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ABSTRACT

To determine the potential environmental persistence and toxic effects of agent simulants Diethyl Malonate (DEM) and Methyl Salicylate (MS), plants, soils, earthworms, and soil microbial populations were exposed to projected aerosolized simulant concentrations of ~100 (low) and ~1000 (high) mg/m^3. Both simulants exhibited biphasic residence times on foliar and soil surfaces following aerosol exposure. Half-times of DEM on soil and foliar surfaces were 1 to 3 h and 5 to 242 h, respectively, and 1 to 2 h and 5 to 31 h for the MS, respectively. Persistence was longer on the foliar surfaces than that of the soils. Both simulants proved phytotoxic to vegetation with a lower theshold of 1 to 2 $\mu g/cm^2$ for the MS versus that of 10 $\mu g/cm^2$ for the DEM. However, neither significantly affected chloroplast electron transport *in vitro* at concentrations of up to 100 $\mu g/mL$. Results from *in vitro* testing of DEM indicated concentrations below 500 $\mu g/g$ dry soil generally did not adversely impact soil microbial activity, while the theshold was 100 $\mu g/g$ dry soil for the MS. Earthworm bioassays indicated survival rates of 66% at soil doses of 204 μg DEM/cm^2 soil and 86% at soil doses of 331 μg MS/cm^2.

INTRODUCTION

The use of chemical agent simulants and decontaminants at both U.S. and foreign training sites has the potential for producing significant environmental impacts. A previous evaluation of the U.S. Army Chemical Research, Development, and Engineering Center (CRDEC) Environmental Fate and Effects Data Base by Reinbold et al. (1986) identified several simulants requiring additional laboratory data to determine their environmental persistence and acute terrestrial and aquatic toxicity. Included in this listing were the simulants Diethyl Malonate (DEM) and Methyl Salicylate (MS).

Diethyl Malonate is an aliphatic dicarboxylic ester that is liquid at room temperature and has a water solubility of 2.7% at 20°C. It is employed in thermal decontamination studies, shipboard decontamination design, shipboard washdown, contamination transfer, and vehicle entry/exit studies. The compound is relatively chemically stable and is particularly prone to hydrolysis in aqueous environments to yield ethyl alcohol and malonic acid salts.

Methyl salicylate is an organic ester. It is liquid at room temperature and has a water solubility of

0.07% at 20°C. It is used to perform entry/exit tests for collective protection and Nuclear, Biological and Chemical (NBC) shelters. Methyl salicylate can undergo both acidic and alkaline hydrolysis of the ester to produce methyl alcohol and salicylic acid or a salicylic acid salt. Both methyl salicylate and salicylic acid can undergo further biodegradation by aerobic and anaerobic microorganisms.

The objective of this study was to perform an initial determination of potential acute environmental effects and persistence of DEM and MS in soils, plants, earthworms, and soil microbial populations following aerosol and *in vitro* applications.

MATERIALS AND METHODS

Exposure System

Aerosol exposures of thee species of plants (short-needle pine, *Pinus echinata*; tall fescue, *Festuca arundinacea*, 'K-13'; and sagebrush, *Artemisia tridentata*, vaseyana) and two types of soils (Burbank, a sandy, skeletal, mixed, xeric, Torriorthent; and Palouse, a fine-silty, mixed, mesic, Pachic Ultic Haploxeroll) to DEM and MS were conducted in a sealed exposure chamber at the Pacific Northwest Laboratory (PNL) Aerosol Wind Tunnel Research Facility as described in Cataldo et al. (1988). Plants, soils, and deposition coupons were placed into the exposure chamber before each test. The aerosols were generated by applying compressed air to nebulizers containing neat simulants. Exposures were conducted at 21°C and 35% to 40% R.H. for 1 h following which the plants, soils, and deposition coupons were removed and the initial samples obtained.

