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ARTICLE

Microbial Degradation of Clomazone under Simulated California Rice Field Conditions

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Clomazone (trade names Cerano and Command) is a popular herbicide used on California rice fields to control aquatic weeds. Its physicochemical characteristics indicate that it will persist primarily in the water column, where microbial degradation may drive its environmental fate. The objectives were to determine microbial degradation rates and compare the metabolic products under aerobic and anaerobic conditions similar to those in California rice fields during the summer. Time-series samples were extracted and analyzed by LC/MS/MS. Metabolic profiling revealed the following clomazone-derived transitions: *m*/*z* 240 → 125 (clomazone), *m*/*z* 242 → 125 (ring-open clomazone), *m*/*z* 256 → 125 (5-hydroxyclomazone), *m*/*z* 256 → 141 (aromatic hydroxyclomazone), *m*/*z* 268 → 125 (unknown metabolite), and *m*/*z* 272 → 141 (4′5-dihydroxyclomazone). Results indicate an anaerobic half-life of 7.9 days, with ring-open clomazone reaching 67.4% of application at 38 days. Aerobically, clomazone degraded more slowly ($t_{1/2}$ = 47.3 days), forming mostly soil-bound residues. Thus, under summer conditions, clomazone is likely to dissipate rapidly from fields via anaerobic degradation.

KEYWORDS: Clomazone; herbicides; microbial degradation; metabolic profiling; mass spectrometry

INTRODUCTION

Synthetic pesticides are important for rice culture. Their ability to increase crop yield provides farmers with efficient means of food production and allows for production in otherwise unfavorable conditions. Despite these benefits, reduction of ecosystem biodiversity, outbreaks of secondary pests, development of pesticide resistance, and contamination of food and/or the environment are common side effects (*1*). In California, the desire of both state agencies and rice growers alike is that pesticides efficiently dissipate from fields prior to discharge of field water into neighboring rivers or streams.

Clomazone (2-[2-chlorobenzyl]-4,4-dimethyl-1,2-oxazolidin-3-one, **Table 1**, compound **1**) is a herbicide used on California rice fields to control watergrass, barnyard grass, and sprangletop. It is also used elsewhere in the world for many other crops, notably soybeans, cotton, and tobacco (*2*). Clomazone was registered for use in California in 2002 and, as of 2007, has increased to 80,000 lbs active ingredient (a.i.) applied to 160,000 acres (*3*), making it one of the most popular rice herbicides in California. In plants, its mode of action is thought to occur via cytochrome P450 activation followed by carotenoid synthesis inhibition (*4*−*6*). Clomazone is highly water-soluble (1100 mg/L), minimally volatile ($P_v = 1.44 \times 10^{-4}$ mm Hg), resistant to hydrolysis under a wide range of pH values, and weakly sorptive to soil (k_D = 0.47−5.30), with sorption dependent upon organic carbon content (k_{oc} = 300 mL/g) (*7*). These physicochemical characteristics indicate that clomazone is likely to persist primarily in the water column, where microbial degradation is thought to contribute to its environmental fate.

Mervosh et al. (*8*) examined clomazone microbial degradation in an aerobic soil, finding slow degradation ($t_{1/2}$ = 49−58 days); temperature and moisture correlated with rising CO_2 evolution. Liu et al. (*9*) conducted a preparative incubation experiment with clomazone with a variety of microorganisms, screening for their ability to degrade the pesticide. Their results depict all currently known clomazone transformations. Quayle, et al. (*10*) conducted a field dissipation study of clomazone in water and soil in treated rice plots, finding the compound to degrade rapidly, with $t_{1/2}$ = 7.2 days (water) and 14.2 days (soil). Each fraction was found highly correlated to pseudofirst-order degradation kinetics (r^2 = 0.90 and 0.92, respectively). The EPA postulates anaerobic degradation to proceed faster than aerobic, with only one metabolite (*N*-[2-(2-chlorophenyl) methyl] 3-hydroxy-2,2-dimethyl propanamide, ring-open clomazone, compound **2**) reaching concentrations of > 10% of initial application (*11*). In aerobic soil, little metabolite formation is believed to occur (*8*). However, no information is currently available on the microbial degradation of clomazone under simulated California rice field conditions.

