



KINETICS OF ALACHLOR TRANSFORMATION AND IDENTIFICATION OF METABOLITES UNDER ANAEROBIC CONDITIONS

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Abstract—Alachlor is one of the two most commonly used herbicides in the United States. In the environment, little mineralization of this compound has been found to occur, and metabolites of alachlor may be formed and could accumulate. The objectives of this study were to determine the rate of alachlor biotransformation and to identify the transformation intermediates formed under aqueous denitrifying, methanogenic, and sulfate-reducing conditions. Second-order biotransformation coefficients for alachlor were determined to be 7.6×10^{-5} ($\pm 4.0 \times 10^{-5}$), 2.9×10^{-3} ($\pm 1.6 \times 10^{-3}$), and 1.5×10^{-2} ($\pm 1.4 \times 10^{-2}$) $\text{mg VSS}^{-1} \text{day}^{-1}$ under denitrifying, methanogenic, and sulfate-reducing conditions, respectively. Acetyl alachlor and diethyl aniline were positively identified as transformation products of alachlor under all conditions. In denitrifying reactors aniline was identified as a product of alachlor. When acetyl alachlor was fed as the parent compound, aniline was also identified as a transformation product under methanogenic conditions. This research showed that although alachlor is degraded under denitrifying, methanogenic, and sulfate-reducing conditions, significant concentrations of several metabolites are formed and are only slowly degraded. © 1997 Elsevier Science Ltd.

Key words—alachlor, kinetics, denitrifying, sulfate-reducing, anaerobic, metabolites

INTRODUCTION

Pesticide contamination of groundwater supplies is a serious and growing problem in the United States. More than 600 active chemicals exist that are used to protect crops from target pests (Somasundaram *et al.*, 1991). Pesticides can remain in the soil for a long period of time, or enter the groundwater supply by seeping through the soil column. Biological transformation of these chemicals to one or more metabolites often occurs with unknown and unmonitored results. In order to develop systems to manage these contaminants and formulate intelligent policies to regulate or restrict their use, an understanding of the biological reactions that these compounds undergo under different conditions is essential.

Hallberg (1987) found alachlor concentrations in the Midwest commonly ranging from 0.1 to $10.0 \mu\text{g l}^{-1}$ with peaks of greater than $500 \mu\text{g l}^{-1}$ resulting from point-source contamination. The National Alachlor Well Water Survey (NAWWS) began work in 1987 to assess the extent of alachlor contamination in private domestic wells. In the 6

million wells surveyed, 1% contained alachlor, with 1200 exceeding the Maximum Contaminant Level (MCL) as set by the EPA (Holden *et al.*, 1992). Alachlor is a probable human carcinogen (EPA Health Advisory Summary, 1989), and the MCL for drinking water has been set at $2 \mu\text{g l}^{-1}$.

Biological mineralization of alachlor has been observed by only a few researchers, in general, at quantities of less than 15% of the initial herbicide concentration. Sun *et al.* (1990) investigated the transformation and mineralization potential of an enrichment culture with alachlor as the sole carbon source under aerobic conditions; after 14 days, only 12% of the ^{14}C ring-labeled alachlor was recovered as $^{14}\text{CO}_2$, but none of the parent compound remained. Alachlor transformation in sewage and lake water was investigated by Novick and Alexander (1985). After a 6-week experimental period, alachlor was not mineralized, but 21% and 10% of the initial alachlor was transformed to organic products in sewage and lake water, respectively. A later study by Novick *et al.* (1986) found that in 30 days, less than 8% of fed alachlor was mineralized in aerobic soil suspensions. Isensee (1991) found no alachlor transformation in subsurface groundwater samples unless active microorganisms were also present. Each of these studies demonstrates that alachlor is not readily mineralized, although the parent compound is often transformed to unidentified products.

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The kinetics of alachlor transformation has also been investigated by several researchers. Pothuluri *et al.* (1990) determined first-order biotransformation constants for alachlor in aerobic and anaerobic subsurface soil samples to be 0.0025 ± 0.0004 to $0.0027 \pm 0.0004 \text{ day}^{-1}$, and $0.0014 \pm 0.0003 \text{ day}^{-1}$, respectively. Beestman and Deming (1974) found that alachlor disappearance followed first-order kinetics and half-lives in the field of 4.0 and 7.3 days for two types of soils. Wilber and Parkin (1995) developed second-order rate constants to describe the transformation of alachlor in aqueous systems under denitrifying, methanogenic, and sulfate-reducing conditions; these were 6.5×10^{-5} , 1.6×10^{-4} , and $4.7 \times 10^{-5} \text{ l mg VSS}^{-1} \text{ h}^{-1}$, respectively. Since mineralization does not appear to occur readily under a variety of conditions, transformation products are most likely forming and could be accumulating. Therefore, the purpose of this research was to evaluate the biotransformation rates of alachlor and identify its potential metabolites under denitrifying, methanogenic, and sulfate-reducing conditions in aqueous, biological systems.

MATERIALS AND METHODS

Stock system set-up

Three aqueous fill-and-draw stock reactors containing mixed bacterial cultures fed alachlor were developed. One stock reactor was maintained under denitrifying conditions, one under methanogenic conditions, and one under sulfate-reducing conditions. The three stock reactors consisted of a continuously stirred 9-l Pyrex bottle fitted with a rubber stopper. Two glass tubes were connected to clamped Silastic tubing for exhaust ports for each reactor. One of the exhaust ports in each reactor was attached to two water-filled side-port serum bottles in series to allow for gas release from the reactors while maintaining anaerobic conditions. A Luer-lock Teflon syringe connected to Teflon tubing was used for the injection and withdrawal of material in the three reactors. All stoppers and tubing were sealed with a Dow silicon sealant to eliminate any air leaks into the system. The reactors were kept closed to the atmosphere and evacuated with an 20/80 mix of CO_2 /argon gas every other day to maintain an anaerobic headspace of 1 l.

