mineralization rates are positively correlated to pH (r = 0.75, r = 0.71, respectively), while 3CBA and DCP degradation are negatively correlated (r = −0.83, r = −0.81, respectively). All correlations are significant at p < 0.01. There were no significant correlations between mineralization rates of any of the substrates and water content or organic content. After addition of fungi, mineralization either increased or remained the same suggesting that mineralization is not performed by fungi.

**Sensitivity to higher concentrations**

Four chemicals were studied at higher concentrations – 2,4D and CBA at 250, 500, 750, and 1000 μg/g. PCP: at 100, 150, 500 μg/g and DCP: at 100, 300, 500, 750 μg/g. The experiments ran up to 71 days, being terminated when no further changes in mineralization were noted (i.e., when curves had leveled off or showed constant slopes if linear). Figure 3 illustrates the average amount of chemical mineralized at the end of the experiments. Agricultural soils show very high levels of 2,4-D at all concentrations tested, and Highland Creek mineralization amounts improve at high concentrations. Springwater soils show a drop in mineralization with concentration that is statistically significant, as do Graythorpe soils at the highest concentration tested. The picture is quite the opposite when mineralization of 3CBA is analyzed. The rural forest areas show the highest mineralization amounts at all concentrations, and mineralization inhibition at higher concentrations is not statistically significant. The Highland Creek soils and agricultural soils both show consistently lower CBA mineralization amounts, and statistically significant inhibition above 500 μg/g. The responses to DCP are more similar to CBA – higher mineralization in rural versus soils across the range, but inhibition is evident in all soils except the
Highland Creek soils. PCP mineralization is strongly inhibited by concentrations above 100 to 150 μg/g in all soils tested. Highland Creek soils show much more tolerance to high PCP concentrations than do agricultural soils.

The effect of the different concentrations is more dramatically illustrated by looking at the amount of time each soil took to degrade 25% of the chemical (Figure 4). The higher concentrations typically pushed the mineralization curves to the right, lengthening the lag phase, or changed the curves from logistic to linear, both these effects leading to longer times before 25% mineralization could be achieved. Once again, the clear differences between chemicals and the unique nature of the Highland Creek soils are illustrated. The forest soil degraders are clearly relatively insensitive to high concentrations of both CBA and DCP, while they are quite sensitive to high levels of 2,4-D. The agricultural soils show exactly the opposite sensitivities, with a particularly odd reaction to DCP. The poorest mineralization rates were seen for the lowest concentrations, the highest for intermediate concentrations. Highland Creek behaves like an agricultural soil with respect to 2,4-D and CBA, but like a forest soil with respect to DCP. Of the five soils, those from Highland Creek can degrade the highest concentrations of PCP, followed by the soils of Springwater, while Graythorpe and the agricultural soils exhibit equivalent sensitivities to high concentrations of PCP.

**PCP mineralization and pH effects**

A more intensive sampling of Graythorpe soils was carried out in order to examine differences in chemical metabolism in soils found directly under different vegetation types and to examine the effects of artificially raised pH. CBA and PCP mineralization experiments were carried as usual on three replicate samples taken from under seven vegetation types. Soils were sampled within a metres radius of particular tree types or directly under smaller plants such as ferns and herbaceous growth. All sites exhibited the same soil profile. pH was raised in the solution applied to the soils to pH 12 by adding 50 μL of 1 M NaOH to the 0.5 ml solution of PCP or CBA added to the soil. Trials showed that the pH of the soils was raised to about 8–9.5 in this manner. Results are summarized in Figure 5, which shows the amount of 3CBA degraded after 32 days (when the curves have leveled off) and the amount of PCP degraded after 58 days. pH treatment had no statistically significant effect on CBA degradation in any of the samples, although in every case the mean mineralization rate was higher in the more alkaline samples. There is no difference in CBA mineralization rates between samples. For PCP,
pH has no significant effect, but there are significant differences between samples. When all the replicates are regressed against soil pH, organic content, or moisture content, no significant correlations were found, suggesting that abiotic factors are not responsible for these site differences.

Discussion

This work clearly demonstrates that all soils do not have the same capacity to degrade anthropogenic chemicals. This is particularly important for the modeling of chemical fates in the environment. It is also important to the study of the evolution of biodegradative pathways. In the past, contaminated systems or soils from unspecified systems have been used for enrichment experiments that lead to the isolation of chloroaromatic degraders. It is likely that we are failing to detect the wider diversity of organisms and genetic solutions to the problem of chloroaromatic contamination that probably exists because of our relatively narrow sampling habits. For instance, Kamagata et al. (1997) have shown that 2,4-D degraders isolated from uncontaminated soils collected from various locations are alpha-proteobacteria closely related to Bradyrhizobium, in contrast to the more typical Ralstonia Burkholderia and Sphingomonas species isolated from agricultural soils.

