Biodegradability of Atrazine, Cyanazine and Dicamba under methanogenic condition in three soils of China

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Abstract

Persistence and degradation of the herbicides Atrazine, Cyanazine and Dicamba were measured in laboratory microcosms incubated under methanogenic condition using three soils of China. Results showed that Atrazine was more resistant to degradation than Cyanazine and Dicamba for the 300 days of incubation. Between 30% and 40% of the initially introduced chemicals were found to be not recoverable through solvent extraction of the incubated soils. Our results also indicated that the half-life of these herbicides in the three soils generally followed: Atrazine > Cyanazine > Dicamba. Biodegradation of Cyanazine and Dicamba was further substantiated by establishing enrichment cultures in which the degradation of the respective herbicides could be accelerated by the microorganisms. Our results suggest that biodegradation of xenobiotics can be established through enrichment culture transfer technique and non-extractability of chemicals should be taken into account in evaluation of chemicals fate and risk.

Keywords: Atrazine; Cyanazine; Degradation; Dicamba; Enrichment culture; Herbicide; Methanogenic condition; Metabolic pathway

1. Introduction

Herbicides frequently detected in shallow ground water of the United States include Atrazine, Cyanazine (Kolpin et al., 2000a, 2001) and Dicamba (Caux et al., 1993; Kolpin et al., 2000a, 2001). These herbicides are also widely used in other countries particularly the developing ones including China. Information on degradability of these herbicides and possible presence of their degradation intermediate products in the environments is crucial in assessing environmental impact and risk from the chemical applications. Since herbicides are mostly transported after the initial application through run-off water (Leonard, 1990), residual chemicals are exposed to anoxic and strictly anaerobic conditions. Surprisingly, little information is available on the biodegradability of the herbicides under anaerobic conditions considering the wide utilization and dispersal of them in the environments (Gu et al., 1992, 2001). Most current available information about the fate of these chemicals is based on the aerobic environments (Kuhn and Suflita, 1989; Bollag and Liu, 1990; Mandelbaum et al., 1995; Chung et al., 1996; Larsen et al., 2001; Mehmannavaz et al., 2001). As a result, only a limited number of investigation examining the biodegradability of chemicals under anaerobic environments (Kuhn and Suflita, 1989). Based on the knowledge of herbicide mobility, it is clear that herbicides move away from target sites into non-target environments and especially

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anoxic conditions (Wolfe et al., 1990; Crawford et al., 1998; Pavel et al., 1999; Larsen and Aamand, 2001). In many cases, the environments receiving the chemicals are anaerobic and the existing results may mislead interpretation in toxicological risk and environmental fate assessment.

It is clear that very little is known about the biodegradability of Atrazine and Cyanazine under anoxic or methanogenic conditions in soils. One previous investigation showed that Atrazine was most resistant to degradation under nitrate-reducing condition followed by Cyanazine using several wetland soils in Virginia of the United States 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. Because of this fact, degradation of this class of chemicals is significantly different from the homocyclic aromatic compounds (Berry et al., 1987; Gu and Berry, 1991, 1992; Gu et al., 2002; Wang et al., 2002). To properly assess the potential of biodegradation of N-substituted agrochemicals in anoxic soils, information regarding the biodegradative capabilities by the anaerobic microorganisms inhabiting the subsurface environments is needed. Quantitative data of herbicide degradation in anoxic soils will provide the important information needed for accurately assessment of contamination and toxicity. In addition, enrichment cultures of microorganisms capable of degrading any specific chemical enable further investigation of the mechanisms involved and the biochemical pathways.

The objective of this study was to evaluate the degradability of Atrazine, Cyanazine and Dicamba in three soils from China incubated under methanogenic condition at two incubation temperatures. Enrichment cultures of microorganisms capable of degrading selective chemicals were also achieved.

2. Materials and methods

2.1. Soils and sampling

Three soils representing the major soil types under different climatic conditions were chosen and sampled from three locations in southern, central and northern China and correspondingly a Red soil was collected from Dongguan, Guangdong; a Brown soils from Kaifeng, Henan; and a Black soil from Shenyang, Liaoning. All soils were under submerged conditions at the time of sampling and the sediment slurry was collected to narrow necked bottle for storage and transport to the laboratory. The physical and chemical properties of the soils were analyzed according to the standard methods (Klute, 1982; Page, 1982), and physical and chemical properties are listed in Tables 1 and 2.

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 obtained from Sigma (St. Louis, MO) at 97% or higher purity available. They were initially dissolved in methanol to make a stock solution and 100 ml were transferred to each serum bottle during setting up of the experiments. The serum bottles receiving the herbicide solution were put under a positive pressure of pure N2 till complete evaporation of methanol. Subsequently, anaerobic salt medium (as described below) and soil slurry were dispensed into the serum bottles and the bottles were sealed with tert-butyl rubber stoppers and aluminum crimp seals. Contents in the bottles were further mixed on a shaker by gentle motion.

