Degradation of Dicamba by an Anaerobic Consortium Enriched from Wetland Soil

RONALD H. TARABAN,1 DUANE F. BERRY,1,* DAVID A. BERRY,2 AND HUBERT L. WALKER, JR.3

Department of Crop and Soil Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061,1 and Berry & Associates Inc., Dexter, Michigan 481302

Received 11 January 1993/Accepted 4 May 1993

The biodegradability of dicamba was investigated under anaerobic conditions with a consortium enriched from wetland soil. Degradation proceeded through an initial demethylation reaction, forming 3,6-dichlorosalicylic acid, followed by reductive dechlorination, forming 6-chlorosalicylic acid. The consortium, consisting of a sulfate reducer, three methanogens, and a fermenter, was unable to mineralize the aromatic ring.

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is a chemically stable postemergence herbicide commonly used to control weeds in cereal grain crops and broadleaf weeds in pastures and rangelands. Because it exists primarily in a dissociated anionic form in soils, dicamba is highly mobile and susceptible to dispersal through both runoff and leaching (3, 5, 12), often entering nontarget environments such as streams and rivers, sediments, and groundwater. Many of these nontarget environments are anaerobic.

Although little is known about the fate of dicamba under anaerobic conditions, its fate under aerobic conditions has been extensively investigated. Dicamba is probably degraded in moist soils through biologically mediated processes (15–17). Studies by Smith (15, 16) have shown that dicamba is readily transformed to 3,6-dichlorosalicylic acid (3,6-DCSA) in soil. Krueger et al. (7) established that dicamba could be degraded by pure cultures of bacteria under aerobic conditions. They isolated eight species of bacteria from soil and water samples with long histories of dicamba exposure that were capable of using dicamba as the sole carbon source. They also found that the primary metabolite of dicamba in soil was 3,6-DCSA.

Microbially mediated reductive dehalogenation of chlorinated aromatic compounds is now widely recognized as a process that plays a crucial role in the restoration of contaminated anoxic environments (8, 11). Over the past 10 years, several organic chemicals, including the halobenzoates (6, 18), chlorinated phenols (1, 2), and the polychlorinated biphenyls (13), have been studied with respect to reductive dehalogenation. Kuhn and Sulfita (8) reported that aryl reductive dehalogenation has been observed for a few pesticides. Biodegradation of the phenoxyalkanoic acid herbicide, 2,4-dichlorophenoxy acetic acid, similar in herbicidal activity to dicamba and equally mobile, has been evaluated under methanogenic conditions in environmental samples including pond sediment aquifer material (4) and sewage sludge (10). Under methanogenic conditions, 2,4-dichlorophenoxy acetic acid is known to undergo reductive dehalogenation, forming products such as 2-, and 4-chlorophenoxyacetate (8).

Although most studies have examined degradation of dicamba under aerobic conditions, to our knowledge none have determined the degradative pathway under strict anaerobic conditions. The present study was conducted to elucidate the degradative pathway of dicamba by using a methanogenic dicamba-degrading consortium enriched from wetland soil. The soil inoculum consisted of a Lawnes sandy loam (fine-loamy, mixed, nonacid, thermic family of Typic Sulfisols) collected from a site located in Surry County, Va. Forty milliliters of soil slurry (containing 1.4 g of solids) was added to a 160-ml serum bottle with 60 ml of deoxygenated mineral salts medium and 9.3 µmol of dicamba. The mineral salts medium, containing 1 ml of a 0.1% resazurin solution, was prepared as described by Boyd et al. (2). Analytical-grade dicamba (purity, 98.7%), purchased from Chem Services Inc., West Chester, Pa., was used without further purification.

The medium was autoclaved for 15 min and maintained under N2 that was previously passed through hot (300°C) copper fillings to remove traces of O2. During transfer of the soil slurry inoculum and medium, serum bottles were maintained under a positive pressure of O2-free N2. Thick butyl rubber stoppers and aluminum crimp seals were used to close the serum bottles. The dicamba-degrading consortium was transferred every 6 to 8 weeks (over a 2-year period) on the basis of substrate depletion. Five milliliters of dicamba-depleted culture was transferred to 160-ml serum bottles along with 95 ml of fresh mineral salts medium (without resazurin) containing 0.1 mmol of dicamba and 0.04% yeast extract (filter sterilized). Transfer cultures (incubated at 25°C up to the third transfer and at 35°C thereafter) were maintained under a gas phase consisting of 80% N2 and 20% CO2.

The enrichment culture technique was used to isolate the sulfate reducer from the dicamba-degrading consortium. The enrichment medium was a mineral salts medium containing bromoethanesulfonic acid (20 mM), sulfate (20 mM), and 0.04% yeast extract. Slant agar tubes (Bellco anaerobic culture tubes [25 by 50 mm], Bellco Glass Inc., Vineland, N.J.) were inoculated with the enrichment culture following the third transfer. The gas phase in the culture tubes consisted of 80% N2 and 20% CO2. After 3 weeks of incubation at 35°C, selected colonies were transferred to serum bottle cultures.

