Impact of paired planting of sunflower [*Helianthus annuus* L.] and Indian mustard [*Brassica juncea* (L.) Czern.] on lead phytoextraction

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Abstract

Lead (Pb) is a common and persistent soil pollutant that has been shown to have significantly negative neurological impacts, even at extremely low exposures. Soils contaminated from leaded gasoline and paint present a significant risk to the public, especially children. While many solutions involve removing or sealing soil, phytoextraction is a technique where plants are grown to accumulate soil contaminants and leave the soil intact. The goal of this research was to determine the ability of mixed versus individual plantings of sunflower (*Helianthus annuus* L.) and Indian mustard (*Brassica juncea* (L.) Czern.) to accumulate Pb from contaminated soils. A greenhouse study was conducted with three experimental groupings of plants: only sunflowers; only mustard; and ½ sunflower & ½ mustard. Plants were grown in 1100 ppm Pb-contaminated yard soil collected from Burlington and amended to 3:1 soil:perlite ratio. After 60 days, plants were harvested and the root and above-ground tissues were analyzed for Pb content by ICP-AES following microwave-assisted nitric acid digestion. Sunflowers had average shoot tissue concentrations of 60 mg/kg Pb while mustard plants only had 35 mg/kg, regardless of whether each species was grown together or separately. Average dry weight for sunflowers was 0.60 g, while average mustard dry weight was 0.40 g. There were no significant differences in dry weight between treatments. There is no apparent benefit of mixed planting these two species in terms of the amount of Pb accumulated in the plant tissues. In fact, when sunflower is grown with mustard there is significantly less Pb in the sunflower plants than when the sunflower is grown alone. This appears to be due to a Pb concentration only half as high in the roots of sunflowers grown with mustard than sunflower-only plantings. Further studies focusing especially on other species in mixed plantings, planting densities, and longer time scales may yield innovative insights or solutions to our persistent and toxic legacy of lead pollution.

Introduction

Lead (Pb) contamination is a significant problem for many urban areas due to its prevalence in gasoline and house paint through the 1970's. Despite the fact that Pb in many applications has been banned in the US for over 30 years, Pb contamination of soils is still a problem because Pb is highly insoluble and tends to form highly stable adsorption complexes at normal soil pH. Pb can cause serious human health problems such as IQ loss and serious neurological damage at low concentrations, especially in growing children (Canfield et al., 2003; Lanphear, 2005). These health effects can occur via two main exposure pathways: ingestion or inhalation of Pb dust from surfaces with leaded paint; and ingestion or inhalation of Pb within soils (Butcher, 2009; Canfield et al., 2003). There are several methods already used to reduce exposure from Pb-painted surfaces, but dealing with exposure to leaded soil is more difficult. Most methods to reduce exposure to soil Pb, such as removing the soil or installing a physical cover are either expensive or do not directly address the indefinite presence of Pb in soil. The process of phytoextraction - using plants to accumulate Pb in their tissues - has been shown to have some

promise in removing Pb from soils and compares favorably with other techniques in terms of cost and complexity (Marques et al., 2009; Sheoran et al., 2009).

Many phytoextraction studies use chelating agents -compounds which form aqueous complexes with cations- or acids to solubilize Pb. These chelating agents form complexes with Pb cations which in some cases is more available to the plant root and in other cases simply keeps a higher proportion of soil Pb in the aqueous phase (Parra et al., 2008). Ethylenediaminetetraacetic acid (EDTA), a synthetic chelating agent, has been shown to be one of the most effective chelating agents and increases Pb accumulation in some plants 2 to 50-fold and total water-soluble Pb 5 to 500-fold (Evangelou et al., 2007; Parra et al., 2008). However, the use of strong chelating agents or acids can lead to leaching and possibly groundwater pollution (Miretzky & Fernandez-Cirelli, 2008; Saifullah et al., 2009) though this is disputed (Zhao et al., 2011). While generally less effective, low molecular weight acids such as citric and malic acid are released by the roots of a number of plants and contribute to increasing accumulation of trace elements in the soil. These compounds have also been shown to increase Pb accumulation by varying, but significant amounts (Evangelou et al., 2007; Parra et al., 2008). While the exact mechanisms by which Pb and other heavy metals enter plant roots are still somewhat unclear, several have been proposed. The pathway by which chelating agents are thought to increase Pb uptake by plants is desorption of Pb from soil surfaces, which enhances diffusion through the soil solution towards root systems. The dissociation of this complex to the Pb^{2+} form near the roots allows Pb^{2+} to be taken up by the roots (Wang et al., 2007).

