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Biodegradation of the Herbicides Atrazine, Cyanazine, and Dicamba by Methanogenic Enrichment Cultures from Selective Soils of China

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Herbicides are frequently detected in shallow groundwater of the United States and elsewhere, and the dominant chemicals include atrazine, cyanazine and dicamba (Kolpin et al., 2000a). These agro-chemicals are also widely used in the developing countries including China. Information on degradability of these herbicides and possible presence of their degradation intermediates in the environments is crucial in assessing environmental impact of the chemical application and the long-term ecological impact from their residual concentrations (Schrüüman and Markert, 1998). Since herbicides are mostly transported through run-off water after the initial application (Leonard, 1990), residual chemicals are often exposed to anoxic and strictly anaerobic conditions of the sediments or wetland. Under these conditions limited by the available molecular oxygen, both anoxic and strictly anaerobic microorganisms are mainly responsible for further transformation and metabolism of organic pollutants by natural microorganisms (Zehnder and Stumm, 1988). Surprisingly, little information is available on the biodegradability of the herbicides under anaerobic conditions considering the widely utilization and dispersal of them in the environments. Most current available information is based on the aerobic environment (Bollag and Liu, 1990). As a result, only a limited number of investigations had examined the biodegradability of chemicals under anaerobic environments (Gu et al., 1992; 2002; Kuhn and Suflita, 1989).

It is apparent that very little is known about the biodegradability of atrazine, cyanazine and dicamba under nitrate-reducing and methanogenic conditions in anoxic soils of China. One previous investigation showed that atrazine was most resistant to degradation under nitrate-reducing conditions followed by cyanazine using several Virginia's wetland soils in the east coast of the USA as inocula (Gu et al., 1992). It is important to note that both atrazine and cyanazine are heterocyclic aromatic compounds with nitrogen (*N*) substituting carbon of the aromatic ring. As a result of this, degradation of this class of chemicals has been proven to be different from the homocyclic aromatic compounds (Berry et al, 1987; 1991; Gu and Berry, 1991; 1992; Gu et al., 2003).

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Dicamba is a chlorinated aromatic compound and its degradation is hindered by the presence of chlorine molecules. To properly assess the potential for biodegradation of these agrochemicals in anoxic soils, information regarding the biodegradation capabilities by the anaerobic microorganisms inhabiting the subsurface environments is needed. Quantitative data of herbicide degradation in anoxic soils will provide important information for accurate assessment of contamination and toxicity. In addition, enrichment cultures of microorganisms capable of degrading any specific chemical will enable further investigation of the mechanisms involved and the organisms' biochemical pathway. The objective of this study was to evaluate the degradability of atrazine, cyanazine and dicamba in enrichment cultures obtained from three soils of China under methanogenic conditions.

MATERIALS AND METHODS

Three soils representing the major soil types under different climatic conditions were chosen and sampled in the People's Republic of China. All three soils were under submerged condition at the time of sampling and the sediment slurry was collected to narrow neck bottles for storage and transport to the laboratory. The physical and chemical properties of the soils were reported elsewhere (Gu et al., 2003).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), cyanazine (2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methyl-propionitrile and dicamba (3,6-dichloro-2-methoxybenzoic acid) were commercial compounds obtained at highest purity available. They were initially dissolved in methanol to make a stock solution and 100µl were dispensed to each serum bottle. The serum bottles with herbicide solution were put under a continuous flow of pure N_2 till the completely evaporation of methanol. Subsequently, the mixture of anaerobic salt medium and soil slurry was dispensed into the serum bottles.

