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COMPREHENSIVE RESEARCH ON RICE
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PROJECT TITLE: The Microbial Degradation of Pesticides Important to Rice Culture

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OBJECTIVES AND EXPERIMENTS CONDUCTED BY LOCATION TO ACCOMPLISH OBJECTIVES:

Objective I. To identify soil amendments that significantly effect the microbial dechlorination of thiobencarb associated with Delayed Phytotoxicity Syndrome (DPS). Emphasis will be placed on the role of incorporated carbon (straw) in both DPS susceptible and non-DPS susceptible soils.

Objective II. To characterize the time taken for oxidative degradation of the toxic dechlorinated product, deschlorothiobencarb, following a simulated emergency field draining. Emphasis will be placed on the role of incorporated carbon.

Objective III. To identify the soil concentration of deschlorothiobencarb that induces dwarfing in rice plants.

Objective IV. To explore potential soil treatments that will reduce the dwarfing of rice plants following thiobencarb application. Emphasis will be placed on chemicals (such as methoxyphenone or BNA-80) that either delay or completely inhibit the onset of DPS.

SUMMARY OF 2001 RESEARCH (MAJOR ACCOMPLISHMENTS) ACCOMPLISHMENTS BY OBJECTIVE:

Objective I. Thiobencarb is the suspected cause of DPS in rice plants. While the definitive toxic agent appears to be deschlorothiobencarb, soil conditions contributing to its formation have not been clearly identified. Two California rice field soils with different physical-chemical properties were examined for the ability to reductively dechlorinate thiobencarb. One soil from the Eastern Sacramento Valley (Mathews/Van Dyke Farms) has historically displayed DPS, while the

second, from the Western Valley (Canal Farm), has not shown susceptibility to DPS. In addition, to determine the influence of organic carbon on deschlorothiobencarb production, various amounts of rice straw were incorporated into each soil.

Soils were crushed and passed through a 2 mm sieve, followed by the addition of varying amounts of rice straw (0.0, 0.33 and 2.0 weight %) to mimic field conditions. A 0.33%-straw amendment represented a disced field, and 2.0%-straw represented a rolled field. Straw/soil mixtures (4g) were aliquoted into test tubes (16 x 100 mm), flooded with 5 mL of well-water and homogenized. Headspaces were purged, and the tubes were tightly sealed with caps and wrapped in aluminum foil to avoid photolysis. The soil was pre-incubated in a dark nitrogen gas tent at 27.5 °C for 7 days, after which 50 µL of 0.8 µg/µL thiobencarb in methanol was added to each sample and homogenized to achieve a 10-ppm concentration. The samples were placed in a dark, nitrogen gas-filled environmentally-controlled culture chamber where the temperature was cycled between 30°C (16 h) and 27°C (8 h) to mimic day-night field conditions in the Central Valley. Replicates (n=3) of each sample type were extracted after designated periods (0, 5, 10, 15, 20, 30, 45, 60, and 90 days after thiobencarb addition). Controls contained 4.0g of soil with 2.0%-straw amendment and were autoclaved at 120°C for 90 min prior to incubation. On each extraction day the redox potentials were measured in both the sample replicates and in the redox controls to verify anaerobic conditions.

Soil extractions included the addition of 1 mL of methanol (pesticide grade) to each tube, followed by 5 mL of hexane; they were then vortexed at high speed for 3 min. Following centrifugation at 500 g for 5 min, the supernatant was collected. Soils were re-extracted twice more with 5 mL of hexane and the extract solutions were combined, dried over anhydride magnesium sulfate column, and concentrated to 5 mL under nitrogen gas.

Analyses were performed on a gas chromatograph (GC) coupled to a mass spectrometer. The GC was equipped with a ZB-50 capillary column (30 m x 0.25 mm ID x 25 µm film thickness). Helium (0.6 mL/min) was the carrier gas, the injector temperature was 250 °C, and the detector temperature was 280 °C. The GC oven was initially set at 100 °C for 1 min, then ramped at 20 °C/min to 270 °C and held there for 1 min. The mass spectrometer was operated in the selective ion monitoring mode.

