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Comparison of the Effects of Arsenic (V), Cadmium (II), and Mercury (II) Single Metal and Mixed Metal Exposure in Radish, *Raphanus sativus*, Fescue Grass, *Festuca ovina*, and Duckweed, *Lemna minor*

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A number of abandoned mine sites in the Boise National Forest have been shown to be contaminated with mixtures of heavy metals including Hg, Cd, As, and Pb (Gillerman et al. 1999; Ellis and Eslick 1997). Understanding the effects of mixtures of these metals in plants would be useful. Some investigations of the effects of heavy metal mixtures on plants are beginning to appear in the literature, but much more is needed (Lam, et al. 1999). Studies showed mixed metal treatment of different willow clones resulted in differences in metal accumulation and toxicity (Landberg and Greger 2002). Other studies showed mixed metal treatment of an aquatic macrophyte resulted in differences in metal accumulation (Huebert and Shay 1992).

Studies with single metal exposure to plants have shown that a large number of plants chelate metals with a family of peptides derived from glutathione, called phytochelatins (PCs), ranging in molecular weight from about 3 kD to 10 kD (Grill et al. 1985). Several enzymes lead to the production of PCs, such as glutathione reductase, (GR), and PC synthetase. These enzymes show increased activity in plants exposed to heavy metals (Schickler and Caspi 1999).Tolerance to several metals in metal-tolerant plants involves PCs (Kneer and Zenk 1992). Other plants show metal tolerance that does not involve PCs (de Knecht et al. 1994). Another group of metal binding proteins, the metallothioneins (MTs), have a molecular weight of about 10 kD. MTs probably contribute to metal tolerance in some plants (Tomsett et al. 1989). These proteins are thought to bind and regulate Cu and Zn in the cell but it is not known how readily metallothioneins sequester heavy metals in the plant cell (Steffens 1990). Heavy metals also bind to various membrane and soluble proteins/polypeptides other than PCs or MTs. This variation in protein targets for heavy metal binding may account for the varied levels of metal tolerance observed among plants.

A number of studies have shown that single metal exposure to plants increases the quantity of free radicals produced by the mitochondria (Noctor and Foyer 1998). Plants often increase free thiol levels to scavenge the free radicals and maintain the redox potential in the cell. In addition to its role in PC production, GR is one of the enzymes in the oxidative stress response pathway contributing to the increase of free thiol levels.

Much of what is known about the heavy metal tolerance in plants is based on single metal studies. As stated earlier, quite often, contaminated sites have several metals. This study focuses on the effects of multiple metal exposures in several plants. The heavy metal-soluble protein binding patterns and total metal

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accumulation in radish, fescue grass, and duckweed receiving a variety of single and mixed metal treatments involving Cd, As, Pb, and Hg are investigated. Also, several oxidative stress responses in radishes receiving Cd and/or Hg are evaluated. Metal availability to plants from water and soils differs significantly, so an aquatic plant (duckweed) is included with terrestrial plants (radish and fescue grass) in the study. Radish often accumulates heavy metals when grown on sludge fertilized agricultural land and this represents a concern for public health. Fescue grass is often used to remediate agricultural land and disturbed abandoned mine sites, while duckweed is frequently found at abandoned mine sites. The focus of this study is to compare the parameters following single metal treatment to those observed following mixed metal treatment.

MATERIALS AND METHODS

Radish (*Raphanus sativus*) seeds for the studies were purchased from Albertsons Groceries and fescue grass (*Festuca ovina*) seeds were obtained from the Boise National Forest Service. Duckweed (*Lemna minor*) was from Carolina Biological Supply. $Cd(NO_3)_2$, $Hg(CH_3COO)_2$, $NaAsO_2$ and $Pb(NO_3)_2$ were obtained from JT Baker. Stock solutions of $Cd(NO_3)_2$, $Hg(CH_3COO)_2$, $NaAsO_2$ and $Pb(NO_3)_2$ were prepared in deionized water and used to supply metals in which fescue grass seedlings and duckweed were grown. Glutathione reductase (GR), NADPH, and 5,5'-dithiobis(2-nitrobenzoic acid), DTNB, were obtained from Sigma, St. Louis, MO. Unless otherwise stated, all other reagents were from Fisher Scientific.