Feed solution

The three stock reactors contained 8 l of media designed to simulate groundwater under denitrifying, methanogenic, or sulfate-reducing conditions (Wilber and Parkin, 1995). The reactors were fed the required electron acceptor in excess ($20 \text{ mg l}^{-1} \text{ KNO}_3$ as N, $260 \text{ mg l}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $310 \text{ mg l}^{-1} \text{ NaHCO}_3$, respectively); this created conditions in which the electron donor became the limiting substance in cell growth. The hydraulic retention times for the reactors were 20, 40, and 40 days for the denitrifying, methanogenic, and sulfate-reducing conditions, respectively. The cultures used in stock reactor start-up were either denitrifying organisms that had been previously exposed to alachlor and atrazine (Wilber and Parkin, 1995) or laboratory stock cultures of methanogenic and sulfate-reducing organisms that had been fed acetate only (no alachlor). The denitrifying stock reactor was operated for over 120 days prior to the start of experiments. The methanogenic and sulfate-reducing cultures were maintained for approximately one year prior to the commencement of experiments.

The three stock reactors were fed daily with alachlor ($100 \mu\text{g l}^{-1}$), a phosphate buffer, and glacial acetic acid at a concentration of about 65, 200, and 55 mg l^{-1} for the denitrifying, methanogenic, and sulfate-reducing reactors, respectively. These concentrations of acetic acid were chosen to maintain a target biomass concentration of approximately $200\text{--}300 \text{ mg l}^{-1}$ in the denitrifying and methanogenic reactors. The sulfate-reducing reactor was fed less acetate to prevent a large quantity of SO_4^{2-} from being reduced to H_2S and HS^- , which are inhibitory to the organisms. A small amount of ferrous chloride was added to the methanogenic and sulfate-reducing reactors to precipitate excess HS^- (0.12 and 0.2 mg l^{-1} , respectively), and 0.92 mg l^{-1} sodium sulfide was added to the methanogenic reactor to reduce the media to a low, methanogenic redox potential. Reactors were kept in the dark at a temperature of 20°C . Resazurin, a color-indicator of redox potential, was added to the denitrifying reactor to indicate whether denitrifying conditions were maintained. This compound can be used to colorimetrically differentiate between aerobic (blue), denitrifying (pink), and anaerobic conditions (colorless).

Experimental design

Control experiments were carried out in smaller 2-l versions of the 9-l stock reactors. One phosphate-buffered denitrifying media control, two resazurin and denitrifying media controls (0.112 mg l^{-1} resazurin and 1.15 mg l^{-1} resazurin), one mercuric-chloride-killed denitrifying biological control, and one deionized water (DI) control were employed. Each of these control experiments was carried out in a batch format. An initial dose of alachlor at a concentration of $50\text{--}100 \mu\text{g l}^{-1}$ was added to each reactor and allowed to mix for approximately 45 min, and then samples for herbicide analysis were taken from the reactor at various time intervals.

Four denitrifying batch experiments were carried out in 2-l Pyrex bottles constructed in the same manner as the 9-l denitrifying stock reactor. These experiments in smaller reactors were carried out to evaluate the effect of various biomass concentration changes without affecting the 9-l stock reactor. To seed the 2-l reactors, samples from the larger reactor were taken and centrifuged. The supernatant was discarded, and the pellet was added to fresh groundwater media in the reactors. Acetate was added (520 mg l^{-1}) as well as excess KNO_3 (125 mg l^{-1} as N). The bacteria were allowed to grow for approximately 1 week. An initial alachlor dose of $100 \mu\text{g l}^{-1}$ was added to each reactor, allowed to mix for approximately 45 min, and then sampled for herbicide analysis at various time intervals.

Batch experiments involving the methanogenic and sulfate-reducing cultures were carried out in the 9-l stock reactors. These were carried out in the same manner as those described for the 2-l denitrifying batch experiments.

Second-order rate coefficients for the denitrifying systems were calculated by assuming a linear change in biomass. Because the organisms were allowed to grow for approximately one week prior to the start of the experiment and acetate was continually present, the organisms were beyond the lag phase and had not reached the stationary growth phase. A constant rate of biomass change with time was therefore assumed. Alachlor transformation with time was expressed by:

$$\frac{dC}{dt} = -kCX,$$

where C is alachlor concentration, t is time, k is the second-order rate coefficient, and X is biomass. Biomass change with time was:

$$\frac{dX}{dt} = Y_{\text{obs}},$$

where Y_{obs} is the yield. The yield was calculated from the change in volatile suspended solids (VSS) from each 2-l denitrifying reactor over the course of the experiment.

The expression for biomass change was integrated and substituted into the expression for alachlor transformation, which was then integrated. The final integrated expression was:

$$-\ln\left(\frac{C}{C_0}\right) = k\left(\frac{Y_{\text{obs}} + X_0}{2Y_{\text{obs}}}\right),$$

where C_0 is the initial alachlor concentration and X_0 is the initial biomass concentration. This equation can be used to plot the appropriate data, generating k as the slope.

Second-order rate coefficients for the methanogenic and sulfate-reducing systems were calculated by assuming that biomass remained constant; biomass in these systems changed by less than 15%. These were calculated as described in Wilber and Parkin (1995).