The production of deschlorothiobencarb in the Eastern Valley soil (no-straw amendment) occurred after 20 days, and the half-life of thiobencarb was 19.6 days (Fig. 1A). The Western Valley soil (no-straw amendment) did not produce deschlorothiobencarb until after 35 days (Fig. 1B). Based on the concentration of deschlorothiobencarb produced it is possible to calculate a minimum amount of conversion of thiobencarb to deschlorothiobencarb. The results are as follows: 59% for Eastern Valley soil and 53% for Western Valley soil.

With straw at 0.33%, representing a disced field, formation of deschlorothiobencarb in the Eastern Valley soil occurred after a 5-day lag, and the thiobencarb degradation half-life was 11.2 days (Fig. 1C). The Western Valley soil began producing deschlorothiobencarb at 20 days, with a thiobencarb degradation half-life of 27.7 days (Fig. 1D). Minimum conversion of thiobencarb to deschlorothiobencarb was 74% and 67% for Eastern and Western Valley soil respectively.

Finally, the 2.0 %-straw amendment, representing a rolled field, in the Eastern Valley soil showed a marked decrease in the amount of thiobencarb; its half-life was 5.7 days, with no

apparent lag period for deschlorothiobencarb formation (Fig. 1E). Conversely, the Western Valley soil generated deschlorothiobencarb at 35 days with a half- life of 31.2 days (Fig. 1F). The minimum conversion of thiobencarb to deschlorothiobencarb was 70% and 33% for Eastern and Western Valley soils, respectively.

Deschlorothiobencarb was produced in both soils, with and without straw amendments. Although Western Valley rice fields have not shown DPS symptoms, our experiments indicated deschlorothiobencarb formation is possible. This result is contrary to previously published studies, where deschlorothiobencarb production was limited to specific soil conditions and amendments of organic substances [1]. However, in previous studies anaerobic conditions were not tightly controlled or verified using redox measurements.

Our results show a significant delay in deschlorothiobencarb formation between the two soils. Overall the Eastern Valley samples, which are prone to DPS, displayed lag times that ranged from 0-20 days, while the Western Valley samples displayed lag times that ranged from 20-35 days. In the Eastern Valley samples, the delay was correlated with the percentage of straw amendments (Figs. 1A, 1C, 1E). The overall magnitude of deschlorothiobencarb production was similar for all three straw amendments. In addition, thiobencarb began to degrade from day zero via a zero-order decay rate. However, in the Western Valley samples the relationship between straw amendments and lag time was not as clear. The 0.33%-straw amendment exhibited a delay of 20 days, while both the no-straw and 2.0%-straw amendments had lag times of 35 days (Figs. 1B, 1D, 1F). This relationship between straw content and lag time is complicated in that both the no-straw and 0.33%-straw amendments showed little degradation of thiobencarb before day 15, whereas the 2.0%-straw amendment exhibited an almost immediate degradation of thiobencarb.

Although dechlorination can occur abiotically, the observation that autoclaved soil did not produce deschlorothiobencarb during the 90-day incubation period indicates that the product was reduced via microbial activity. For years it has been known that anaerobic microorganisms can reductively dehalogenate aromatic rings [2-8].

In terms of field management, rolling straw (2.0% amendment) onto rice fields would produce the most rapid degradation of thiobencarb, regardless of microorganism or soil type. In the case of the Eastern Valley soil the larger the amount of organic carbon the faster the formation of deschlorothiobencarb. The best-case scenario for the Eastern Valley farmers would be to remove straw from the field, which should prolong the effects of the herbicide while delaying the dechlorination of thiobencarb, hence minimizing DPS.

Objective II. This objective was designed to: 1) determine the rate of formation of deschlorothiobencarb in a flooded California field; and 2) determine the rate of dissipation of deschlorothiobencarb after emergency field drainage, such as when DPS symptoms first appear.