Microbes oxidize organic compounds in the presence of terminal electron acceptors, and reduce them in a sequential series dependent upon redox potential, E_h, as alternative electron acceptors are utilized (*12*). Flooded rice fields are a mosaic of anaerobic soil with aerobic micro sites, and under these conditions, clomazone degradation rate and metabolite identity may

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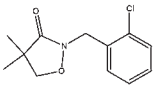
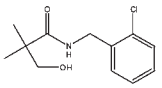
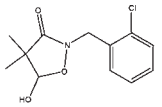
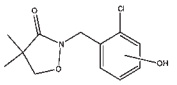
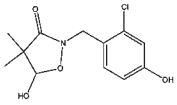
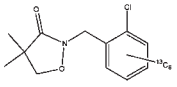
Table 1. Observed Mass Transitions and Corresponding Structures, Mass Shift, and Average Spike Recovery ($N = 3$)

Compound	Name	Structure	Mass Transition (m/z)	Mass Shift (from clomazone)	Spike 0.1 μg/mL	Recovery 1.0 μg/mL
1	Clomazone		240 / 125	$[M+H]^+$	91.7	82.3
2	Ring-Open Clomazone		242 / 125	$[M+2+H]^+$	99.3	104.8
3	5-hydroxyclomazone		256 / 125	$[M+16+H]^+$	59.1	101.3
4	Aromatic hydroxyclomazone		256 / 141	$[M+16+H]^+$	105.8	91.6
5	4',5-dihydroxyclomazone		272 / 141	$[M+32+H]^+$	46.4	102.6
6	$^{13}C_6$ Clomazone		246 / 131	$[M+6+H]^+$		

differ. Knowledge of soil degradation kinetics is paramount to determining how long the floodwater should be held.

Recent advances in liquid chromatography−tandem mass spectrometry (LC/MS/MS) provide the sensitivity and selectivity required for metabolic and kinetic modeling in soils and decrease dependency on radiolabeled standards. The objectives of this study were to determine the major biotransformation products formed under aerobic and anaerobic conditions and determine the kinetics of clomazone biodegradation processes, using LC/MS/MS. Experiments were conducted via sacrificial time-series microcosms at 30 °C, typical of summertime highs in California's Sacramento Valley.

MATERIALS AND METHODS

Chemicals and Soils. Clomazone and metabolites were provided gratis by FMC Corporation (Philadelphia, PA); full-scan mass spectrometric analysis revealed no contamination. Uniformly aromatic-labeled $^{13}C_6$ clomazone was purchased from IsoSciences, LLC (King of Prussia, PA), and acetonitrile and methanol were obtained from Sigma-Aldrich (St. Louis, MO).

The soil was an Esquon-Neerdobe thermic clay loam from the California Rice Research Station in Biggs, CA (*13*) and was characterized by the UC Agriculture & Natural Resources (ANR) Analytical Laboratory at UCD. This revealed the soil to contain 0.71% organic matter and 0.41% organic carbon. Soil was sampled from 0 to 10 cm, sieved through a 2-mm mesh, and stored in plastic bags at 4 °C. Experiments were conducted within 6 months of sampling.

Microcosm Experiments. In 60-mL serum vials, 12.5 g of soil was added. For anaerobic experiments, 50 mL of autoclaved distilled water was added, while for aerobic experiments, water holding capacity was adjusted to 50% with autoclaved distilled water. Anaerobic vials were preincubated at 30 °C in the dark for 14 days (such that $E_h < -200$ mV) and aerobic for 7 days, prior to clomazone addition. On day 0, 100 μg of clomazone (technical grade) was added to each vial and returned to the incubator. The application was representative of the 0.6 lbs/acre regulatory rate. For metabolic profiling, six treatments, three controls (triply autoclaved soil), and three blanks for each time point were extracted over 40 days (anaerobic) or 60 days (aerobic). For target analysis, five treatments, three controls, one blank, one blank spike, and one water spike were extracted at 0, 3, 6, 12, 19, 26, 38, 60, and 83 days.