Herbicide measurement

Reactor samples were measured for alachlor and its transformation products using a solid-phase extraction technique (SPE) with octadecyl silane PrepSep C-18 columns from Fisher Scientific. Column preparation consisted of the following rinses: two 10-ml ethyl acetate, one 4-ml methanol, and one 4-ml DI. The 100-ml reactor sample was centrifuged at approximately 2000 rpm for 20 min; the supernatant was passed through the column. The column was then air dried and eluted twice with 1 ml of ethyl acetate. Extractions were conducted on the same day as sample collection.

Extracted samples were analyzed for alachlor, diethyl aniline, acetyl alachlor, and aniline by gas chromatograph (Hewlett-Packard 5890A) using an electron capture detector (GC-ECD) and a nitrogen-phosphorous detector (GC-NPD). Both detectors used a fused silica capillary column (J & W) with a DB-5 stationary phase. The inlet was operated in a split mode, with a split ratio of 40:1 and a septum purge of 3 ml min⁻¹. The purge valve was open for the entire run. Helium was the carrier gas at a flow of 1.5 ml min⁻¹. The makeup gas was an argon/methane (95:5%) mixture. The oven temperature was 200°C, and the run length was 15 min. Five-microliter injections were used. Detection limits were approximately 5 µg l⁻¹ for alachlor.

Four external standards between 5 µg l⁻¹ and 100 µg l⁻¹ were prepared by making 100-ml dilutions in organic-pure water (Barnstedt) with an initial solution of alachlor (150 mg l⁻¹) in water prepared gravimetrically. These were extracted and analyzed in the same manner as the samples. Standards made gravimetrically (into ethyl acetate) were used to check extraction efficiency. The extraction efficiency of the SPE for alachlor was determined to be greater than or equal to 95%.

Mass spectrometer analysis

A quadrupole high-resolution (VG Trio-1) mass spectrometer (MS) with GC inlet and electron ionization was used to identify metabolites formed in the degradation of alachlor. Extracted samples were evaporated to dryness and the residue dissolved in 20 µl of ethyl acetate. Two-microliter injections were used. The run conditions were as follows: 45°C for 5 min, ramped at a rate of 5°C min⁻¹ to 250°C, and held for 4 min. Detection limits for aniline were approximately 1 µg l⁻¹ with the extraction method used.

Other analytical methods

Acetate concentrations in the samples were measured using a GC (Hewlett Packard 5890A) with a flame ionization detector (FID). A packed column (Supelco) of 60/80 Carbowax with 0.3% Carbowax and 0.1% H₃PO₄

was used. Filtered samples were acidified to a pH of approximately 3, using several drops of 88% formic acid. Standards were made in a similar manner using dilutions of a sodium acetate solution prepared gravimetrically. Injections were 1 µl. The detection limit was approximately 5 mg l⁻¹. The method outlined in Standard Methods (APHA, 1992) was used to measure volatile suspended solids (method 2540 G). Dissolved sulfide was measured using the iodometric method described in Standard Methods (method 4500-S²⁻ E).

RESULTS AND DISCUSSION

Alachlor biotransformation, denitrifying conditions

Alachlor biotransformation followed a second-order pattern in these systems, with biomass calculated as changing linearly over the period of the experiment (28, 31, 35, and 35 days for reactors 1, 2, 3, and 4, respectively). As previously mentioned, resazurin was added to indicate whether denitrifying conditions were maintained in the small 2-l experimental reactors and the 9-l stock reactor. Resazurin is a common redox indicator; however, it was found to be involved in the abiotic transformation of alachlor (5.0×10^{-2} mg res⁻¹.day⁻¹ ± 5.4×10^{-2} mg res⁻¹.day⁻¹), as evidenced by the transformation of this compound in the abiotic reactor containing resazurin, whereas no alachlor transformation was seen in the DI control or the phosphate-buffered media control (Fig. 1). Second-order transformation coefficients for biologically mediated alachlor transformation are given in Table 1.

The pseudo-first-order transformation coefficient for resazurin-influenced alachlor transformation was determined in the same manner as described in Wilber and Parkin (1995); however, the pseudo-first-order coefficient was divided by the concentration of resazurin initially added to the reactor. Resazurin concentration was assumed to remain constant throughout the experiments. This assumption was not verified. The rate of abiotic alachlor transformation due to resazurin was subtracted from the overall transformation coefficient. This was done by subtracting the pseudo-first-order transformation rate coefficient for the resazurin reaction from the overall pseudo-first-order rate coefficient; this was then divided by the changing biomass concentration in the reactors over the experimental period. This was assumed to be representative of the portion of herbicide transformation that was a result of biotransformation alone.

Biotransformation rate coefficients for alachlor were somewhat different for the different biomass concentrations tested (reactors 1, 3, and 4 in Table 1). However, considering the variability of the analytical methods used and the corrections made for the effect of resazurin, the differences are not thought to be significant. The high variability in k gives rise to the relatively large standard deviation reported. Values for k are very similar to those

