ACUTE ENVIRONMENTAL TOXICITY AND PERSISTENCE OF DEM, A CHEMICAL AGENT SIMULANT: DIETHYL MALONATE

Final Report

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DEM is not phytotoxic at foliar mass loading levels of less than $10 \mu\text{g}/\text{cm}^2$. However, severe damage is evident at mass loading levels in excess of $17 \mu\text{g}/\text{cm}^2$. Tall fescue and sagebrush were more affected than was short-needle pine, however, mass loading levels were markedly different. Regrowth of tall fescue indicated that the effects of DEM are residual, and growth rates are affected at higher mass loadings through the second harvest.

Results from in vitro testing of DEM indicated concentrations below $500 \mu\text{g}/\text{g}$ dry soil generally did not negatively impact soil microbial activity. In fact, Palouse soil dehydrogenase activity was initially enhanced by the addition of DEM at 25 to $500 \mu\text{g}/\text{g}$. Short-term effect of DEM (2-3 days incubation) was more profound on soil dehydrogenase activity ($\text{EcD}_{50} \approx 2500 \mu\text{g}/\text{g}$) than on soil phosphatase activity ($\text{EcD}_{50} > 2500 \mu\text{g}/\text{g}$), indicating soil dehydrogenase is more susceptible to DEM than soil phosphatase. However, no enzyme inhibition or enhancement was observed after 28 days incubation, indicating the effect is transient in nature with a possibility of recovery.

Results of the earthworm bioassay indicate survival to be 86 and 66% at soil doses of 107 and $204 \mu\text{g DEM}/\text{cm}^2$, respectively.

FOREWORD

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EXECUTIVE SUMMARY

The purpose of the following chemical simulant studies is to assess the potential acute environmental effects and persistence of diethyl malonate (DEM). Studies were designed to provide baseline data related to the effects of DEM on terrestrial organisms, namely plant, soil microorganisms and earthworms, and to assess the relative depuration from environmental surfaces. Deposition velocities for DEM to soil surfaces ranged from 0.04 to 0.2 cm/sec, for low and high air concentrations. For foliar surfaces, deposition velocities ranged from 0.0002 cm/sec at low air concentrations to 0.05 cm/sec for high dose levels. Collection efficiency was lowest for short-needle pine and greatest for sagebrush and tall fescue.

DEM deposited to soil and foliar surfaces is rapidly lost through volatilization processes. The residence times or half-lives of DEM deposited to soils was 2 h for the fast component and 5 to 16 h for the residual material. DEM deposited to foliar surfaces also exhibited biphasic depuration. The half-life of the short residence time component ranged from 1 to 3 h, while the longer time component had half-times of 16 to 242 h. Volatilization and other depuration mechanisms reduce surface contaminant levels in both soils and foliage to less than 1% of initial dose within 96 h.

DEM is not phytotoxic at foliar mass loading levels of less than 10 $\mu\text{g}/\text{cm}^2$. However, severe damage is evident at mass loading levels in excess of 17 $\mu\text{g}/\text{cm}^2$. Tall fescue and sagebrush were more affected than was short-needle pine, however, mass loading levels were markedly different. Regrowth of tall fescue indicated that the effects of DEM are residual, and growth rates are affected only at higher mass loadings through the second harvest. At higher dose levels *in vitro* studies failed to indicate any effects of DEM on photosynthesis/respiration or electron transport at solution concentrations of 100 $\mu\text{g}/\text{mL}$.

Results from *in vitro* testing of DEM indicated concentrations below 500 $\mu\text{g}/\text{g}$ dry soil generally did not negatively impact soil microbial activity. Short-term effects of DEM were more profound on soil dehydrogenase activity than on soil phosphatase activity, indicating soil dehydrogenase is more susceptible to DEM than soil phosphatase. However, no enzyme inhibition or enhancement was observed after 28 days incubation, indicating the effect is transient in nature with a possibility of recovery.

Results of the earthworm bioassay indicate survival to be 86 and 66% at soil doses of 107 and 204 $\mu\text{g DEM}/\text{cm}^2$, respectively. At higher dose level, activity or mobility was judged to be affected in over 50% of the individuals.

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ACUTE ENVIRONMENTAL TOXICITY AND PERSISTENCE OF
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1.0 INTRODUCTION

Army Regulation (AR) 200-2 integrates environmental considerations into Army plans and programs. As implemented by the U.S. Army Materiel Command, this regulation requires environmental analyses and documentation for all items developed by subordinate research and development commands. CDREC as the developer is responsible for research to generate environmental data on chemical simulants. The purpose of the research effort is to determine the environmental fate and effects of simulants in terrestrial and aquatic systems. These data are used to support environmental issues on simulant use in testing, trialing, and training.

Chemical simulants are substances whose characteristics partially resemble selected physical and chemical properties of chemical agents. Simulants are used in testing or trialing to determine the performance of equipment. Simulants are also used in training troops to operate equipment, and to perform in a chemically contaminated environment.

The use of chemical agent simulants and decontaminants at both U.S. and foreign training sites has the potential for producing significant environmental impacts. Only limited data on the chemistry and behavior of these materials are available (Reinbold et al. 1986, Howard et al. 1985).

The CRDEC Environmental Fate and Effects Data Base is a compilation of physical, chemical, toxicological and environmental data of selected simulant agents. This data base also contains available data on chemicals employed for decontamination of simulant and agent contaminated equipment. The computerized data base can be accessed through the CRDEC Data Management Office. Reinbold et al. (1986) used this data base to conduct a hazard ranking of simulants. Based on this study, simulants were identified that required laboratory data to determine their environmental persistence and acute terrestrial and aquatic toxicity. Based on the rankings on Reinbold et al. (1986) simulants were selected from those shown in Table 1.1 that required further laboratory data to determine their environmental persistence and acute terrestrial and aquatic toxicity.

Table 1.1 LIST OF CHEMICAL AGENT SIMULANTS AND DECONTAMINANTS CURRENTLY IN USE BY U.S. ARMY

Chemical Material	CAS Number
<u>Simulant Agents</u>	
Bis (2-ethylhexyl) 2-ethylhexyl phosphonate	126-63-6
Bis (2-ethylhexyl) phosphonate BIS)	3658-48-8
n-Butyl mercaptan (BUSH)	109-79-5
2-chloroethyl ethyl sulfide (CEES)	693-07-2
Diethyl adipate	141-28-6
Di (2-ethylhexyl) phthalate (DOP)	117-81-7
Diethyl hydrogen phosphonate (DEHP)	762-04-9
Diethyl malonate (DEM)	105-53-3
Diethyl phthalate (DEP)	84-66-2
Diethyl pimelate	2050-20-6
Diethyl sebacate (DES)	110-40-7
Diisopropyl fluorophosphate (DFP)	55-91-4
Diisopropyl methylphosphonate (DIMP)	1445-75-6
Dimethyl adipate (DMA)	627-93-0
Dimethyl hydrogen phosphonate (DMHP)	868-85-9
Dimethyl methylphosphonate (DMMP)	756-79-6
Diprophylene glycol monomethyl ether (DPGME)	34590-94-8
Ethanol	64-17-5
Ethyl chloroacetate (ECA)	105-39-5
Biethyl mercaptosuccinate, 0,0-dimethyl phosphorodithioate (Malathion)	121-75-5
Methyl salicylate	119-36-8
Diethyl p-nitrophenyl phosphate (Paraoxon)	311-45-5
Diethyl p-nitrophenyl thiophosphate (Parathion)	56-38-2
Polyethylene glycol 200 (PEG 200)	25322-68-3
Triethyl phosphate (TEP)	78-40-0
Trimethyl phosphate (TMP)	512-56-1
<u>Decontaminants</u>	
Diethylenetriamine	111-40-0
Perchloroethylene	127-18-4
Phenol	108-95-2
Chlorobenzene	108-90-7

The objective of these studies is to determine the potential acute environmental effects and persistence of various classes of agent simulants. This report is one of a series, and it addresses just one of the chemical agent simulants listed in Table 1.1, namely diethyl malonate (DEM). Previous reports (Van Voris et al., 1987; Cataldo et al., 1988; Cataldo et al., 1989a; Cataldo et al., 1989b) described the environmental effects and chemical fate of diisopropyl methylphosphonate (DIMP), diisopropyl fluorophosphate (DFP), and 2-chloroethylethyl sulfide (CEES), bis (2-ethylhexyl) phosphonate (BIS), and triisopropyl phosphite (TIP).

1.1 CHEMICAL PROPERTIES

The simulant, diethyl malonate (DEM) has a viscosity and elastic shear similar to the chemical agents. Thus, it is employed in thermal decontamination studies, shipboard decontamination design, shipboard washdown, contamination transfer, and vehicle entry/exit studies. While a relatively limited data base exists on the fate and effects of DEM, the documents of Bennett et al. (1984) and Howard et al. (1985) contain pertinent information concerning the chemical characteristics, stability, and toxicity of DEM.

DEM, the chemical formula for which is $\text{CH}_2(\text{COOC}_2\text{H}_5)_2$, is an aliphatic dicarboxylic ester. It is liquid at room temperature and has a water solubility of 2.7% at 20°C. Its important physical properties include a boiling point of 199°C, a viscosity of 1.86 centistokes at 25°C, and a vapor pressure of 0.249 mm Hg at 20°C. The compound is relatively chemically stable, and is particularly prone to hydrolysis in aqueous environments to yield ethyl alcohol and malonic acid salts.

1.2 ENVIRONMENTAL FATE AND EFFECTS

The environmental persistence of DEM is dependent on its hydrolysis half-life, which includes acid hydrolysis to ethyl alcohol and malonic acid. DEM is subject to microbial biodegradation as is malonic acid (Pinkham et al., 1978). No reliable data are available regarding the persistence or effects of DEM in the terrestrial environment.

1.3 OBJECTIVES

The purpose of these studies is to provide information about the environmental behavior and effects of the agent simulant, DEM. The scope of these efforts is restricted to assessment of impacts on a limited number of terrestrial organisms based on contact toxicity and the chemical persistence of the simulant in soils and on vegetative surfaces. These studies include only cursory evaluation of major decomposition products.

2.0 MATERIALS AND METHODS

The following methodology and experimental protocols were used in evaluating the environmental effects and chemical fate of DEM applied as an aerosol. Exposure tests were performed under dynamic conditions using a sealed chamber supplied with filtered air and a mixing fan to ensure uniform aerosol concentrations. Plants and soils were exposed to DEM aerosols in the chamber, and aerosols were characterized for air concentration and subsequent calculation of deposition velocities. Exposed plants and soils were sampled periodically following each test and results analyzed to provide dose and persistence information. A study of the influence of DEM on soil microorganisms was performed by amending soils with liquid DEM.