Other studies have examined the extent to which mycorrhizae increase the uptake of Pb in plants that they colonize. These symbiotic fungi are hosted by a vast majority of plants and have been shown to increase nutrient uptake, especially trace elements (Alford et al., 2010). These same functions can be utilized to increase Pb accumulation in some plants due to the fact that lead's soluble form, Pb^{2+}, is similar in size and charge to a number of essential plant micronutrients such as Ca^{2+} (Alford et al., 2010). Other microbes, such as plant-growth-promoting rhizobacteria, have been shown to increase plant growth and accumulation of some heavy metals. These bacteria have been shown to increase root length and fine-hair production, as well as produce plant-growth promoting hormones such as indole-3-acetic acid (Alford et al., 2010). These changes to plant growth increase the accumulation of heavy metals and may lead to more effective phytoextraction. While a number of studies examined the extraction rates of a particular plant species, and some sought to determine the benefits of utilizing symbiotic fungi (Alford et al., 2010; Punamiya et al., 2010) and bacteria (Zhuang et al., 2007) to facilitate this process, only one study to date has examined the potential effectiveness of the coordinated use of multiple plant species to remove Pb from soils.

Wu et al.'s multi-species phytoextraction greenhouse study examined species richness and total Pb accumulation (2005). Wu and his colleagues found a significant positive relationship between the number of orchard weed species in a community and the total accumulation of Pb. While it was found that treatments with greater plant variety (two to six species per pot) accumulated more Pb, the only explanatory factor discussed was total biomass. The increase in biomass shown by Wu et al. (2005) may be due to the efficient and complementary use of root space by species possessing slightly different root structures and growth rates. Other studies of multiple crop plant interactions have shown increased plant growth and health, and certain

pairings have been shown to increase productivity over single-species plantings (Al-Dalain, 2009; Ofori & Gamedoagbao, 2005). The efficient use of root space would suggest that the total root mass of the plant community remains in contact with a greater proportion of the soil solution (and hence solubilized Pb) than any one type of root structure. Some pairings of plant species may show increased ability to take up Pb due to production of root exudates (organic acids or other organic compounds released into the soil by plant roots) which may solubilize Pb (Nascimento & Xing, 2006) and make it more available to the roots of all of the plants in the plot. Root exudates may also indirectly increase Pb solubility by feeding microorganisms which can solubilize Pb or promote plant growth (Nascimento & Xing, 2006).

The goal of this project was to determine if sunflower (*Helianthus annuus* L.) and Indian mustard (*Brassica juncea* (L.) Czern.) grown in conjunction demonstrate greater accumulation of Pb from soil than either species can accumulate individually. Dry weights of shoot and root tissues as well as the tissue Pb concentrations for both tissue types were measured to determine the effect of single- and dual-species plantings on both species.

Methods

Soil Collection and Treatment

Pb-contaminated yard soil from around several old, wood-framed houses in Burlington was mixed to provide a sufficient volume for this experiment. 0.06 m^3 were collected on site from three separate areas located along the drip line of a volunteer's house after removing the surface vegetation. Another 0.06 m^3 had been collected in a similar fashion for an earlier Pb remediation project, but had not been used. The soil most recently collected was sieved to 5 mm and the other soil was sieved to 2 mm. Both were mixed together thoroughly with a trowel and stored at room temperature for several days. Soil was tested for total Pb content using microwave-assisted nitric acid extraction per USEPA Method 3051 and was determined to contain 1100 mg/kg by Perkin-Elmer Optima 3000 DV Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Waltham, MA, USA).