Simulation of methanogenic conditions was generated by using an artificial salt medium amended with a reducing agent (Na_2S) as described earlier (Gu and Berry, 1991; 1992; Gu et al., 2002). The medium consisted of the following (g/l): KH_2PO_4 0.27, K_2HPO_4 0.35, NH_4Cl 0.1, $MgCl_2 \bullet 6H_2O$ 0.1, $CaCl_2 \bullet H_2O$ 0.073, $FeCl_2 \bullet H_2O$ 0.02, and 1.0 ml of a trace metal solution as described elsewhere (Gu et al., 2002) along with 1.0 ml of 0.1% (final concentration 0.1 µg/ml) resazurin redox indicator. The soil slurry was added to the mineral salt medium to form a soil slurry suspension (40:60, v/v) and the pH was adjusted to 7.0. The mixture was stirred and sparged with O_2-free N_2 while100 ml mixture were transferred to each 160ml serum bottle previously received 100µmole of herbicide (atrazine, cyanazine or dicamba). Following transfer of the mixture, the serum bottles were sealed with thick butyl-rubber stoppers and capped with aluminum crimp seals. Serum-bottle microcosms were incubated stationary in the dark at either 15 or 25°C. The experiments were set up in triplicate for each treatment. Controls consisted of a set of herbicide-amended autoclaved sterile control (the herbicide was added to the soil slurry following sterilization).

Mixture (1 ml) was withdrawn periodically from serum bottle microcosms using syringes. Samples were placed in clean glass vial and stored at −10°C until analysis. In preparation for high-performance liquid chromatography (HPLC) analysis, culture samples were thawed, mixed with methanol (1:1), centrifuged (13,000×), and filtered through Gelman (Ann Arbor, Michigan) 0.2-μm-pore-size Acrodisc membrane filers. Methanol was used in sample preparation to ensure that herbicides were not adsorbed to membranes. Samples were analyzed on an HPLC system (Agilent Technologies, California) consisting of a diode array detector and quaternary pump. Separation of atrazine and cyanazine were achieved by using an 18 cm Supelco sil 5-μm particle LC-18-DB column. Methanol and water (70:30, v/v) delivered at a flow rate of 1 ml/min, was used as the mobile phase in the HPLC analysis of atrazine and cyanazine while methanol, water and acetonitrile-water-glacial acetic acid (60:39.5:0.5, v/v) (10:25:65, v/v) for dicamba. Quantification of atrazine, cyanzine and dicamba was accomplished by the external standards method at wavelengths of 240, 259 and 271nm, respectively.

When degradation of herbicide was observed, further enrichment transfer was carried out to substantiate the biodegradation (Gu and Berry, 1991; 1992). Enrichment cultures were established by adding 20ml of the active wetland soil microcosms showing disappearance of parent compound, along with 80 ml of freshly prepared mineral salt medium, to a 160 ml serum bottle containing the appropriate herbicide. The enrichment transfers can be performed as often as necessary to obtain herbicide-degrading consortium composed of only a few effective microorganisms using the herbicide as the sole source of carbon and energy.

Quantitation of methane produced was measured by injection 50μL of the headspace gas into a gas chromatography (5890II Hewlett Packard Co., California) equipped with a thermal conductivity detector and fitted with a Porapak N column (1.8 m, 80/100 mesh) as described before (Gu et al., 1992). Column temperature was maintained at 50°C and detector temperature at 150°C. Flow rate of the carrier gas Helium was 20 ml/min.

RESULTS AND DISCUSSION

Disappearance of atrazine was observed in microcosms regardless of inoculation and temperature, and the disappearance was slowed down after the first 60 days when an initial 40% loss was observed (data not shown). This is in general agreement with a previous observation on wetland soils from USA (Gu et al., 1992). Degradation of atrazine has been reported under different environmental conditions (Caux et al., 1993; Chung et al., 1996; Crowford et al., 1998; Larsen and Aamand, 2002; Larsen et al., 2001; Ragge et al., 1999). However, two soils from central and northern China showed temperature-dependant disappearance of cyanazine over time and one of them is presented in Fig. 1. The initial rate of disappearance was high and, after 60 days of incubation, the temperature effect became more pronounced between 15 and 25°C.