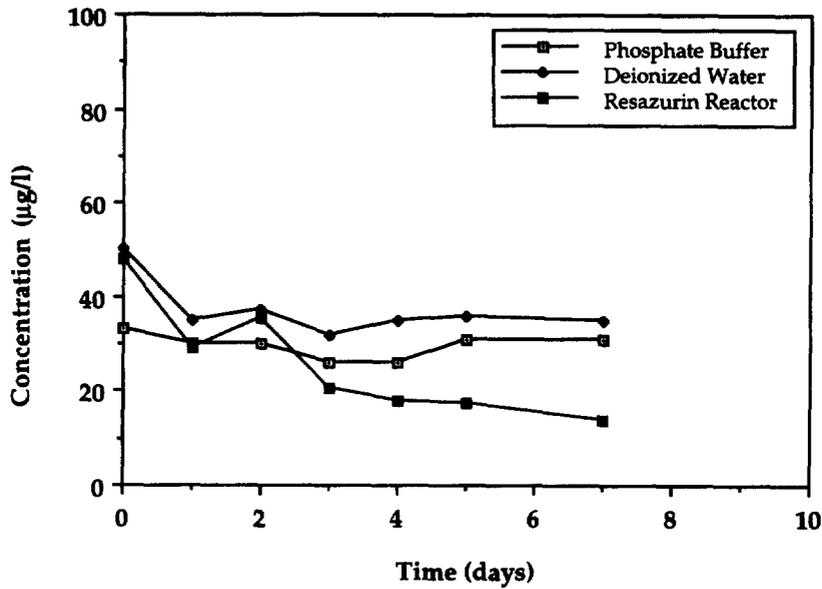


Fig. 1. Transformation of alachlor in the phosphate buffer media control, the deionized water control, and a resazurin-containing abiotic reactor under denitrifying conditions.

determined by Wilber and Parkin (1995), also given in Table 1.

Alachlor transformation, methanogenic conditions

Alachlor underwent rapid transformation under methanogenic conditions. Sequential alachlor-spiked batch experiments were carried out in the 9-l stock reactor with no groundwater media exchange between experiments. As seen in Fig. 2, the rate of alachlor transformation decreased with experiments 1–3. This decrease was thought to be because of a buildup of a toxin(s), perhaps one or more metabolites of alachlor, or some other metabolic byproduct. At the end of the third experimental period, acetate utilization and methane production had become negligible; however, alachlor transformation continued at a reduced rate.

Sulfide concentrations, at approximately 25 mg l⁻¹ in the methanogenic reactor, were well below levels thought to be inhibitory (150–200 mg l⁻¹) (Maillacheruvu *et al.*, 1993) throughout the four experimental periods. Acetic acid levels were maintained (> 5 mg l⁻¹) throughout the experiments.

After the conclusion of the last experiment, the reactor was decanted down to 1 l and fed new groundwater media with no added alachlor for approximately 60 days. No recovery of the bacterial population was observed after this period, as defined by acetate utilization and methane production. This observation lends credence to the hypothesis that decreased bacterial activity was due to a toxic, and not simply inhibitory, substance(s).

An abiotic reaction occurred with the bisulfide ion, resulting in the transformation of alachlor. Levels of bisulfide were sufficiently low to have a minimal effect on the overall rate of alachlor transformation; however, this portion was removed from the overall rate coefficient. This was done by subtracting the pseudo-first-order transformation rate coefficient for the bisulfide reaction from the overall pseudo-first-order rate coefficient; this was then divided by the average biomass concentration in the reactors over the experimental period. The second-order transformation rate coefficient for the abiotic reaction of alachlor with the bisulfide ion ($1.5 \times 10^{-3} \text{ l mg}^{-1} \text{ day}^{-1}$) was taken from Wilber and Parkin (1995).

Table 1. Second-order transformation coefficients for alachlor under denitrifying conditions

Reactor number	Experimental variables		Second-order transformation coefficient, <i>k</i> (corrected for resazurin-mediated transformation) (l mg VSS ⁻¹ day ⁻¹)
	Average biomass (as mg l ⁻¹ VSS)	Resazurin (mg l ⁻¹)	
1	376	1.15	1.3×10^{-4}
2	309	1.15	8.1×10^{-5}
3	90	0.112	4.2×10^{-5}
4	189	0	5.0×10^{-5}
Average	—	—	$7.6 \times 10^{-5} (\pm 4.0 \times 10^{-5})$
Literature value (Wilber and Parkin, 1995)	—	—	6.45×10^{-5}

± represents one standard deviation from the mean of separate trials.

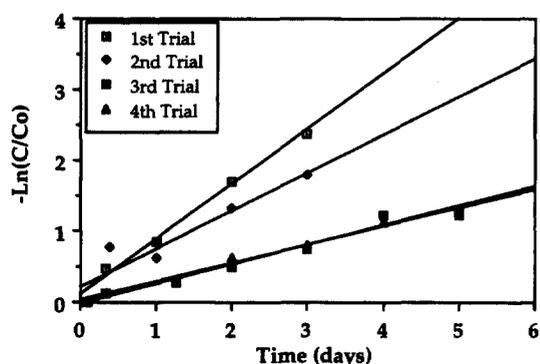


Fig. 2. Determination of pseudo-first-order transformation rate coefficients for alachlor in the methanogenic system (R^2 values for the lines are 0.991, 0.895, 0.979, and 0.985, for trials 1–4, respectively).

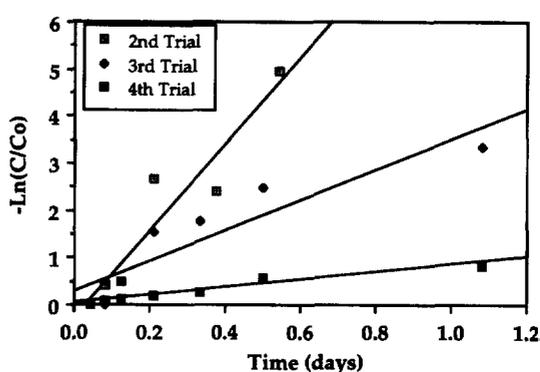


Fig. 3. Determination of pseudo-first-order transformation rate coefficients for alachlor in the sulfate-reducing system (R^2 values for the lines are 0.893, 0.847, and 0.954, for trials 2–4, respectively; too few points gathered for trial 1 for coefficient determination).

