Dynamics of carbofuran-degrading microbial communities in soil during three successive annual applications of carbofuran

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Received 4 October 1999; received in revised form 3 May 2000; accepted 29 May 2000

Abstract

The influence of repeated annual field applications of the insecticide–nematicide carbofuran on carbofuran-degrading microbial communities was studied at a site in Florida that exhibited enhanced degradation toward the chemical. Three successive annual applications of carbofuran at 4.5 kg ha$^{-1}$ y$^{-1}$ did not result in an increase in the size of the microbial community capable of mineralizing uniformly ring-labeled $^{14}$C-carbofuran (carbofuran-ring degraders) in surface soil (0–15 cm depth). After the second annual application, however, the community capable of mineralizing carbonyl-labeled $^{14}$C-carbofuran (carbofuran hydrolyzers) in the treated surface soil was significantly larger than that after the first annual application. Communities of methylamine utilizers in treated and untreated soils were much larger than the communities of carbofuran phenol degraders, but not statistically different from the sizes of carbofuran hydrolyzers. In addition to no increase in the number of carbofuran ring degraders in the treated site during three consecutive annual applications of carbofuran, laboratory addition of 10 mg carbofuran g$^{-1}$ to treated and untreated soils collected in 1995 did not result in an increase in the number of carbofuran ring degraders. This suggests that degradation of the ring structure of carbofuran in soil is a cometabolic process. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Carbofuran; Most-probable-number; Carbofuran ring degraders; Carbofuran hydrolyzers; Enhanced soil

1. Introduction

It has been known since 1981 that repeated field applications of the insecticide–nematicide carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) cause enhanced degradation of the chemical in the soil (Felsot et al., 1981; Read, 1983; Camper et al., 1987; Turco and Konopka, 1990). As a consequence of enhanced degradation, there may be a loss in the efficacy of the pesticide to control target pests, resulting in crop failure (Felsot et al., 1981; Racke and Coats, 1990). Enhanced degradation of pesticides in soils is a microbial process (Racke and Coats, 1990). There are two schools of thought on the mechanism by which microorganisms in soil develop enhanced degradation. One is as a consequence of repeated applications of a pesticide, where an increase in the number of microorganisms capable of degrading the chemical results in more rapid degradation of the chemical compared to untreated soil. Evidence supporting this hypothesis is based on the observations that after addition of the phenoxy herbicide 2,4-D (Ou, 1984; Holben et al., 1992; Ka et al., 1994) or the organophosphate insecticide isofenphos (Racke and Coats, 1987), the numbers of soil microorganisms capable of degrading the chemicals increased substantially. The other hypothesis is that repeated applications of a pesticide result in an increase in the enzyme activity, but not community size, specifically toward the degradation of the pesticide. Evidence for the second hypothesis is supported by the findings that the sizes of EPTC-degrading communities in soils with or without a history of field applications of the herbicide were similar (Moorman, 1988).

At present, published information on the changes of carbofuran-degrading community sizes in soils after development of enhanced degradation toward carbofuran is mixed. Hendry and Richardson (1988) and Dzantor and Felsot (1989, 1990) pointed to an increase in the number of carbofuran degraders in soils with enhanced degradation compared to the number of the indigenous carbofuran degraders. Racke and Coats (1988), Merica and Alexander (1990), Scow et al. (1990) and Robertson and Alexander (1994) failed to link an increase in the number of carbofuran degraders with the number of carbofuran applications in enhanced soils.

There are likely to be three groups of microorganisms that may be involved in the degradation of carbofuran in soils.
and uniformly ring-labeled (URL) 14C-carbofuran (specific activity, 131 MBq mmol−1), and uniformly ring-labeled (URL) 14C-carbofuran (specific activity, 229 MBq mmol−1) were provided by FMC Corporation (Princeton, NJ). Prior to use, the two 14C-labeled compounds were purified to >98% radiopurity by thin-layer chromatography (TLC) using preparative silica gel G TLC plates (Ou et al., 1982, 1985). URL 14C-carbofuran phenol was obtained by alkaline hydrolysis of URL 14C-carbofuran overnight at 70°C, and was purified by preparative TLC to >98% radiopurity. Analytical grade methylamine hydrochloride (>99% purity) was purchased from Aldrich (Milwaukee, WI).

