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Effect of Metals and Other Inorganic Ions on Soil Microbial Activity: Soil Dehydrogenase Assay as a Simple Toxicity Test

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Most assays used to evaluate the effects of potential pollutants on soil microbial activity are either lengthy, spanning several weeks or months, or require the use or development of sophisticated equipment or complex incubation systems. For example, Babich et al. (1983) measured respired CO_2 from soils incubated up to 28 days to obtain "ecological dose" values for Cd and Zn. Drucker et al. (1979) have used both released CO_2 and the enumeration of microbial populations on selective growth media to investigate the effects of 17 metals on soil microbial activity over several weeks of exposure. The effect of Cd on carbon mineralization in coniferal soil and litter microcosms incubated for 24 days was examined by Bond et al. (1976). Although these techniques have contributed greatly to our understanding of the effects of metals and other pollutants on soil microbial populations, they are not readily amenable to the rapid analysis of the increasing numbers of new chemicals. Simple and time efficient assays would greatly facilitate future evaluations of chemicals potentially toxic to soil microorganisms.

The purpose of this report is not to introduce a new assay but to illustrate the utility of the previously developed soil dehydrogenase assay as an effective primary test for assessing the potential toxicity of chemicals to soil microbial activity. The soil dehydrogenase assay that was introduced by Lenhard (1956) and has gone through several modifications (Casida et al. 1964, Klein et al. 1971) is both simple and efficient. In this manuscript we describe our use of the soil dehydrogenase assay in determining the effects of a number of potential toxic inorganic ions on soil microbial activity. The ions include Cu^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, NH_4^+, Cd^{2+}, Cr^{3+}, F^-, AsO_4^{3-}, BO_3^{3-} and SO_4^{2-}.

MATERIALS AND METHODS

Soil dehydrogenase activity was assayed by the method of Klein et al. (1971) as modified by us. Assays were initiated by adding

0.5 mL of a 0.5% (w/v) glucose solution to 25 mL centrifuge tubes containing one gram of air-dried, alfalfa-enriched or unenriched soil that had been amended with 0.2 mL of a 3% (w/v) solution of 2,3,4-triphenyl tetrazolium chloride (TTC). Test compounds were added as part of the glucose solution. To control for assay components that absorbed at 485 nm, a reagent blank was prepared by replacing the TTC solution with distilled water. Assay and control tubes were incubated at 27°C in the dark. At the end of 24 hr, 10 mL of methanol was added to each tube and the tubes were then thoroughly mixed to efficiently extract TTC-formazan formed during the incubation. After separating the extracting solution from the soil by centrifugation at 12,000 g, the supernatant solution was decanted and its absorbancy was determined at 485 nm with a Spectronic 20 colorimeter. Soil dehydrogenase activity, expressed as μg TTC-formazan produced per gram soil per 24 hr, was quantified by comparison of adsorbance values to a standard curve (0 to 30 μg per mL methanol) prepared with reagent grade TTC-formazan. All assays were duplicated.

The soil was a composite sample taken from Section 24 of the Rocky Mountain Arsenal. Before sampling, all plant forms and organic matter within the O horizon were removed. Upon receipt at Pacific Northwest Laboratory, the soil was air dried, sieved (<2 mm) and mixed before use. The soil contained 1.30% (w/w) organic carbon.

Enriched soil was prepared in the following manner. A uniform mixture of air-dried soil (400 g) and alfalfa (4 g) were placed in a 1 L Erlenmeyer flask where 87 mL of distilled water was evenly distributed over the mixtures surface to bring the soil moisture to 22% of the moisture holding capacity. The moist soil mixture was incubated in the dark for 6 days after which the "enriched soil" was placed in a large pyrex dish and air dried.

All chemicals are analytical reagent grade and were used without further purification. Standard solutions of all test compounds were prepared in distilled waters. Ammonium sulfate, zinc sulfate ($7H_2O$), sodium sulfate, nickel (II) sulfate ($6H_2O$), cadmium (II) nitrate ($4H_2O$), and sodium borate ($10H_2O$) were obtained from J. T. Baker Co. Chromium (III) sulfate (XH_2O) was obtained from Mallinkrodt. Sodium fluoride was obtained from Aldrich. Copper (II) sulfate ($5H_2O$) was obtained from Fisher Scientific and magnesium sulfate from MCB Manufacturing Chemists. Sodium arsenate ($7H_2O$), 2,3,5-triphenyl tetrazolium chloride (TTC) and 2,3,5-triphenyltetrazolium formazan (TTC-formazan) were purchased from Sigma. Glucose was purchased from Difco Laboratories.