Experimentally determined second-order rate coefficients are listed in Table 2 for alachlor along with values from Wilber and Parkin (1995) for comparison.

Alachlor transformation, sulfate-reducing conditions

Alachlor was also transformed quickly in the sulfate-reducing reactor. Sequential alachlor-spiked batch experiments were carried out in a similar manner to those in the methanogenic reactor. As seen in Fig. 3, the rate of alachlor transformation decreased with each successive experiment. The decrease in transformation rates of alachlor in the sulfate-reducing reactor was also thought to be because of a buildup of a toxin(s). At the end of the final experimental period, acetate utilization had ceased. However, alachlor transformation also continued in this reactor, albeit at a reduced rate.

Sulfide concentrations, at about 30 mg l^{-1} in the sulfate-reducing reactor, were well below reported inhibitory levels ($150\text{--}200 \text{ mg l}^{-1}$) (Maillacheruvu *et al.*, 1993) throughout the four experimental periods. Sulfide levels were low in the sulfate-reducing reactor because only small amounts of acetate and sulfate were added to the reactors to prevent an

inhibitory build-up of sulfide. Acetic acid levels were maintained ($> 5 \text{ mg l}^{-1}$) throughout the experiments.

After the conclusion of the last experiment, the sulfate reducing reactor was also decanted in a manner similar to the methanogenic reactor. No recovery of the bacterial population was observed in this reactor, as defined by acetate utilization.

The portion of the rate coefficient due to the abiotic reaction with the bisulfide ion was subtracted from the overall rate coefficient in the same manner as that described for the methanogenic reactor. These alachlor transformation rate coefficients are listed in Table 2 along with values from Wilber and Parkin (1995) for comparison.

Metabolites, denitrifying conditions

Many peaks were detected with the GC-NPD and GC-ECD that were consistently present in the reactors under all electron acceptor conditions. Some of these peaks increased and/or decreased with time, but could not be identified. Several metabolites were positively identified. In the reactors with both resazurin (at concentrations of 1.15 mg l^{-1} or 0.112 mg l^{-1}) and organisms present, aniline, acetyl alachlor, and diethyl aniline (Fig. 4) were positively identified as intermediates in the transformation of alachlor. Acetyl alachlor appeared on day 2 of the experiment and had disappeared by day 4. Diethyl aniline was also identified in the reactors containing resazurin and bacteria. Aniline, identified and quantified by GC-MS, appeared in the denitrifying reactors containing resazurin plus acetate-utilizing organisms between days 12 and 17 of the experiments. Aniline concentration dropped below detection limits ($\approx 1 \mu\text{g l}^{-1}$) by day 35, the last day of the experiment (data not shown). At the maximum measured aniline concentration, 14% of the initial alachlor added had been transformed to aniline. Because aniline may be undergoing subsequent

Table 2. Second-order transformation coefficients for alachlor under methanogenic and sulfate-reducing conditions

Conditions	Second-order transformation coefficient, k ($\text{l mg VSS}^{-1}\text{day}^{-1}$)
Methanogenic, Trial 1	4.8×10^{-3}
Methanogenic, Trial 2	3.4×10^{-3}
Methanogenic, Trial 3	1.6×10^{-3}
Methanogenic, Trial 4	1.6×10^{-3}
Methanogenic Average	$2.9 \times 10^{-3} (\pm 1.6 \times 10^{-3})$
Sulfate-Reducing, Trial 2	3.0×10^{-2}
Sulfate-Reducing, Trial 3	1.1×10^{-2}
Sulfate-Reducing, Trial 4	2.7×10^{-3}
Sulfate-Reducing Average	$1.5 \times 10^{-2} (\pm 1.4 \times 10^{-2})$
Methanogenic, literature value (Wilber and Parkin, 1995)	8.50×10^{-5}
Sulfate-reducing, literature value (Wilber and Parkin, 1995)	1.60×10^{-4}

± represents one standard deviation from the mean of separate trials.