2.1 EXPOSURE SYSTEM

Aerosol exposures of plants and soils to DEM were conducted in a sealed exposure chamber at the Pacific Northwest Laboratory (PNL) Aerosol Wind Tunnel Research Facility. The chamber and test system used were an improved version of the system used to conduct experiments with DIMP and DFP (Van Voris et al., 1987), CEES (Cataldo et al., 1988), and BIS (Cataldo et al., 1989a). Improvements to the test system prior to the DEM tests included doubling the output capacity of aerosol generation, addition of four new aerosol sampling ports, resealing all ports and windows, and reconfiguring the exhaust scrubber and filters.

The exposure chamber, shown in Figure 2.1, was used to contain the aerosol exposures. The exposure region of the chamber was 0.40 m² with a height of 0.6 to 0.9 m. Chamber volume was 0.37 m³. A small electric mixing fan was mounted above the grated floor to provide slow horizontal mixing within the chamber. Mixing velocities were estimated from observations of plant leaf movement to be less than 1 m/sec. Lighting to promote plant respiration during tests was provided by an incandescent lamp mounted at the back window of the chamber.

The exposure chamber was operated at a negative air pressure of 1 cm-H₂O during test and purge periods to contain the DEM aerosols. HEPA-filtered air was supplied to the chamber at a flow rate of about 70 Lpm. The air was first passed through the aerosol generation chamber located on the side of the chamber (Figure 2.1), to serve as a flow for DEM aerosol, and then introduced to the chamber below the level of the metal grating floor panel. Exhaust was drawn from the top of the chamber, passed through a dual-stage liquid scrubber, filters, a flowmeter, and to the facility's exhaust system. In addition to the carrier air flow, about 14 Lpm was introduced at the aerosol generator. The leak rate of the chamber was determined from vacuum tests to be 0.04 ± 0.02 Lpm.

FIGURE 2.1. ACUTE-DOSE AEROSOL EXPOSURE CHAMBER FOR DEM TESTS

2.2 EXPOSURE PROTOCOL

Three tests of DEM aerosols were performed (DEM-01, -02, and -03). DEM-01 was performed as a trial test, and tests DEM-02 and -03 were actual plant and soil exposure tests. Plants, soils, and deposition coupons (47 mm glass fiber filter substrate) were placed into the test section prior to each test. At least one-half of the metal grating floor, itself with a porosity of ~90%, was left uncovered to ensure good aerosol mixing within the chamber. Deposition coupons were suspended horizontally within the plant canopy. Rubber access doors on both the exposure and generation chambers were sealed before each test using clamps. Sealant grease was not required because of the very low leak rate at -1 cm-H₂O. The lamp and mixing fan were turned on and the carrier air flow established. Aerosols were generated by applying compressed air to nebulizers. Tests were 60 min in duration, followed by purge periods of 10 min to allow the chamber to clear of aerosol.

Generation of DEM aerosol was tested during the trial test and rates were adjusted to provide concentrations during the subsequent plant and soil exposures equal to the target concentrations of 150 and 1500 mg/m³. Samples of the aerosol were obtained during the tests using both gas sampling syringes (grab samples) and bubblers to trap DEM present in known volumes of the chamber atmosphere. From these samples, air concentrations of DEM aerosols were determined.

Immediately following the purge period, plants and soils were removed from the chamber and the first samples were obtained. After sampling, plants were placed in a growth chamber for subsequent sampling and observation. Deposition coupons were also removed from the chamber and placed into vials of methylene chloride.

The exposure system was decontaminated after tests DEM-01 and DEM-03 by contacting all surfaces with copious amounts of 0.5 N NaOH. The system was then rinsed with water and allowed to dry prior to subsequent tests. Components of the aerosol generation system were also decontaminated and cleaned. No chamber cleaning was performed between the low and high dose tests DEM-02 and DEM-03 because the tests were performed sequentially on the same day and residual contamination from the low dose test were determined to be of no significance to the high dose test.

Test conditions are listed in Table 2.1. Temperature and relative humidity were measured at the inlet to the chamber.

TABLE 2.1. CONDITIONS DURING DEM AEROSOL EXPOSURE TESTS

Test	Date	Temperature (°C)	Relative Humidity (%)	Duration		Flow Test (Lpm)	Rate Purge (Lpm)
				Test (min)	Purge (min)		
DEM-01	9/26/89	21	48	60	10	74	69
DEM-02	9/28/89	24	35	60	10	84	65
DEM-03	9/28/89	25	35	60	10	85	83

Flow rates in the system during the tests were determined as the sum of all flows using a vane anemometer to measure the inlet carrier air flow and a flowmeter for the compressed air used in the aerosol generator. The small leak rate (0.04 Lpm) did not significantly influence air flow in the test system.

2.3 AEROSOL GENERATION

DEM aerosols were generated intermittently or continuously during tests using one or two ABCO No. 535092 polyethylene nebulizers. Neat DEM was poured into the 25-mL reservoir of the nebulizers. The nebulizers were placed into the aerosol generation chamber and monitored through a window during all tests. A single nebulizer was operated continuously using 10 psi filtered compressed air during trial test DEM-01. Results of that test indicated the need to increase aerosol generation rates at least two times; therefore, a second nebulizer was added and connected in parallel to the first nebulizer. Air pressure was also increased to 15 psi to increase the rate of aerosol generation.

Nebulizers were selected to generate DEM aerosols for the current study because they provided easily controllable steady generation rates of undiluted DEM droplets in the micrometer size range. The generation process could be started or stopped within less than 0.5 sec and remained unaffected by repeated intermittent periods of use. In the nebulizers, aerosols of DEM were produced by aspirating liquid from the reservoirs by the suction force supplied by the expanding compressed air jets. The liquid was then spread as a thin film around a spherical obstruction in the air jet, atomized by shear forces at the edges of the sphere, and dispersed in the turbulent flow downstream of the sphere. Only small droplets were produced, large droplets were impinged on the outlet tube of the nebulizers and drained back into the reservoirs. Because the liquids were composed of neat DEM, no fractional evaporation and solution concentration

occurred because of this refilling process, and the generation process efficiently produced only air-suspended droplets and vapor. Other generation methods that were considered included spray atomization and vaporization/condensation; however, no other method provided sufficient control over chemical purity or aerosol concentration.

Because the rate of aerosol generation from nebulizers is dependent on liquid properties such as viscosity, as well as the specific nebulizer geometry, the rate of generation for DEM was determined in trial test DEM-01. During that test, generation of DEM was continuous and 10 psi compressed air was supplied to the system to provide similar conditions to those used previously for other agent simulants. Because DEM was the fifth simulant generated for exposure tests, a comparison of generation rates from the same nebulizer for several chemicals was made, and is shown in Table 2.2. Rate of generation is seen to increase with decreasing molecular weight (and decreasing vapor pressure). Exceptions to this include H₂O, possibly because of its strong polarity, and DEM, for unknown reasons. Because of slight differences between the two nebulizers used in the DEM tests, rates of generation from the second nebulizer were seen to be 1.3 to 1.8 times greater than those from the first nebulizer (the one used in all previous tests); the range of differences between the two nebulizer generation rates was attributed to different liquid properties of the various tested materials.

TABLE 2.2. AEROSOL GENERATION RATES FOR NEAT CHEMICAL AGENT SIMULANTS AND OTHER LIQUIDS USING ANABCO NO. 535092 NEBULIZER AT 10 psi AND ~STP

Chemical	Molecular Weight	Viscosity (centistokes)	Vapor Pressure (mm-Hg)	Density (g/mL)	Rate of Generation (mL/min)
BIS	~308	6.6	0.000058	0.94	0.19 ± 0.01
DIMP	180		0.17	0.98	~0.3
DEM	160		0.25	1.055	0.19
DFP	184		0.58	1.055	~0.4
CEES	125		3.4	1.070	0.40 ± 0.03
H ₂ O	18		17.5	1.000	0.11 ± 0.02
Ethanol	46		~45	0.79	0.52 ± 0.03
Methanol	32		~100	0.79	0.56 ± 0.02

Rates of DEM aerosol generation during the current exposure tests are shown in Table 2.3. Again, the rate during trial test DEM-01 was less than that during the subsequent tests because only one nebulizer was used and because it was operated at a compressed air pressure of 10 psi rather than 15 psi. No accumulation of droplets or other deposits occurred in the aerosol generation chamber during the tests, indicating aerosol generation efficiency was not influenced by generation rate. An interesting difference between the aerosol generated during the low dose and those generated during the high dose tests was noted; during the low dose test a droplet aerosol was visible in the generation chamber, but not in the exposure chamber, however, during the highest dose test (DEM-03), a dense DEM-droplet aerosol was visible in both locations. Accordingly, a light haze was observed in the exposure chamber during the first high dose test (DEM-01) when aerosol was generated at an average rate 3.5 times greater than DEM-02, and 3.2 times less than DEM-03.

TABLE 2.3. AEROSOL GENERATION DURING DEM EXPOSURE TESTS. A single operated at 15 psi during DEM-02 and DEM-03. Test durations were 60 min.

Test	Dose	Generation Period	No. of Nebulizers	Generation Rate (g/min)	Test-Average Generation Rate (g/min)
DEM-01	Trial/High	Continuous	1	0.18	0.18
DEM-02	Low	Intermittent ^(a)	2	0.50	0.052 ^(b)
DEM-03	High	Continuous	2	0.57	0.5

(a) Intermittent generation consisted of 12 periods 30 sec of generation followed by 270 sec of non-generation.

(b) Test average generation rate obtained by multiplying the instantaneous generation rate by the ratio of time on to time off.

2.4 AEROSOL CHARACTERIZATION

Procedures for DEM aerosol generation were determined for initial target concentrations of 100 and 1000 mg/m³. Because it was not clear that it would be possible to obtain adequate amounts of DEM for analysis from plant and soil samples, especially after hours and days of depuration, results of trial test DEM-01 (aerosol concentration ~ 500 mg/m³) were used to provide aerosol generation methods for slightly increased target DEM air concentrations of 150 and 1500 mg/m³. (A small mass of DEM evaporated within 8 to 16 h at STP). Measurements were

performed to characterize the concentration of DEM aerosol in the exposure chamber during the tests using bubbler, syringe, and deposition coupons samples.

2.4.1 Bubbler Samples

Two Ace Glass Co. No. 7529 smog bubblers were used to obtain time-averaged samples of DEM aerosol mass concentration during all tests. The bubblers were operated with methylene chloride solvent to trap DEM. Sampling periods were 10 to 20 min in duration, and bubblers were used twice during the trial test, four times during DEM-02, and five times during DEM-03. In addition, one pre-test bubbler sample was obtained prior to DEM-02 to provide a control for analysis. The bubblers were operated in series during trial test DEM-01 to test for DEM carry through. After carrythrough was shown to be limited to less than 4% (DEM in the second bubblers was less than the detectable limit, 25 times the detectable limit was collected in the leading bubblers), single bubblers were operated during the subsequent tests.