Initial Planting

To prepare the soil for potting, one part perlite and three parts soil were combined and mixed in order to reduce soil compaction, and increase air holding capacity of the soil. Approximately 0.004 m^3 of this soil/perlite mixture was allotted to each of the 18 circular 0.004 m^3 pots used. The pots were then watered lightly and seeds were planted in each. Six pots were planted with four sets of two sunflower seeds each (*Helianthus annuus* L. 'Velvet Queen') with a goal of having four evenly spaced seedlings. Six more pots were planted with four sets of three mustard seeds each (*Brassica juncea* L. var. Southern Giant Curled). The last six pots were planted with two sets of two sunflower seeds and two sets of three mustard seeds each. The greenhouse was kept between 21 and 27 ºC, and no artificial lighting was used. After watering three times a week for two weeks and a high rate of germination failure, the few plants which had germinated were cut at soil level and removed from the pots. The pots were allowed to dry out for one week.

Second Planting

Since the initial planting resulted in such a high rate of germination failure, perhaps due to Pb toxicity as well as overwatering, a second planting was attempted using germination trays filled with the UVM greenhouse's Fafard 3B media. Two trays were filled with Fafard 3B media. One was seeded with 50 sunflower seeds (*Helianthus annuus* L. 'Hallo') and the other was seeded with approximately 70 mustard seeds. Both trays were then covered in a dusting of Fafard 3B media and watered with Jack's Pure Water LX fertilizer (17-4-17 diluted to 100 ppm N). These were allowed to germinate and grow for seven days before transplanting into pots with Pb-contaminated soil. Fafard 3B plugs were gently shaken to remove majority of media, but in order to avoid shocking the plants, not all was removed. Four sunflowers were transplanted into each of six replications of the sunflower-only treatment pots. Four mustard plants were transplanted into each of the six replications of the mustard-only treatment pots. Two sunflower and two mustard plants were transplanted into each of the six replications of the mixed treatment pots. Once potted, the plants were watered three times a week and grown for an additional 53 days, for a total of 60 days from germination. The goal was to have reasonably sized plants and to take advantage of the higher tissue concentration of Pb in younger plants suggested by the findings of Adesodun et al. (2010). Day length ranged from 13 to 15 hours over the growing period due to the seasonal changes in day length.

Plant Harvest and Analysis

All plants were harvested over the course of two days. Each plant was carefully uprooted to retain as much root mass as possible. The roots were shaken to remove loose soil, and then swirled in a beaker of standing reverse osmosis (RO) water. The roots were then rinsed in fresh RO water, swirled in the beaker once more, and rinsed again until visually confirmed clean. Once the root system was clean, the plant stem was cut where it had intersected the soil surface. All 144 plant sections were placed into individual folded tinfoil pieces and oven dried at 60 ºC for 72 hours.

Once dried, plant samples were weighed and ground using the Udy cyclone mill. Ground plant tissues from each pot were aggregated by plant and tissue type, yielding 48 samples. All of the samples were nitric acid digested following USEPA method 3051, and analyzed for Pb concentration with an ICP-AES. Due to the low sample mass for a number of aggregate samples (some as low as 0.10 g), solutions were only partially diluted (to volumes as low as 10 mL) instead of the standard 50 mL to ensure machine-detectable levels of Pb.

Data Analysis

To test for the significance of treatment-level differences, the per-pot totals for dry weight of the shoot, root, and whole plant (shoot + root) tissues were calculated for sunflower-only, mustard-only, and mixed treatments. Pb concentrations of the per-pot aggregate samples of shoot, root, and whole plant tissues were also calculated for each of the three treatments. Total Pb accumulation of the shoot, root, and whole plant tissues were calculated for each treatment on a per-pot basis (Table 1). While running descriptive statistics on each data set, one extreme outlier was found: the shoot tissue Pb concentration of the second mixed treatment pot. This data point

and the three data points that were calculated from it were removed from the data set before further statistical tests were performed. ANOVAs of the total shoot, root, and whole plant dry weights of the three treatment types were performed, followed by Tukey's HSD test to determine if there were significant differences between pairs of treatments. ANOVAs of the tissue Pb concentration and total Pb accumulation were run in a similar manner. Significance was accepted at $p<0.05$. JMP 9.0.2 was used to perform all of the analyses (SAS Corp. 2010).