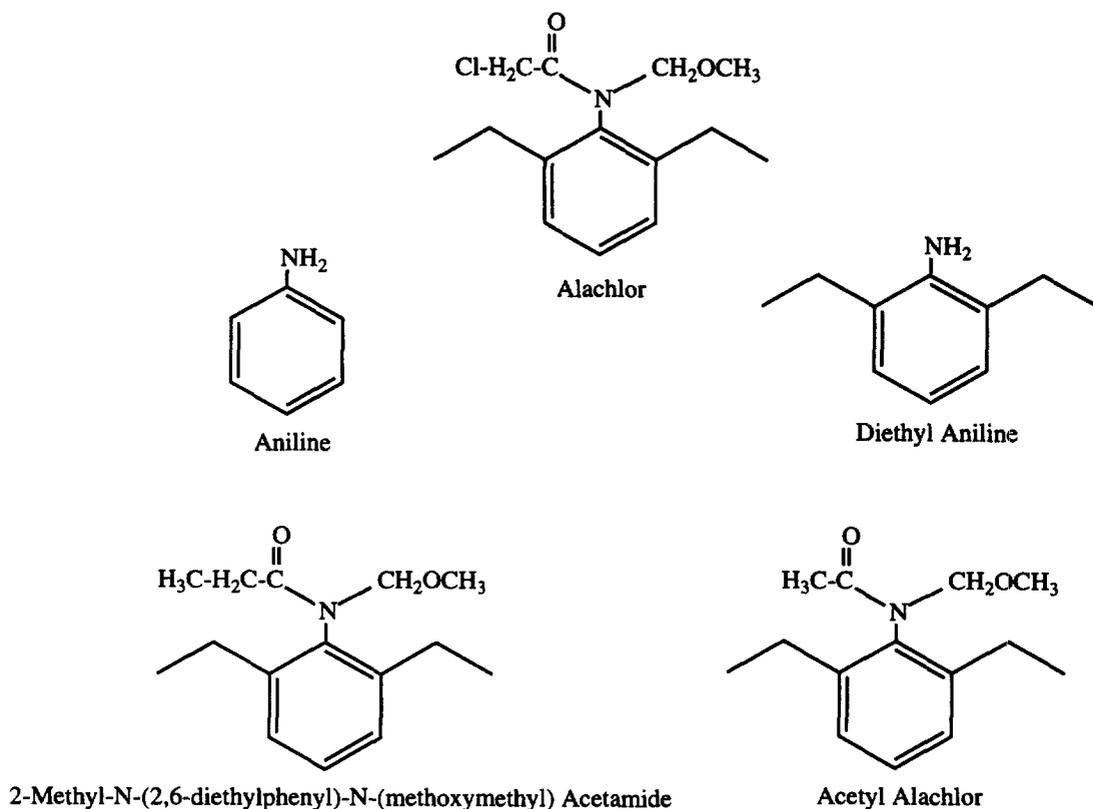


Fig. 4. The structure of alachlor and the metabolites identified under denitrifying, methanogenic, and sulfate-reducing conditions.

transformation once it is formed, more than 14% of the alachlor fed may have been converted to aniline. Aniline is very reactive and is a human poison that is considered a severe health hazard. The presence of aniline in groundwater as a result of alachlor transformation is possible, especially from highly concentrated point-source spills; however, aniline is known to covalently bind with organic matter such as humic acids, and this could effectively remove the compound from the environment (Weber *et al.*, 1996).

One 2-l reactor contained acetate-utilizing organisms with no resazurin. Different metabolites were produced in this reactor than in the other systems. Aniline was not detected in this reactor, suggesting that this substance may be formed via an abiotic reaction mediated by resazurin, may result from coupled abiotic and biological transformation steps, or may have been formed and subsequently transformed too quickly to be detected. Because certain key intermediates in the transformation of alachlor could be absent in this purely biological system, transformation would not necessarily follow the same pathway as that in a system containing resazurin or other compounds which can facilitate electron transfer, such as vitamin B₁₂ (Chiu and Reinhard, 1995) or other natural organic compounds.

Becker and Freedman (1994) found that catalytic quantities of cyanocobalamin increased both the rate of chloroform transformation, as well as perhaps altering the transformation pathway, when added to a culture of methanogenic organisms. Although resazurin is not present in the environment, transition metal cofactors and humic materials often are. These compounds are often facilitators of electron transfer and therefore could act in a similar manner in the environment as resazurin did in the laboratory. When these compounds are present in small, catalytic quantities in the presence of organisms, they could speed, or alter, the transformation pathway of alachlor.

Few other researchers have investigated the transformation products of alachlor. Isensee (1991) investigated the transformation of alachlor in subsurface vadose zone samples. Only one product, hydroxyalachlor, was identified as a metabolite of alachlor, although other unidentified polar transformation products were detected. Sun *et al.* (1990) detected four unidentified metabolites of alachlor in an aerobic enrichment culture, and Novick and Alexander (1985) observed six organic products of alachlor in sewage and four organic products in lake water. Products such as aniline, diethyl aniline, and acetylalachlor have not been identified as products

of alachlor transformation; however, these compounds could have been present in the above studies and gone unidentified.

Metabolites, methanogenic conditions

Acetyl alachlor, diethyl aniline, and 2-methyl-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl) acetamide were identified in the methanogenic reactor, these compounds are shown in Fig. 4. Two-methyl-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl) acetamide was preliminarily identified on the GC-MS, but no standard was available for purchase to confirm this metabolite using elution time data. This compound was not monitored over time. Acetyl alachlor was positively identified under methanogenic conditions on both the mass spectrometer and the GC-NPD. The concentration profile generated on the GC-NPD for acetyl alachlor is shown in Fig. 5. Diethyl aniline formation was also observed under methanogenic conditions and was identified and quantified on the GC-NPD. The concentration profiles generated on the GC-NPD for diethyl aniline are shown in Fig. 5 as well.

The acetyl alachlor concentration appears to remain fairly constant over time. This metabolite was found in the reactor approximately 1 h after alachlor addition, indicating that there is most likely a background level that is not transformed and is present from previous alachlor additions. A portion of alachlor could have been biologically transformed to acetyl alachlor and remained as this product while the rest of alachlor was transformed via other metabolites.

Diethyl aniline is formed quickly and is then slowly transformed, dropping below detection limits in the final 5 days of the experiment. Assuming that a

number of different transformation pathways exist for alachlor, diethyl aniline is most likely representative of only a portion of the alachlor initially added. At the highest observed concentration of diethyl aniline, it represented 9% of the initial alachlor added to the system in the methanogenic reactor. Here, it appears that diethyl aniline is formed from a quickly transformed compound, either alachlor itself, or a very short-lived transient. Diethyl aniline itself appears to be a more slowly transformed metabolite, and therefore may be expected to be present for a longer period of time in the environment.

Later experiments were performed where acetyl alachlor was added as the parent compound under methanogenic conditions. These experiments were carried out with a new acetate enrichment culture that had not been previously exposed to alachlor or acetyl alachlor. One hundred micrograms per liter of acetyl alachlor was added to the reactor to begin the experiment. Figure 6 shows the overall loss of acetyl alachlor over time. After 14 h of measurements, acetyl alachlor could no longer be detected; however, GC analysis revealed the presence of aniline (Fig. 6). Aniline concentrations increased from below the detection limit (around $5 \mu\text{g l}^{-1}$ on the GC-NPD) to approximately $11.5 \mu\text{g l}^{-1}$ during the first 10 h of the experiment, which corresponds to the disappearance of acetyl alachlor.