Radishes were either grown in sifted soil mixed with Cd (890 nmol/g soil) and/or Hg (499 nmol/g soil) or in agar with a range of concentrations of Cd (89 μM-267 μM) and Hg (50 μM-150 μM). These plants were grown under grow lights with a 12 h light/dark cycle. For growth in soil, seeds were planted and grown for 5 weeks. A mixture of shoots and leaves were harvested and analyzed. For growth in agar, radish seedlings were grown and harvested after 10-12 days. Fescue grass seeds were placed in an aerated solution of metals and grown for 5 days with a 12 h light/dark cycle.

Duckweed was maintained in an aquarium. Portions were removed and placed in 300 mL of water containing 5 mL of Miracle Gro nutrient solution. The metals, Cd, Hg, or As were added to the solution individually or in combination ranging from 25 – 80 μM in concentration. Plants were incubated for 3 days at room temperature with a 12 h light/dark cycle. The plants were then removed, blotted, weighed, and analyzed.

One gram of fresh weight of plant material was homogenized in 5 mL of 0.05 M sodium acetate (pH 8.0) and 0.2 % sodium azide. The homogenate was centrifuged at 10,000 x g for 15 minutes and the soluble fraction removed and analyzed for metal or enzyme activity or subjected to Sephadex gel filtration. Five mL of soluble fraction were placed on a Sephadex G-75 column (1.6 cm x 60 cm column) and eluted with a flow rate of 1 mL/min with the homogenization buffer. Eluates were collected in 3 mL fractions and analyzed for protein according to the method of Lowry (Lowry et al. 1951) and heavy metals as described below in Materials and Methods.

Whole plant samples, soluble fractions, and gel filtration fractions were digested in strong acid in compliance with EPA method 3050 A. Digested samples were analyzed for Hg using the mercury cold vapor method 245.1, EPA-625/6-74-003a with a Thermo Jarell Ashe Atomic Absorbance spectrometer. Digested samples

were analyzed for Cd by inductively coupled plasma spectrometry on a Varian Liberty 100 instrument. Arsenic and lead were analyzed by Atomic Absorbance spectrometry using a graphite furnace.

Glutathione reductase (GR) activity was measured in whole soluble fractions obtained from radishes grown in an agar matrix as described above. Since most oxidative stress studies have involved terrestrial plants, it was decided to initiate these studies with radishes (Noctor and Foyer 1998). GR (EC 1.6.4.2) activity was measured by following the decrease in absorbance at 340 nm (ε_{340} = 6220 $M^{-1}cm^{-1}$) due to NADPH oxidation (Schickler and Caspi 1999).

Free sulfhydryl levels were measured using a modification of the procedure of Riddles et al. (1983). A 1.5 mM DTNB solution in 83 mM potassium phosphate, pH 7.4 was incubated with radish supernatant (~100 μg protein). The displacement of the 5-thio-2-nitrobenzoic acid from DTNB by free sulfhydryls was followed spectrophotometrically at 412 nm (ε_{412} = 14.15 $mM^{-1}cm^{-1}$) using a Varian Cary 100 Bio spectrophotometer (Riddles et al. 1983).