Eastern Valley soil (Mathews/Van Dyke Farms) was sieved to 2 mm and was stored refrigerated, until use. The same methods described above were used, with minor modifications. Some 4 g of moist soil plus 80 mg rice straw were placed in 16 X 100 mm test tubes. Tubes were flooded with 5 mL of well water and homogenized. After purging with nitrogen gas, tubes were tightly capped and covered in aluminum foil. To establish anaerobic conditions, soil was pre-incubated for 7 d in the dark on a 30°C/25°C day/night cycle. Soil was then spiked with thiobencarb at a rate of 38.9 nmol/g and tubes were again purged with nitrogen gas and incubated in the dark with

the day/night temperature cycle as described above. Enough tubes were prepared to allow triplicate analysis at each time point.

At 0, 1, 3, 6, and 12 days after thiobencarb addition, soils were analyzed for thiobencarb and deschlorthiobencarb. On days 3, 6 and 12, water was drawn off of 9 tubes each, which were returned, uncapped to the incubator to mimic an emergency field drainage. Three dry tubes were analyzed each week for 3 weeks post water-removal. Water removed from tubes was analyzed separately for mass balance.

For deschlorothiobencarb half-life determination, tubes of soil and water were prepared as above. Twelve days after spiking with thiobencarb, water was removed and tubes were returned, uncapped, to the incubator. At 0, 0.5, 1, 2, 4 and 7 days post water-removal, soils were analyzed for thiobencarb and deschlorothiobencarb. All soil and water samples were extracted and analyzed described above.

The rate of deschlorothiobencarb formation in soil was determined by plotting ln C/ln C_0 vs time for days 6, 12, 19, and 26 days after thiobencarb spiking (no detectable deschlorothiobencarb was formed prior to day 6). The r^2 value of 0.97 for the regression line indicates a first-order reaction. The slope of the line gives the rate constant, k, of 0.324 wk^{-1}. This is not a rate constant that can be applied generally, because it was derived under conditions intended to produce maximum deschlorothiobencarb in a short time (susceptible soil, 2% rice straw).

The thiobencarb degradation rate in flooded, anaerobic conditions was determined by plotting ln C/ln C_0 vs. time. The regression line indicates that the process is first-order, with $k = 1.14$ wk^{-1}. The half-life for thiobencarb can be calculated, $t_{1/2} = \ln 2/k = 0.6$ wk, or 4.2 days.

Rates of dissipation of deschlorothiobencarb varied depending upon when water was removed from the flooded system. Figure 2 compares results obtained when water was removed 3, 6, and 12 days after spiking with thiobencarb. Deschlorothiobencarb was not detectable 3 d after spiking, but during the first week without water deschlorothiobencarb rose to 5 nmol/g, and then degraded to 0.9 nmol/g during the following week. Some 7.6 nmol/g of deschlorothiobencarb had formed 6 d after thiobencarb spiking, and in the first week after water removal, the level spiked to 17.5 nmol/g, then dropped to 1.7 nmol/g after two weeks without water. When water was removed 12 d after spiking, 11.5 nmol/g of deschlorothiobencarb had formed. The level rose to 14.6 nmol/g and degraded to 2.1 nmol/g after two weeks without water. In all cases, deschlorthiobencarb levels were reduced more than 80% after two weeks without water.

Objective III. Goal of this study was to determine at what concentration deschlorothiobencarb causes delayed phytotoxicity syndrome (DPS) in rice plants.

Since no measurements currently exist describing the no effect level (NOEL) and median effect level (ED50) of deschlorothiobencarb in rice, a series of pilot experiments were set up to evaluate the most efficient way to conduct a toxicity study. First, bench space was obtained at the UCD Orchard Park Greenhouse facility. Initially, pre-germinated rice seeds were cultured in glass jars containing some 200 grams autoclaved soil each. After the plants reached the 3- to 4-leaf stage aqueous deschlorothiobencarb was applied to produce a homogenous mixture doing of the soil. Rice plants were thinned out to give a total of four plants per jar. Twenty-four hours after dosing, a soil plug was taken from each jar, weighed, then extracted (using the same

methods as described above). However, chemical analysis (see above) indicated that the applied deschlorothiobencarb might have volatized prior to incorporating into the soil.