Simulant Chemistry

For DEM all analyses were performed on a Hewlett-Packard Model 5890A gas chomatograph (Hewlett-Packard Instruments, Palo Alto, CA) interfaced to a Hewlett-Packard Model 5970A mass spectrometer using DEM as an internal standard. Methyl salicylate and salicylate in sample extracts were determined by reversed-phase chomatography on a C-18 column (Waters Associates, Milford, MA). Acetic acid was added to the mobile phase to suppress the ionization of salicylic acid and the salicylic acid esters resulting in increased retention times for these species. The separated components were detected by their ultraviolet absorption at 304 nm by a Waters 490E detector operated at a sensitivity of 0.008 a.u.f.s. Integrated peak areas, provided by a Hewlett-Packard model 3390A integrator, formed the basis for quantification.

Estimation of Dose to Plant and Soil Surfaces

Leaf tissue (duplicate samples from different places within the canopy) contaminated with simulant were placed in glass vials containing 10 mL of high-purity, distilled-in-glass methylene chloride for the DEM, or acetonitrile for the MS, 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 Li-Cor LI-3000 (Li-Cor Instruments, Lincoln, NB) leaf area meter. The foliar mass loading was calculated as μg contaminant/cm^2 leaf surface.

Thee subsamples of each soil sample were removed from Petri dishes exposed to aerosols using a cork borer (sample area was 3 x 0.95 cm^2), and the samples were placed into a 25-mL tared Corex centrifuge tube with a Teflon-lined screw cap. Five milliliters of solvent 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 x g for 10 min at 25°C. 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. Mass loading was calculated as μg contaminant/cm^2. All sample extracts from the tests were kept frozen at -80°C before analysis.

Phytotoxicity

Assessments of visual phytotoxicity resulting from foliar contamination with DEM or MS were based on the development of visual (gross) toxicity symptoms. Quantitation of these effects is based on a Modified Daubenmire Rating Scale (Daubenmire 1959) where the ratings go from 0 to 6 with a rating of 0 indicating no obvious effect over the control plants and 6 indicating that between 95% to 100% of the foliage is affected.

The basis of the gross phytotoxic symptoms of DEM and MS 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).

Soil Microbial Assays

Soils described above were amended with either DEM or MS to final concentrations ranging from 0 to 2500 μg/g 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 effects of DEM and MS on soil microbial activities were then evaluated *in vitro* by measuring the activity of the two soil enzymes, soil dehydrogenase and soil acid phosphatase, at selected times post-amendment (see text) using a modification

of the method of Tabatabai (1982). All activities were measured in triplicate and mean values compared with that of the control soil (non-simulant treated) and expressed as a percentage of the control. The EcD_{50}, an expression of the ecological dose of simulant causing 50% inhibition (Babich et al. 1983) was calculated for each soil and enzyme from response curves depicting percentage inhibition with concentration at the sampling time given.

Soil Invertebrate Measurements

An earthworm (*Eisenia fetida*) bioassay system was used to elucidate toxicity of the DEM and MS to soil invertebrates. 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 six worms. Five replicate plates were exposed to the aerosol in each test series Effects scored included both earthworm mortality and activity. Mass loading or dose was determined on similar soil plates without worms.

RESULTS AND DISCUSSION

Simulant Depuration

Plants and soils contaminated with aerosolized simulant were sampled at intervals over a 5-day period. The depuration plots of both DEM and MS from plants and soils were biphasic. A typical curve, in this case for MS depuration from sagebrush, tall fescue, and short-needle pine foliage, is given in Figure 1. Slopes derived from the semi-log plots of these values as a function of post-exposure time allowed for calculation of the environmental half life ($t_{1/2}$) of the simulants on the different surfaces and are presented along with the mass loading values in Tables 1 (plants) and 2 (soils). The initial rapid loss phase had a half-life of 1.3 to 3.3 h for the DEM and 0.9 to 2.4 h for the MS on sagebrush, tall fescue, and short-needle pine foliage (Table 1). The longer depuration component had half-lives of 45, 243, and 17 h for sagebrush, short-needle pine, and tall fescue, respectively, for the DEM and 31, 10, and 6 h, respectively, for the MS. At most, only 1 to 4 $\mu g/cm^2$ (sagebrush) of the simulants remained on the foliar surfaces after 4 days. In the case of the short-needle pine (Table 1), depuration of the DEM apparently persisted much longer than the MS on the same tissue and at approximately the same mass loading.

