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Initial transformation step of dicamba by a sulfate-reducing consortium enriched from sediment of the Pearl River of China

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ABSTRACT

Dicamba is a readily detectable chemical in run-off and groundwater of agricultural areas. Biotransformation of dicamba was investigated in laboratory anaerobic microcosms incubated at two temperatures (15 and 25 °C) under sulfate-reducing condition using sediment from the Pearl River of Southern China as a source of microorganisms. Initial results of sulfate-reducing microcosms showed that dicamba was transformed by 86% in 120 days of incubation at 25 °C compared with the sterile controls. In comparison, only <60% of the initial dicamba concentrations disappeared at 15 °C. Subsequent enrichment cultures were established from the initial microcosms and accelerated transformation of dicamba was observed in the enrichment transfer cultures by 30 days of incubation for 95% and 78% removal at 25 and 15 °C, respectively. Through enrichment transfer technique, the lag phase and the time required for dicamba biotransformation were all shortened from the initial microcosms, indicating the enrichment of microorganisms capable of transforming dicamba. Addition of 0.1% yeast extract enhanced the dicamba biotransformation by the dicamba-transforming consortium. Transformation of dicamba was further investigated by incubating ^{14}C-ring labeled dicamba with the dicamba-transforming consortium. Results confirmed that dicamba was transformed by the consortium but no substantial amount of $^{14}CO_2$ was recovered, suggesting that the initial degradation was an demethoxylation reaction removing the *o*-methyl group forming 2-hydroxy-3,5-dichlorobenzoic acid. Our results suggest that dicamba is quickly transformed through demethoxylation, but further degradation of the transformation intermediate is much slower in the environment.

1. Introduction

Herbicides, including atrazine, cyanazine (Kolpin et al., 2000, 2001) and dicamba (Caux et al., 1993; Kolpin et al., 2000), are used globally in agriculture. They are common environmental contaminants in the developing countries including China, particularly Southern China where intensive cultivation of several rotations of crops per year requires heavy input of chemicals. Since herbicides are mostly transported after the initial application through run-off water horizontally and vertically (Leonard, 1990), residual chemicals are mainly transported to anoxic and strictly anaerobic conditions in the sediment (Crowford et al., 1998; Larsen and Aamand, 2001; Pavel et al., 1999; Wolfe et al., 1990). Available information about the degradation and transformation of these chemicals are mostly based on biological and biochemical reactions of predominantly aerobic environments (Bollag and Liu, 1990; Chung et al., 1996; Kuhn and Suflita, 1989; Larsen et al., 2001). As a result, only a limited number of investigations have examined the biodegradability of agrochemicals under anaerobic environments (Gu et al., 2003a, b; Kuhn and Suflita, 1989). It is apparent that a large discrepancy exists between the wide utilization and dispersal of them in the environments and degradability of them under anaerobic conditions (Gu et al., 1992, 2001, 2003a, b). Information about the fate of these chemicals and possible degradation intermediate products in the environments is crucial in assessing environmental impact and risk, and ecosystem safety from the application of these chemicals.

Dicamba is a post-emergence broadleaf herbicide that interferes with normal auxin function of the plants. Production of dicamba in the United States was more than 4 million kg in 1990 (Gianessi and Puffer, 1991). Dicamba is easily degraded under nitrate-reducing and methanogenic conditions in laboratory microcosms using several wetland sediments from Virginia, USA as inocula (Gu et al., 1992). It was also degraded in sulfate-reducing and methanogenic conditions using a drainage sediment from a golf course in New Jersey, USA as inoculum, but no degradation was observed under neither denitrifying nor iron reducing conditions (Milligan and Häggblom, 1999). Quantitative data of herbicide degradation in

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anoxic soils may be variable due to the nature of the testing system used and more accurate assessment of chemical degradability needs to be achieved through modification of the available methods. The technique of enrichment transfer of cultures of microorganisms capable of degrading specific chemicals enables further investigation of the mechanisms involved, construction of the biochemical pathways and the bacteriology (Gu, 2008; Gu and Berry, 1991, 1992; Gu et al., 1992; Lin et al., 2006).

The objectives of this study were to evaluate the degradability of dicamba in microcosms containing marine sediment incubated under sulfate-reducing conditions at two temperatures, further enrichment for bacteria capable of degrading dicamba, and characterize the initial degradation reaction using ^{14}C-ring labeled dicamba.