Flow rates and sample durations were monitored and recorded during each sample. Flow rate was controlled at about 0.3 Lpm and monitored using a Singer Instrument Co. Dry Test Meter Model 115. Post-test sample volumes were removed from the bubblers using pipets to avoid contact with the halocarbon grease at the top seal of the bubblers. The sample volumes were then measured and recorded. Subsequent analysis for DEM was determined as concentration of DEM in the sample liquid. Determination of aerosol mass concentration in the exposure chamber was then calculated by multiplying the concentration of DEM in the bubbler liquid by total volume of chamber atmosphere sampled.

2.4.2 Syringe Samples

Four grab samples were also obtained during each exposure test (DEM-02 and DEM-03). Samples were obtained by drawing 60 mL of exposure chamber atmosphere into a 100-mL gas sampling syringe constructed of glass and Teflon. Samples were drawn at recorded periods through a 10-cm large-bore stainless steel needle. About 10 mL of methylene chloride were then drawn through the needle into the syringe, and the mixture of aerosol and solvent were contacted by shaking and swirling the syringe for about 2 min. The resulting solution was then replaced into the original sample vial. The syringe was cleaned by rinsing with clean solvent between sampling periods.

Two samples collected during DEM-03 were rinsed three times each and all solutions analyzed to determine the efficiency of removal of sampled DEM from the syringe. Residual in the second rinse was $3 \pm 2\%$ of the mass sampled. Residual DEM in the third rinse was not

detectable, indicating that less than 0.1% of the DEM was not removed after two rinses. Data obtained was increased by 3% (as, unlike the bubblers, an absolute carrythrough value was determined), multiplied by the ratio of the solvent volume analyzed to that collected to determine the total mass of DEM collected, and then multiplied by the gas sample volume to determine aerosol mass concentration in the exposure chamber.

2.4.3 Deposition Coupons and Deposition Velocity

Four deposition coupons were placed in the exposure chamber within the plant canopy during tests DEM-02 and DEM-03. The 47-mm glass fiber deposition coupons (Gelman Type A/E) were suspended horizontally on spring holders. The total area available for deposition, including both top and bottom but not the area covered by the spring holder, was 34.0 cm². Deposition coupons were collected after the purge period and placed into vials containing 10 mL of methylene chloride.

Results of the deposition coupon samples were used to obtain the deposition velocity of DEM to a surrogate surface for comparison with rates determined for plant and soil samples. The total mass of DEM on each coupon was divided by the product of the chamber concentration, coupon area, and test duration to determine deposition velocity.

2.5 SIMULANT CHEMISTRY

2.5.1 Synthesis of the Dimethyl Malonate Internal Standard

Dimethyl malonate was chosen as an internal standard because of its obvious chemical similarity to diethyl malonate as well as its anticipated similar but slightly lower retention time on the polar analytical column. Dimethyl malonate was synthesized from malonic acid by standard esterification procedures. Briefly, synthesis involved mixing 200 mL methanol, 150 mL toluene, 4 drops of concentrated sulfuric acid and 2.00 g malonic acid (0.0192 moles) in a 500-mL round-bottomed flask. The solution was stirred by means of a magnetic stir bar under reflux conditions overnight at which time the methanol was stripped from the reaction mixture by means of a rotary evaporation apparatus. The remaining toluene solution was washed two times with 100-mL portions of 5 % aqueous NaHCO₃ followed by a wash with 100 mL of saturated aqueous NaCl. The toluene layer was dried by the addition of anhydrous Na₂SO₄. After removal of the Na₂SO₄ by filtration, the toluene was evaporated under reduced pressure. The remaining dimethyl malonate product was a colorless oil whose identity was later confirmed by GC/MS studies.

2.5.2 GC/MS Separation of Dimethyl Malonate and Diethyl Malonate

All analyses were performed on a Hewlett-Packard Model 5890A gas chromatograph interfaced to a Hewlett-Packard Model 5970A mass spectrometer. Separations were conducted on a 30 m x 0.255 mm i.d. DB-Wax fused silica column (J & W Scientific, Folsom, CA). The column contained a 0.25- μ m polar film of crosslinked carbowax stationary phase. Samples were introduced to the column through 1.0- μ L splitless injections. After an initial hold at 40 °C for 2 min, the column was temperature programmed from 40 to 220 °C at a rate of 6 °C/min. During chromatographic runs, nominal resolution mass spectra were collected by ramping the quadrupole mass spectrometer from 40 to 600 amu at a rate of 200 amu/sec.

A total ion current chromatogram for an injection of diethyl malonate (Aldrich Chemical Co., Milwaukee, WI) is presented in the top of Figure 2.2. The retention time of 14.5 min was observed for this compound under the chromatographic conditions employed. The 70-eV mass spectrum of diethyl malonate is shown in the bottom of Figure 2.2. This spectrum contains a predominant fragment ($m/e = 115$) arising from the loss of ethoxy from the parent compound.

FIGURE 2.2. TOTAL ION CURRENT CHROMATOGRAM (TOP) AND SPECTRUM (BOTTOM) OF DIETHYL MALONATE

The total ion current chromatogram of synthetic dimethyl malonate gave a single peak. This chromatogram is shown in the top of Figure 2.3 with the resulting mass spectrum presented in the bottom of the Figure 2.3. The retention time of dimethyl malonate (13.0 min) was sufficiently different from diethyl malonate to allow use of dimethyl malonate as an internal standard. The mass spectrum obtained from the synthetic dimethyl malonate matches literature spectra (Heller and Milne, 1978), thus assuring synthesis of the correct compound. The spectra of dimethyl malonate shows a predominant loss of methoxy from the parent compound giving rise to the fragment at m/e of 101.

FIGURE 2.3. TOTAL ION CURRENT CHROMATOGRAM (TOP) AND SPECTRUM (BOTTOM) OF SYNTHETIC DIETHYL MALONATE

2.5.3 Quantification of Diethyl Malonate by Selected Ion Monitoring

During the course of this study organic extracts of samples were examined for the concentration of diethyl malonate. Extracts from several of these samples (most notably sagebrush and short-needle pine leaf tissues) were expected to contain high concentrations of indigenous oils (mostly terpenes and sesquiterpenes). Due to the difficulties in attempting to accurately quantitate diethyl malonate in these highly complex extracts, selected ion monitoring was utilized to increase the selectivity of the analysis. The ions which were monitored were m/e of 115 (specific for diethyl malonate) and m/e of 101 (specific for the dimethyl malonate internal

standard). The dwell time utilized was 100 m/sec at an electron multiplier voltage of 200 volts above the perfluorotributylamine tune voltage (2000 volts). The sampling rate was in excess of the minimum criterion set forth by Matthews and Hayes (1976) for accurate integration of chromatographic peaks during selected ion acquisition. The chromatographic separation conditions remained identical to those previously described. An additional advantage of selected ion monitoring was an increase in sensitivity over the scanning mode. The increase in sensitivity was especially important when monitoring time-course samples since the concentration of analyte contained in the extracts became increasingly diminished.

A standard curve for diethyl malonate was prepared as described below. A concentrated stock solution of dimethyl malonate was prepared in methylene chloride (500 μ L dimethyl malonate/25.0 mL methylene chloride). A series of diethyl malonate standards, ranging in concentration from 10 to 110 ppm, were prepared in methylene chloride. A 10.0-mL aliquot of each standard was spiked with 25.0 μ L of the internal standard stock solution. The standard solutions were run under the selected ion monitoring conditions described above. By plotting the ratio of the diethyl malonate integrated peak area to that of the internal standard as a function of diethyl malonate concentration, a standard calibration curve was obtained. The standard curve is presented in Figure 2.4. The detection limit of diethyl malonate under the selected ion monitoring conditions was approximately 125 pg/injection.

FIGURE 2.4. STANDARD CURVE FOR DIETHYL MALONATE

2.5.4 Sample Extraction and Analysis

All samples generated during this study were immediately extracted into approximately 10 mL of methylene chloride. Sample vials were sealed with Teflon lined caps to prevent evaporation and immediately transported to the chemical analysis laboratory. Each sample was promptly spiked with 25.0 μ L of internal standard stock solution, thoroughly mixed, transferred to crimp-top autosampler vials, and expediently analyzed. Comparison of the diethyl malonate/dimethyl malonate peak areas in each sample to the calibration curve shown in Figure 2.4 formed the basis for quantification throughout the analysis.

An example of the increased selectivity afforded by selective ion monitoring over the normal scanning acquisition is presented in Figure 2.5. The total ion current chromatogram of an extract of diethyl malonate exposed short-needle pine, prior to the addition of the internal standard is shown in the top of Figure 2.5. The peak at 14.5 min is due to diethyl malonate.

FIGURE 2.5. TOTAL ION CURRENT CHROMATOGRAM (TOP) AND SELECTED ION CURRENT CHROMATOGRAM (BOTTOM) FOR DIETHYL MALONATE EXPOSED SHORT-NEEDLE PINE LEAVES

The remaining peaks are due to terpenes and sesquiterpenes which were co-extracted from the pine needles. It is clear that large quantities of these natural oils could lead to severe interference in the accurate determination of diethyl malonate. The bottom of Figure 2.5 shows the chromatographic profile of the same short-needle pine extract after the addition of internal standard and under the selected ion monitoring acquisition parameters utilized by this study. The marked increase in selectivity is apparent as only two chromatographic peaks, due to the internal standard (retention time of 12.9 min) and diethyl malonate, are present. This enhanced selectivity allows for far greater accuracy in quantification of diethyl malonate.

2.6 ESTIMATION OF DOSE TO PLANT AND SOIL SURFACES

Leaf tissue (duplicate samples from different places within the canopy) contaminated with DEM were placed in glass vials containing 10 mL of high-purity, distilled-in-glass methylene chloride, and extracted for 10 min. The vials were fitted with Teflon-lined screw caps. Following extraction, the tissues were removed from the vials and leaf areas were measured using a Licor LI-3000 leaf area meter. The foliar mass loading was calculated as ng contaminant/cm² leaf surface.

Three subsamples of each soil sample were removed from Petri dishes exposed to aerosols using a cork borer (sample area was 3 x 0.95 cm²), and the samples were placed into a 25-mL tared Corex centrifuge tube with a Teflon-lined screw cap. Five mL of methylene chloride were added to the soil sample, and the tube was vigorously shaken for 1 min before the solid and liquid phases were separated. Soil samples were centrifuged at 8000 xg for 10 min at 25°C, using a Beckman model J2-21M centrifuge. The solvent was transferred to a clean vial, and the soil samples were air dried for 16 h and dried at 60°C for 8 h. Tared tubes were reweighed to obtain the dry weight of the soil and mass loading was calculated (ng contaminant/g soil). All sample extracts from the tests were kept frozen at -80°C before analysis.