Degradation experiments involving the dicamba-degrading consortium were initiated by transferring 5 ml of substrate-depleted enrichment culture and 5 ml of yeast extract-maintained culture to three 160-ml serum bottles containing 90 ml of freshly prepared minerals salts medium (without sulfate) plus yeast extract (0.04%) and 0.1 mmol of dicamba. Two of the three serum bottle cultures were used for metabolite isolation purposes. A sterile control was prepared.

---

* Corresponding author.
by adding 10 ml of sterilized inoculum (autoclaved three times over 4 days) to 90 ml of mineral salts medium prepared as mentioned above. The cultures were incubated in the dark at 35°C. The headspace gas in the microcosm experiments consisted of either 80% N₂ and 20% CO₂ or 80% H₂ and 20% CO₂.

Samples of culture medium (0.5 ml) were collected periodically and stored frozen in glass vials. Dicamba and metabolite concentrations in the culture medium were determined by high-performance liquid chromatography (HPLC).

Serum bottle cultures were filtered (no. 1 filter; Whatman, Inc., Clifton, N.J.), acidified (pH 2.0), and extracted three times with 10 ml of ether. The combined extracts were evaporated to 10 ml, dried by passage through Na₂SO₄, and evaporated to 0.2 ml under N₂. Thin-layer chromatography with 0.25-mm-thick precoated silica gel plates (Fisher Scientific, Fairlawn, N.J.) was used to separate components. The mobile phase used to separate 3,6-DCSA (Rf = 0.44) (Berry & Associates, Inc., Dexter, Mich. [purity, 97%]) consisted of CH₃Cl-acetic acid (30:1 [vol/vol]). The mobile phase used to separate 6-chlorosalicylic acid (6-CSA; Rf = 0.59) (Berry & Associates, Inc. [purity, 97%]) was CHCl₃-acetic acid (30:1 [vol/vol]). A UV lamp was used to visualize 3,6-DCSA and 6-CSA. Metabolites were extracted from silica gel with methanol. A portion of the methanol extract was evaporated under N₂ to dryness in preparation for mass spectrometry (6-CSA only), whereas the remainder was used to determine the UV spectrum and HPLC retention time. Prior to mass spectral analysis, 6-CSA was derivatized with diazomethane, forming methyl-6-chloro-2-methoxy benzoate. A Beckman DU-7 scanning spectrophotometer (Beckman Instruments, Inc., Irvine, Calif.) was used to determine UV spectra. Mass spectral data used to confirm the identity of the methylated derivative of 6-CSA were obtained by using a 7070E-HF high-resolution mass spectrometer (VG Analytical, Manchester, United Kingdom) with a direct insertion probe with an electron energy of 70 eV.

In preparation for HPLC analysis, culture samples were thawed and mixed with methanol (1:1), and the mixtures were centrifuged (14,000 × g) and filtered through Gelman (Ann Arbor, Mich.) 0.2-μm-pore-size Acrodisc membrane filters. Samples were chromatographed on an LDC analytical HPLC system consisting of a CM 400 multiple-solvent delivery system with a model 3100 variable wavelength detector set at 271 nm. A 25-cm Whatman C-18 Partisphere column was used to separate components. The mobile phase was 31.7:7.5:58:4:2:4 acetronitrile:methanol:water:acetic acid.

The appearance of the demethylated metabolite, 3,6-DCSA, initially detected as an unidentified peak in HPLC chromatograms, was noted at 35 days, corresponding to the time at which the maximum rate of dicamba disappearance was observed (Fig. 1). Demethylation of dicamba was rapid, occurring without any apparent lag period. By day 20, 3,6-DCSA production had reached its maximum level, whereas by day 60 the concentration had decreased to undetectable levels. The disappearance of 3,6-DCSA was positively correlated with the appearance of another metabolite, first detected as an unidentified peak in HPLC chromatograms, which was subsequently identified as 6-CSA. Although the dicamba-degrading consortium was able to reductively dehalogenate 3,6-DCSA, it was unable to dechlorinate 6-CSA, with the result that this metabolite accumulated in the culture medium. We did not detect a loss of dicamba or production of the metabolites 3,6-DCSA and 6-CSA in the autoclaved sterile control.