Since flooding proved to be an inefficient way to dose, it was determined that several injections directly into the soil around the rice plants might provide enough homogeneity of dosing while insuring soil incorporation of the deschlorothiobencarb. However, upon chemical analysis of the soils it was determined that the resultant lack of homogeneity was not sufficient to provide for adequate dosing of the rice plants.

Finally, it was decided to try hydroponic culturing of the rice plants in individualized tubes (one seedling/tube). This allowed for the efficient, homogeneous dosing of the rice plants upon reaching the 3- to 4-leaf stage. Using simulated field conditions for temperature and light-dark cycling (see above) a pilot was set up using the following rangefinding doses:

15.0 ug/mL (the highest amount measured in soils described in Objective 1)
7.5 ug/mL
3.75 ug/mL
1.88 ug/mL
0.0 ug/mL (controls)

Rice plants were observed for decreased growth, color changes, and increased tillering, and initial results indicate the NOEL may be somewhere between the two highest doses, which correlates with the highest deschlorothiobencarb levels measured in our simulated field study (Objective 1). A complete toxicity assessment will be done during the next year, now that the hydroponic dosing system has been developed.

Objective IV. Since the correlation of organic carbon with formation of DPS-causing deschlorothiobencarb was so strong, it was decided to focus more of our efforts on determining what natural soil characteristicss were most likely involved so that natural conditions most likely to produce DPS could be avoided.

To characterize the soils, samples of the two soils (used in Objective 1, above), plus a number of other soils (both DPS prone and DPS resistant), were sent to the Division of Agriculture and Natural Resources (DANR) Analytical Laboratory, where a number of soil analyses were performed. Ca and Mg were quantified using inductively coupled plasma-atomic emission spectroscopy (ICP-AES), while K and Na were quantified using atomic absorption spectrometry (AAS). Iron was extracted using diethlenetriminepentacetyic acid (DPTA) [9] and quantified by AAS. The cation exchange capacity (CEC) was calculated from barium displacement of cations [10], with barium concentration being determined by ICP-AES. Total nitrogen and carbon were determined by a modified Dumas method [11] using a Carlo-Erba 1500. Total Kjeldahl Nitrogen was determined by wet oxidative digestion and quantification of ammonium by the diffusion-conductivity method [12]. Nitrates and ammonium were extracted with KCl and determined by the diffusion-conductivity method [12]. Organic matter was determined using the Walkley-Black method [13] using a UV/VIS spectrometer (600 nm). Phosphate values were determined by the Olsen method [14] and reaction with *p*-molybdate and flow injection analysis. Finally, particle size analysis was determined by a hydrometer [15].

Upon analysis, the Eastern valley soil was considered a loam because its composition was within 7 to 27% clay, 28 to 50% silt, and less than 52% sand [16], while the Western Valley soil was considered a silty-clay due to its relatively high amounts of clay and silt (Table 1). These soils were found to be comprised of very different amounts of iron, organic carbon, total nitrogen, potassium, calcium, magnesium, sodium and ammonia. However, not enough different soil types or analyses were performed to statistically determine that differences in their characteristics were correlated to formation of DPS. In general, the Eastern Valley soil described in Objective 1 had smaller amounts of organic carbon, total nitrogen, ammonia, potassium, calcium, magnesium, and sodium. Previous studies, comparing 17 soils, indicated that soils lacking proper conditions did not show deschlorothiobencarb formation [17]. Moon *et al.* [18] observed that dechlorination activity was correlated to high amounts of phosphorus, iron and the ratio of carbon to nitrogen in the soil. Comparing these factors in the two soils there is no clear trend. The Western Valley soil has a high carbon to nitrogen ratio but a lower amount of iron and phosphorus. However, the heterogeneity and high variability within soil samples leave room for more investigation as to the necessary nutrient balance to produce the least amount of deschlorothiobencarb. We plan to obtain additional soil samples from DPS-prone and resistant areas and further investigate the correlation between characteristics and DPS production during the next year.