2.7 PLANT EFFECTS

The effects of diethyl malonate on vegetation were explored by studying evidence of phytotoxicity and metabolic effects in three species: the short-needle pine (*Pinus echinata*), tall fescue (*Festuca arundinacea*, 'K-13') and sagebrush (*Artemisia tridentata*, vaseyana).

2.7.1 Gross Phytotoxicity

Assessments of phytotoxicity resulting from foliar contamination with DEM were based on the development of visual toxicity symptoms. These symptoms included leaf burn, blade die-

back, necrotic spotting, and chlorosis (Table 2.4). Quantitation of these effects is based on a Modified Daubenmire Rating Scale (MDRS) (Daubenmire, 1959), a discussion of which is included in the results section of this report.

2.7.2 Metabolic Effects

The basis of the observed whole plants phytotoxicity of DEM was further investigated using two *in vitro* systems: 1) the effects of the simulant on photosynthesis (oxygen evolution) and dark respiration (oxygen uptake) in intact leaf segments; and 2) the effects of the simulant on specific photochemical reactions and electron transport chains in isolated chloroplasts. Methods were described previously (Cataldo et al., 1988)

TABLE 2.4. CODING FOR MODIFIED DAUBENMIRE RATING SCALE AND ASSOCIATED PHYTOTOXICITY SYMPTOMS

Symptom/Intensity	Description
<u>Modified Daubenmire Rating Scale</u>	
0	no obvious effects over controls
1	5% of plant foliage affected
2	between 5%-25% of foliage affected
3	between 25%-50% of foliage affected
4	between 50%-75% of foliage affected
5	between 75%-95% of foliage affected
6	between 95%-100% of foliage affected
<u>Phenotypic Responses</u>	
OGA	old growth affected
NGA	new growth affected
O&NGA	old and new growth affected
TB	tip or leaf edge burn
LBD	leaf burn and leaf drop
NS	necrotic spotting
LD	leaf abscission or needle drop
Chl	chlorosis
BD	blade dieback
LC	leaf curl
W	wilting
GD	growing tip dieback
D	plant dead
F/SA	floral or seed/fruit abortion
(value)	indicates the length in cm that needles or leaves exhibit dieback or burn

2.8 SOIL MICROBIAL ASSAYS

2.8.1 Microbial Assays

Two soils were used to evaluate the microbiological effect of the agent simulant, diethyl malonate (DEM). They were Burbank (sandy, skeletal, mixed, xeric, Torriorthent), a sandy loam soil found at the Hanford area of Washington, and Palouse (fine-silty, mixed, mesic, Pachic Ultic Haploxerolls), a silt loam soil typical of eastern Washington agricultural areas. Selected physical and chemical properties for these two soils are listed in Table 2.5. Soils were amended with DEM (Aldrich Cat. No. D9775-4, Lot No. 03403MV, 99% pure) to final concentrations ranging from 0 to 2500 $\mu\text{g g}^{-1}$ dry soil. Soil moisture was adjusted to 26% and 23% for the Palouse and Burbank soil, respectively. Amended soil was then incubated at 22°C in the dark. The effect of DEM on soil microbial activities was then evaluated *in vitro* by measuring the activity of the two soil enzymes, soil dehydrogenase and soil acid phosphatase.

TABLE 2.5. SELECTED PROPERTIES OF SOILS USED IN THE DEM STUDIES

Soil Property	Burbank Sandy Loam	Palouse Silt Loam
% Sand	45.1	1.1
% Silt	51.4	77.5
% Clay	4.0	21.4
% Ash	98.0	93.8
pH (100% field capacity)	7.4	5.4
Organic carbon (%)	0.5	1.7
Sulfur (%)	0.053	0.043
Nitrogen (%)	0.061	0.16
Total P ($\mu\text{g/g}$)	2400	3770
Phosphate-P ($\mu\text{g/g}$)	4.8	5.8
Carbonate/Bicarbonate (%)	<0.1	<0.1
Ammonium-N ($\mu\text{g/g}$)	6.1	18.3
CEC (meq/100 g)	5.5	23.8

At 3 and 28 days after the DEM amendment, soil samples were assayed for dehydrogenase activity by a modification of the method of Tabatabai (1982). An aliquot of soil (2.0 g wet weight) was first mixed with 0.3 mL of CaCO_3 (3%, w/v), 0.15 mL of glucose (3%, w/v), and 0.375 mL of the substrate, 2,3,5-triphenyltetrazolium chloride (3% w/v). After incubation at 22°C for 24 h, 10 mL of methanol was added to the soil to extract the product, 2,3,5-triphenylformazan (TPF). The solution was mixed thoroughly, centrifuged, and the absorbance of the supernatant determined at 485 nm. Soil dehydrogenase activity, expressed as μg of TPF produced per gram of dry soil per 24 h, was determined by comparing absorbance values to a standard curve prepared with reagent grade TPF and methanol.

Soil acid phosphatase activity was measured at 2 and 28 days after the addition of DEM. A modified procedure of Tabatabai (1982) was used for this study. One gram of soil (wet weight) was placed in 15-mL centrifuge tubes with 4 mL of modified universal buffer (MUB) added. MUB consisted of tris(hydroxymethyl)amino methane, 2.42 g; maleic acid, 2.32 g; citric acid, 2.80 g; boric acid, 1.256 g; 1M NaOH, 97.6 mL; in 1000 mL final volume with the pH adjusted to 6.5 with 0.1M HCl. One mL of para-nitrophenol phosphate (0.025M prepared with MUB buffer) was added to each tube. The tubes were stoppered, vortexed and incubated for one hour at 37°C. One mL of 0.5M CaCl_2 and 4 mL of 0.5M NaOH were then added to stop the reaction. The mixtures were centrifuged at 12,000 x g for 10 min and the supernatant absorbance measured at 400 nm with a spectrophotometer. Phosphatase activity was determined by comparing these values to a standard curve prepared with reagent grade para-nitrophenol and expressed as μg of para-nitrophenol produced per gram of soil per hour.

All dehydrogenase and phosphatase activities were measured in triplicate and mean values compared with that of the control soil (not DEM-treated) and expressed as a percent of the control.

2.9 SOIL INVERTEBRATE MEASUREMENTS

An earthworm (*Eisenia fetida*) bioassay system was used to evaluate the toxicity of the DEM. An artificial soil containing 350 g sand, 100 g Kaolin and 50 g dried peat moss (adjusted to pH 6.5 with CaCO_3), was used for the earthworm exposures. Worms were fed twice weekly with fermented alfalfa, and soil moisture adjusted to 35% of dry weight. These soil tests used 70 g of the artificial soil (placed in 100 x 25 mm Petri plates), containing 6 worms. Five replicate plates were used for each test series as noted in the text. The tests were terminated after 12 days, and effects observed over this period. Effects scored included both earthworm mortality and activity. Mass loading or dose was determined on similar soil plates without worms.

2.10 STATISTICAL ANALYSIS

Statistical analyses involved calculation of average $\pm$ SD. Significance was determined using the Student one-tailed t-test (Zar, 1974).

Statistical analysis involved calculation of average $\pm$ SD. Significance was determined using the Student's *t*-test (1974).

3.0 RESULTS AND DISCUSSION

3.1 AEROSOL CHARACTERIZATION

The concentration of DEM aerosols in the exposure chamber were measured by two methods, bubbler and syringe samples, that provided similar results. DEM concentration during the low dose test (DEM-02) was greater than the 150 mg/m³ target value and the concentration during the high dose test (DEM-03) was slightly less than the 1500 mg/m³ target value. This was attributed to possible differences in the droplet/vapor partition between the two aerosols. Deposition velocities to surrogate (glass fiber substrate) surfaces indicated a large relative increase in deposition rate during the high dose test that also supported the conclusion that the droplet/vapor partition was different at low and high DEM aerosol concentration.

3.1.1 Aerosol Concentration

Results of bubbler and syringe samples are shown in Tables 3.1 and 3.2. Average concentrations during the two exposure tests were 318 mg/m³ (DEM-02) and 1140 mg/m³ (DEM-03). Results of the syringe samples obtained during DEM-02 averaged 420 mg/m³, but were not considered representative of average conditions because they were obtained shortly after 30-sec generation periods, at times when greater than average aerosol concentrations were likely present in the exposure chamber. As generation was continuous during DEM-03, both bubbler and syringe samples were considered representative and were averaged to determine the DEM aerosol mass concentration. The individual average concentrations were 1045 mg/m³ (bubbler) and 1240 mg/m³ (syringe). The single sample obtained during a purge period, B3A during DEM-03, indicated a higher than expected concentration of 1230 mg/m³. This may have been caused by the volatilization of droplets deposited to surfaces within the exposure chamber.

The DEM concentration present during DEM-02 was 2.1 times greater than anticipated, and that during DEM-03 was 1.3 times less than anticipated. This occurred even though the relative generation rate of DEM was 9.7 (~10) times greater during the high dose test. These results, coupled with observation of droplet mists during both DEM-01 and DEM-03 but not during DEM-02, and deposition rate measurements discussed in the following section, indicated that the partition between droplet and vapor states present in the DEM aerosols was not similar for all tests. It is likely that the vapor phase of the aerosols would deposit to plant, soil, and chamber surfaces at a slower rate than would DEM droplets because the droplets would deposit under the influence of gravitational and diffusional forces not affecting the diffusion of vapor.

TABLE 3.1. RESULTS OF BUBBLER SAMPLES OBTAINED DURING DEM AEROSOL TESTS

Test	Sample ID	Sample Period (min)	Gas Volume (L)	Solvent Volume (mL)	DEM Conc. ($\mu\text{g/mL}$)	DEM Aerosol Concentration (mg/m^3)
DEM-01	B1A	20.0	6.7	42.3	74.9	470
	B1B	20.0	6.7	46.0	0.0	0
	B2A	50.5	5.9	43.3	69.1	510
	B2B	50.5	5.9	46.8	0.0	0
DEM-02	B1A	7.5	4.6	42.5	26.7	250
	B1B	27.5	4.4	42.6	31.0	300
	B2A	47.5	4.3	41.5	47.1	460
	B2B	65.0	2.8	44.7	16.4	260
DEM-03	B1A	5.0	3.0	44.8	56.8	850
	B1B	20.0	2.9	44.3	82.0	1270
	B2A	35.0	2.9	45.4	51.3	800
	B2B	50.0	2.8	45.1	78.2	1260
	B3A	65.0	2.7	45.6	73.7	1230

TABLE 3.2. RESULTS OF SYRINGE SAMPLES OBTAINED DURING DEM AEROSOL TESTS

Test	Sample ID	Sample Period (min)	Gas Volume (mL)	Solvent Volume (mL)	DEM Mass (μg)	DEM Aerosol Concentration (mg/m^3)
DEM-02	S1	16.0	60	10	27.6	470
	S2	31.0	60	10	24.4	420
	S3	41.5	60	10	27.1	460
	S4	51.5	60	10	19.3	330
DEM-03	S1	16.5	60	10	89.6	1540
	S2a	31.5	60	10	72.6	1250
	S2b(a)	31.5	60	10	3.36	56
	S2c	31.5	60	10	0.00	0
	S3a	43.5	60	10	51.5	880
	S3b	43.5	60	10	0.59	10
	S3c	43.5	60	10	0.00	0
	S4	54.0	60	10	75.8	1300

(a) S2b, S2c, S3b, and S3c aerosol concentration results did not include 3% correction for carry through h (described in Section 2.2). All other aerosol concentrations do include a multiplying factor of 1.03.