2.2. Methanogenic conditions and simulation

Methanogenic condition was simulated by using an artificial salt medium amended with a reducing agent (Na2S). Serum bottles (160 ml internal capacity) were used in the setting up of experiments. The medium consisted of the followings (g/l): KH2PO4 0.27, K2HPO4 0.35, NH4Cl 0.1, MgCl2·6H2O 0.1, CaCl2·H2O 0.073, FeCl2·H2O 0.02, and 1.0 ml of a trace metal solution along with 1.0 ml of 0.1% (final concentration 0.1 µg/ml) resazurin as a redox indicator. The specific components of the salt medium and trace metals were previously described in detail (Gu and Berry, 1991, 1992). The medium was autoclaved first for 15 min. to remove
dissolved O₂, then cooled under a positive pressure of flowing stream of high purity N₂, which was passed through heated copper filings (300 °C) first to remove traces of O₂. After cooling, 1.2 g NaHCO₃ and 0.12 g Na₂S·9H₂O were added to the medium and the pH of the medium was adjusted to 7.0±0.2. The soil slurry and the mineral salt medium were added into each serum bottle in the ratio of 20:80 (v/v). The mixture was stirred and sparged with O₂-free N₂ and the serum bottles were sealed with thick butyl-rubber stoppers and capped with aluminum crimp seals. Serum-bottle microcosms were incubated stationary at either 15 or 25 °C in the dark. The experiments were set up in triplicate. The positive controls consisted of a set of serum bottles containing herbicide amendment after several successive autoclaving and another set of negative controls contained no herbicide addition.

2.3. Sampling and analysis

Mixture (1 ml) was withdrawn periodically (usually about 2 weeks) from each serum-bottle microcosm using a syringe fitted with a hypodermic needle. Samples were immediately placed in clean glass vial and stored at −10 °C until analysis. Prior to high-pressure liquid chromatography (HPLC) analysis, samples were thawed, mixed with methanol (1:1), centrifuged (13000×g), and filtered through Gelman Science 0.2-μm-pore-size Acrodisc membrane filters (Ann Arbor, Michigan). Methanol was used in sample preparation to ensure that herbicides would not adsorb to membranes at significant quantities. Samples were analyzed on an Agilent 1100 Series HPLC system (Agilent Technologies, California) consisted of a diode array detector and quaternary pump. Separation of Atrazine and Cyanazine were achieved by using a 18 cm Supelco® sil 5-μm particle LC-18-DB column (Supelco Park, Bellefonte, Pennsylvania). Mobile phase consisted of methanol and water (70:30, v/v) delivered at a flow rate of 1 ml/min for analysis of Atrazine and Cyanazine. Quantification of Atrazine and Cyanazine was accomplished by the external standards method at wavelengths of 240 and 259 nm._dicamba was quantified on the same HPLC system using a mobile phase consisting of 10:25:65 [methanol:water:(acetonitrile–water–glacial acetic acid in 60:39.5:0.5, v/v/v)] at a flow rate of 1 ml/min and wavelength of 271 nm.

2.4. Enrichment cultures

When degradation of herbicide was observed in appreciable amounts within reasonably time period, further substantiation of biodegradation was carried out. An enrichment culture was established by adding 20 ml of inoculum from an active microcosm to 80 ml of freshly prepared mineral salt medium in a 160 ml serum bottle containing the appropriate herbicide. The enrichment transfers can be performed as often as necessary to obtain a herbicide-degrading consortium composed of only a few effective microorganisms. Enrichment culture technique can be used to establish whether an organic compound can serve as a carbon and energy source. Both the herbicide in liquid culture and the CH₄ gas formed in the headspace were monitored together with the positive and negative controls.

2.5. Headspace gas analysis

Quantification of methane produced was measured by injection 50 μl of the headspace gas into a gas chromatography (5890a 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.

2.6. Scanning electron microscopy

Approximately 1 ml of the enrichment culture was filtered through 0.2-μm-pore-size membrane filter (Gelman Science, Ann Arbor, Michigan) and immersed in 3% glutaraldehyde–0.1 M sodium cacodylate buffer overnight. The solution was previously filtered through a 0.2-μm-pore-size membrane filter (Gelman Science, Ann Arbor, Michigan). The samples were subsequently washed with 0.2 M sodium cacodylate three times, fixed in 1% OsO₄–0.1 M sodium cacodylate for 3 h, and rinsed with Na cacodylate and deionized water, respectively. Dehydration of the prepared samples was accomplished in an ethanol–distilled water series. Samples were held in 100% ethanol and sealed in glass vials until they were critical point dried in liquid CO₂. Immediately after critical drying, samples were coated with gold–palladium and viewed under a Leica Cambridge S440 scanning electron microscope (Cambridge, UK).