PUBLICATIONS OR REPORTS: Two scientific manuscripts are currently being prepared.

1. Schmelzer, K. R., Johnson, C. S., Viant, M. R., Williams, J. F., Tjeerdema, R. S., and Crosby, D. G. Influence of organic carbon in the reductive dechlorination of thiobencarb (Bolero) in California rice field soils. In preparation for *J. Agric. Food. Chem.*

2. TenBrook, P. L., Schmelzer, K. R., Palumbo, A., Williams, J. F., Tjeerdema, R. S., and Crosby, D. G. Degradation of deschlorothiobencarb in drained California rice field soils. In preparation for *J. Agric. Food. Chem.*

CONCISE GENERAL SUMMARY OF CURRENT YEAR'S RESULTS:

1. An investigation into the cause of Delayed Phytotoxicity Syndrome (DPS) in rice was initiated, as DPS has been a serious problem in some parts of the eastern Central Valley of California for many years. DPS appears to be caused by deschlorothiobencarb, produced via the dechlorination of the rice herbicide thiobencarb (Bolero) by anaerobic soil bacteria.

2. In soils modeled after typical flooded California rice field conditions, dechlorination of thiobencarb occurs within as little as 5 days after herbicide application. Production of deschlorothiobencarb is directly correlated to organic carbon (straw) content of the soil, as increased straw content stimulates earlier deschlorothiobencarb production – as little as 5 days after thiobencarb application.

3. In rice field soils modeling emergency water drainage after an observation of DPS, it was discovered that during the first week following drainage soil concentrations of deschlorothiobencarb continue to increase, as anaerobic conditions continue to exist for some time. Only after one week do soils become aerobic enough that deschlorothiobencarb is oxidatively degraded. Therefore, signs of DPS would not likely begin to reverse until more than a week has passed following emergency field drainage.

4. While still preliminary, hydroponic rice plant toxicity rangefinding experiments indicate that the no effect level (NOEL) of deschlorothiobencarb is probably between 7.5 and 15 ug/mL. This concentration range was also found produced by bacteria in our modeled anaerobic soil experiments, thus correlating not only the production of deschlorothiobencarb to anaerobic conditions, but also toxic effects to rice at the same concentrations.

5. While soil organic carbon content is strongly correlated with production of deschlorothiobencarb, thus also likely DPS, other natural soil characteristics may also either enhance or inhibit the formation of DPS. However, an insufficient number of soils and samples were analyzed this past year to statistically draw any conclusions. This work will be continued next year.

6. When considering rice field management, the obvious recommendation is to minimize the amount of straw incorporation prior to flooding the fields. In addition, when performing an emergency field drainage, assume at least a one week delay, and probably more, before the signs of DPS begin to reverse.