If a greater relative concentration of vapor to droplet mass was present during the low dose test, more of the aerosol would likely be suspended in the exposure chamber and less would be deposited to surfaces. On the other hand, if the greater concentrations present during the high dose tests caused a relative increase in the fractional portion of the aerosol mass consisting of droplets, then deposition rates would increase and aerosol concentrations would be less than those anticipated.

3.1.2 Deposition Velocity to Surrogate Surfaces

Rates of DEM aerosol deposition to surrogate glass fiber substrate suspended in the exposure chamber were determined. The average deposition velocities for the low dose (DEM-02) and the high dose (DEM-03) tests were 0.00085 ± 0.00011 and 0.0066 ± 0.0018 cm/sec, respectively. The deposition velocity during the high dose test was 7.8 times greater than that during the low dose test. These results support the speculations of variations in the

vapor/droplet partition of the DEM aerosols as a function of mass concentration discussed in the previous section.

Rates of deposition to the surrogate surfaces within the exposure chamber were uniform. The variation in the mass of DEM deposited to four deposition coupons during the low dose test was 13%, variation during the high dose test was 27%.

3.2 MASS LOADING ON AND DEPOSITION VELOCITIES TO FOLIAR SURFACES AND SOILS

A variety of physical and environmental factors combine to determine the rate at which atmospheric pollutants transfer from the air column, through the near surface boundary layer, to a receptor surface. These factors, which include various atmospheric conditions such as wind speed and humidity, aerosol diameter, vapor pressure, and the physical and chemical structure of the receptor surface, combine to determine the net deposition to a surface. Two different removal mechanisms operate on atmospheric pollutants, one for gas-phase compounds and one for compounds found in the aerosol phase. Although the two deposition mechanisms depend on different parameters, the net deposition to a surface can be calculated when an average gas/aerosol deposition velocity is known. A deposition velocity, which is normally expressed as V_d in units of cm/sec, is analogous to a mass transfer coefficient and describes the rate at which an atmospheric pollutant is deposited on a given surface. Without the deposition velocity, the dosing levels to a receptor surface cannot be predicted.

To quantify the relationship between chemical dosage and damage or effect, the aerosol concentration, deposition velocity, and surface exposure time must all be known to estimate the total surface deposition, or mass loading (ML). Because of the complexity of deposition processes, specific deposition velocities are rarely known, and therefore must be either measured directly or calculated. In this study we have directly measured surface ML by subsampling a known area of an exposed surface, and then extracting and quantifying any deposited chemical species. Subsequently, these data were combined with the measured aerosol concentrations and exposure times to compute the deposition velocity. The formula for calculating V_d is presented in Equation 1.

$$V_d \text{ (cm/sec)} = \frac{\text{ML (ng compound/cm}^2 \text{ leaf)}}{\text{aerosol conc (ng compound/m}^3\text{)}} \times \frac{1 \times 10^6 \text{ cm}^3}{\text{m}^3} \times \frac{1}{\text{exposure time}} \quad (1)$$

Quantifying the deposition process for specific receptor surfaces permits a comparison of the relative importance of atmospheric variables and canopy and receptor surface (plant and soil) characteristics to the net deposition efficiency. The following subsections discuss the ML of DEM to vegetative and soil surfaces, and the calculated deposition velocities are presented. All reported soil and plant results were obtained from exposure tests DEM-02 (318 mg/m³) and DEM-03 (1145 mg/m³).

3.2.1 Vegetative Surfaces

Air concentrations of DEM for the low dose exposure averaged 320 mg/m³, while concentrations averaged 1140 mg/m³ for the high dose treatment. Exposure duration was 60 min. Foliar mass loading ranged from 0.2 to 12 µg/cm² in DEM-02, and from 17 to 193 µg/cm² in DEM-03 (Table 3.3). Short-needle pine exhibited the lowest mass loading, while sagebrush and tall fescue had the highest. Differences in mass loading for sagebrush and tall fescue are not significant ($P \geq 0.05$). Mass loading levels between the high and low dose exposures were substantially higher than the factor of four difference in air concentration, indicating a possible loss at the low dose levels due to volatilization.

Calculated deposition velocities for plant canopies, based on the average air concentration and mass loading levels, are provided in Table 3.3. For the low dose treatment, V_d was lowest for the pine (0.0002 cm/sec), and a factor of 50 higher for sagebrush and tall fescue (0.007 to 0.010 cm/sec). At higher exposure concentrations, V_d values increase by a factor of 3 to 25. Collection efficiency remains less for the pine than the other two plant species. This is likely related to the canopy structure, and turbulence within the canopies. Differences in deposition velocity for sagebrush and tall fescue are not significant ($P \geq 0.05$). The lower values for V_d at the lower air concentration may be indicative of a higher rate of volatilization at lower mass loading levels.

TABLE 3.3. AVERAGE FOLIAR MASS LOADING AND DEPOSITION VELOCITIES FOR DEM ON VEGETATIVE SURFACES DURING LOW- AND HIGH-DOSE EXPERIMENTS (n=3)

Treatment/Species	Mass Loading ($\mu\text{g}/\text{cm}^2 \pm \text{SD}$)	Deposition Velocity ($\text{cm}/\text{sec} \times 10^3 \pm \text{SD}$)
Low Dose (DEM-02)		
Short-Needle Pine	0.20 $\pm$ 0.12	0.17 $\pm$ 0.11
Sagebrush	11.69 $\pm$ 0.54	10.14 $\pm$ 0.47
Tall Fescue	8.32 $\pm$ 4.45	7.22 $\pm$ 3.86
High Dose (DEM-03)		
Short-Needle Pine	17.08 $\pm$ 2.94	4.16 $\pm$ 0.72
Sagebrush	114.79 $\pm$ 29.44	27.97 $\pm$ 7.18
Tall Fescue	193.56 $\pm$ 63.41	47.16 $\pm$ 15.45

3.2.2 Soil Surfaces

Soil mass loading levels, following 60 min exposure at the low dose were 45 and 93 $\mu\text{g}/\text{cm}^2$ for Burbank and Palouse soil, respectively (Table 3.4). At the high dose level, mass loading levels were approximately 750 $\mu\text{g}/\text{cm}^2$, and there was no significant difference between soils ($P \geq 0.05$). As with the foliage, mass loadings in the high dose treatment were approximately 14 times greater than the increase in air concentration between the low and high dose (factor of four). This would again indicate that there is substantial volatilization occurring at low deposition levels.

Deposition velocities calculated for the low dose treatment were 0.04 cm/sec and 0.08 cm/sec for Burbank and Palouse, respectively. These increased to 0.18 cm/sec in the high dose treatment. Again, these values should be similar and independent of air concentration, if no other factor, such as volatilization, were influencing the system.

TABLE 3.4. AVERAGE SOIL MASS LOADING (AND DEPOSITION VELOCITIES) FOR DEM ON SOIL SURFACES DURING THE LOW- AND HIGH-DOSE EXPERIMENTS (n=3)

Treatment/Soil	Mass Loading ($\mu\text{g}/\text{cm}^2 \pm \text{SD}$)	Deposition Velocity ($\text{cm}/\text{sec} \times 10^3 \pm \text{SD}$)
Low Dose (DEM-02)		
Burbank	45.31 $\pm$ 3.26	39.33 $\pm$ 2.83
Palouse	93.40 $\pm$ 7.68	81.08 $\pm$ 6.67
High Dose (DEM-03)		
Burbank	770.45 $\pm$ 386.28	187.73 $\pm$ 94.12
Palouse	722.77 $\pm$ 226.88	176.11 $\pm$ 55.28

3.3 PHYTOTOXICITY

3.3.1 Gross Phytotoxicity

The determination of foliar toxicity effects can present several problems. While the plants used for this study were of natural genetic stock, individual differences in physiological variability and toxicity response within each test species could be expected to occur. Further, under both field conditions and in the stirred exposure system employed for these studies, where air movement occurs along a given vector (i.e., wind direction), substantial amounts of deposition to canopies can occur irregularly depending on canopy structure and density and the presence of back eddies. Finally, the most cost-effective and consistent manner in which to quantify damage to vegetation must be considered. Taking these issues into account, a nonparametric grading system, a modified Daubenmire rating scale (MDRS) (Daubenmire, 1959), was used as a damage index for the evaluation of contact toxicity.

The criteria used to compile the MDRS are described in Table 2.4. These criteria were used to describe the extent of visual damage to the plants caused by the given exposure period at each concentration of DEM. Damage can be visualized and subjectively quantified using any one or more of the listed symptoms. Also, the intensity of foliar damage can be further quantified by determining the physical length of the needle or leaf damage. The data generated are nonparametric and represent an estimate of foliar damage.

Table 3.5 provides the gross toxicity data for the three plant species exposed to DEM (foliar exposure only). At the low dose, 320 mg/m³ for 60 min, none of the three plant species exhibited any phytotoxicity following foliar contamination. Neither old nor new growth was affected. At the higher dose, 1140 mg/m³ for 60 min, there was a rapid and severe onset of toxicity. Two days following exposure, chlorosis (Chl), wilting (W), leaf curl (LC) and tip burn/necrosis (TB) were evident. The extent or intensity of damage was most severe in the grass, tall fescue. Sagebrush and short-needle pine were affected to a lesser extent.

TABLE 3.5. PLANT SYMPTOMOLOGY TO DEM EXPOSURE. Symptomology Determined at 2 and 21 Days Post-Exposure.

Treatment/Species	Days Post-Exposure	Toxicity Response(a)	
		Damage Index (DR Scale)	Symptomology
Low Concentration (DENM-02)			
Tall Fescue	0	1.0	O&NGDH(b)
	2	1.0	O&NGDH
	21	1.0	O&NGDH
Short-Needle Pine	0	1.0	O&NGDH
	2	1.0	O&NGDH
	21	1.0	O&NGDH
Sagebrush	0	1.0	O&NGDH
	2	1.0	O&NGDH
	21	1.0	O&NGDH

(a) Daubenmire Scale and symptomology definitions (Table 2.4).