RESULTS AND DISCUSSION

Metal exposure in these studies led to modest reductions in plant growth (10-30%) as measured by plant height or fresh weight. Radish plants had levels of Hg and Cd uptake proportional to the exposure concentrations (Table 1). However, duckweed accumulated similar amounts of Cd and Hg, even though it was exposed to nearly twice the concentration of Cd as Hg. The Hg and Cd levels measured with radishes are consistent with those measured for other terrestrial plants (Ellis and Eslick 1997; Barman et al. 2000). Even though duckweed was exposed to significantly lower levels of Cd and Hg it accumulated about 2 to 5 times as much Cd or Hg as did radishes (Table 1). This difference is most likely accounted for by the difference in metal uptake between terrestrial plants and duckweed. Terrestrial plants uptake trace metals mainly by the root system in a soil matrix, whereas aquatic plants such as duckweed may uptake trace metals by roots ***and*** leaves giving rise to higher tissue concentrations. In contrast to the similar levels of Cd and Hg uptake by duckweed As was found at significantly higher levels after single exposure (Table 1). This finding suggests that As may be taken up and sequestered by duckweed via a different mechanism than is Hg and Cd.

Gel filtration profiles of soluble fractions from control and all treated plants exhibited two distinct molecular weight fractions. One fraction, designated the peak I fraction, contained proteins ranging in molecular weight from 60 kD to ≥ 100 kD. The smaller proteins/polypeptides found in the peak II fraction ranged in molecular weight from 3 to 10 kD. Based on molecular weights, it is likely that the peak I fraction consists of proteins and that the peak II fraction contains PCs (Grill et al. 1985; Pickering et al. 2000; Rauser 1995). Protein concentrations found in peak I and peak II were about equal in radish, whereas in duckweed the peak I protein concentration was only about 20% of peak II.

Metal distributions among the soluble proteins (peak I fraction) and polypeptides (peak II fraction) differed. Radishes and duckweed exposed to only Cd showed 75 to 100% of soluble Cd concentrated in the peak II fraction (Table 2). Similar studies with fescue grass and willow (*Salix sp.*) showed a similar fractionation pattern with Cd (*unpublished results*). This pattern of Cd distribution is consistent with the findings of Fette et al. (1994), who found about 84 % of the

Cd in low-molecular weight fraction (peak II fraction) and 16% in proteins of greater molecular weight in the roots of water hyacinth after 7 days exposure. Arsenic exposed to duckweed accumulated in the peak II fraction with no detectable levels found in peak I (Table 2). PCs with molecular weights in the 3-10 kD range (peak II) have been shown to participate in heavy metal accumulation in other plants species (Fette et al. 1994; Grill et al. 1985; Rauser 1995). The low-molecular weight Peak II fraction seemed to accumulate the bulk of Cd in radish, fescue grass and duckweed as well as nearly all of the As and Hg that was accumulated by duckweed. It is likely that PCs play an important role in the accumulation of these metals by these plants.

Table 1. Metal accumulation in whole plants after single and multiple metal exposures.[a]

Single Exposure	Accumulation (nmol metal/gram fresh weight) Single Metal[b]	Mixed Exposure	Accumulation (nmol metal/gram fresh weight) Mixed Metal[b]	% Change in Accumulation[c]
Radish				
499 nmol Hg/g soil	20 ± 2 (Hg) n=3	499 nmol Hg/g soil 890 nmol Cd/g soil	25 ± 3 (Cd) n=3	38 % reduction (Cd)*
890 nmol Cd/g soil	40 ± 4 (Cd) n=3			
Fescue Grass				
44 nmol Cd /g soil	18 ± 3 (Cd) n=3	44 nmol Cd/g soil 72 nmol Pb/g soil	14 ± 2 (Cd) n=3	22% reduction (Cd)
72 nmol Pb/g soil	46 (Pb) n=2		6.9 (Pb) n=2	85% reduction (Pb)
Duckweed				
44 μM Cd	93 ± 7 (Cd) n=6	44 μM Cd 25 μM Hg	231 ± 9 (Cd) n=6	148 % increase (Cd)*
25 μM Hg	90 ± 4 (Hg) n=6		103 ± 5 (Hg) n=2	14% increase (Hg)
67 μM As	641 ± 27 (As), n=6	67 μM As 25 μM Hg	214 ± 13 (As) n=4	67% reduction (As)*

[a]Plants were grown as described in MATERIALS AND METHODS. *P<0.05 (Mann-Whitney U test) significant difference between metal accumulation after single metal exposure compared to accumulation after mixed metal exposure (Siegel 1956). [b]Metals in parentheses indicate the metal that was measured in each study. [c]% Change = [(value for mixed exposure – value for single exposure)/ value for single exposure] x 100.