Because both simulants have a significant vapor pressure, volatilization likely accounts for the observed losses in the rapid phase while the slower loss rate seen in the second phase may represent

losses resulting from volatilization, and both abiotic and biotic decomposition. It is also likely that the cutin and wax surface layers of the foliage affect sorption and thus the subsequent volatilization of the simulants (Baker 1980).

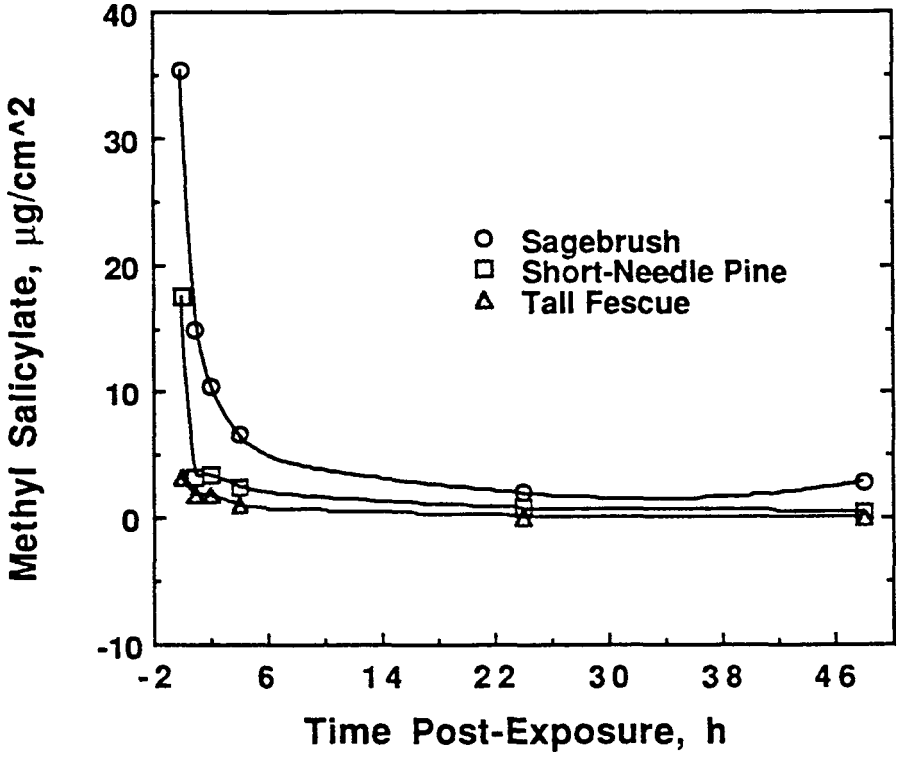

Figure 1. Depuration of MS from Sagebrush, Short-Needle Pine, and Tall Fescue Foliage Over Time

Even though the amounts of mass loading were greater, the loss of the material from the soils was more rapid than that from the foliage (Table 2). There appeared to be differences between the soil types with the Palouse, which has a higher organic-matter content than the Burbank (1.7% versus 0.5% organic carbon) exhibiting a longer second phase. For example, of the 370 µg MS/cm^2 initially deposited to the Palouse soil, only 51 µg/cm^2 remained after 2 days, while in Burbank soil, less than 5 µg MS/cm^2 remained after 2 days. Sorption of the simulant to the soil organic matter may have contributed to this difference.

Table 1. Foliar Mass Loading and Depuration of Simulants DEM and MS Following Exposure to Aerosol.

Simulant	Species	Foliar Mass Loading, µg/cm^2	Half-Life ($T_{1/2}$), h	
			Rapid	Slow
DEM	Sagebrush	114.8	2.18	45.1
	Short-Needle Pine	17.08	1.29	242.54
	Tall Fescue	19.36	3.31	16.99
MS	Sagebrush	35.4	1.13	37.43
	Short-Needle Pine	17.44	0.85	19.8
	Tall Fescue	3.72	2.37	3.25

Table 2. Soil Mass Loading and Depuration of Simulants DEM and MS Following Exposure to Aerosol.