Table 1 Diagram of the nine multi-group comparisons to test for treatment-level effects. The dependent variables are in the left-most column and the three tissue categories are in the top column. Inside each cell of the table are the three treatments used in each ANOVA.

	Shoot	Root	Whole Plant (Shoot + Root)
Dry Weight (Pot Total)	Sunflower-Only Mustard-Only Mixed	Sunflower-Only Mustard-Only Mixed	Sunflower-Only Mustard-Only Mixed
Tissue Pb Concentration (Aggregate of Each Tissue Type Per Pot)	Sunflower-Only Mustard-Only Mixed	Sunflower-Only Mustard-Only Mixed	Sunflower-Only Mustard-Only Mixed
Total Pb Accumulation (Pot Total)	Sunflower-Only Mustard-Only Mixed	Sunflower-Only Mustard-Only Mixed	Sunflower-Only Mustard-Only Mixed

To test for species-level effects, the average individual dry weights of the shoot, root, and whole plant dry weights were calculated for four 'treatments': sunflower-only, mustard-only, sunflower-mixed, and mustard-mixed. The use of the average individual dry weight was necessary due to the population of four individuals of each species in each of the single-species pots and the presence of only two individuals of each species in each of the dual-species pots. Pb concentrations of the per-pot aggregate samples of shoot, root, and whole plant tissues were calculated on a per-species basis for each pot. Average individual plant Pb accumulation of the shoot, root, and whole plant tissues were calculated on a per-species basis for each pot (Table 2). While running descriptive statistics on each data set, two extreme outliers were found: the shoot tissue Pb concentration of the sunflower and the mustard in the second mixed treatment pot. These data points and the six data points that were calculated from it were removed from the data set before further statistical tests were performed. ANOVAs of the total shoot, root, and whole plant dry weights of the four 'treatment' types were performed, followed by Tukey's HSD test to determine if there were significant differences between pairs of treatments. ANOVAs of the tissue Pb concentration and total lead accumulation were run in a similar manner. Significance was accepted at $p<0.05$.

Table 2 Diagram of the nine multi-group comparisons to test for species-level effects. The dependent variables are in the left-most column and the three tissue categories are in the top column. Inside each cell of the table are the four 'treatments' used in each ANOVA.

	Shoot	Root	Whole Plant (Shoot + Root)
Dry Weight (Individual Average per Pot)	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed
Tissue Pb Concentration (Aggregate of Each Tissue Type per Species for Each Pot)	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed
Total Pb Accumulation (Individual Average per Pot)	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed	Sunflower-Only Mustard-Only Sunflower-Mixed Mustard-Mixed

Results and Discussion

Plant Dry Weight

Sunflower-only treatments were found to have a total shoot dry weight of 2.460 g per pot, mixed treatments were found to have a total shoot dry weight of 1.917 g, and the mustard-only treatments were found to have a total shoot dry weight of 1.703 g (Table 3). In an ANOVA of the three treatments, the sunflower-only treatment had significantly higher shoot dry weight than either the mixed or mustard treatments. The mixed and mustard treatments did not have significantly different dry weights. The sunflower treatment was also found to have a significantly higher whole plant (shoot + root) dry weight of 3.110 g, than either the mixed treatment (2.275 g) or the mustard treatment (1.938 g). The sunflower treatment root dry weight of 0.650 g was significantly different from the mustard treatment root dry weight of 0.235 g. The mixed treatment root dry weight of 0.358 g was not significantly different from either the sunflower-only or mustard-only treatments.