Chemical Extraction. For metabolic profiling, samples were removed from the incubator, and 50 mL of water added to aerobic samples. Samples were shaken for 1 h, centrifuged at 3500 rpm for 15 min, filtered through a Whatman #1 filter (Fisher Scientific), and decanted. An additional 50 mL of water was added to the soil; it was again extracted and the extracts combined. As per Zanella et al. (*14*), the filtrate was acidified to pH 3 with concd H_3PO_4, and concentrated using solid-phase extraction (SPE) cartridges (3 mL, 300 mg C18 Bakerbond; JT Baker, Paris, KY). Each column was sequentially washed with 1 mL of methanol, 1 mL of water, and 1 mL of pH 3 water (acidified with concd H_3PO_4). The sample was loaded on column, allowed to dry for 10 min, sequentially eluted with 1 mL of methanol and 2 mL of acetonitrile, evaporated to near-dryness under N_2, and resuspended in methanol to 0.5 mL. For each time point, aliquots of treatments, controls, and blanks were combined prior to analysis.

For target analysis, samples were extracted sequentially with water and acetonitrile to remove water-dissolved and soil-bound fractions, respectively. On each extraction day, samples were removed from the incubator, and 50 mL of water was added to aerobic samples. Samples were shaken for 1 h and centrifuged at 3500 rpm for 15 min. The supernatant was decanted and filtered through a 0.2 μm acrodisk filter (Nalgene USA), shaken, a 0.5 mL aliquot amended with internal standard (compound **6**), and stored for analysis in a −20 °C freezer. Acetonitrile (25 mL) was added to the centrifugate and shaken for 16 h. The extract was then filtered through a Whatman #1 filter, a 1-mL aliquot solvent exchanged to 10:90 methanol/water by evaporation to near-dryness and resuspension, filtered through a 0.2 μm filter, amended with internal standard (compound **6**), and stored for analysis at −20 °C.

Chemical Analysis. Metabolic profiling was accomplished using a Acquity UPLC system (Waters, Milford, MA) coupled to an API 4000 Qtrap hybrid triple quadruple linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with LightSight software. The column used was a 150 mm × 3 mm i.d., 3 μm Luna phenyl-hexyl column (Phenomenex, Torrance, CA). The mobile phases were as follows: A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile. After a 2-min isocratic run at 3% B, a gradient to 60% B was concluded at 6 min and then ramped to 95% B for up to 15 min. All injection volumes were 10 μL, column temperature was 50 °C, and the flow rate was 0.3 mL/min. Samples were run in positive ion mode.