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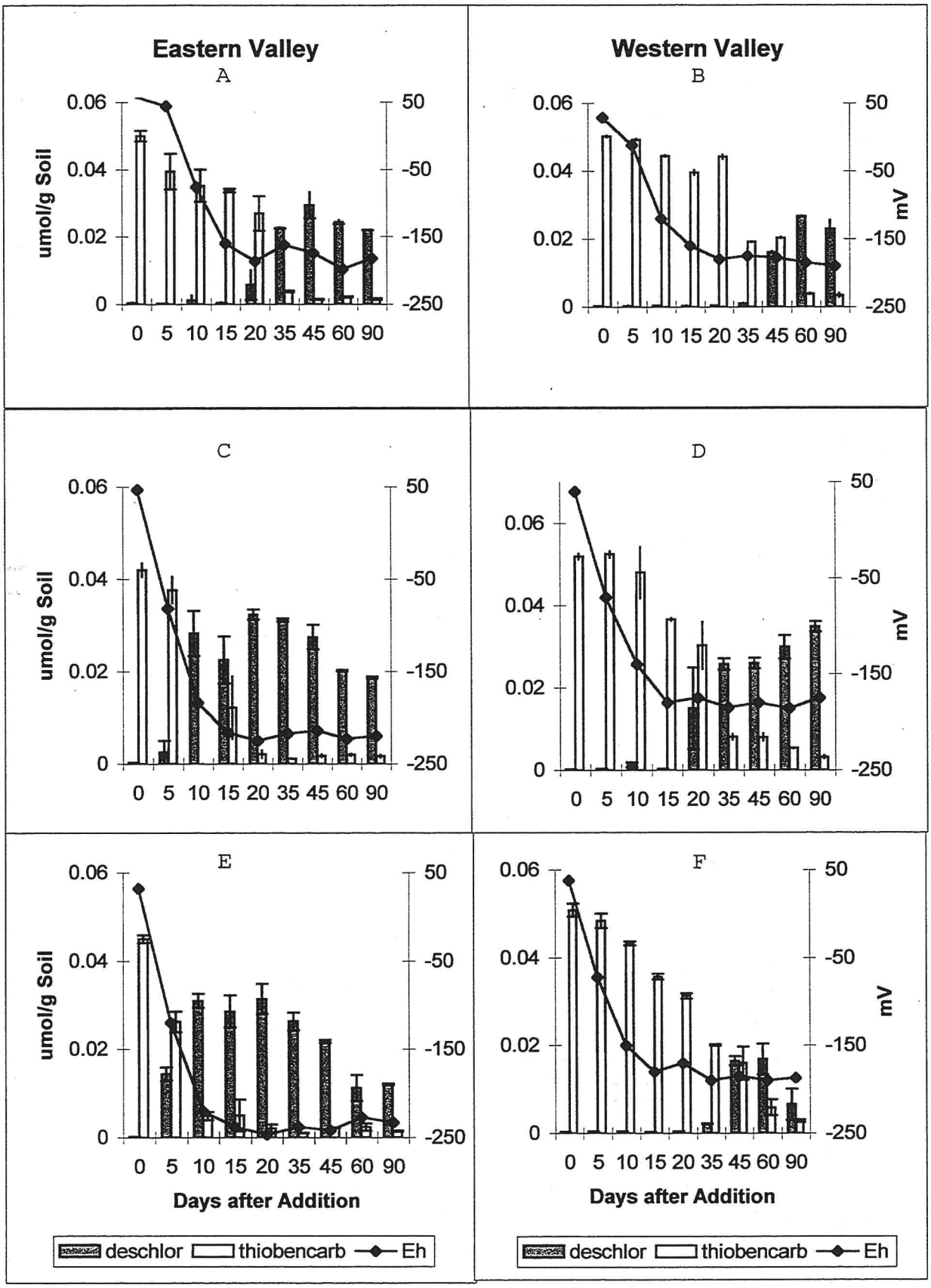
Table 1. Soil Characteristics

Parameters	Eastern Valley Soil	Western Valley Soil
pH	6.2	6.2
EC mmhos/cm	1.70	1.05
Fe ppm	247	216
CEC meq/100g	19.4	45.8
OM %	2.12	2.87
C-Org %	1.23	1.66
N-TOT %	0.11	0.19
C-Tot %	1.08	2.05
TKN %	0.111	0.161
NH4-N ppm	15.1	31.0
NO3-N ppm	73.4	0.9
P ppm	18.9	11.9
Exch-K. meq/100g	0.3	0.7
Exch-Ca meq/100g	5.9	17.5
Exch-Mg meq/100g	3.6	14.0
Exch-Na meq/100g	0.2	2.4
Sand %	36	4
Silt %	48	49
Clay %	16	47

Figure 1. Changes in thiobencarb and deschlorothiobencarb over time. Graphs A and B, no straw; graphs C an D, 0.33% straw; Graphs E and F, 2% straw.