Individual sunflowers were not found to have significantly different shoot dry weights when grown alone, an average of 0.615 g, or mixed with mustard, an average of 0.598 g (Table 3). In the same four-treatment ANOVA (separating the weights of the mixed sunflower and mustard treatment into two 'treatments'), mustard was not found to have significantly different shoot dry weight when grown alone, an average of 0.426 g, or mixed with sunflower, an average of 0.365 g. Both sunflower treatments were not significantly different in either root dry weight or whole plant dry weight. Mustard treatments were not significantly different from each other in either root dry weight or whole plant dry weight.

A three-treatment ANOVA of all of the individual tissues showed a significant difference between the shoot dry weight of sunflower of 0.617 g per plant and the shoot dry weight of mustard of 0.426 g per plant (Table 3). The mixed treatment shoot dry weight of 0.479 g was not significantly different from either the sunflower-only shoot dry weight or the mustard-only shoot

dry weight per plant. The whole plant sunflower dry weight of 0.741 g was significantly different from both the mixed and mustard whole plant dry weights of 0.569 g and 0.485 g, respectively. The mixed and mustard treatment whole plant dry weights were not significantly different.

A four-treatment ANOVA of all of the individual tissues showed that the sunflower-only shoot dry weight of 0.617 g was not significantly different from the sunflower-mixed shoot dry weight of 0.595 g (Table 3). The shoot dry weight of mustard grown alone, 0.426 g, was not significantly different from the shoot dry weight of mustard grown mixed with sunflower, 0.363 g. The sunflower-mixed shoot dry weights were not significantly different from the mustard-only shoot dry weights. The root and whole plant dry weights of sunflowers grown alone were not significantly different from sunflowers grown mixed with mustard. The root and whole plant dry weights of mustard grown alone were not different than those of mustard grown mixed with sunflower.

Table 3 Plant dry weights grouped by treatment type for both per-pot dry weight means and individual plant dry weight means. The significance level of each ANOVA is given above each set of dry weight means in italics. Different letters to the right of each set of dry weights denote significant differences between groups from a post-hoc Tukey's HSD test. Significance accepted at $p<0.05$

	Treatment		Shoot (mg/kg) (Mixed 2 excluded)	Root (mg/kg)	Whole Plant (mg/kg) (Mixed 2 excluded)
Pot total dry weight means n=18		*p*	***0.0005***	***0.0337***	***0.0018***
	Sunflower-Only		2.460 (±0.757)A	0.650 (±0.387)A	3.110 (±0.980)A
	Mixed		1.917 (±0.494)B	0.358 (±0.783)AB	2.275 (±0.558)B
	Mustard-Only		1.703 (±0.734)B	0.235 (±0.104)B	1.938 (±0.831)B
Individual dry weight means n=24		*p*	***<0.0001***	***0.0237***	***0.0001***
	Sunflower-Only		0.615 (±0.188)A	0.163 (±0.097)A	0.778 (±0.244)A
	Sunflower-Mixed		0.598 (±0.131)A	0.110 (±0.024)AB	0.726 (±0.150)A
	Mustard-Only		0.426 (±0.181)B	0.059 (±0.028)B	0.485 (±0.207)B
	Mustard-Mixed		0.365 (±0.138)B	0.070 (±0.010)B	0.440 (±0.156)B
Individual dry weight means n=72		*p*	***0.0105***	***<0.0001***	***0.0025***
	Sunflower-Only		0.617 (±0.228)A	0.123 (±0.049)A	0.741 (±0.258)A
	Mixed		0.479 (±0.189)AB	0.090 (±0.037)B	0.569 (±0.220)B
	Mustard-Only		0.426 (±0.222)B	0.059 (±0.033)C	0.485 (±0.251)B
		p	***0.0009***	***<0.0001***	***0.0002***
	Sunflower-Only		0.617 (±0.228)A	0.124 (±0.049)A	0.741 (±0.258)A
	Sunflower-Mixed		0.595 (±0.116)AB	0.112 (±0.025)A	0.707 (±0.133)A
	Mustard-Only		0.426 (±0.222)BC	0.068 (±0.033)B	0.485 (±0.251)B
	Mustard-Mixed		0.363 (±0.140)C	0.059 (±0.030)B	0.431 (±0.160)B