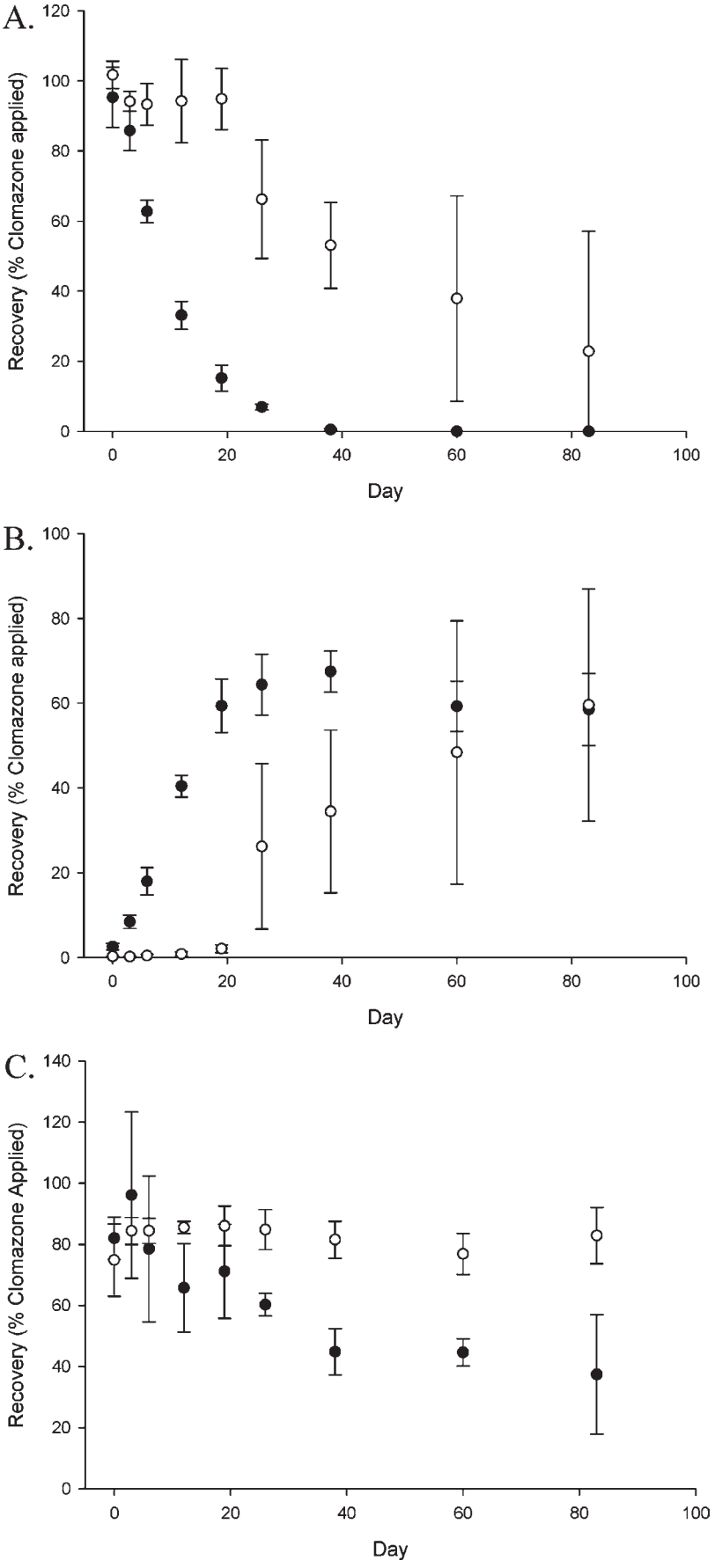


Figure 1. Percent recovery of clomazone and compound **2** (expressed as percent of clomazone-applied) for treated soil (●) and autoclaved controls (○) under anaerobic conditions (**A**), showing anaerobic ring-open formation (**B**), and under aerobic conditions (**C**). Data points are mean values ± 1 SD (controls, $n = 3$; treatments, $n = 5$).

For target analysis, samples were quantified using a HP1100 HPLC coupled to an Applied Biosystems API 2000 LC/MS-MS with electrospray ionization interface, run in positive ion mode. Chromatography was performed on a 50 mm × 2.1 mm i.d., 5 μm Titan phenyl column with a 10 mm × 2 mm i.d. guard column of the same material (Peeke Scientific, Sunnyvale, CA). The mobile phases were as follows: A, 0.1% sodium acetate in water; B, 0.1% sodium acetate in methanol. After a 1-min isocratic run at 10% B, a gradient to 40% was concluded at 1.5 min, ramped to 50% B at 4 min, ramped to 95% B at 5 min, and held for 13 min. All injection volumes were 10 μL, column temperature was 50 °C, and the flow rate was 0.25 mL/min. Declustering potential, entrance potential, and collision energy were optimized for each analyte. Samples were run in MRM mode, scanning for transitions depicted in **Table 1**.