Simulant	Species	Soil Mass Loading, µg/cm^2	Half-Life ($T_{1/2}$), h	
			Rapid	Slow
DEM	Burbank	175	1.22	5.4
	Palouse	175	1.97	16.51
MS	Burbank	378	0.61	22.0
	Palouse	369	1.04	91.35

Gross Phytotoxicity

Both simulants proved phytotoxic to vegetation with a lower theshold of 1 to 2 $\mu g/cm^2$ for the MS versus that of 10 $\mu g/cm^2$ for the DEM for 1% to 5% visible damage to the foliage of the plants. Sagebrush and tall fescue exhibited the most foliar mass loading and concomitant damage in those plants exposed to the DEM and the MS. These loading differences may be attributed in part to the leaf blade orientation and surface texture of these species as opposed to that of the pines. Severe symptomology was evident at MS concentrations 2 (sagebrush, 46 versus 115 $\mu g/cm^2$) to 40 (tall fescue, 4 versus 194 $\mu g/cm^2$) times less than comparable damage with DEM (Table 3), indicating that MS was the more phytotoxic of the two.

Table 3. Average Foliar Mass Loading (± s.d., N=6) at Low and High Aerosol Concentrations of DEM and MS and Subsequent Phytotoxicity at 21 Days Post-Exposure.

Simulant	Species	Foliar Mass Loading, $\mu g/cm^2$	Damage Index[a]
DEM	Short-Needle Pine	0.20±0.12	1.0
		17.08±2.94	3.0
	Sagebrush	11.69±0.54	1.0
		114.79±29.44	5.0
	Tall Fescue	8.32±4.45	1.0
		193.56±63.41	6.0
MS	Short-Needle Pine	0.77±0.35	1.0
		6.11±1.51	2.0
	Sagebrush	1.85±0.72	1.0
		46.00±6.58	6.0
	Tall Fescue	1.30±1.15	1.0
		4.19±1.61	5.0

[a] Modified Daubenmire Scale.

Metabolic Effects on Plants

Technical reasons during the exposure series with the DEM prevented O_2 electrode measurements of the tissues exposed directly to the aerosol. Subsequent, *in vitro* experiments (data not shown) showed that plant tissues (leaf slices) exposed to 100 $\mu g/mL$ of DEM for 15 min failed to show any adverse effects using polarographic methods. However, these data could not be directly compared to

the data obtained for the aerosol exposed MS tissues, and therefore the MS result will be presented separately.

Tissue samples from MS-exposed leaves indicated that immediately following exposure, a reduction in both the photosynthetic and dark respiratory rate was observed in all plants proportional to the exposure concentration. In the case of the sagebrush (Tables 4 and 5), at the high concentrations the net phototsynthetic rate decreased to zero (Table 4) while the respiratory rate (Table 5) declined to less than 10% of the controls. However, within 2 days a recovery was apparent and within 2 weeks both rates had nearly returned to the control level in the surviving plants (one plant that had died in the high-dose experiments was not sampled). This recovery was aided by the production of new leaves during this time. Similar responses were observed in the other two species.

Table 4. Average Net Photosynthesis (O_2 Evolution) (±s.d., N=4) of Leaf Segments Expressed as Percentage of Control Plant Rates Measured at Various Intervals Post-Exposure from MS.

Species	Day Post-Exposure	Net Photosynthesis	
		Low Dose	High Dose
Sagebrush	-2	100.0±0.0	100.0±0.0
	0	60.4±21.1	-35.3±30.3
	2	94.1±26.5	-11.2±15.0
	5	116.5±14.9	12.8±1.9
	14	118.0±14.6	51.6±30.8
Short-Needle Pine	-2	100.0±0.0	100.0±0.0
	0	85.9±8.2	58.7±12.1
	2	46.4±21.9	38.7±3.2
	5	52.9±26.3	78.6±19.2
	14	56.7±25.4	77.1±19.1
Tall Fescue	-2	100.0±0.0	100.0±0.0
	0	60.5±6.7	-3.5±17.8
	2	106.2±10.2	17.1±4.94
	5	105.9±23.6	63.2±5.5
	14	99.0±23.6	95.1±15.8

The damage and apparent loss of photosynthetic capability (shown for the MS tissues and projected for the DEM tissues) may have been caused by either direct contact phytotoxicity to the surface of the leaves (thereby damaging the leaf area capable of photosynthesis), indirect metabolic damage to sensitive biochemical pathways within the cells of the leaf, or a combination of both. In addition to the polarographic analysis, and in an attempt to resolve these possibilities, dry matter accumulation over two harvests (cuttings) of tall fescue were measured. The exposed tissue was then eliminated after the first harvest making evident any residual effects in the second. For both simulants there were slight but significant ($P>0.05$) decreases in the dry weight of the foliage from the plants exposed to the high

concentrations of simulants as opposed to the controls after 60 days of growth (data not shown). This indicated that there may be a slight residual effect but that the majority of damage may have been contact in nature.