Plant Tissue Pb Concentration

A three-treatment ANOVA showed that the sunflower-only treatment shoot Pb concentration of 64.5 mg/kg was significantly higher than the mustard-only or mixed treatment shoot Pb concentrations of 37.2 mg/kg and 41.5 mg/kg, respectively (Table 4). The mustard-only and mixed treatment shoot concentrations of Pb were not significantly different. Root concentrations of Pb were not significantly different between any two of the three treatments and ranged from 274 mg/kg in the mixed treatments to 457 mg/kg in mustard-only treatments. Whole plant tissue Pb concentrations were not significantly different and ranged from 80.7 mg/kg in mixed treatments to 115 mg/kg in sunflower-only treatments.

Pb was present in the shoot tissues of the sunflower-only treatment at 64.5 mg/kg, and was present in shoot tissues of the sunflower-mixed treatment at 60.6 mg/kg (Table 4). A four-treatment ANOVA showed that these two treatments were not significantly different. Pb was present in the shoot tissues of the mustard-only treatment at 37.2 mg/kg and in the shoot tissues of the mustard-mixed treatment at 28.4 mg/kg (no significant difference), but these treatments had significantly lower Pb concentrations than the sunflower-only and sunflower-mixed treatments. Mustard plants had significantly less Pb in their shoot tissues than sunflower plants. This is important to note because the above-ground tissues are often the most practical parts of the plants to harvest to remove Pb from the site. This suggests that it is better to plant sunflowers if the above-ground tissues are the only tissues being removed. It should also be noted that mustard plants grown with other mustard plants have the highest concentration of Pb in their shoot tissues among all treatments, and are significantly higher than the sunflower in the mixed treatment. None of the whole plant tissue Pb concentrations were significantly different, but ranged from 78.7 mg/kg in mustard-mixed treatments to 115 mg/kg in sunflower-only treatments. Root tissue Pb concentrations of the sunflower-only, mustard-only, and mustard-mixed treatments were not significantly different. Root tissue Pb concentrations of the sunflower-only, sunflower-mixed, and mustard-mixed treatments were not significantly different.

Table 4 Pb concentrations of tissue aggregates for each treatment. The significance level of each ANOVA is given above each set of dry weight means in italics. Different letters to the right of each set of dry weights denote significant differences between groups from a post-hoc Tukey's HSD test. Significance accepted at $p<0.05$

	Treatment		Shoot (mg/kg) (Mixed 2 excluded)	Root (mg/kg)	Whole Plant (mg/kg) (Mixed 2 excluded)
Pb concentration n=18		*p*	***0.0010***	*0.0556*	*0.0728*
	Sunflower-Only		64.5 (±11.9)A	307 (±92)A	115 (±27)A
	Mixed		41.5 (±7.4)B	274 (±74)A	80.7 (±9.1)A
	Mustard-Only		37.2 (±5.6)B	457 (±215)A	85.9 (±22.6)A
Pb concentration n=24		*p*	***<0.0001***	***0.0347***	*0.0765*
	Sunflower-Only		64.5 (±11.9)A	307 (±92)AB	115 (±26)A
	Sunflower-Mixed		60.6 (±8.7)A	200. (±78)B	81.9 (±2.4)A
	Mustard-Only		37.2 (±5.6)B	457 (±215)A	85.9 (±22.6)A
	Mustard-Mixed		28.4 (±14.2)B	325 (±81)AB	78.7 (±29.7)A

Total Pb Accumulation

Total Pb taken up by shoot tissues of each of the three treatments were not significantly different, but ranged from 0.061 mg in mustard to 0.161 mg in sunflower (Table 5). Sunflower shoots had 2.6 times the Pb when compared to mustard plant shoots, but the p-value for significance was 0.0647. Total Pb taken up by the root tissues of each of the three treatments were not significantly different than any of the other treatments but ranged from 0.092 mg in mustard to 0.188 mg in sunflower. Sunflower roots accumulated two times greater Pb than mustard roots but the p-value for significance was 0.0574. Whole plant Pb accumulation of the sunflower treatment was 0.349 mg, which was significantly greater than the mustard treatment whole plant Pb accumulation of 0.154 mg. The mixed treatment whole plant Pb accumulation of 0.223 mg was not significantly different from the whole plant Pb accumulation of either of the other two treatments.