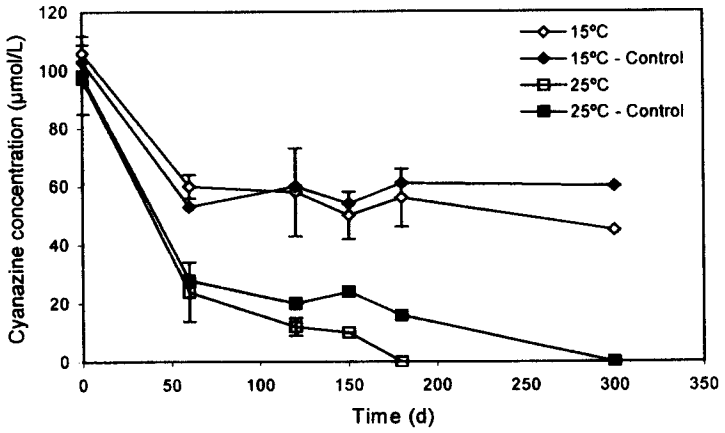


Figure 1. Fate of cyanazine in soil microcosms containing soil from central China incubated at 15 and 25°C under methanogenic conditions

Consistent observation of parent herbicide disappearance in the biologically active and the sterile controls is due to the adsorption of chemical on the solid surfaces and possible involvement of abiotically chemical reaction accounting for the transformation of herbicides. Organic compounds are reactive with clay minerals and other soil constituents (Alexander, 1995; Luthy et al., 1997; Zielke et al., 1989). During the entire 300 days of incubation, there was a general distinguishable difference between the biologically active microcosms and the sterile ones after the first 60 days and this difference can be attributed mostly to the biological activity (Fig 1).

Portion of the applied herbicides may become unavailable shortly after application. In the current investigation between 40-50% of atrazine or cyanazine were not recoverable from the aliquot solution in the first 150 days. A previous investigation on atrazine, cyanazine and dicamba also showed loss of herbicides during incubation under both nitrate-reducing and methanogenic conditions using three wetland soils from Virginia, USA (Gu et al., 1992; Pavel et al., 1999). In that study, complete disappearance of the herbicides was observed in a small number of microcosms including the sterile controls. Subsequently, microcosms of the controls and the biologically active ones were extracted with dichloromethane and the extractants were further analyzed for the presence of herbicide parent compounds. However, no herbicide could be detected in the extracts suggesting that atrazine and cyanazine may be transformed in microcosms even under sterile condition and such alteration of the herbicides may not be a result of microbial activities. Such information should be taken into account in assessment of herbicide toxicity and the degradation by biological and abiological processes.

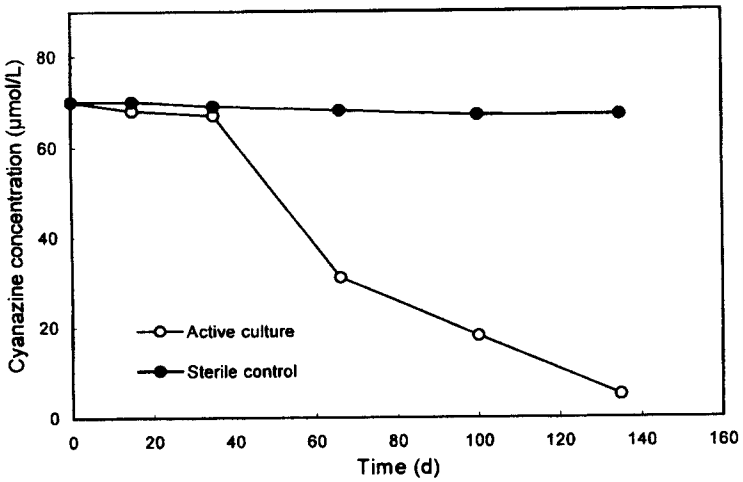


Figure 2. Fate of cyanazine in the 8th enrichment microcosms containing initially a soil from central China incubated at 25°C under methanogenic conditions

Clay surface catalyzed reaction and hydrolysis may be responsible for such non-biological processes (Alexander, 1995; Zielke et al., 1989). At the same time, herbicides have a tendency to be transformed quickly after they are in the environment. This initial decrease of their availability is very important in evaluating their potential impact on the environment and also on the bioavailability of the compounds over time (Alexander, 1995; Caux et al., 1993; Luthy et al., 1997). In addition, degradates of herbicides may be more persistent than the parent compounds and in such situation the environmental toxicological assessment should be carried out by including the toxicity of degradation intermediates.