Soluble protein binding of Hg was similar to Cd in duckweed, but different in radishes (Table 2). Radishes exposed to mercury resulted in 0.50 μM Hg accumulations in peak I compared to 0.050 μM in peak II (Table 2). Although much of the data suggests an important role for PCs in accumulating heavy metals in plants, it seems as though PCs have a diminished role in Hg accumulation by radish and that Hg binding to proteins in the higher molecular weight peak I

fraction is instead observed. Several enzymes critical for metal binding such as γ-glutamyl synthetase and glutathione synthetase have molecular weights that would place them in the peak I fraction.

The terrestrial plants examined consistently showed a reduced individual metal accumulation when mixed metals were presented (Table 1). Following Hg/Cd exposure, radish exhibited a statistically significant reduction of overall Cd tissue concentration compared to Cd only exposure (Table 1). Fescue grass showed a modest reduction of Cd tissue concentration and a large reduction of Pb accumulation when a Cd and Pb combination was introduced to the plants (Table 1). The aquatic plant, duckweed, showed both an increase and a decrease of a particular metal accumulation when mixed metals were presented (Table 1). Mixed metal treatment led to an ***increase*** in duckweed accumulation of Cd and Hg as compared to single metal treatment (rather than the decrease observed in the terrestrial plants examined). However As concentration in duckweed was reduced significantly when plants were exposed to a Hg/As mixture. This finding in duckweed is consistent with that from a similar study with Zn/Cd mixed exposure (Huebert and Shay 1992). It was found that the presence of Zn altered the uptake of Cd by duckweed. Interestingly, high levels of Zn increased Cd uptake, while low zinc levels decreased Cd uptake. It is clear that metal accumulation by both terrestrial and aquatic plants after single metal exposure is altered in the presence of additional metals.

Cadmium binding to soluble proteins in radishes exposed to a Hg/Cd mixture (Table 2) which is the reverse of that observed after single exposure to Cd (Table 2). In the presence of Hg, Cd distribution becomes more like that observed for Hg in radish. Mixed metal exposure in duckweed resulted in all metals accumulating in the peak II fraction as is observed following single metal exposure.

Table 2. Metal (μM) accumulation in homogenate eluate fractions from radishes and duckweed. [a]

Plant, growth condition[b]	Metal Measured	N	**peak I** mean (S.D.)	**peak II** mean (S.D.)
Radishes, 890 nmol Cd/g soil	Cd	4	0.36 (0.05)	1.07 (0.09)
Radishes, 499 nmol Hg/g soil	Hg	4	0.50 (0.01)	0.050 (0.025)
Radishes, 499 nmol Hg/g soil /890 nmol Cd/g soil	Cd	3	0.37 (0.04)	0.010 (0.004)
Duckweed, 36 μM Cd	Cd	3	<0.044	2.0 (0.3)
Duckweed, 80 μM Cd	Cd	9	<0.044	4.1 (0.3)
Duckweed, 20 μM Hg	Hg	3	0.01 (0.02)	1.8 (0.1)
Duckweed, 45 μM Hg	Hg	3	0.05 (0.02)	3.9 (0.1)
Duck weed, 67 μM As	As	5	>0.067	14.4 (0.4)
Duckweed, 25 μM Hg/44 μM Cd	Cd	4	0.06 (0.03)	10.8 (0.2)
Duckweed, 25 μM Hg/67 μM As	As	2	>0.067	4.0 (0.5)

[a] $P<0.05$ (Mann-Whitney U test) significant difference between relative peak I and peak II metal accumulations (Siegel 1956). [b] Radishes were grown in soil with the specified concentration of metal and duckweed was grown in metal solutions.