Although aniline was not detected as a metabolite when alachlor was added to the methanogenic stock reactor, it could be potentially present below the compound detection limit. When acetyl alachlor was added at a concentration of $100 \mu\text{g l}^{-1}$, aniline appeared at a concentration of about $11 \mu\text{g l}^{-1}$, which is very close to the detection limit for aniline. When alachlor was added at a concentration of $100 \mu\text{g l}^{-1}$,

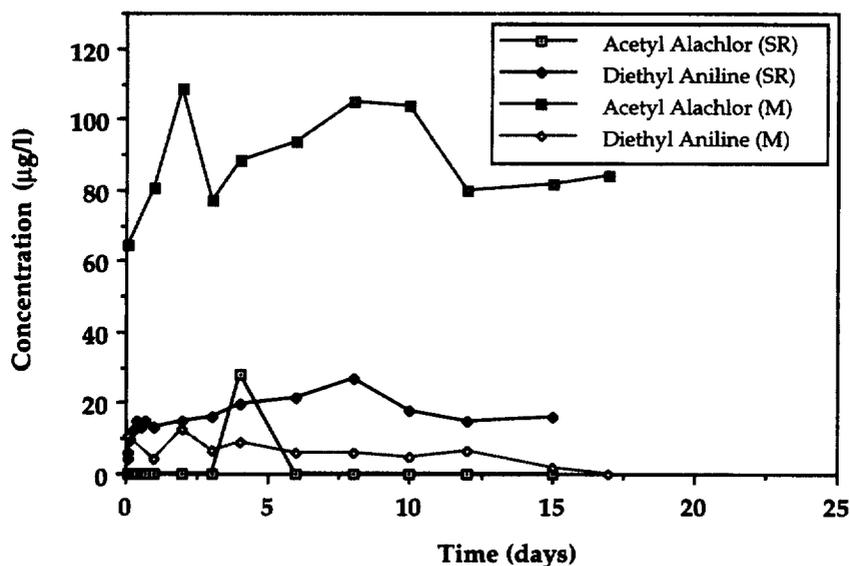


Fig. 5. Formation and transformation of diethyl aniline (in $\mu\text{g l}^{-1}$) and acetyl alachlor (in units of $\mu\text{g l}^{-1}$ as alachlor) in the methanogenic and sulfate-reducing reactors.

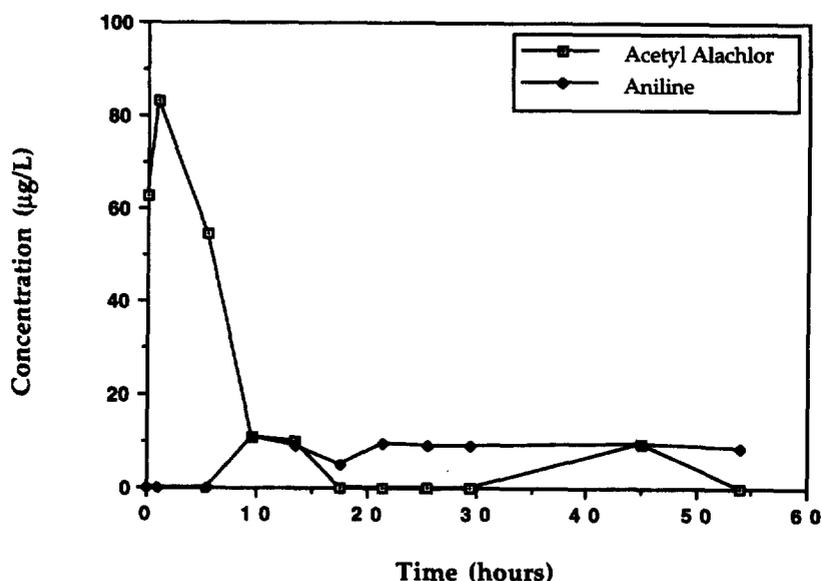


Fig. 6. Concentration profile showing the transformation pattern of acetyl alachlor and subsequent formation of aniline in the methanogenic reactor (fed acetyl alachlor).

even if there was 100% conversion to acetyl alachlor (which was not observed in experiments described in this work), only about $87 \mu\text{g l}^{-1}$ acetyl alachlor would be present in the system. Because aniline was so close to the detection limit when acetyl alachlor was fed at a concentration of $100 \mu\text{g l}^{-1}$, if only $87 \mu\text{g l}^{-1}$ acetyl alachlor was fed, it is possible that the aniline concentration would be below detection limits. Therefore, aniline could have been present when alachlor was the parent compound but, because of experimental conditions, was never detected in the reactor.

Metabolites, sulfate-reducing conditions

Acetyl alachlor, diethyl aniline, and 2-methyl-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl) acetamide were also identified in the sulfate-reducing reactor (Fig. 4). Under sulfate-reducing conditions, acetyl alachlor was positively identified on both the mass spectrometer and the GC-NPD. Diethyl aniline was identified on GC-NPD. The concentration profiles with time for acetyl alachlor and diethyl aniline under sulfate-reducing conditions are given in Fig. 5.

Acetyl alachlor only appears at one sampling point in the GC-NPD chromatograms. Acetyl alachlor was also detected on the GC-MS, but samples were not checked for the compound over time, so no true knowledge about the behavior of acetyl alachlor with time can be gained from these results. Wilber and Parkin (1991) also identified acetyl alachlor as a product of alachlor transformation under sulfate-reducing conditions.