Data Analysis. Metabolic profiling was performed using the API 4000 Qtrap, whose hybrid Q3/linear ion trap allows many more transitions scanned than the API 2000 with higher sensitivity and MS^n capability. LightSight software was used to measure response levels of clomazone and products. This software has an embedded database of metabolic LC/MS/MS transition shifts for phase I and II metabolic processes, similar to those outlined by Holcapek, et al. (*15*). Clomazone-derived metabolites were identified by observing the mass shift from the parent and presence of diagnostic chlorine fragmentation (M + 2). To confirm that the transitions observed resulted from microbial degradation, treatment response was measured relative to that of the control and blanks.

For target analysis, quantification was via internal standard calibration with $^{13}C_6$ clomazone (compound **6**), the internal standard for all analytes. Water was the solvent for anaerobic standards, but due to ion suppression in aerobic samples at low (< 0.1 μg/mL) levels, aerobic standard curves were matrix-matched. R^2 (quadratic regression) exceeded 0.99 for all analytes. The limit of quantification for each analyte was 0.02 μg/mL (20 ng on column) or 1% of initial application in the water fraction. Responses observed below this level were recorded but not quantified.

Statistical Analysis. For clomazone and metabolite quantification, a pseudofirst-order degradation model was assumed. Dissipation half-lives ($t_{1/2}$), the time taken for clomazone to be reduced to 50% of its original value, were determined from regression analysis of log mean pesticide concentration versus time (days). Differences between treatment and control were determined using an ANCOVA test for equality of slope at 95% confidence.

RESULTS

LC/MS/MS Fragmentation Mechanism. When undergoing LC/MS/MS fragmentation, clomazone was found to fragment as *m/z* 240 → 125, with cleavage to a 2-chlorobenzyl moiety believed to be the Q3 base peak. This is consistent with previous LC/MS/MS analysis of clomazone (BfR Germany, unpublished data). **Table 1** depicts mass transitions and their respective mass shifts from clomazone which gave a significant response. Anaerobically, three metabolic transitions were found: *m/z* 242 → 125, *m/z* 256 → 125, and *m/z* 256 → 141. The first two were identified as compounds **2** and **3**, as confirmed by HPLC retention time. However, the *m/z* 256 → 141 retention time differed from that of the 3′ aromatic hydroxyclomazone standard. This leads us to believe that hydroxylation occurred at a different, unspecified aromatic position (compound **4**). Aerobically, five metabolic transitions were found: *m/z* 242 → 125, *m/z* 256 → 125, *m/z* 256 → 141, *m/z* 272 → 141, and *m/z* 268 → 125. The first three are identical to those identified in the anaerobic microcosms. The *m/z* 272 → 141 peaks were unresolved (not shown), potentially due to hydroxylation at various aromatic positions, similar to that with compound **4**. Nonetheless, the retention time was similar to that of compound **5**. The latter $[M + 28 + H]^+$ is an unknown; potential candidates include diketo formation at unspecified positions, dimethylation, or ethylation. Since no rational precursor to diketo was observed, it is an unlikely metabolite.

Metabolite Quantification. The results of spike recovery on soil surface are depicted in **Table 1**. Clomazone and its metabolites fortified at both 0.1 μg/mL and 1.0 μg/mL (water concentration basis) gave good, reproducible responses, with the possible exceptions of compounds **3** and **5**, whose responses were observed consistently low, possibly due to soil sorption or ion suppression. Reported concentrations were corrected to 1.0 μg/mL spike recovery efficiency (**Table 1**). A pilot study of the flooded soil revealed the samples to be highly anaerobic, $E_h < -200$ mV after 14 days of incubation (data not shown). This result is similar to that of Gunasekara et al. (*15*), who achieved anaerobic status in soil after only 7 days.