When four groups were analyzed, shoot Pb accumulation per plant was almost identical in sunflower only and sunflower mixed treatments, at 0.0383 mg and 0.0380 mg (Table 5). Shoot Pb accumulation per plant was found not to be significantly different between the mustard only and mustard mixed treatments, at 0.0167 mg and 0.0100 mg respectively. Root Pb accumulation per plant was not significantly differently between any two treatments but ranged from 0.0220 mg in sunflower mixed treatments, to 0.0483 mg in sunflower only treatments. Whole plant Pb accumulation per treatment was highest in the sunflower only treatments (Figure 1). The sunflowers grown in sunflower only treatments were found to have significantly higher whole plant Pb accumulation than any plants grown in other treatments (Table 5). The most important finding of this study is that sunflower grown with mustard plants accumulate less Pb than sunflowers grown with other sunflowers. This appears to be due to a decrease in Pb root concentration in sunflowers grown with mustard plants compared to sunflowers grown with other sunflowers. Mustard plants appear to reduce Pb concentration in sunflower plants (primarily roots) when grown together.

Table 5 Total Pb accumulation for each treatment. The significance level of each ANOVA is given above each set of dry weight means in italics. Different letters to the right of each set of dry weights denote significant differences between groups from a post-hoc Tukey's HSD test. Significance accepted at $p<0.05$

	Treatment		Shoot (mg) (Mixed 2 Excluded)	Root (mg)	Whole Plant (mg) (Mixed 2 Excluded)
Total Pb Accumulation per Pot n=18		*p*	*0.0647*	*0.0574*	***0.0189***
	Sunflower-Only		0.161 (±0.070)A	0.188 (±0.087)A	0.349 (±0.117)A
	Mixed		0.132 (±0.072)A	0.101 (±0.038)A	0.223 (±0.093)AB
	Mustard-Only		0.061 (±0.020)A	0.092 (±0.027)A	0.154 (±0.037)B
Total Pb Accumulation per Plant n=24		*p*	***0.0001***	*0.0593*	***0.0003***
	Sunflower-Only		0.0383 (±0.0172)A	0.0483 (±0.0232)A	0.0867 (±0.0273)A
	Sunflower-Mixed		0.0380 (±0.0045)A	0.0220 (±0.0130)A	0.0600 (±0.0141)B
	Mustard-Only		0.0167 (±0.0052)B	0.0250 (±0.0055)A	0.0400 (±0.0089)B
	Mustard-Mixed		0.0100 (±0.0000)B	0.0240 (±0.0134)A	0.0340 (±0.0134)B