Our results suggest no selection for organisms able to degrade atrazine or its analogues in forest soils, while atrazine spraying has resulted in its mineralization in agricultural soils. Our data are in agreement with a study by Bariuso and Hansot (1996), where less than 4% mineralization of atrazine was observed in unexposed plots. The low and constant
mineralization rate observed in the forest soils is likely caused by the absence of specific microorganisms able to use the atrazine ring as a growth substrate, rather than unfavorable conditions in the soil. Similarly the high rates of mineralization of PCP seen in the agricultural soils could be explained by the use of the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile). Sphingomonas chlorophenolica (previously Pseudomonas) sp. strain ATCC 39723 metabolizes PCP and bromoxynil likely using the same enzymatic pathway; hence, some PCP degraders may be selected for by this herbicide (Trupp et al. 1992).

The PCP-mineralization capability of some samples from the treated systems is a new finding. The Greythorpe and Springwater soils that were generally poor at degrading PCP were slightly acidic. The significant correlation between PCP mineralization and pH led us to wonder if the mineralization rates were mostly determined by PCP availability and/or toxicity. PCP adsorbs onto soil more strongly under acid conditions, when it is protonated, more hydrophobic, and less available to microorganisms (McAllister et al. 1996). Similarly the toxicity of PCP increases at low pH because of an increase in the concentration of the undissociated form (Stanlake and Fenn 1982). However, the experiments performed with increased pH show that the site where the soil was sampled has a much more dramatic effect than does the pH amendment. Further studies are underway on these sites exhibiting good PCP degradation.
Another new finding of this study is the differences between the soils with respect to DCP degradation. This compound can be naturally occurring in uncontaminated systems (Grinstead 1994) and clearly degrades of this compound exist in forested ecosystems. However, the ability of the agricultural soils to mineralize DCP was limited and extremely sensitive to high concentrations. This finding is curious given the high 2,4-D mineralization rates of the agricultural soils and the fact that most, if not all, known 2,4-D degraders metabolize this herbicide via DCP (Higgison 1990). This result might suggest that uptake of
DCP is problematic for agricultural 2,4-D degraders, and/or that the chemicals are processed by different populations in these soils. Similarly, most known 2,4-D degraders are also able to metabolize 3CB, often with the same enzymes. However, again 3CB is relatively poorly degraded by agricultural soils, especially at concentrations above 500 mg kg−1 dry soil, in spite of the healthy populations of 2,4-D degraders. The fact that the two systems behave in completely opposite ways with respect to these chemicals underscores the cautious extrapolation of the laboratory behaviour of isolated degraders to the real world.

Overall, when the forest soils are compared to the agricultural soils, they exhibit higher mineralization and sorption of 3CB and DCP, roughly equivalent mineralization levels of 4-CP and 2,4-D and, at some sites, equivalent TCP mineralization rates. The selection pressure for the evolution of degraders of all five of the chlorophenols in uncontaminated systems is still unknown. The absence of a mixed vegetation on the agricultural soils may explain their overall lower versatility. Conventional cultivation practices are known to decrease the mass and diversity of carbon in soil (Schulten et al. 1995). It has also been found that genetic diversity of microorganisms increases with greater habitat variability (McArthur et al. 1988). The greater amount and diversity of organic matter in forest soil could explain the greater catabolic diversity of microorganisms in forest soils compared to cultivated soils.

The use of forest systems as buffer strips has been proposed by Ewing and Ebbinghaim 1995. They noted improved 2,4-D and atrazine degradation in coniferous forest soils relative to deciduous forest or grassland soils. The results obtained in this study provide strong support for this idea. We have found that rural forest ecosystems are able to mineralize a number of chlorophenolics well and in some cases better than contaminated systems. The capabilities of the highland Creek Ravine forest soils are a blend of exposed and uncontaminated soils, and show a superior ability than either soil type to degrade PCP, but a decreased ability to utilize CBA and DCP. If forested buffer strips are to be advocated, we must learn about how much buffer strips change in response to greater levels of exposure to anthropogenic chemicals.

Conclusions

Preexposure of soils to atrazine is required before mineralization is likely to occur. No preexposure is required for mineralization of 2,4-D, 4CP, DCP, 3CB, or TCP to take place, although exposure to a suite of herbicides including 2,4-D and bromoxynil enhances 2,4-D and TCP mineralization. Exposure to the same suite of herbicides seems to impair the ability of soils to mineralize 3CB and DCP. Close proximity to urban development seems to enhance 2,4-D and TCP mineralization, but impairs 3CB degradation in forest soils. TCP mineralization in uncontaminated forest soils is site specific.

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