2. Materials and methods

2.1. Site and sediment sampling

Sediment was collected at a sampling site (113°45′, 22°27′) of Pearl River in P.R. China. Surface sediment under constant submerged conditions was collected using a Wildo® Ponar Grab (Wildlife Supply Co., Buffalo, NY) as described earlier (Gu et al., 2002) at the time of sampling. Water depth was 6.3 m on June 3, 2003 and the sediment slurry was collected into narrow-necked glass bottles full and sealed for storage and transport back to the laboratory for immediate processing.

Dicamba, 3,6-dichloro-2-methoxybenzoic acid, was obtained from Sigma–Aldrich (St. Louis, MO) at 99.7% purity. The chemical was initially dissolved in HPLC grade methanol to make a stock solution and 100 μl of the solution wastransferred to each serum bottle before setting up the experiments to achieve a target concentration of 100 μmol per serum bottle. The serum bottles (160 ml) receiving the herbicide stock solution were put under a positive pressure of pure N_2 until complete evaporation of the methanol prior to dispensing medium into the bottle.

2.2. Sulfate-reducing conditions and simulation

The medium used for culturing sulfate-reducing bacteria was described by Widdel and Bak (1992) with modifications in the current experiments. Sulfate-reducing conditions were simulated by using an artificial salt medium amended with a reducing agent (Na_2S). Serum bottles (160 ml internal capacity) were used in the setting up of experiments. The medium consisted of the following (g/l): KH_2PO_4 0.27, NH_4Cl 0.25, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 0.1, KCl 0.5, Na_2SO_4 4.0, $FeCl_2 \cdot H_2O$ 0.02, and NaCl 7.0. Rezazurin was used as a redox indicator for visual observation of potential contamination of oxygen in the serum bottle. The medium was autoclaved first for 15 min to remove dissolved O_2, then cooled under a positive pressure of a flowing stream of high purity N_2, which was passed through heated copper filings (300 °C) first to remove traces of O_2. After cooling of the medium, 1.0 ml of a trace metal solution was added, the specific composition of which was described earlier (Widdel and Bak, 1992), followed by addition of 1.2 g/l $NaHCO_3$ and 0.12 g/l $Na_2S \cdot 9H_2O$ to the medium; the pH of the medium was adjusted to 7.4 ± 0.2. The sediment slurry in the ratio of 10:90 (dry weight, w/v) was added into the medium flask and the anaerobic condition of the medium was maintained. Then the anaerobic mixture containing both the synthetic sulfate-reducing medium (as described above) and sediment slurry on a stirrer to achieve homogeneity of the contents was dispensed into each serum bottle. After purging the bottles with pure N_2 for 10 min, serum bottles were sealed with butyl rubber stoppers and then aluminum crimps under ambient conditions. Contents in the serum bottles were further mixed on a shaker by gentle shaking to facilitate the dissolution of the dicamba completely in the bottle at room temperature in the dark. Internal pressure in the serum bottles was equal to ambient condition, and the N_2 purging prevented the oxygen contamination of the serum bottles. Serum-bottle microcosms were incubated stationary at either 15 or 25 °C in the dark. The experiments were set up in triplicate for each treatment. The positive controls consisted of a set of serum bottles containing herbicide amendment after several successive autoclavings, and another set of negative controls contained no herbicide addition.

Activity of sulfate reduction was measured by detection of H_2S in serum bottles over time of incubation. Serum bottles were purged with high purity N_2 with a hypodermic needle through the butyl rubber stopper, and a second needle allowed exiting air to pass through a NaOH trap. Aliquot in serum bottle was taken and distilled after acidification and the evolved H_2S reacted with *N,N*-dimethyl-*p*-phenylenediamine to form methylene blue, which was measured at 600 nm spectrophotometrically (APHA, 1999).