Table 5. Average Dark Respiration (O_2 Uptake) (±s.d., N=4) of Leaf Segments Expressed as Percentage of Control Plant Rates Measured at Various Intervals Post-Exposure from MS.

Species	Day Post-Exposure	Dark Respiration	
		Low Dose	High Dose
Sagebrush	-2	100.0±0.0	100.0±0.0
	0	46.5±22.6	47.1±5.4
	2	80.3±10.9	13.5±24.6
	5	68.5±32.6	51.1±12.1
	14	139.4±2.1	102.1±6.9
Short-Needle Pine	-2	100.0±0.0	100.0±0.0
	0	54.1±11.7	37.8±18.4
	2	52.8±12.6	49.5±18.9
	5	49.5±2.2	64.5±3.2
	14	90.3±22.1	142.4±3.1
Tall Fescue	-2	100.0±0.0	100.0±0.0
	0	71.1±11.4	14.3±17.8
	2	104.4±10.9	43.2±25.4
	5	88.6±1.1	86.7±1.4
	14	67.6±19.6	86.5±5.1

To confirm this, *in vitro* studies were undertaken using isolated spinach (*Spinacea oleracea*) chloroplasts to determine potential effects on photosynthetic electron transport. The results of these studies (given in Table 6) indicate that no apparent effect was evident on the electron transport system (whole chain, Photosystem I, and Photosystem II) at simulant concentrations up to 100 µg/g. This again appears to support the concept of the immediate contact toxicity with low residual effects to vegetation from the two simulants tested.

Table 6. Interaction of 100 µg/g (ppm) DEM or MS with Isolated Spinach Chloroplasts[a].

Simulant	Electron Transport System,% Control±SD (n=3)		
	Whole Chain	Photosystem I	Photosystem II
DEM	99.4±13.1[b]	107.5±10.6[b]	94.9±10.5[b]
MS	101.3±8.9[b]	100.5±3.5[b]	97.38±4.5[b]

[a] *In vitro* amendment of simulant, exposure duration approximately 5 min.

[b] Not significant based on two-tailed t-test, P>0.1.

Soil Microbial Effects of Simulants

Inhibition of enzymes, which drive key metabolic reactions in microbial cells is a probable underlying cause of chemical toxicity to soil 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 and MS toxicity toward soil microorganisms and soil biochemical processes.

The EcD_{50}, an expression of the ecological dose of DEM causing 50% inhibition (Babich et al., 1983), was calculated for each soil and enzyme from response curves; results are given in Table 7. For both Burbank and Palouse soils, the EcD_{50} after 3 days incubation was roughly 2500 µg/g and after 28 days incubation, it increased to greater than 2500 µg/g, indicating a recovery trend as a function of time for soil dehydrogenase activity. The EcD_{50} of DEM on soil phosphatase is far greater than the highest DEM concentration tested (2500 µg/g) and at day 28 appeared to be higher than at day 3, again indicating a recovery trend as a function of time for soil phosphatase activity.

The EcD_{50} of MS for soil dehydrogenase activity in Burbank soil stayed roughly the same after 42 days, and EcD_{50} for Palouse soil increased from 1338 µg/g to being no longer inhibited by MS. In fact soil dehydrogenase activity was greatly stimulated with increased incubation time. These findings suggested that the effect of MS on soil dehydrogenase activity was transient in nature and that soil microorganisms might be able to metabolize this simulant, resulting in an increase in their activity. Results also indicate that Palouse soil was less effected than Burbank soil.