After observing the disappearance of cyanazine in the initial microcosms amended with the soil from central China incubated at 25°C (Fig. 1), enrichment culture was established. A relatively stable concentration of cyanazine in the sterile controls of the enrichment culture further suggests that microbial degradation was the contributor to the significant decrease of herbicide concentrations (Fig. 2). Since degradation intermediates of cyanazine were detected in groundwater, their transformation is likely to be mediated by microorganisms (Kolpin et al., 2000a; 2000b). In a similar way, dicamba-degrading enrichment was obtained and the degradation ability was sustained using microcosms initiated on a soil from northern China and the results on the 9th transfer are presented (Fig. 3). Clearly enrichment culture is a very useful technique in investigation the mechanisms of degradation, microorganisms involved, and the microbial ecology of degradation. Biodegradation of resistant herbicide can be substantiated by enrichment technique, and microorganisms responsible for degradation may be obtained.

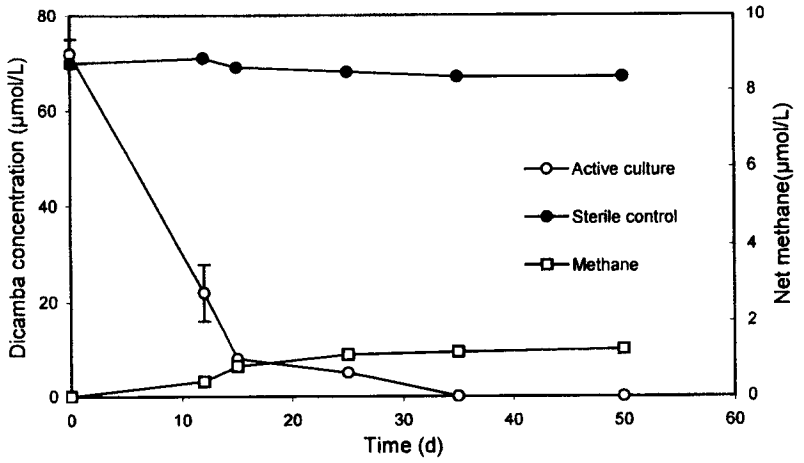
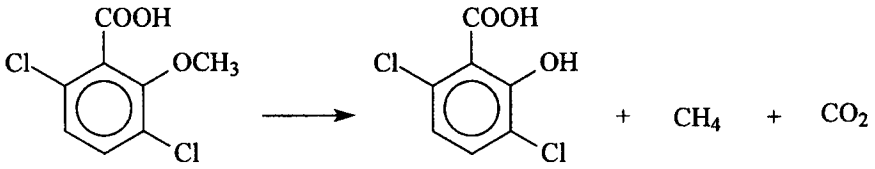


Figure 3. Degradation of dicamba and subsequent production of methane in an enrichment culture (9th transfer) obtained from the initial inoculum of a soil from northern China



Dicamba

2-hydroxy-3,5-dichlorobenzoic acid

Figure 4. Proposed pathway of dicamba degradation elucidated using ^{14}C ring-labeled-dicamba in enrichment culture (14th transfer) obtained from a soil of northern China

Enrichment process can significantly reduce the half-life of the chemical in the microcosms. It should be pointed out that enrichment transfer may also result in the depletion of living factors or nutrients necessary for the growth of the microorganisms because minimum salt medium does not provide other organic molecules other than the dicamba. When such situation is resulted from the capability of microorganisms, the needed cofactors or living factors should be incorporated into the medium for the successful growth of microorganisms.

Using the ^{14}C labeled dicamba (ring label), radioactivity in the aqueous phase was not significantly changed over the time of incubation indicating the structure of aromatic ring was not cleavaged. At the same time a slight build up of nonradioactive methane was observed on GC. From the information, it is reasonable to speculate that the degradation taking place in the enrichment culture follows the pathway illustrated in Fig. 4. Using the same enrichment, reconfirmation results were also obtained (Taraban et al., 1993).

In summary, atrazine is more recalcitrant to biodegradation than cyanazine under methanogenic condition in our study. Soil types and incubation temperatures have a strong influence on the degradation of herbicides, but initial disappearance of chemical may be due to the non-biological processes. Enrichment culture technique offers a potential for obtaining the microbial community capable of degrading the chemical and also the possibility to substantiate the degradation processes.

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