3. Results and discussion

3.1. Fate of Atrazine, Cyanazine and Dicamba

Disappearance of both Atrazine and Cyanazine was observed immediately after the setting up of the microcosms in both the inoculated and the autoclaved controls containing each of the three soils as inocula (data not shown). The initial rates of disappearance were higher with the Red and Brown soils than with the Black soil particularly within the first 150 days of incubation. Since the trend of disappearance for both herbicides was observed in both the biologically active microcosms and the sterile controls (data not shown), involvement of
chemical reaction and sequestration may be responsible for the loss of herbicides. Organic compounds are reactive with clay minerals and soil organic matter, and the bonding strength increases over time (Stevenson and Fitch, 1986; Zielke et al., 1989; Wolfe et al., 1990; Luthy et al., 1997; Loiseau and Barriuso, 2002). In addition, Atrazine can be hydrolyzed by Pseudomonas species to hydroxyatrazine and Cyanuric acid before fully mineralized (Mandelbaum et al., 1995).

At least 30% of Atrazine remained in the microcosms even after 300 days of incubation (data not shown). Non-extractable residues of Atrazine are believed to be associated with the <20 µm fraction of organic matter mostly and between 20% and 50% of them were actually intact Atrazine and its degrades (Loiseau and Barriuso, 2002). 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 150 days and this difference can be attributed mostly to the biological activity. Observed decreases of Cyanazine concentrations were greater in microcosms incubated at 25°C than those at 15°C (Fig. 1), further extends the possible microbial involvement to herbicide transformation. Since Cyanazine is structurally similar to Atrazine, it is not surprising to observe similarity trends of chemical disappearance between the two. It was also apparent that Atrazine was quite resistant to microbial transformation because more than 30% of the initially introduced concentration remain to be detectable even after 300 days of incubation. Completely disappearance of Cyanazine was only observed in microcosms amended with the Brown soil incubated at 25°C, not at 15°C (data not shown). After the initiation of the experiment, it took 180 days for the complete disappearance of Cyanazine in the solution phase of the microcosms.

Portion of the applied herbicides may become unavailable shortly after application due to chemical transformation and physical adsorption in the presence of organic matter and clay minerals (Zielke et al., 1989; Alexander, 1995; Luthy et al., 1997). 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). In that study, completely disappearance of the herbicides was observed in a small number of microcosms including the sterile controls.

In the current investigation, Atrazine and Cyanazine concentrations of the sterile controls showed significant loss over time but they were not detectable from the aliquot solution or sediment materials by solvent extraction (data not shown). Extracts of dichloromethane and ethyl acetate were further analyzed for the presence of Atrazine or Cyanazine, but no detectable concentrations of the herbicides could be found. Coupling the results of a previous study and the present one, it suggested that Atrazine and Cyanazine may be transformed in microcosms even under sterile condition and such degradation of herbicides may not related to microbial activities. Entrapment in micropores is another mechanism could be involved. Since significant quantities of the Atrazine and Cyanazine were lost in these microcosms, such information should be taken into account in assessment of herbicide toxicity and the degradation by biological and abiological processes (Alexander, 1995). In addition, clay surface catalyzed chemical reaction and hydrolysis may be responsible for such non-biological loss of chemicals (Luthy et al., 1997). This initial decrease of their parent compound concentrations is very important in evaluating their potential impact on the environment and also on the bioavailability of the compounds over time (Alexander, 1995). In addition, degradation of herbicides may be more persistent than the parent compounds (Kolpin et al., 2000a,b) and in such situation the environmental toxicological assessment should be carried out by including the toxicity of degradation intermediates.

3.2. Degradation by enrichment cultures

Complete disappearance of Cyanazine was initially observed in the microcosms amended with the Brown soil at 25°C (data not shown). The decrease of Cyanazine concentrations was almost linear over the 180 days of incubation. Enrichment culture was subsequently established by transferring 20 ml of the initial slurry in the original microcosm to new microcosms containing 80 ml of freshly made salt medium and 100 µm of Cyanazine. In the enrichment process, degradation of Cyanazine was also observed with a relatively stable concentration of Cyanazine in the sterile controls, further suggesting that microbial degradation was responsible for the significant decrease of herbicide concentrations (Fig. 2). Enrichment culture technique is a widely used microbio-