In this study, plants that were exposed to heavy metals exhibited a notable oxidative stress known to result from heavy metal exposure (Nocter and Foyer 1998). If heavy metal derived oxidative stress was triggered in the plants, then

increased levels of the components of the antioxidant response machinery would be predicted (Nocter and Foyer 1998). Glutathione reductase (GR) is one of several plant enzymes in a pathway that scavenges free radicals formed by heavy

Table 3. Glutathione reductase (GR) activity and thiol concentration levels in radish homogenate following Hg and/or Cd.[a]

Metal Exposure,[b]	% GR activity[c]	thiol concentration, μM, mean (S.D.)[d]
no metal	100	0.10 (0.03)
50 μM (Hg)	223	
89 μM (Cd)	200	
100 μM (Hg)	185	
178 μM (Cd)	200	
150 μM (Hg)	300	
267 μM (Cd)	330	
499 nmol/g soil (Hg)		0.12 (0.02)
890 nmol/g soil (Cd)		0.11 (0.01)
100 μM (Hg) + 89 μM (Cd)	127	
100 μM (Hg) + 178 μM (Cd)	133	
100 μM (Hg) + 267 μM (Cd)	183	

[a] GR activities and thiol concentrations were determined as described in MATERIALS AND METHODS and were compared to untreated control seedlings. The difference between metal treated and nontreated samples are expressed as a percent of the control. *P<0.05 (Mann-Whitney U test) significant difference between GR activity in plants with individual and mixed metal exposures (Siegel 1956). [b]Metal used in treatment is list in parentheses. [c]n=5 for the single metal exposure and n=3 for the mixed metal exposure studies. All growths were done in agar. [d]n=3 for no metal control and 890 nmol Cd/g soil and n=4 for 499 nmol Hg/g soil studies. All growths were done in soil.

metals interacting with mitochondria and its activity is known to be elevated in response to heavy metal exposure (Schickler and Caspi 1999). For radishes grown in agar, exposure to 50 - 100 μM Hg or 89 - 178 μM Cd resulted in a 2-fold increase in GR activity and growth in 150 μM Hg or 267 μM Cd resulted in a 3-fold increase (Table 3). Despite differences in soluble protein metal binding patterns for Cd and Hg, the antioxidant response of increased GR activity as a result of Cd or Hg exposure was nearly identical in radishes. GR activity in radishes receiving a Cd/Hg mixture had about 50% of the increase observed for radishes receiving only Hg or Cd. Such increases in GR activity probably increases the availability of GSH for PC synthesis which increases in the presence of heavy metals (Grill et al. 1985). Further, it is possible that the decreased response of GR in radishes receiving the mixture of Cd and Hg could lead to a reduced PC level and thus account for the lower Cd accumulation observed in the plants receiving Cd from a mixture compared to those receiving Cd only. Despite the observed increases in GR activity, the overall thiol levels were unaffected by metal treatment (Table 3). This finding is consistent with those of other studies (Maier et al. 2003). Though no changes in response to heavy metal exposure were

measured in total thiol levels, it is possible that the GSH fraction of the total thiol was changed. Total GSH levels were not measured in this study, however others have found that the percentage of GSH of total thiols was decreased in response to Cd metal exposure (Meuwly and Rauser 1992). The observed decrease in the fraction of GSH/total thiol was attributed to a decrease in overall GSH levels and compensating increase of total thiol levels in Maize seedlings treated with Cd (Meuwly and Rauser 1992).

This study suggests that effects of mixed heavy metals significantly alter heavy metal accumulation and antioxidant responses in plants. Understanding the mechanistic basis for these differences, particularly with respect to transport processes and extent of metal accumulation, will be important in better modeling the full impact of heavy metal contamination on the environment, one which includes both aquatic and terrestrial ecosystems.

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