Diethyl aniline increased slowly in concentration under sulfate-reducing conditions until day 28, when it began to slowly decrease in concentration.

This suggests that diethyl aniline is formed from another slowly transformed compound. At the highest observed concentration of diethyl aniline, it represented 20% of the initial alachlor added to the sulfate-reducing reactor.

CONCLUSION

This research showed that although alachlor is biologically transformed under denitrifying, methanogenic, and sulfate-reducing conditions, significant concentrations of several metabolites are formed and are only slowly transformed. Nitrate, sulfate, and bicarbonate concentrations in groundwater vary widely across the United States. All of these compounds are found in groundwater supplies, and microenvironments where denitrifying, sulfate-reducing, or methanogenic organisms could thrive do exist. In regions where herbicide contamination is a problem, such as the Midwest, nitrate concentrations are often high. In Iowa, concentrations typically vary between 0 and 100 mg l^{-1} as N for nitrate, and 0 and 1900 mg l^{-1} for sulfate; average nitrate (as N) and sulfate concentrations for Iowa are 6.2 mg l^{-1} and 130 mg l^{-1} , respectively (Hallberg, 1987). Bicarbonate is often present (Hallberg, 1987). Therefore, denitrifying, sulfate-reducing, or methanogenic conditions could be present where anaerobic zones in the groundwater occur. Little work has been carried out on the identification of alachlor metabolites; however, many researchers have detected organic products formed upon the transformation of alachlor (Isensee, 1991; Sun *et al.*, 1990; Novick and Alexander, 1986; Novick *et al.*, 1985; Wilber and Parkin, 1995). This research

demonstrated the formation of several metabolites of alachlor; acetyl alachlor, diethyl aniline, aniline, and 2-methyl-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide under different electron acceptor conditions. Some of these compounds were readily degradable; however, other compounds appeared to be relatively long-lived. Furthermore, in the methanogenic and sulfate-reducing reactors, repeated alachlor spikes to the reactors resulted in some form of toxicity to the organisms, slowing the transformation rate of alachlor substantially and eventually halting all acetate-utilization and methane production. This could point to a potential, previously unmonitored, environmental problem.

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REFERENCES

- APHA (American Public Health Association), American Water Works Association, and Water Pollution Control Federation (1992) Standard Methods for the Examination of Water and Wastewater, 18th edition. American Public Health Association, Washington DC 2-58 and 4-127.
- Becker J. G. and Freedman D. L. (1994) Use of cyanocobalamin to enhance anaerobic biodegradation of chloroform. *Environ. Sci. Technol.* **28**, 1942-1949.
- Beestman G. B. and Deming J. M. (1974) Dissipation of acetanilide herbicides from soils. *Agron. J.* **66**, 308-311.
- Chiu P.-C. and Reinhard M. (1995) Metalloenzyme-mediated reductive transformation of carbon tetrachloride in titanium (III) citrate aqueous solution. *Environ. Sci. Technol.* **29**, 595-603.
- Environmental Protection Agency (EPA) (1989) Health Advisory Summary, January, 1989.
- Hallberg G. R. (1987) Agricultural chemicals in ground water: extent and implications. *Am. J. Alternative Agric.* **2**, 3-15.
- Holden L. R., Graham J. A., Whitmore R. W., Alexander W. J., Pratt R. W., Liddle S. K. and Piper L. L. (1992) Results of the National Alachlor Well Water Survey. *Environ. Sci. Technol.* **26**, 935-943.
- Isensee A. R. (1991) Dissipation of alachlor under *in situ* and stimulated vadose zone conditions. *Bull. Environ. Contam. Toxicol.* **46**, 519-526.
- Maillacheruvu K. Y., Parkin G. F., Peng C. Y., Kuo W.-C., Oonge Z. I. and Lebduchka V. (1993) Sulfide toxicity in anaerobic systems fed sulfate and various organics. *Water Environ. Res.* **65**, 100-109.
- Novick N. J. and Alexander M. (1985) Cometabolism of low concentrations of propachlor, alachlor, and cycloate in sewage and lake water. *Appl. Environ. Microbiol.* **49**, 737-743.
- Novick N. J., Mukherjee R. and Alexander M. (1986) Metabolism of alachlor and propachlor in suspensions of pretreated soils and in samples from ground water aquifers. *J. Agric. Food Chem.* **34**, 721-725.
- Pothuluri J. V., Moorman T. B., Obenhuber D. C. and Wauchope R. D. (1990) Aerobic and anaerobic degradation of alachlor in samples from a surface-to-groundwater profile. *J. Environ. Qual.* **19**, 525-530.
- Somasundaram L., Coats J. R., Racke K. D. and Shanbhag V. M. (1991) Mobility of pesticides and their hydrolysis metabolites in soil. *Environ. Toxicol. Chem.* **10**, 185-194.
- Sun H. L., Sheets T. J. and Corbin F. T. (1990) Transformation of alachlor by microbial communities. *Weed Sci.* **38**, 416-420.
- Weber E. J., Spidle D. L. and Thorn K. A. (1996) Covalent binding of aniline to humic substances. 1. Kinetic studies. *Environ. Sci. Technol.* **30**, 2755-2763.
- Wilber G. G. and Parkin G. F. (1991) Transformation of pesticides under anaerobic conditions. In *In situ and On Site Bioreclamation* (Edited by Hinchee R. and Olfenbuttel R.), pp. 385-402. Butterworth-Heinemann, Stoncham, MA.
- Wilber G. G. and Parkin G. F. (1995) Kinetics of alachlor and atrazine biotransformation under various electron acceptor conditions. *Environ. Toxicol. Chem.* **14**, 237-244.