![Fig. 1. A representative graph showing the fate of Cyanazine in the Brown soil microcosms incubated at 15 and 25°C under methanogenic conditions.](image_url)
logical method in investigating the mechanisms of degradation, microorganisms involved, and the microbial ecology of degradation (Gu and Berry, 1991, 1992; Gu et al., 2002). Our data showed that biodegradation of resistance herbicide can be substantiated by enrichment technique, and degradative microorganisms may be enriched for further investigation of the biodegradation pathways and/or the isolation of microorganisms responsible for the transformation of specific compounds. Unfortunately, no Atrazine-degrading enrichment culture could be established in this study, and reason may be due to the fact that degradation of Atrazine is a very slow process especially under methanogenic condition (Gu et al., 1992; Rügge et al., 1999).

In a similar manner, Dicamba-degrading enrichment was also achieved and degradation of the chemical was substantiated in subsequent enrichment (Fig. 3). After four successive transfers, the degradative capability was still active and more than 90% of the initial added chemical were transformed within the first 14 days. Dicamba was shown to be degraded by microorganisms of various environments including wetland soils (Gu et al., 1992; Pavel et al., 1999) and agricultural soil (Gu et al., 2001). Since the degradation did not result in appreciable amounts of net CH₄ production in the headspace, metabolic pathway is then proposed as shown in Fig. 4. Similar to previous observation, the initial reaction during Dicamba degradation is a de-methoxylation process (Gu et al., 1992) and the methyl group can be used by acetogens to form acetate. The small amounts of acetate are used for maintenance of the microbial community. The culture aliquot was prepared for scanning electron microscopy observation and morphologically diverse populations of microorganisms were observed (Fig. 5) because fermenting bacteria, acetogen and methanogens are co-existing in culture. There are at least four different morphological types of bacteria in the enrichment culture. However, subsequent degradation reactions need to be further elucidated to understand mineralization of Dicamba.

### 3.3. Half-life of herbicides

One important information from this study was the half-life values of these herbicides in each of the three...
soils under methanogenic condition were calculated based on the assumption that the disappearance rates follow the first-order kinetics. It is apparent that half-life values were generally smaller at 25 °C than 15 °C except for the data with Red soil (Table 3). In this study, the half-life values were 217–330 days at 15 °C and 165–315 days at 25 °C for Atrazine; 157–189 at 15 °C and 47–187 at 25 °C for Cyanazine; and 29–161 at 15 °C and 22–28 at 25 °C for Dicamba (Table 3). Relatively speaking, the half-life values of Cyanazine were slightly shorter than those of Atrazine except for the complete degradation observed with Brown soil. Similarly, using the data available, a half-life value of 34 days ($r^2 = 0.975$) was obtained with a lag phase of 40 days for the Cyanazine-degrading enrichment culture indicating remarkable reduction of half-life value through enrichment transfer.

### 4. Conclusions

Atrazine is more recalcitrant to biodegradation than Cyanazine under methanogenic condition and the half-life value of Atrazine is significantly longer than that of Cyanazine and Dicamba. Soil types and incubation temperatures have a strong influence on the degradation of the herbicides, but initial disappearance of chemical may be due to the non-biological processes. It is important that non-biological and biological processes can be differentiated through enrichment culture technique. Degradation of Dicamba is initially a de-methoxylation reaction under methanogenic condition.

### Acknowledgement

This project was financially supported by the Chinese Academy of Sciences.

### Table 3

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<tr>
<th></th>
<th>Red soil</th>
<th>Brown soil</th>
<th>Black soil</th>
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<tbody>
<tr>
<td><strong>Atrazine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 °C</td>
<td>266.6 (0.799)</td>
<td>216.6 (0.870)</td>
<td>330.1 (0.751)</td>
</tr>
<tr>
<td>25 °C</td>
<td>315.1 (0.723)</td>
<td>165.0 (0.920)</td>
<td>247.6 (0.826)</td>
</tr>
<tr>
<td><strong>Cyanazine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 °C</td>
<td>188.8 (0.704)</td>
<td>176.3 (0.744)</td>
<td>156.7 (0.888)</td>
</tr>
<tr>
<td>25 °C</td>
<td>187.3 (0.820)</td>
<td>46.8 (0.969)</td>
<td>161.2 (0.906)</td>
</tr>
<tr>
<td><strong>Dicamba</strong></td>
<td></td>
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<tr>
<td>15 °C</td>
<td>29.0 (0.970)</td>
<td>161.2 (0.806)</td>
<td>34.1 (0.896)</td>
</tr>
<tr>
<td>25 °C</td>
<td>21.7 (0.955)</td>
<td>27.9 (0.983)</td>
<td>25.0 (0.858)</td>
</tr>
</tbody>
</table>

### References