EC_{50} values, defined as the concentration of test compound resulting in a 50% reduction in dehydrogenase activity, were obtained

from a nonlinear least-squares curve fit of individual data sets to the exponential equation:

$$Y = a\,[1 - e^{-bd}] \qquad (1)$$

where Y equals the percentage inhibition observed for a given compound concentration, d equals the compound concentration, the parameter a is the asymtotic value represented by 100 percent inhibition and b is the dose dependent rate parameter. Values for a and b were obtained using a COMP-A BASIC language nonlinear least-squares curve fitting program (Thomas et al. 1977). EC_{50} values were calculated by setting Y equal to 50 and solving Equation (1) for d.

Because the data was evaluated as percent inhibition the values for the parameter a should not have been greater than 100 and only in the one case of Zn^{2+} (unenriched soil) did this occur (Table 1). Except for Cr^{3+} (unenriched) t-tests of the parameters were significant (data not shown).

Table 1. Values for the parameters a and b determined from a nonlinear least squares fit of Equation (1).

	Parameters	
Cation	a	b
Cu^{2+} A[a]	93.99	0.025
B[b]	98.04	0.013
Ni^{2+} A	99.47	0.0035
B	92.72	0.0055
Zn^{2+} A	96.27	0.0023
B	145.94	0.00084
Cd^{2+} A	96.25	0.0044
B	84.10	0.02257
Cr^{3+} A	100.36	0.0018
B	78.62	0.038

a) Soil was enriched with 1% alfalfa (see Materials and Methods).
b) Soil was not enriched.

RESULTS AND DISCUSSION

Dehydrogenases are the major representatives of the oxidoreductase enzymes. They catalyze the oxidation of substrates producing electrons that can enter the cells electron transport system (ETS). Specific dyes such as TTC that intercept the flow of electrons can be used as indicators of ETS activity. During the process, TTC is reduced to TTC-formazan, an insoluble red precipitate, that can be extracted by organic solvents and quantified by absorption spectroscopy. The soil dehydrogenase assay is simple and requires no expensive equipment other than a centrifuge and spectrophotometer.

A significant correlation between soil dehydrogenase activity and soil respiration has been reported (Stevenson 1959, Stevenson 1962, Frankenberger, Jr. and Dick 1983). The earlier correlations led Casida and coworkers (1964) to suggest that the dehydrogenase assay would be useful in evaluating the effect of toxic chemicals on soil microbial activity. Since then, however, only a few investigators have used dehydrogenase activity as a soil toxicity assay. Broecker and Zahn (1977) found in their investigations of the toxicity of 3,5-dichlorophenol to activated sludge that comparable results were observed with the dehydrogenase and respiration assays. In studies of the effects of organic and inorganic toxicants on biological purification systems, Lenhard (1963) observed that the dehydrogenase assay was more sensitive to heavy metals (Ag, Hg, Cr) than organic compounds. Bremner and Tabatabai (1973) have found that the assay is susceptible to interference by nitrate and other inorganic soil constituents. More recently Doelman and Haanstra (1979) have found that dehydrogenase activity was affected by Pb in a similar way to soil respiration measured with a Gilson respirometer.

We have extended these studies to investigate the toxic effects of a number of metals and inorganic ions (Tables 2 and 3). The inhibition of soil dehydrogenase activity was investigated in an air-dried, unenriched soil and the same soil enriched for microbial activity with 1% (w/w) alfalfa. The enriched soil was found to have 3-4 times the soil dehydrogenase activity of the unenriched soil.

Table 2 shows the inhibition of soil dehydrogenase by inorganic cations. The metal ions Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Cr^{3+}, normally found in trace concentrations in pristine soils, were significant inhibitors of soil dehydrogenase activity in both the enriched and unenriched soil. The EC_{50} values for the trace metals ranged from a low of 13 ppm for Cr^{3+} (unenriched soil) to a high of 346 ppm for Zn^{2+} (unenriched soil). Inhibition of dehydrogenase activity in

Table 2. Inhibition of Soil Dehydrogenase Activity by Inorganic Cations.