2.3. Sampling and analysis

Sample slurry of the initial enrichment culture or subsequent enrichment transfer cultures (1 ml) was withdrawn periodically (usually about 20 days for initial enrichment culture, and 5 days for the transfer cultures) from each serum bottle microcosm using a syringe fitted with a hypodermic needle. Samples were immediately placed in clean glass vial with cap and stored at −20 °C until analysis. Prior to high-performance liquid chromatography (HPLC) analysis, samples were thawed, mixed with methanol (1:1), centrifuged (13,000 × *g*), and filtered through 0.2-μm-pore-size Acrodisc membrane filters (Pall Gelman Science, Ann Arbor, MI). Methanol was used in sample preparation to ensure that herbicides would not adsorb to membranes at significant quantities. Sample filtrates were analyzed on an Agilent 1100 Series HPLC system (Agilent Technologies, CA) consisting of a diode array detector and quaternary pump. Separation of dicamba was achieved using an 18 cm Supelco® sil 5-μm particle LC-18-DB column (Supelco Park, Bellefonte, PA). The mobile phase consisted of 10:25:65 (v/v) of methanol/water/(acetonitrile/water/glacial acetic acid in 60:39.5:0.5, v/v) at a flow rate of 1 ml/min and quantification was carried out at 271 nm. External standards were used and the concentrations were linear between 5 and 200 μM (r^2, 0.986; $n = 4$).

2.4. Enrichment cultures

When disappearance of dicamba in the initial enrichment microcosms was observed to be >80% after 120 days of incubation, enrichment transfer cultures (1st enrichment transfer) were established by inoculating 10 ml of content from the initial microcosm to 90 ml of freshly prepared medium in a new 160 ml serum bottle containing the same concentration of dicamba as the initial microcosms. The enrichment transfer cultures were performed four times to obtain a stable dicamba-degrading consortium containing only four morphologically different types of microorganisms involved in the degradation based on significant disappearance of dicamba in serum bottles. Enrichment transfer culturing techniques have been used to confirm the ability of microorganisms utilizing an organic compound as a carbon and energy source (Gu, 2008; Lin et al., 2006).

2.5. Yeast extract supplementation

One set of serum bottles was set up to investigate the effect of yeast extract (Difco Labs, Detroit, MI) on transformation of dicamba. The culture medium composition was identical to the medium used in enrichment cultures described above. Yeast extract was added at 0.01% and 0.1% in the medium inoculated with the 4th enrichment transfer culture as inoculum. The serum bottles were incubated at 25 °C in the dark. Dicamba concentration in the serum bottle was analyzed. Triplicate serum bottles were prepared for each treatment and also the controls.

2.6. ^{14}C-labeled dicamba and incubation

Smaller serum bottles (60 ml internal volume) containing 30 ml of freshly prepared sulfate-reducing medium and 5 ml of the 4th enrichment culture were used in this part of the investigation. Ring labeled 0.20 μCi/mg dicamba (Sigma, St. Louis, MO) and non-labeled dicamba were used to achieve a final concentration of 100 μM. In this test, a carbon dioxide trap (1.5 ml polypropylene Eppendorf centrifuge tube without cap containing 0.2 ml 1 M NaOH) was attached to the butyl rubber stoppers from inside the serum bottle as shown in Fig. 1. All serum bottles were incubated at 25 °C. Each time, two serum bottles were taken for analysis at 5-day intervals for radioactivities in aliquot after removal of both CO_2 and biomass, dissolved CO_2, and microbial biomass. The NaOH trap in each serum bottle was removed from the bottle and CO_2 collected liberated first and then absorbed for scintillation counting as described in the following. Aliquot in serum bottles was acidified with HCl to collect the soluble CO_2 for counting. After centrifugation (13,000 × *g*), the supernatant was also counted for the radioactivity from dicamba and possible degradation intermediates in solution phase.

Samples for liquid scintillation were prepared using modified methods of Kuyper and Aghdaohi (1972), and Mullins and Eaton (1977) to distill $^{14}CO_2$ from NaOH trap and culture medium. Five hundred microliters of 80% ethanolamine were placed in the bottom of a standard 20 ml scintillation vial and spread over the inner surface by tipping and rotating the vial. Five hundred microliters of the sample NaOH or culture medium was dispensed into a glass thimble, and then the thimble was placed into the scintillation vial. A Teflon-lined cap containing an 18 gauge needle was inserted into the arm of the thimble and screwed loosely into place. A 1 ml syringe containing

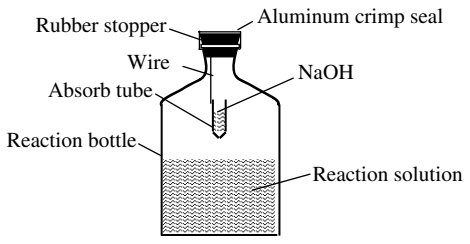

Fig. 1. Schematic diagram of the modified serum bottle for radiolabelled assessment of dicamba transformation under sulfate-reducing conditions.