(b) New growth developing healthy.

TABLE 3.5. (cont.) PLANT SYMPTOMOLOGY TO DEM EXPOSURE. Symptomology Determined at 2 and 21 Days Post-Exposure.

Treatment/Species	Days Post-Exposure	Toxicity Response ^(a)	
		Damage Index (DR Scale)	Symptomology
High Concentration (DEM-03)			
Tall Fescue	0	3.0	W
	2	6.0	W, TB (13.0)
	21	6.0	W, Chl, LC, TB (16.0)
Short-Needle Pine	0	1.0	O&NGDH
	2	2.0	Chl, TB (6.0)
	21	3.0	Chl, TB (5.0)
Sagebrush	0	1.0	O&NGDH
	2	3.0	LC, W
	21	5.0	Chl, LC, GD

(a) Daubenmire Scale and symptomology definitions (Table 2.4).

(b) New growth developing healthy.

Due to the severity of damage from contact toxicity with DEM, residual effects were assessed using tall fescue. The objective of the residual plant effects study was to determine whether there is any lasting effect of foliar toxicants on root systems which were not directly exposed. Control and treated plant canopies were harvested at 30 days post-exposure and dry matter production noted. Cropped plants were allowed to regrow for a second harvest. The results provided in Table 3.6 indicate a significant reduction in biomass following foliar exposure, but only at the high dose treatment. At low dose levels, no residual effect on dry matter production is observed for the second harvest. However, in the high dose treatment a residual effect is apparent, and growth reduction is significantly different than the controls ($P \leq 0.1$).

TABLE 3.6. EFFECTS OF DEM ON THE REGROWTH (DRY-MATTER PRODUCTION) OF FOLIARLY EXPOSED TALL FESCUE PLANTS AT 30 AND 60 DAYS POST-EXPOSURE

Treatment	Dry-Matter Production	
	30 Days Post- Exposure	60 Days Post- Exposure
(Avg. g dry wt. $\pm$ variance, n=2)		
Control	1.35 $\pm$ 0.24	1.14 $\pm$ 0.09
Low Concentration	1.41 $\pm$ 0.16(b)	1.11 $\pm$ 0.04(b)
High Concentration	0.62 $\pm$ 0.03(c)	0.71 $\pm$ 0.21

(a) See Table 3.3 for rates of foliar mass loading.

(b) Not significant.

(c) Significant at $P \leq 0.05$ using a one-tailed t-test.

3.3.2 Metabolic Effects on Plants

Plant tissues, exposed to 100 $\mu\text{g/mL}$ of DEM for 15 min, failed to show any adverse effects using polarographic methods. Likewise, isolated chloroplast fractions failed to demonstrate any effect of DEM on whole chain or photosystems I or II *in vitro*. Based on the fact that the foliage of exposed plants, particularly at the high dose level, were heavily contaminated to the point of wetness, and the fact that polarographic methods are unable to be employed at high DEM concentrations, no resolution of the metabolic effects of DEM was possible.

3.4 EFFECTS ON SOIL MICROBES

The inhibition of enzymes which drive key metabolic reactions in microbial cells is likely the underlying cause of toxicity of chemicals to the microorganisms. Microbial dehydrogenase enzyme systems catalyze the oxidation of organic material and fulfill an important role in the soil carbon cycle. The assay of soil dehydrogenase activity is a general indicator of the potential activity of the soil microbial population (Skujins, 1976). Phosphatases, which can exist extracellularly, are a broad group of enzymes that cleave esters and anhydrides of phosphates from complex organophosphates and are believed to be important in the mineralization of this element from soil organic matter (Ramirez-Martinez, 1968). Thus, these two enzyme activities were used in this study to assess DEM toxicity towards soil microorganisms and soil biochemical processes.

The extent of DEM inhibition of soil enzymatic activities was dependent on soil type, DEM concentration, and length of incubation. The effect of DEM on soil dehydrogenase activity is shown in Table 3.7. Three days after the addition of DEM, dehydrogenase activity in Burbank soil was not affected at DEM concentrations of 25 to 500 $\mu\text{g/g}$ dry soil, but was decreased to 55% of the control level at 2500 $\mu\text{g/g}$.

TABLE 3.7. THE EFFECT OF DEM ON BURBANK AND PALOUSE SOIL DEHYDROGENASE ACTIVITIES

DEM Concentration ($\mu\text{g/g}$)	Dehydrogenase Activities % of Control ^(a)			
	Burbank Soil		Palouse Soil	
	Incubation Time, Days		Incubation Time, Days	
	3	28	3	28
0	100 (13)	100 (6)	100 (4)	1000 (6)
25	103 (9)	87 (12)	146 (5)*	104 (17)
100	109 (13)	84 (4)*(b)	159 (7)*	116 (15)
500	112 (10)	8 (9)*	155 (4)*	116 (13)
2500	55 (7)*	77 (5)*	44 (3)*	77 (9)

(a) Mean ($\pm$ standard deviation), $n=3$.

(b) *Denotes significantly different from control (0 μg DEM/g dry soil) based on student's t-test, $P \leq 0.05$.

After 4 weeks' incubation time, DEM concentration greater than 100 $\mu\text{g/g}$ caused inhibition in dehydrogenase activity, however, the degree of impact decreased to 77% at the highest DEM concentration tested. In Palouse soil, soil dehydrogenase activity was substantially enhanced (146-159% of control) 3 days after the addition of DEM at concentrations of 25 to 500 $\mu\text{g/g}$, but was inhibited to 44% by 2500 $\mu\text{g/g}$ DEM concentration. However, the impact was transient in nature, with recovery to the level of control soil after 28 days. The only exception was with the 2500 $\mu\text{g/g}$ treatment, which remained inhibited. The EcD_{50} , an expression of the ecological dose of DEM causing 50% inhibition (Babich et al., 1983), were calculated from response curves in Figure 3.1. For both Burbank and Palouse soils, the EcD_{50} after 3 days incubation was roughly 2500 $\mu\text{g/g}$ and after 28 days incubation, it increased to greater than 2500 $\mu\text{g/g}$, indicating a recovery trend as a function of time for soil dehydrogenase activity.

FIGURE 3.1. RESPONSE CURVE FOR THE EFFECT OF DEM ON BURBANK AND PALOUSE SOIL DEHYDROGENASE ACTIVITIES

Soil phosphatase activity was not affected by DEM at 25-500 µg/g. Only at concentration of 2500 µg/g, did soil phosphatase activity decline moderately to 78% in Burbank soil and to 85% in Palouse soil. However, after 28 days, activity recovered to the level of control soil (Table 3.8). Curves illustrated in Figure 3.2 indicated that EC_{50} of DEM on soil phosphatase is far greater than the highest DEM concentration tested (2500 µg/g). EC_{50} at day 28 appeared to be higher than at day 2, again indicating a recovery trend as a function of time for soil phosphatase activity.

TABLE 3.8. THE EFFECT OF DEM ON BURBANK AND PALOUSE SOIL PHOSPHATASE ACTIVITIES

DEM Concentration ($\mu\text{g/g}$)	Dehydrogenase Activities % of Control(a)			
	Burbank Soil		Palouse Soil	
	(Incubation Time, Days)		(Incubation Time, Days)	
	2	28	2	28
0	100 (9)	100 (4)	100 (4)	100 (0.4)
25	98 (11)	111 (11)	103 (3)	97 (4)
100	101 (10)	102 (4)	113 (4)*	98 (9)
500	110 (9)	103 (14)	91 (3)*	88 (6)
2500	78 (7)*(b)	104 (4)	85 (4)*	96 (2)

(a) Mean ($\pm$ standard deviation), $n=3$.

(b) * Denotes significantly different from control (0 μg DEM/g dry soil) based on Student's t-test, $P \leq 0.05$

FIGURE 3.2. RESPONSE CURVE FOR THE EFFECT OF DEM ON BURBANK AND PALOUSE SOIL PHOSPHATASE ACTIVITIES

The data show that, in general, concentrations of DEM at 25 to 500 µg/g do not significantly impact soil enzymatic activities. Activities were slightly impacted only at concentrations greater than 500 or 2500 µg/g. In fact, Palouse soil phosphatase activity was enhanced by DEM at 25-500 µg/g. Both soil dehydrogenase and phosphatase activities recovered to the level of control soil after 28 days, indicating the effect was not persistent. The aliphatic structure of DEM [CH₂(COOC₂H₅)₂] suggests this compound will likely be degraded by soil microorganisms and enhance microbial activities at low concentrations; but becomes toxic at concentrations higher than 2500 µg/g.

3.5 TERRESTRIAL PERSISTENCE

3.5.1. Persistence of DEM in Soil

Soils contaminated in the high dose test for 60 min were sampled at intervals over a 4-day period. Samples were extracted for total DEM and the environmental half-life determined. Data points were plotted, and regressions performed to determine loss rates. The depuration plots of DEM in both Palouse (Figure 3.3) and Burbank soil (Figure 3.4) were biphasic. Half-times for Palouse were 2 and 16 h; while for Burbank soil half-times were 1.2 and 5.4 h. Since DEM has a significant vapor pressure, volatilization likely accounts for the observed losses. After 4 days, of the 750 $\mu\text{g DEM/cm}^2$ deposited to soils, only 2 $\mu\text{g DEM/cm}^2$ remains in the Palouse soil. In Burbank, less than 0.1 $\mu\text{g/cm}^2$ remains after 4 days.

FIGURE 3.3. DEPURATION OF DEM IN PALOUSE SOIL

FIGURE 3.4. DEPURATION OF DEM IN BURBANK SOIL

While the initial rapid loss of DEM likely represents volatilization of surface deposited material, the slower loss rate seen in the second phase may represent losses due to both volatilization, and abiotic and biotic decomposition. Also it is likely that a fraction of the deposited DEM may be sorbed or bound to exchange sites thus limiting rates of volatilization.

3.5.2. Persistence of DEM on Foliar Surfaces

Depuration rates for plant canopies is based on the mass loadings determined for DEM-03, the high dose treatment (Figure 3.5.). As with the soils, depuration of DEM from contaminated foliar surfaces is biphasic. The rapid loss phase has a half-time of 1 to 3 h for sagebrush, tall fescue and short-needle pine. The longer depuration component had half-times of 17, 45 and 242 h, for tall fescue, sagebrush and pine, respectively. After 4 days, only 1 to 3 $\mu\text{g DEM/cm}^2$ remains associated with foliar surfaces. It is likely that the surface composition of foliar surfaces, waxes and cutin, affect sorption and thus subsequent volatilization of DEM.