Figure 1 shows the degradation of clomazone and the appearance of compound **2** under anaerobic conditions, and clomazone degradation under aerobic conditions in Biggs soil. Each data point represents the sum of water-dissolved and soil-bound fractions of each sample averaged over five replicates (treatment) or three replicates (control), with error bars ± 1 SD. Data is presented as percent of application on a molar basis. Under anaerobic conditions, clomazone degraded rapidly, forming primarily compound **2**, which reached 67.4% abundance at 38 days. Aerobically, clomazone degraded more slowly, with 37.4% remaining at 83 days. Trace amounts of compounds **3** and **4** were observed in anaerobic samples, and compounds **3**, **4**, and **5** were found in aerobic samples. All products of hydroxylation in both aerobic and anaerobic samples were observed below quantification limits, constituting < 1% of the application amount. No other metabolites were found at concentrations above 0.02 μg/mL in either the water or the acetonitrile fractions.

Pseudofirst-Order Degradation Model. The treatment data for aerobic and anaerobic clomazone degradation are plotted in **Figure 2**, on a semilog scale, with C_0 = 100%. Anaerobic and aerobic samples reached equilibrium at 38 and > 83 days, with half-lives calculated at 7.9 and 47.3 days, respectively. Because of some degradation observed in the anaerobic control samples after 19 days, two regressions are plotted that include data through 19 and 38 days for both treatments and controls. However, for both aerobic and anaerobic conditions, differences between treatment and control response were highly significant ($p < 0.0001$).

DISCUSSION

Clomazone degrades via microbial processes readily under anaerobic conditions but only slowly under aerobic conditions; both processes appear to be pseudofirst order. The anaerobic conversion of clomazone to compound **2** was evident immediately (**Figure 1A**), with the metabolite appearing at 3 days, reaching a maximum of 67.4% clomazone application at 38 days, and the concentration slowly decreasing. Clomazone has been shown to be persistent in aerobic soils, and in the southeastern US, it has been observed to readily volatilize if fields are not kept moist (*11*). No aerobic metabolites were observed above LOQ.

Some clomazone degradation and compound **2** formation were observed in anaerobic samples after 19 days, possibly due to the stimulation of spores resistant to autoclaving, which has been previously documented in rice field soil (*16*, *17*). To illustrate the potential impact this may have on first-order kinetic calculations, two regression lines are plotted in **Figure 2**: clomazone degradation through (A) 19 days (no observed parent degradation) and (B) 38 days (equilibrium time). A and B produce $t_{1/2}$ = 7.9 and 8.0 days, respectively, but differ in calculated degradation rates at later time points. At 14 days (recommended holding time), A produces an estimate of 26.2% clomazone remaining, while B calculates 19.4%. Note that significant degradation occurred in the cases of both A and B, and with similar calculated values, this discrepancy is negligible within the 14 days clomazone is actually held in the field. However, this discrepancy widens at later time points. Nonetheless, soil sterility remains problematic, as studies that attempt