2.2. Field plots, carbofuran treatments and soil sampling

Field plots at an experimental site located in the northeast of Florida (60 km northeast of Gainesville, FL) were treated with carbofuran annually in March or April from 1992 to 1996 at a rate of 4.5 kg ha−1. This site had been under continuous potato cultivation for more than 10 y. Control (untreated) plots at this site had never been treated with carbofuran or any structurally similar pesticides, but received fertilizers at rates identical to the treated plots. Soil samples were collected from three plots at two depths (0–15 and 45–60 cm) 4 weeks after annual applications of carbofuran in three consecutive years (third, fourth and fifth annual applications in 1994, 1995 and 1996, respectively). Soil samples from each depth were combined and mixed. Soil samples were stored in plastic bags in the dark at 4°C and used within 3 months after collection. All soil samples were classified as Ellzy fine sand (sandy, siliceous, hyperthermic Arenic Ochraqualfs). Key soil properties of the samples have been reported by Trabue et al. (1997).

2.3. 14C-most-probable-number (MPN) assay

A 14C-MPN technique similar to that used by Ou (1984) to determine 2,4-D-degrading microbial community sizes in soils was employed to determine the sizes of microbial communities capable of degrading carbofuran or carbofuran phenol. URL or CBL 14C-carbofuran were used to determine community sizes capable of mineralizing the aromatic ring structure or hydrolyzing the carbamate linkage of carbofuran. URL 14C-carbofuran phenol was used to estimate the numbers of microorganisms capable of mineralizing the phenolic structure of carbofuran phenol.

One gram of soil (oven-dry weight basis) was transferred to a sterile capped MPN tube containing 9 ml of a minimal mineral medium, including 10 µg tryptone ml−1 (Ou, 1984). The medium also contained 10 µg ml−1 of technical grade carbofuran and 16.7 Bq ml−1 of URL 14C-carbofuran, CBL 14C-carbofuran or URL 14C-carbofuran phenol. Five replicates of successive 5- to 10-fold dilutions were made. All tubes were incubated in the dark at 28°C for 28 d. Control MPN tubes were treated as described above, except that soil was not added to the tubes. At the end of the incubation, 0.1 ml of concentrated HCl was added to each tube, and after mixing, they were kept in a fume hood for 4–6 h. Afterwards, 0.5 ml of the MPN media was assayed for 14C-activity by liquid scintillation counting (LSC). Tubes were scored as positive when ≥70% of the initial 14C-activity remained in solution. 14C-activity in all control 14C-MPN tubes was unchanged after 28 d.

To determine community size changes after laboratory treatment of carbofuran, 100 g of soil (oven-dry weight basis) were added to a 250 ml Erlenmeyer glass flask with a cotton plug and mixed with 1.0 mg of technical grade carbofuran to give a concentration of 10 µg g−1. The flasks were incubated in the dark at ambient temperature...
(23 ± 2°C), and once a week their weights were checked, sterile deionized water was added to compensate for any water loss. Periodically, 1 g of soil was removed and placed in a sterile MPN tube containing 9 ml of the MPN medium (including 10 µg tryptone ml⁻¹), technical grade carbofuran (10 µg ml⁻¹), and either URL ¹⁴C-carbofuran, CBL ¹⁴C-carbofuran or URL ¹⁴C-carbofuran phenol. Community sizes were estimated as above.

### Table 2

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>MPN</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated soil (1994)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–15</td>
<td>1.61 × 10⁵bc</td>
<td>4.87 × 10⁴</td>
<td>5.31 × 10⁵</td>
</tr>
<tr>
<td>45–60</td>
<td>2.78 × 10⁶</td>
<td>8.42 × 10⁵</td>
<td>9.18 × 10⁶</td>
</tr>
<tr>
<td>Untreated soil (1994)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–15</td>
<td>1.61 × 10⁶bc</td>
<td>4.87 × 10⁴</td>
<td>5.31 × 10⁵</td>
</tr>
<tr>
<td>45–60</td>
<td>3.45 × 10⁷bc</td>
<td>1.05 × 10⁶</td>
<td>1.14 × 10⁷</td>
</tr>
<tr>
<td>Treated soil (1995)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–15</td>
<td>1.23 × 10⁷</td>
<td>2.27 × 10⁶</td>
<td>6.68 × 10⁷</td>
</tr>
<tr>
<td>Untreated soil (1995)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–15</td>
<td>4.99 × 10⁵ab</td>
<td>9.21 × 10⁴</td>
<td>2.70 × 10⁶</td>
</tr>
</tbody>
</table>

* Values followed by the same letter are not significantly different (P < 0.05).