FIGURE 3.5. DEPURATION OF DEM DEPOSITED TO PLANT CANOPIES OF SAGEBRUSH (SB), TALL FESCUE (TF), AND SHORT-NEEDLE PINE (SNP)

3.6 EFFECT OF DEM ON EARTHWORM SURVIVAL

The earthworm bioassay was performed using artificial soil having contamination doses of 107 and 204 $\mu\text{g DEM}/\text{cm}^2$. Earthworms were evaluated for survival and activity at 1, 7 and 12 days post-exposure. In the low dose treatment, survival rate was 86% after 12 days, with a slight overall loss in activity. At the high dose level, survival decreased to 66%, and activity was judged to be affected in over 50% of the individuals.

TABLE 3.9. INFLUENCE OF SOIL DEPOSITED DEM ON THE SURVIVAL OF EARTHWORMS (*Eisenia foetida*). Artificial Soils (70 g) and Worms Exposed to DEM and Held for 12-Days Post-Exposure.

Treatment	Days Post-Exposure	Soil Mass Loading ($\mu\text{g}/\text{cm}^2$)	Earthworm	
			Survival	Condition
		(Avg $\pm$ SD, n=3)		
Control	1	0	30/30	Active
	7		30/30	Active
	12		30/30	Active
Low Concentration (DEM-02)	1	107.5 $\pm$ 65.0	30/30	Active
	7		27/30	Moderately Active
	12		26/30	Moderately Active
High Concentration (DEM-03)	1	204.1 $\pm$ 39.6	30/30	Active
	7		23/30	Sluggish to Active
	12		20/30	Sluggish to Active

4.0 CONCLUSIONS

Analysis of the results from the low and high air concentration test generations indicates that air concentrations of DEM will influence the proportion of aerosolized material that persists in the liquid droplet versus gaseous state. Thus, the vapor pressure of DEM is high enough to result in mixed gas and liquid phases, and in the low dose test vapor dominated, while in the high dose test liquid droplets dominated. This affected the overall rate of deposition to surfaces. Deposition velocities for DEM to soil surfaces ranged from 0.04 to 0.2 cm/sec, for low and high air concentrations. For foliar surfaces, deposition velocities ranged from 0.0002 cm/sec at low air concentrations to 0.05 cm/sec for high dose levels. Collection efficiency was lowest for short-needle pine and greatest for sagebrush and tall fescue.

The volatility of DEM results in a rapid loss from soil and foliar surfaces. The residence times or half-lives of DEM deposited to soils was 2 h for the fast component and 5 to 16 h for the residual material. DEM deposited to foliar surfaces also exhibited biphasic depuration. The half-life of the short residence time component ranged from 1 to 3 h, while the longer time component had half-times of 16 to 242 h. Volatilization and other depuration mechanisms reduce surface contaminant levels in both soils and foliage to less than 1% of initial dose within 96 h.

DEM is not phytotoxic at foliar mass loading levels of less than 10 $\mu\text{g}/\text{cm}^2$. However, severe damage is evident at mass loading levels in excess of 17 $\mu\text{g}/\text{cm}^2$. Tall fescue and sagebrush were more affected than was short-needle pine, however, mass loading levels were markedly different. Regrowth of tall fescue indicated that the effects of DEM are residual, and growth rates are affected at higher mass loadings through the second harvest. At higher dose levels *in vitro* studies failed to indicate any effects of DEM on photosynthesis/respiration or electron transport at solution concentrations of 100 $\mu\text{g}/\text{mL}$.

Results from *in vitro* testing of DEM indicated concentrations below 500 $\mu\text{g}/\text{g}$ dry soil generally did not negatively impact soil microbial activity. In fact, Palouse soil dehydrogenase activity was initially enhanced by the addition of DEM at 25-500 $\mu\text{g}/\text{g}$. Short-term effects of DEM (2-3 days incubation) were more profound on soil dehydrogenase activity ($\text{EcD}_{50} = 2500 \mu\text{g}/\text{g}$) than on soil phosphatase activity ($\text{EcD}_{50} > 2500 \mu\text{g}/\text{g}$), indicating soil dehydrogenase is more susceptible to DEM than soil phosphatase. However, no enzyme inhibition or enhancement was observed after 28 days incubation, indicating the effect is transient in nature with a possibility of recovery. Although *in vitro* testing usually gives a good indication of dose response and may be representative of a field exposure, spill or waste disposal situation, it is

not a true representation of actual aerosol deposition. Factors such as volatility, mass loading, wind speed and relative humidity can vary so that when used as a simulant for environmental testing, the influence of DEM on soil microbial and biochemical activity may be different following an actual depositional event.

Results of the earthworm bioassay indicate survival to be 86 and 66% at soil doses of 107 and 204 $\mu\text{g DEM/cm}^2$, respectively. At higher dose level, activity or mobility was judged to be affected in over 50% of the individuals.

5.0 QUALITY ASSURANCE AND QUALITY CONTROL

At Pacific Northwest Laboratory (PNL), quality control (QC) is "the system of activities to provide a quality product," and quality assurance (QA) is "the system of activities to provide assurance that the quality control system is performing adequately." The prime responsibility for QA/QC is placed on the Program Manager, Principal Investigator, and the Task Leaders; however, the QA audit function is maintained outside of the project and is not directly supported by the project. This audit function is performed by Rob Cuello, the Earth and Environmental Sciences Center's senior Quality Assurance Officer, and is outside the control of the Program Manager or the Principal Investigator.

5.1 QUALITY ASSURANCE

The objective of the QA program for the wood pole study is to ensure that the production of data are precise and accurate (within the stated acceptance criteria), representative, comparable, traceable, and defensible. To ensure these conditions are met, PNL's quality assurance program is envisioned to consist of the following elements:

- Procedure review and approval [of Standard Operating Procedures (SOP's)]
- Personnel training
- Pretest preparation
- Quality Control of sampling function and analysis function
- QA audits
- Feedback and corrective action (if necessary).

5.2 QUALITY ASSURANCE TRAINING

The activities of the QA training program include: introducing personnel to the QA program, identifying training elements, preparing and training supervisory and test personnel, implementing QA audits, and conducting refresher QC training or on-the-job training as needed.

The project team will review the procedures that govern the collection and interpretation of field and/or laboratory data and will familiarize themselves with what audit procedures are to be used. Supervisory personnel will require some training to become knowledgeable of the QA procedures to be employed.

5.3 PROCEDURE REVIEW AND APPROVAL (SOP)

Quality control guidelines for a particular program are developed by the Task Leaders and approved by the Principal Investigator for all standard operating procedures (sampling, analysis and reporting), instruction, specifications, equipment, and so forth. All SOP's for sampling and analysis are described in writing, and each procedure is reviewed to determine what quality control steps will be incorporated. Each team member will use the procedures outlined in the SOP's to prevent contamination, to provide the proper size sample, to assure proper taxonomic identification, to provide proper kind and number of blanks, to maintain standardization of measuring equipment, and to guarantee the keeping of useable records.

Quality assurance also ensures that the documentation system provides for and maintains a current configuration (latest revision) of procedures being used on the program. Whenever it is evident that data being obtained are not sufficiently accurate or appropriate for the intent of the program, the sampling and/or analytical SOP must be modified after the review and approval by the Project Manager. The modifications are then incorporated into the revised SOP's.

5.4 LABORATORY RECORD BOOKS

Work performed on any given project are documented in laboratory record books issued to the project personnel. Permanent records on each book are maintained by PNL and include the project number under which the book was issued, the Record Book number, and the name of the staff member to whom the book was issued. A signature sheet for each record book is kept on file with PNL, and an assignment form inside the front cover of the book is used to indicate the project work number and the individual to whom the book was assigned. Staff members assume full responsibility for the use and security of the books while the books are in their possession. At the end of the project, the books are returned to PNL for archiving.

5.5 SAMPLE IDENTIFICATION AND TRACEABILITY

A system of accountability is used to control the number and variety of samples and the quality of data generated on a research project. The Principal Investigator helps design the initial system and, since he or she works most closely with the experiments, helps monitor the system's effectiveness. Quality assurance at this stage of the project is used to assure that the samples are complete and appropriate. Therefore, the QA officer has the following responsibilities:

- Participate in the development of a system for keeping records of samples and for introducing proper QA steps to assure dependability of the system.
- Review sample log system and implementation forms. (The forms provide information on samples collected and indicate the work to be done on the samples.)
- Monitor the sample identification system to ensure proper labeling of samples, proper dispersements of samples for analysis, and proper quality control of spikes, blanks, and duplicates.

The Principal Investigator and the Task Leaders are responsible for seeing that the documentation is done either by themselves or by the team members. A team member is usually assigned the responsibility of handling the samples and storing, retrieving, dispersing, and maintaining records of those samples.

5.6 QUALITY CONTROL

The key quality control operations that may be emphasized in a particular program are procurement QA, standardization/calibration, sampling, and analysis.

5.7 PROCEDURE QUALITY CONTROL

Task Leaders are responsible for ensuring that all procured materials (e.g., samples, collection containers) conform to appropriate specifications. They also are responsible for ensuring that reagents and chemicals with limited shelf life are identified and used within the specified expiration date.

5.8 SAMPLING QUALITY CONTROL

A sampling information document developed by the Task Leader is used to detail the kind of samples to be taken, the locations where samples are taken, the time and duration of sampling, the size of samples to be taken, and other pertinent information on the conditions that are useful to the sampling team. From this information, the sampling team will select the labor hours and apparatus necessary to carry out the sampling task and will follow the appropriate SOP.

The sampling team then develops a QA plan that includes:

- sampling information forms
- lists of apparatus, reagents, supplies
- pre-sampling calibrations
- on-site checks of apparatus
- post-sampling calibrations.

5.9 ANALYTICAL QUALITY CONTROL

Analytical quality is monitored through function checks and control checks. Function checks are performed by the analyst to verify the stability and validity of the sample and the performance of the analytical equipment. Sample validity is assessed in terms of spoilage, container integrity, amount of specimen, sample identification, sample blanks, and other general appearance such as condition of filters or uniformity of sample collections across a filter.

The analytical equipment is checked in terms of calibration and performance of calibrating standards; the latter is part of the permanent record of the analysis. It is recommended that calibration standards that span the working range in factors of two should be run through the entire analysis system at least four times. This develops information on precision and detection limits where appropriate.

Control checks are made by analyzing samples provided by the Task Leader. These samples include blanks, duplicates, spikes, and, if available, standard reference materials in quantities that depend on the total number of samples assigned and on the level of accuracy needed in the analysis. These control samples are introduced into the system in such a manner that the analyst will not give them particular attention.

As a rule, large batches of samples (25 samples or more) should have a control (spike, blank, or replicate) sample in every five samples. A small batch of samples (up to five samples) may have more control samples than real samples.

For large and continuous amounts of samples, control charts of performance of duplicates and spikes must be maintained. This allows the analyst and Task Leader to know when the system is out of control, which part of the system is the probably cause, and when and what corrective action is to be taken.