Cation		Cation Concentration (ppm) 0	30	150	300	500	1,000	3,000	5,000	EC_{50}
		Dehydrogenase Activity (Percentage of Control)[a]								
Cu^{2+}	A[b]	104	50	-[d]	9	6	4	0	0	29
		96	47	-	18	11	4	3	0	
	B[c]	100	78	-	12	6	6	0	0	53
		100	65	-	12	6	6	0	0	
Mg^{2+}	A	98	101	92	101	95	106	96	99	>5,000
		102	101	101	107	98	96	98	95	
	B	100	111	119	97	94	101	112	118	>5,000
		100	103	94	93	104	88	78	87	
Ni^{2+}	A	91	86	71	32	11	8	1	0	114
		109	86	69	32	11	6	1	0	
	B	93	61	52	31	6	6	0	0	77
		107	61	56	31	19	6	0	0	
Zn^{2+}	A	101	93	63	55	33	15	4	3	177
		99	93	66	56	34	16	3	3	
	B	100	98	87	72	51	17	0	0	346
		100	101	87	67	45	20	0	0	
NH_4^+	A	97	101	79	74	62	71	86	84	>5,000
		103	109	72	59	80	86	77	84	
	B	104	96	89	93	102	104	119	129	>5,000
		96	109	111	111	114	115	95	138	
Cd^{2+}	A	101	96	47	32	11	7	3	0	93
		99	99	48	34	11	6	3	0	
	B	100	50	32	14	8	0	0	0	20
		100	55	41	8	5	0	0	0	
Cr^{3+}	A	98	105	83	67	33	6	2	3	216
		102	106	83	69	35	6	2	3	
	B	100	46	32	23	6	0	0	0	13
		100	46	41	23	6	0	0	0	

a) Percent of control values were calculated by dividing the µg of TTC-formazan produced in the presence of test compound by the average of two control values for each experiment. The reported control values represent the individual values of two controls divided by their average.

b) Soil was enriched with 1% alfalfa (see Materials and Methods).

c) Soil was not enriched.

d) Experiment not done.

Table 3. Inhibition of Soil Dehydrogenase Activities by Inorganic Anions.

Anion		Anion Concentration (ppm) 0	30	150	300	500	1,000	3,000	5,000
		Dehydrogenase Activity (Percentage of Control)[a]							
F^-	A[b]	100	92	101	124	136	137	175	160
		100	107	115	124	131	137	158	151
	B[c]	102	110	99	114	118	148	133	65
		98	95	114	118	122	139	138	75
AsO_4^{3-}	A	109	97	69	33	26	23	20	19
		91	91	62	37	30	23	21	23
	B	113	70	36	26	29	26	30	33
		87	88	38	30	26	33	27	30
BO_3^{3-}	A	96	100	94	102	102	80	27	28
		104	89	103	100	99	84	32	29
	B	92	108	108	87	87	41	32	10
		108	111	100	97	76	25	10	14
SO_4^{2-}	A	100	-[d]	-	-	108	101	-	84
		100	-	-	-	89	104	-	101
	B	100	-	-	-	134	146	-	120
		100	-	-	-	134	165	-	146

a) Percent of control values were calculated by dividing the µg of TTC-formazan produced in the presence of test compound by the average of two control values determined for a given experiment. The reported control values represent the individual values of two controls divided by their average.
b) Soil was enriched with 1% alfalfa (see Materials and Methods).
c) Soil was not enriched.
d) Experiment not done.

unenriched soil followed the order Cr > Cd > Cu > Ni > Zn whereas with enriched soil inhibition followed the order Cu > Cd > Ni > Zn > Cr.

A marked difference in the EC_{50} values for Cd and Cr was observed between enriched and unenriched soil (Table 2). Chromium (III), which was the most effective inhibitor of dehydrogenase activity in unenriched soil (EC_{50}, 13 ppm), became the least effective inhibitor with enriched soil (EC_{50} = 216 ppm). Cadmium was four times more toxic in unenriched soil than in enriched soil. Apparently the preincubation of the unenriched soil with alfalfa either led to a microbial population more resistant to Cd and Cr or the additional organic matter reduced the availability of the

metals to the microbial populations. The latter possibility is the least attractive, because corresponding effects of organic carbon content were not observed for Cu, Ni and Zn (Table 2). Reduced availability of these metals would have been expected from the work of Babich et al. (1983).

Both Mg^{2+} and NH_4^+, normal components of soil solution, did not inhibit dehydrogenase activity in the unenriched soil. However, in enriched soil ammonium ion concentrations of 150 ppm and greater, showed a small but constant level of inhibition.

Arsenate was the most inhibitory of the anions examined (Table 3). However, inhibition was incomplete, approximately 25% of the dehydrogenase activity in both soils was not sensitive to arsenate inhibition. Complete inhibition of the sensitive fraction occurred at concentrations greater than 300 ppm whereas the resistant fraction was not inhibited up to concentrations of 5000 ppm. Borate inhibited at concentrations greater than 1000 ppm and fluoride and sulfate either did not inhibit or were stimulatory.

Inhibition was not an artifact of the assay caused by the oxidation of TTC-formazan in the presences of inhibitors. When TTC-formazan was incubated under normal assay conditions with sterile soil, the addition of any of the inhibitory anions or cations at 5000 ppm did not reduce the recovery of TTC-formazan.

In this investigation the dehydrogenase assay proved to be an efficient means of determining the toxicity of several inorganic compounds to soil microbial activity. The reproducibility of the assay is evidenced by the small differences observed in replicate determinations (Tables 2 and 3).

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