### 2.4. MPN for methylamine utilizers

An MPN technique that determined the community size capable of utilizing an organic substrate for growth was used for determination of the number of microorganisms capable of utilizing methylamine for growth (Alexander, 1982). The MPN method was similar to the ¹⁴C-MPN assay, except that non-labeled methylamine hydrochloride equivalent to 100 µg methylamine ml⁻¹ was added to the MPN tubes as a sole source of carbon and no tryptone was added. Five replicates of successive 5-fold dilutions were made. Tubes were scored positive if turbidity developed in the MPN solutions in the tubes after 28 d at 28°C. Control tubes, which contained the MPN medium and methylamine, were all devoid of turbidity after 28 d.

### 2.5. Statistical analysis

Numbers of microorganisms per gram soil based on the MPN assays were obtained using the Eureka software (Borland International, Scotts Valley, CA). The MPN and the 95% confidence limits were estimated by the software.

### 3. Results

#### 3.1. Carbofuran degrading communities in field soils

The numbers of microorganisms capable of mineralizing the aromatic ring of carbofuran (ring degraders) in treated surface soils (0–15 cm depth) collected 4 weeks after three annual field applications of carbofuran were not statistically different (Table 1). The MPN in treated subsurface soils (45–60 cm depth) were also not statistically indistinguishable, with the exception of the subsurface soil collected in 1995. The MPN in this soil was significantly larger than the MPN in the other two subsurface soils. The MPN in the treated surface soils were statistically larger than the MPN in the corresponding subsurface soils, with the exception of the surface and subsurface soils collected in 1995. The MPN in the two soils were not statistically different. The MPN in untreated surface soils collected in 1994 and 1995 were not significantly different from each other, nor were they significantly different from the treated surface soils, with the exception of the untreated surface soil collected in 1995. The MPN in this soil was statistically smaller than the MPN in the treated surface soil collected in the same year. The MPN in untreated subsurface soils collected in three consecutive years as well as in untreated surface soils collected in 1996 were zero.

The MPN of microorganisms capable of hydrolyzing the carbamate linkage of carbofuran (carbofuran hydrolyzers) in the treated and untreated soils collected in 1994 and 1995 (Table 2) were three to five orders of magnitude larger than the MPN of carbofuran ring degraders. The MPN of carbofuran hydrolyzers in the treated surface soil collected in 1995 was significantly larger than the MPN in the treated
Table 3
MPN (cells g⁻¹) of methylamine utilizers and carbofuran phenol ring degraders in treated and untreated surface soils collected in 1995; CI — confidence interval

<table>
<thead>
<tr>
<th>Degraders</th>
<th>MPN</th>
<th>95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treated soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylamine</td>
<td>7.93 x 10⁶⁺</td>
<td>2.40 x 10⁶</td>
<td>2.62 x 10⁷</td>
</tr>
<tr>
<td>Carbofuran phenol</td>
<td>1.60b</td>
<td>6.2</td>
<td>41.2</td>
</tr>
<tr>
<td><strong>Untreated soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylamine</td>
<td>4.93 x 10⁶a</td>
<td>1.49 x 10⁶</td>
<td>1.62 x 10⁷</td>
</tr>
<tr>
<td>Carbofuran phenol</td>
<td>8.5b</td>
<td>3.3</td>
<td>21.9</td>
</tr>
</tbody>
</table>

* Values followed by the same letter are not significantly different (P < 0.05).

and untreated surface and subsurface soils collected in 1994, but not significantly different from the MPN in the untreated surface soil collected in 1995. However, the MPN in the untreated surface soil collected in 1995 was not statistically different from the MPN in the treated surface soil, and untreated surface and subsurface soils collected in 1994, but significantly larger than the MPN in the treated subsurface soil collected in 1994. Due to larger than usual precipitation in late winter and early spring in 1995, the water table at the site at the time of sampling was fairly shallow; occurring at 50–55 cm from the soil surface.

3.2. Carbofuran phenol- and methylamine-degrading communities in field soils

The MPN of microorganisms capable of utilizing methylamine in the treated and untreated surface soils collected in 1995 were much larger (5 x 10⁵ times) than the MPN of microorganisms capable of mineralizing the aromatic ring of carbofuran phenol (Table 3). The MPN of methylamine utilizers in the treated and untreated surface soils were not statistically different from each other. It is interesting to point out that the MPN of methylamine utilizers and carbofuran hydrolyzers in the two soils were not statistically different (Tables 2 and 3).