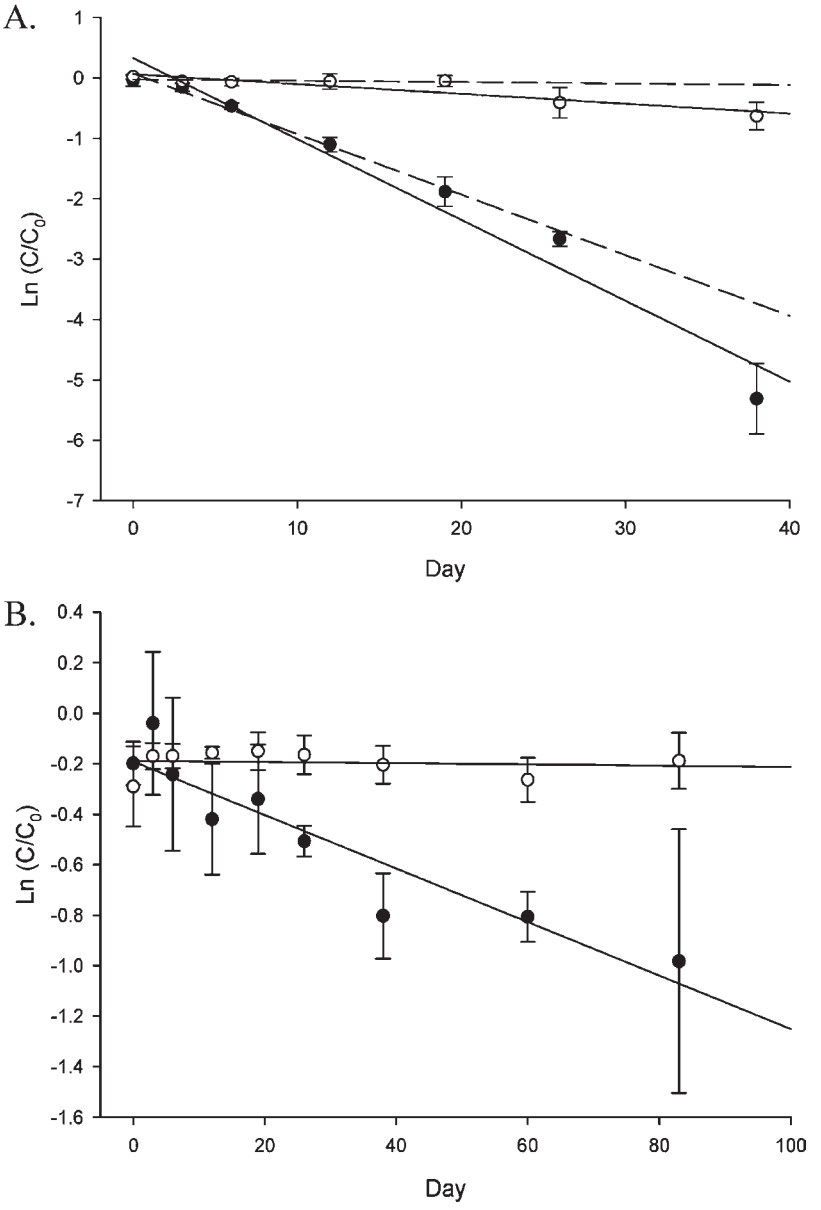


Figure 2. Pseudofirst-order degradation kinetics for clomazone treated soil (●) and autoclaved controls (○) under anaerobic (**A**) and aerobic (**B**) conditions. Anaerobic regression lines are expressed for 0−19 days (dashed line) and 0−38 days (solid line). Data points are mean values ± 1 SD (controls, $n = 3$; treatments, $n = 5$).

to quench microbial degradation without altering physicochemical characteristics often raise more questions than provide answers (*18*).

The strong preference of anaerobes to reduce clomazone is shown by the predominance of reduction at the N−O bond on the oxazolidinone ring, this being more favored than hydroxylation at any position. This preference is a function of both the susceptible functional group and specific enzymes that target them. Soil organic matter and/or reduced inorganic compounds are likely electron donors for this reaction, with clomazone the electron acceptor and compound **2** the reduced result. Anaerobic reduction of N−O bonds in oxazine rings was documented by Klier et al. (*19*), who noted highly consistent, specific reduction at this position and proposed their findings as a synthetic technique. Liu et al. (*9*) proposed a two-step cytochrome-P450-mediated transition of clomazone to compound **2**, with compound **3** as the intermediate. The mechanism of this reaction remains debatable; with a lack of significant compound **3** concentration, compound **2** formation appears to occur as a direct result of clomazone fermentation. A hazard assessment performed by Quayle, et al. (*10*) identified clomazone as presenting a low environmental hazard; however, the toxicity of compound **2** to plants, animals, and the environment is generally unknown, and future work may need to be directed toward this if clomazone is to remain a widely used herbicide.