Effects of Simulants on Earthworm Survival

The earthworm bioassay was performed using artificial soil amended with 107 and 204 µg DEM/cm^2 and 56 and 331 µg MS/cm^2. Earthworms were evaluated for survival and activity at 1, 10, and 21 days post-exposure. The final measurements, taken at 21 days post-exposure, are given in Table 8. In the DEM treatment, survival rate decreased to 66%, and activity was judged to be affected in over 40% of the individuals. In the MS treated samples, survival decreased to 86%, and activity was judged to be affected in only 20% of the individuals. Given the rapid loss though volitilization seen above on other soil surfaces for the MS, it was believed that the worms received only a small exposure even in the high-dose experiments. This may not have been as true for the DEM-treated animals and may be in part responsible for the higher evident toxicity.

Table 7. The Effect of MS on Burbank and Palouse Soil Enzyme Activities Expressed as EcD_{50} Values (μg/g soil)[a].

Simulant	Days Post-Exposure	Dehydrogenase		Phosphatase	
		Burbank Soil	Palouse Soil	Burbank Soil	Palouse Soil
DEM	3	~2500	~2500	>2500	>2500
	28	>2500	>2500	>2500	>2500
MS	1	1195	1338	stimulated	>2500
	42	1250	stimulated	>2500	>2500

[a] EcD_{50} represents the ecological dose that caused a 50% inhibition of the soil enzyme activity.

Table 8. Influence of Soil-Deposited DEM and MS on the Survival of Earthworms (*Eisenia foetida*). Artificial soils (70 g) and worms exposed and held for 21 days post-exposure.

Simulant	Soil Mass Loading, μg/cm^2	Earthworm	
		Survival	Condition
DEM	204.1±39.6	20/30 (66%)	Moderately Active
MS	331.8±93.5	26/30 (86%)	Moderately Active

CONCLUSIONS

The volatility of DEM and MS results in a rapid loss from soil and foliar surfaces. This loss is biphasic in nature and consists of a short-term phase with half lives ranging from 1 to 3 h and a second phase ranging from 3 to >200 h for foliage. The depuration of MS is somewhat faster than that of DEM, but both simulants may be dependent on cuticular composition or soil organic matter contents.

Diethyl Malonate is not phytotoxic at foliar mass loading levels of less than 10 μg/cm^2 and MS at concentrations less than 1 to 2 μg/cm^2 However, severe damage is evident at mass-loading levels in excess of 17 μg/cm^2 DEM and 5 to 40 μg/cm^2 MS and varies with plant species. Tall fescue and sagebrush were more affected than was short-needle pine by both simulants. Regrowth of tall fescue indicated that the effects of both DEM and MS are slightly residual.

In vitro studies showed that photosynthesis and respiration in plants exposed to MS aerosols were

adversely affected. However, recovery occurred within 4 to 14 days as new growth was initiated. Evaluation of the effects of both DEM and MS on photosynthetic electron transport failed to demonstrate any affect on the processes, thus indicating that both DEM and MS may exhibit contact phytotoxicity though disruption of membrane function and cell integrity.

Results from *in vitro* testing of DEM indicated that concentrations below 500 µg/g dry soil generally did not negatively impact soil microbial activity. The EcD_{50} was ~ 2500 µg/g for soil dehydrogenase and > 2500 µg/g for phosphatase activity indicating soil dehydrogenase is more susceptible to DEM than is 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 from *in vitro* testing with MS indicated that concentrations below 100 µg/g dry soil generally did not negatively impact soil microbial activity. In fact, Palouse soil dehydrogenase activity was enhanced by the addition of MS after 42 days incubation. The short term effect of MS (1-day incubation) was more profound on soil dehydrogenase activity (EcD_{50} = 1195 µg/g) than on soil phosphatase activity (stimulated, EcD_{50} could not be calculated), indicating soil dehydrogenase is more susceptible to MS than is soil phosphatase. However, soil enzyme activities increased, recovered, or were enhanced after 42 days incubation, indicating the effect is transient in nature followed by a recovery trend.

Results of the earthworm bioassay indicate a slightly higher toxicity from the DEM at lower initial dosages than those of the MS. The MS however exhibited a higher depuration rate than the DEM and may have had an actual exposure concentration much less than that measured.

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