The identity of the metabolite 3,6-DCSA was confirmed by comparison with an authentic standard by using thin-layer chromatography, HPLC retention time (5.7 min.), and UV absorbance (λmax = 221 nm). The second metabolite was believed to be either 3-CSA (Berry & Associates, Inc. [purity, 97%]) or 6-CSA. Authentic 6-CSA and 3-CSA standards were analyzed by HPLC. The HPLC retention time recorded for 6-CSA was 4.8 min, which corresponded with the retention time observed for the metabolite. The HPLC retention time observed for 3-CSA was 10.3 min. The identity of the second metabolite, 6-CSA, was confirmed on the basis of mass spectral analysis (m/z, 200), corresponding to the mass of the methylated derivative methyl-6-chloro-2-methoxy benzoate. The UV absorbance (λmax = 215 nm) and thin-layer chromatography Rf value also corresponded with the authentic 6-CSA standard. Figure 2 illustrates the pathway for dicamba degradation by the enrichment consortium.

Aryl reductive dehalogenation in natural samples often requires long acclimation periods. The acclimation period may also be a response to the growth of dehalogenating populations (11). The 2-year enrichment process that this consortium underwent presumably provided a substrate-specific environment in which the acclimation period was reduced. In our enrichment culture system, yeast extract was added to ensure that required growth factors were provided and that a high population of microorganisms would be maintained. It also served as a source of nutrients for the consortium.

Pure cultures capable of aryl reductive dehalogenation are rare (11). Shelton and Tiedje (14) isolated a novel sulfidogen, DCB-1, from a methanogenic 3-chlorobenzoate-degrading consortium, enriched from sewage sludge and capable of carrying out reductive dehalogenation. The reductive deha-

![FIG. 1. Degradation of dicamba with subsequent production of 3,6-DCSA and 6-CSA.](image)

![FIG. 2. Proposed pathway for transformation of dicamba (1) to 3,6-DCSA (2) and subsequently to 6-CSA (3) by the methanogenic dicamba-degrading consortium.](image)
logenation capability of DCB-1 (Desulfoxomonile tiedjei) appears restricted to meta-substituted benzoates (9). The finding that a sulfate reducer was responsible for dehalogenation of a chlorinated benzoate in a methanogenic consortium prompted us to investigate the roles of anaerobes in our methanogenic dicamba-degrading consortium.

We were able to isolate a sulfate-reducing, gram-negative, motile rod from the enrichment consortium. Hydrogen sulfide was produced, as evidenced by the odor of spent culture medium when the cells were grown in mineral salts medium containing sulfate, yeast extract, and a headspace gas consisting of H₂ and CO₂. The isolate was able to oxidize lactate; however, acetate was not utilized. Three methanogens were also isolated from the consortium by using H₂, CO₂, and acetate as substrates. The methanogens were tentatively identified by microscopic examination as belonging to the genera Methanotrix, Methanospirillum, and Methanosarcina. The Methanospirillum species and Methanosarcina species were capable of H₂ utilization, with the Methanotrix species and Methanosarcina species being capable of acetate utilization. Initial attempts to isolate the fermenter were not successful. The fermenter, tentatively identified as a Clostridium species by microscopic examination, appears to be syntrophically associated with the methanogens and may depend on these organisms for H₂ removal.

The sulfate reducer was tested for its ability to degrade dicamba. The isolate was cultured in minerals salts medium with yeast extract and a headspace gas of either N₂ and CO₂ or H₂ and CO₂, both with and without sulfate. By omitting sulfate from the medium, we attempted to force the electron flow toward reductive dehalogenation of dicamba. Under the conditions specified above, the isolate was unable to demethylate or dechlorinate dicamba over a 60-day incubation period. We also found that the methanogens were unable to transform dicamba.

The initial demethylation reaction presumably yielding acetate and 2H, which was carried out by the dicamba-degrading consortium, appears to be mediated by the fermenter, which is capable of CO dehydrogenase activity.Dicamba was not demethylated when the consortium was cultured (60-day incubation period) in medium containing yeast extract with a headspace gas of H₂ and CO₂, supporting our contention that the fermenter was responsible for the demethylation reaction. Not surprisingly, hydrogen must be maintained at low concentration levels to allow for demethylation activity. Apparently, demethylation of dicamba is necessary before dechlorination can occur, possibly because the methyl group sterically hinders the dehalogenation reaction. On the basis of the fact that 3-chlorobenzoate is not dechlorinated by the dicamba-degrading consortium, it is reasonable to conclude that reductive dehalogenation is facilitated by the presence of a hydroxyl group positioned ortho to the reaction site.

Reducive dehalogenation is a biologically mediated process that undoubtedly plays an important role in determining the fate of herbicides such as dicamba in anaerobic soils and sediments. Although it is important that we continue to study dehalogenation with pure cultures to gain insight into how microbes catalyze reductive dehalogenation and to elucidate the reaction mechanism, it is also clear that much is to be learned from studying defined enrichment cultures that rely on microbial interactions to bring about reductive dehalogenation. It is likely that aryl-reductive dehalogenation in many natural anaerobic environments results from a mutualistic effort involving a diverse microbial population.

REFERENCES