The numbers of carbofuran phenol ring degraders in the treated and untreated soils averaged 16 and 9 cells g⁻¹ soil, respectively, and were statistically indistinguishable (Table 3). The MPN of carbofuran ring degraders and carbofuran phenol ring degraders in treated and untreated soils were all small (Tables 1 and 3). They were not statistically different in the untreated soil (Tables 1 and 3). But the MPN of carbofuran ring degraders in the treated soil was statistically larger than the MPN of carbofuran phenol ring degraders.

3.3. Growth of carbofuran degraders after a laboratory treatment of carbofuran

Laboratory treatment of carbofuran at 10 µg g⁻¹ to the field treated surface and subsurface soils collected in 1995 did not result in a statistically significant increase in the MPN of carbofuran ring degraders after 28 d of incubation (Fig. 1A). After 14 d, the MPN in the field treated surface soil was significantly smaller than before the laboratory treatment. The MPN of carbofuran ring degraders in the field untreated surface soil did not change after laboratory treatment with carbofuran, with the exception that 14 d after the treatment, the MPN was significantly smaller than before the treatment. In the untreated subsurface soil, carbofuran ring degraders were not detected until 28 d after the laboratory treatment of carbofuran, when 4.5 cells g⁻¹ were detected in this soil.

The MPN of carbofuran-hydrolyzers in the field treated surface soil collected in 1995 was initially very large (1.23 x 10⁷ cells g⁻¹ soil), and after laboratory treatment with carbofuran at a rate of 10 µg g⁻¹ soil, the MPN remained stable during the first 7 d of incubation (Fig. 1B). After 14 d, the MPN declined significantly. The MPN of carbofuran hydrolyzers in the field untreated surface soil did not change during the first 3 d after the laboratory application of carbofuran; however, between days 3 and 7, the MPN increased significantly. After 14 d, it declined to the level statistically similar to that before the treatment.

3.4. Growth of carbofuran phenol degraders and methylamine utilizers after laboratory treatment with carbofuran phenol and methylamine

After laboratory treatment of URL ¹⁴C-carbofuran phenol
at 10 μg g⁻¹ to field treated and untreated surface soils collected in 1995, the MPN of carbofuran phenol ring degraders in these two soils were statistically indistinguishable during the entire 28 d of incubation (Fig. 2A). Even though the average MPN of carbofuran phenol degraders in the surface soil after 28 d was 6.8 times larger than before the laboratory treatment, due to large errors associated with the MPN determination, the two numbers were not statistically different. Results similar to the MPN of carbofuran phenol degraders were obtained for the MPN of methylamine utilizers in treated and untreated surface soils (Fig. 2B). The average MPN in the treated soil 7 d after the laboratory treatment was 10 times larger than before the laboratory treatment, although they were not significantly different.

4. Discussion

In this study, we chose MPN assays over other enumeration techniques. Dilution-plate count methods were inadequate. Although small semi-transparent watery colonies developed on basal mineral-carbofuran agar plates after 5–7 d of incubation, no carbofuran degradation and no growth were observed when biomass of individual colonies was inoculated into the liquid basal mineral-carbofuran medium (Ou, unpublished observations). Methods such as gene probing are not available for those strains. Only one gene involved in carbofuran degradation has been cloned. This gene, the methyl carbamate degrading (mcd) gene, was cloned from Achromobacter sp. WM111 and encodes for a carbofuran hydrolase (Tomasek and Karns, 1989). mcd appears to be not widely distributed among carbofuran degraders in the US. DNA isolated from many soils that have been exposed to carbofuran did not hybridize with this gene (Derk et al., 1993). ¹⁴C-MPN assays at present appear to be the only means for determination of carbofuran-degrading communities in soils.

The sequence of carbofuran degradation in soil is first via hydrolysis to carbofuran phenol and methylamine. Carbofuran phenol is degraded further through aromatic ring cleavage, and eventually to CO₂ and H₂O (Ou et al., 1982; Trabue et al., 1997). Therefore, the same microorganisms that mineralized the aromatic ring of carbofuran were likely also to be responsible for carrying out mineralization of the aromatic ring of carbofuran phenol. It was not clear why the MPN for carbofuran ring degraders was statistically larger than the MPN for carbofuran phenol ring degraders in treated soil collected in 1995 where there was enhanced degradation of carbofuran (Trabue et al., 1997). They were all small in numbers, however.