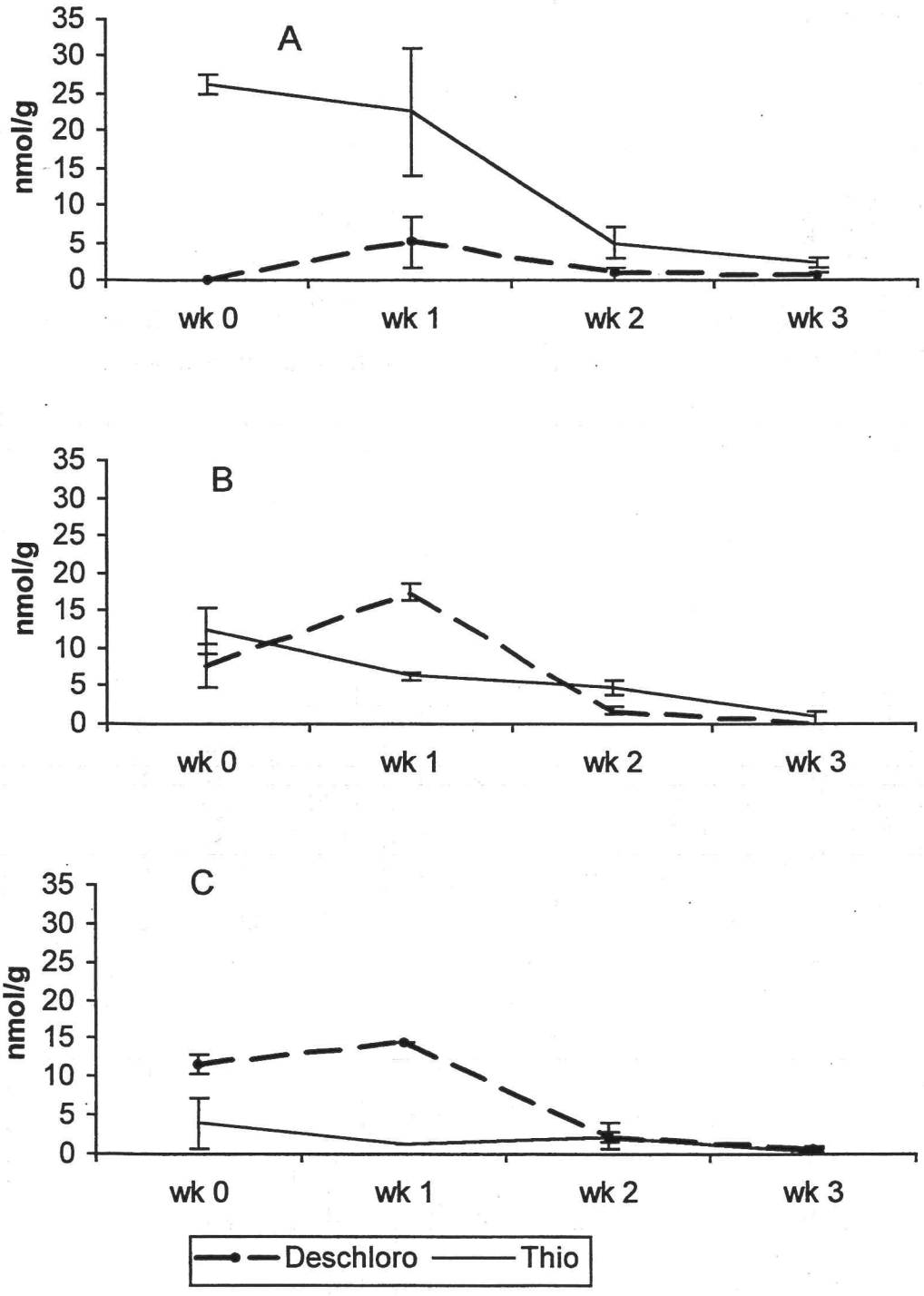


Figure 2. Thiobencarb and deschlorothiobencarb levels after water removal. A) water removed 3 days after thiobencarb application; B) water removed 6 days after thiobencarb application; C) water removed 12 days after thiobencarb application.