Cytochrome-P450-mediated hydroxylation was anticipated to be a major factor in aerobic biodegradation, but this is probably untrue, as no predominant metabolite prevailed in aerobic batches. Small (< 0.02 μg/mL) concentrations of compounds **2**−**5** and an unknown transition (m/z 268.0 → 125.0) were observed in the metabolic profiling experiment. These compounds may be relevant to plant uptake and subsequent metabolic transformation, but from a regulatory viewpoint, their concentrations are not seen as significant. Furthermore, the low rate of metabolism may be explained in part by partitioning effects on bioavailability. **Figure 3** depicts the concentration of clomazone found in the

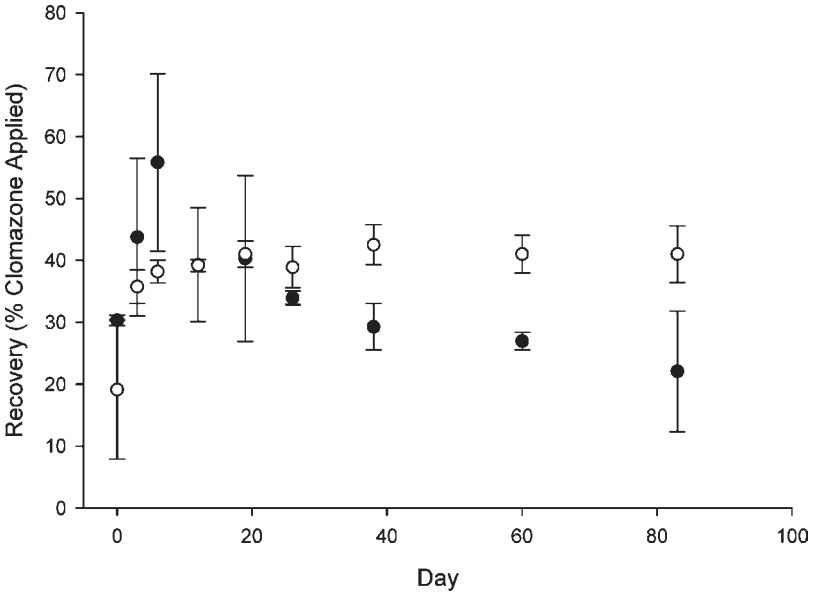


Figure 3. Aerobic acetonitrile extraction (soil-bound residue) recovery for clomazone treated soil (●) and autoclaved controls (○). Data points are mean values ± 1 SD (controls, $n = 3$; treatments, $n = 5$).

acetonitrile (soil-sorbed) fractions of aerobic samples. From 0 to 6 days, an increase in soil partitioning was evident as clomazone equilibrated with the sorption sites. Later, however, the applied concentration decreased due to biodegradation, potentially causing clomazone to desorb, becoming bioavailable. Controls reached steady state after 6 days, and the concentration did not decrease. The rates of sorption between treatment and control are believed to differ due to differences in soil organic matter resulting from autoclaving. Since labile carbon is less bioavailable in aerobic versus anaerobic microcosms, in a carbon-deficient system such as clay loam, soil microbes may degrade the pesticide further, producing fewer observable products. This illustrates the need for a better understanding of the complex biotic interactions to further comprehend how the pesticide is degraded, as soil microbes is too general of a term to be treated as an independent, reproducible reaction catalyst. Future work will be aimed at determining the mass-balance for clomazone biodegradation via use of ^{13}C-labeled clomazone; mineralization rates, biomass turnover, and identification of the active microbes will be determined.

In conclusion, anaerobic degradation in rice fields is likely to significantly contribute to the dissipation of clomazone. The herbicide degraded rapidly to compound **2** under anaerobic conditions but only slowly aerobically, mostly forming soil-sorbed residues. In rice fields, as soil redox potential decreases, clomazone degradation and compound **2** formation rates are expected to increase. These transformations are microbially mediated, as confirmed by comparison with autoclaved controls. This investigation also illustrates the capabilities of LC/MS/MS metabolic profiling and provides regulators and growers alike with the information necessary to make informed decisions on clomazone management.

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