Carbonyl-labeled ¹⁴C-carbofuran was used to determine the number of carbofuran hydrolyzers in soil based on the ¹⁴C-MPN technique. When the carbamate linkage of carbofuran is broken by hydrolysis, ¹⁴C-carbonyl is immediately converted to ¹⁴CO₂, carbofuran phenol and methylamine (Fig. 3). Due to unavailability of furanyl ring-labeled...
14C-carbofuran, the number of microorganisms capable of degrading the furanyl ring could not be determined. However, it is conceivable that carbofuran ring degraders may also have the capacity to mineralize the furanyl ring. We found that URL 14C-carbofuran in treated and untreated surface and subsurface soils collected in 1995 from the same site was rapidly mineralized to 14CO2 (Trabue et al., 1997).

Prior to this study, the treated site had been treated with carbofuran annually for two consecutive years and soil of this site was found to exhibit enhanced degradation toward carbofuran after the second annual treatment (1993) (Ou, unpublished observation). Enhanced degradation was continually observed from 1994 to 1996 (Trabue, Unpublished PhD thesis, University of Florida, 1997; Trabue et al., 1997). Despite the fact that carbofuran was applied to the treated site annually at a rate of 4.5 kg ha−1 for five consecutive years, the MPN of carbofuran ring degraders in the surface soils during the last three annual applications were small and not statistically different. This suggests that mineralization of the ring structure of carbofuran was mainly by cometabolism.

The fact that the MPN of carbofuran hydrolyzers and methylamine utilizers were not statistically different may be fortuitous. However, some carbofuran-degrading bacteria are methylotrophic organisms (Chaudhry and Ali, 1988; Singh et al., 1993; Topp et al., 1993). They were capable of hydrolyzing carbofuran and utilizing methylamine as a sole source of C for growth. It is known that methylotrophic bacteria utilize 1 C compounds, including methane and methylamine, as a sole source of C for growth (Hanson and Hanson, 1996). Large communities of methanotrophs ranging from 106 to 108 cells g−2 soil exist in oxic soils (Hanson and Hanson, 1996). Since methanotrophs are a subgroup of methylotrophs, the sizes of methylotrophic communities in soils should be at least equal or larger than the sizes of methanotrophic communities. In our study, community sizes of methylamine utilizers in the two soils were similar to the reported values for methanotrophs.

Our findings suggest that some carbofuran hydrolyzers and methylamine utilizers may also be methylamine utilizers. Hydrolysis of 4.5 kg carbofuran ha−1 (equivalent to 2 µg g−1) will result in the formation of 280 ng methylamine g−2 soil. Thus, the methylamine will provide 110 ng C and 130 ng N g−1 for serving as C and N sources for methylotrophic bacteria. A typical bacterial cell has a dry mass of 280 fg and consists of 50% C and 14% N (Neidhardt et al., 1990). Thus, 280 ng methylamine g−1 could support from 7.9 × 105 to 3.3 × 106 cells g−1 soil, if N and C are not limiting factors, respectively. Due to the large difference between the lower and upper confidence intervals (Table 3), even an increase of 3.3 × 106 cells g−1 would not result in a statistically significant increase in the community size of methylamine utilizers. In this study, five replicates for each dilution were employed for all MPN experiments, an increase to 10 replicates may provide smaller difference between lower and upper confidence intervals. As a consequence, release of 280 ng methylamine g−1 from hydrolysis of carbofuran may result in a statistical increase in MPN for methylamine utilizers.

Communities of carbofuran ring degraders in the field treated and untreated soils after receiving 10 µg carbofuran g−1 in laboratory did not increase numerically, suggesting that the ring structure of carbofuran did not provide a carbon source for the growth of microorganisms, even though the structure was microbially degraded. This finding was supported by the results of MPN estimations of carbofuran ring degraders in soil from the treated plots during three consecutive annual field applications of carbofuran (Table 1). Similarly, the MPN of carbofuran phenol ring degraders in the treated and untreated soils after receiving 10 µg carbofuran phenol g−1 did not increase numerically as well. Therefore, enhanced degradation of carbofuran in the treated soil (Trabue et al., 1997) may have been due to an increase in enzyme activity responsible for carbofuran ring degradation. Merica and Alexander (1990) also found that the MPN of carbofuran ring degraders did not increase in enhanced soil in 2 and 12 d after receiving 10 µg URL 14C-carbofuran g−1.

In conclusion, our results indicated that communities of microorganisms capable of degrading the ring structure of carbofuran during three consecutive years of carbofuran treatments remained small and stable, and hence the degradation is a cometabolic process.

Acknowledgements

We thank John E. Thomas for technical assistance. Sincere appreciation is extended to David P. Weingartner for field treatments of carbofuran. This study was partially supported by a grant from USDA, Florida Agricultural Experimental Station journal series No. R-06805.

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