5.10 EXPERIMENTAL PROTOCOLS

Study Component: CHEMICAL ANALYSIS OF DIETHYL MALONATE

Purpose: A method was developed to permit rapid and selective analysis of diethyl malonate in a variety of matrices. The method developed utilized dimethyl malonate as an internal standard for the quantification of diethyl malonate. Components were separated by capillary gas chromatography and detected by mass spectrometry operated in the selected ion monitoring acquisition mode.

Analytical Method: The GC/MS method employed a Hewlett Packard 5890A capillary gas chromatograph interfaced with a Hewlett Packard 5970A mass spectrometer. Separations were conducted on a 30 m x 0.255 mm i.d. DB-Wax column (J & W Scientific, Folsom, CA) containing a 0.25- μ m film of stationary phase. The polar polyethylene glycol stationary phase was cross-linked and bonded with the fused silica surface to provide for greater thermal stability as well as to minimize phase stripping during splitless injections. Sample extracts (1.0 μ L) were introduced to the column in the splitless mode with a loading time of 0.6 min. After an initial hold at 40°C for 2 min, the column was temperature programmed at a rate of 6°C/min to a final temperature of 220°C.

Method Calibration: Quantification of diethyl malonate was accomplished by the internal standard method utilizing dimethyl malonate as the internal standard. Data acquisition was in the selected ion mode which had the advantage of increasing the selectivity and sensitivity of the analysis over the normal scanning acquisition mode. Masses were selected on the basis of comprising major fragmentation ions that were free from matrix interferences. Based on these criteria, m/e of 101 (specific for dimethyl malonate) and m/e of 115 (specific for diethyl malonate) were chosen. Data acquisition for these ions was at an electron multiplier voltage of 2200 volts with a dwell time of 100 m/sec. A standard curve was generated by spiking diethyl malonate standards with a constant concentration of internal standard and subjecting these samples to analysis under the conditions described above. By plotting the ratio of the integrated peak areas of the diethyl malonate to that of the internal standard as a function of diethyl malonate concentration, a standard calibration curve was obtained. Comparison of the ratio of diethyl malonate to internal standard integrated peak areas in the sample extracts to the standard curve formed the basis for quantification during this study.

Sample Preparation: Methylene chloride extracts of plant tissues, soils, deposition coupons, and air samples were immediately transported to the chemical analysis laboratory where they were promptly spiked with internal standard. Samples containing the internal standard were transferred to autosample vials fitted with Teflon-lined caps and analyzed for diethyl malonate without further manipulation.

Study Component: QUANTIFICATION OF PLANT METABOLIC EFFECTS OF CHEMICAL SIMULANTS

Purpose: The phytotoxicity of CEES on whole plants was investigated using *in vitro* systems. These included: 1) the effects of the simulant on photosynthesis (oxygen evolution) and dark respiration (oxygen uptake) in intact leaf segments; and 2) the effects of the simulant on specific photochemical reactions and electron transport chains in isolated chloroplasts.

Analytical Methods:

Whole Leaf Measurements

Leaf samples from the different species exposed at high and low concentrations of the chemical were taken prior to, immediately following, and at several intervals after exposure for analysis of oxygen evolution and uptake. Leaves were excised from the plants, placed in moistened paper towels, and maintained at 4°C until assayed. They were then wet with distilled water and sliced with a razor blade into pieces <5 mm in length or diameter. The pieces were transferred to an assay medium consisting of 2 mM CaCl₂, 10 mM sodium bicarbonate, and 20 mM HEPES pH 7.6. Paired tissue samples were taken from this solution and placed directly into paired, water-jacketed (3.9 mL of control media at 20°C) cuvettes. The suspension was continually stirred with magnetic stirrers. The cuvettes were then covered with aluminum foil for dark respiration for approximately 25 min until a steady-state rate was obtained. They were then illuminated with saturating light ($>1200 \mu\text{Einsteins min}^{-2} \text{s}^{-1}$) at 600 nm for an additional 20 min to obtain a steady-state rate of photosynthesis. After illumination, the tissues were removed from the cuvettes, and blotted and dried overnight in a 75°C oven so the dry weight could be obtained. Assays were run in triplicate and the data expressed as $\mu\text{Mol O}_2 \text{ h}^{-1} \text{g dry wt}^{-1}$.

Isolated Chloroplast Measurements

Chloroplasts were isolated from commercially obtained spinach (*Spinacea oleracea*) leaves according to the methods of Walker (1980). Approximately 80 g of leaves with the mid-ribs removed were washed with distilled water and chilled prior to grinding. The leaves were then ground for 10 sec with a sorvall tissue homogenizer in 50 mL of grinding medium, consisting of 0.33 M sorbitol; 10 mM $\text{Na}_4\text{P}_2\text{O}_7$; 5 mM MgCl_2 ; and 2mM sodium ascorbate, pH 6.5 which had been chilled to a slush-like consistency to maintain the grinding temperature around 4°C. The ground material was then filtered through 8 layers of cheesecloth and the filtrate immediately centrifuged at 1500 x g for 90 sec. The supernate was then decanted and the surface of the pellet washed with 1 mL of resuspension mix which was then discarded. The pellet was resuspended in a mixture consisting of 0.3 M sorbitol; 2 mM Na_2EDTA ; 1 mM MgCl_2 ; 1 mM MnCl_2 ; 50 mM HEPES; 10 mM f ; 5 mM PPI; 0.5 mM Pi, pH 7.6. Chlorophyll content was determined according to the method of Arnon (1949): 50 μL of the chloroplast suspension was added to 20 mL of 80% (v/v) acetone and filtered (through No.1 Whatman paper), and the absorbance read at 652 nm. Nine divided by the absorbance gives the volume of the original suspension containing 100 μg of chlorophyll. All procedures were carried out under low light and at 4°C.

Photochemical Assays

Assays were conducted on PS II, PS I, and whole-chain electron transport, measuring oxygen evolution and uptake with a Clark-type electrode (YSI Instruments) in a 1.8-mL volume, water-jacketed cuvette (Gilson Medical Electronics) maintained at 20°C. Stock solution of the CEES was prepared so that addition of 100 μL would equal a final concentration within the cuvette of 1 or 10 ppm. All assays were conducted in paired cuvettes at the same time, with one cuvette serving as a control and the other containing the simulant. The CEES was either added directly to the cuvette prior to illumination (~1 min) or to a chloroplast suspension in a test tube for 1 h prior to transfer to the cuvette for assay. Control chloroplasts were treated in the same manner. Assays were run in triplicate, and all data are expressed in either $\mu\text{Mol O}_2 \text{ h}^{-1} \text{ mg}^{-1} \text{ chl}$ or as % control of the paired assay. The analyses of the three components of the chloroplast electron transport system were performed according to the following methods:

PS II Measurements: Assays were conducted according to the methods of Boyer and Bowen (1970). The assay medium (1.8 mL) consisted of 0.33 M sorbitol; 2 mM Na_2EDTA ; 1

mM MgCl_2 ; 1 mM MnCl_2 ; and 50 mM HEPES, pH 7.6. Sodium 2,6-dichloroindophenol (DCIP), 0.88 mM, was added just prior to the addition of chloroplasts (100 μg). The suspension was then illuminated from the side with saturating light ($>1200 \mu\text{Einsteins min}^{-2} \text{s}^{-1}$) at 600 nm, and the rate of oxygen evolution determined from the initial slope of the electrode output as a function of time.

PS I Measurements: Assays were conducted according to the methods of Keck and Boyer (1974). The assay medium consisted of 1 mM ADP, 1 mM K_2HPO_4 , 0.1 M KCl, 5 mM MgCl_2 , 0.1 mM DCMU, 80 μM DCIP, 1 mM sodium ascorbate, 0.5 mM methyl viologen (MV), 0.5 mM sodium azide (prepared daily), and 100 μg chlorophyll. Assays were illuminated and measured as above.

Whole-Chain (Water to MV) Measurements: Assay conditions were identical to those described for PSI measurements (Keck and Boyer, 1974) except that DCMU, DCIP, and sodium ascorbate were deleted from the medium.

Study Component: INHIBITION OF SOIL ENZYMATIC PROCESSES BY CHEMICAL SIMULANTS

Purpose: The effects of CEES on soil microbial and biochemical activities were evaluated *in vitro* by measuring the activity of two soil enzymes, dehydrogenase and phosphatase.

Dosing and Incubation: Stock solutions of CEES (Aldrich Cat. No. 24264-0, Lot No. KM00903JM) were prepared in distilled water and added to samples of Palouse and Burbank silt loam soils (to final concentrations ranging from 0 to 250 $\mu\text{g/g}$ dry soil) and incubated at 22°C in the dark. All dehydrogenase and phosphatase activities were measured in duplicate and mean values were compared with those of the control soil (not CEES-treated) and expressed as percent of those of the control.

Experimental Methods:

Dehydrogenase Activity

Soil samples were assayed for dehydrogenase activity as described by Tabatabai (1982) immediately following incubation and after 1 week and 4 weeks. Soils amended with CEES (1.5 g dry weight basis) were first mixed with 0.015 g of CaCO_3 ; 0.3 ml of 1% glucose and 0.25 mL of 3% 2,3,5-triphenyltetrazolium chloride (TTC) and

incubated for 24 h at 22°C. Ten mL of methanol was then added to the soil and mixed thoroughly. The mixture was centrifuged and the absorbance of the supernatant at 485 nm was measured using a Beckman DU-50 spectrometer. Soil dehydrogenase activity, expressed as mg of TTC-formazan produced per g of soil/24 h, was quantified by comparing absorbance values to a standard curve prepared with reagent-grade TTC-formazan and methanol.

Phosphatase Activity

Soil phosphatase activity was measured on the CEES-amended soil using the procedure described by Tabatabai and Bremner (1969) as modified by Klein et al. (1979). One g of soil (dry weight) was placed in 15-mL centrifuge tubes with 4 mL of modified universal buffer (MUB), which consists of tris(hydroxymethyl) amino methane, 3.025 g; maleic acid, 2.9 g; citric acid, 3.5 g; boric acid, 1.57 g; 1 N NaOH, 122 mL yielding final volume of pH 8.65. One mL of para-nitrophenol phosphate (0.025 N prepared with MUB) was added to each tube. The tubes were stoppered, vortexed and incubated for 1 h at 37°C. One mL of 0.5 N CaCl_2 and 4 mL of 0.5 N NaOH were then added to stop the reaction. The mixtures were centrifuged at 12,000 g for 10 min, and supernatant absorbance was measured at 400 nm with a spectrophotometer. Phosphatase activity was determined by comparing these values to a standard curve constructed with reagent-grade para-nitrophenol and expressed as μg of para-nitrophenol released per g of soil/h.

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