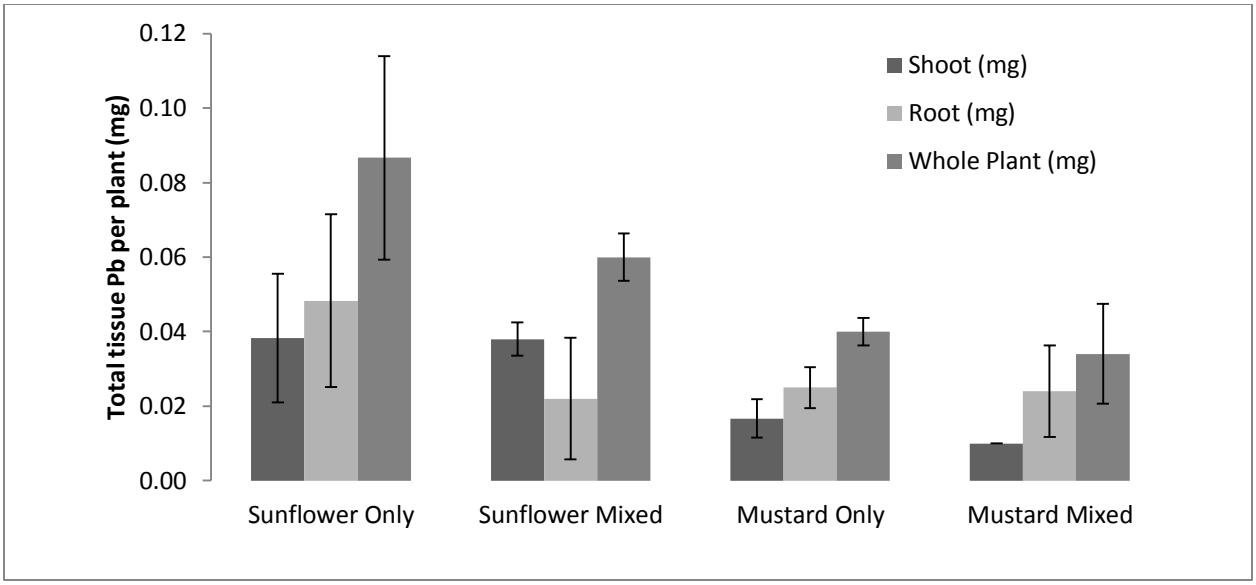

Figure 1 Total Pb accumulation per plant in the shoots, roots, and whole plant. Each end of the error bar denotes one standard deviation.

Comparison of Total Pb Accumulation between Sunflower and Mustard

Sunflowers accumulated a significantly higher concentration of Pb in their shoot tissues than mustard, which, combined with sunflower's significantly greater shoot mass, resulted in more effective total shoot Pb accumulation. The sunflower-only treatment shoot tissue concentration of 64.5 mg/kg after eight weeks of growth agrees with findings of 60.7 mg/kg in stems and 71.5 mg/kg in leaf tissues in a similar experiment by Adesodun et al. (2010). This similarity occurs despite the soil Pb concentration of 1100 mg/kg in this experiment compared to 400 mg/kg in Adesodun et al.'s. While few other studies directly examine Pb accumulation in unconditioned soils, controls from these indicate a similar range of Pb accumulation in sunflowers in a similar soil Pb concentration range (Fassler et al., 2010; Lin et al., 2009; Parra et al., 2008; Solhi et al., 2005). Mustard-only treatment shoot tissue concentrations of 37.2 mg/kg agreed with previous phytoextraction studies' findings of Pb accumulation in mustard and other closely related *Brassica* species (Parra et al., 2008; Purakayastha et al., 2008; Solhi et al., 2005). While sunflower showed greater accumulation of Pb and greater biomass in unconditioned soils than mustard, this trend has not been found in soil or hydroponic studies with longer growth times and/or the addition of chelating agents (Niu et al., 2007; Parra et al., 2008). This may be due to either the relatively small magnitude of Pb accumulation of plants in unconditioned soils compared to soils with higher soluble concentrations of Pb (due to the action of chelating agents). It may also indicate that mustard possesses a greater ability to accumulate Pb across a much larger range of soil Pb concentrations.

Effects of Mixed Plantings

While the dual-species treatment in this study was not shown to increase the biomass or Pb accumulation of either species of individual plants, in a study of mixed plantings of orchard weeds, Wu et al. (2005) showed that mixed plantings did increase total Pb accumulation. While

Wu and his colleagues examined the accumulation of Pb in mixed plantings, they only controlled the total number of individuals in each treatment, not the number of individuals of each species. Plantings of two or more species were shown to increase total Pb accumulation over single-species plantings by Wu et al. (2005)'s study, but this seemed to be due to increase in biomass per unit area and not a synergistic effect between any of the plants. The results of my research support the conclusion that any of the gains seen in mixed plantings are more likely derived from the efficient use of space or access to soluble Pb by the entire plant community and not from any shift in the mechanisms of Pb uptake in either species due to the other's presence. Given the absence of any positive synergistic response between sunflower and mustard, which would most likely arise from the root system, it is likely that the mechanisms by which these two species absorb or adsorb Pb are restricted to a very small radius about the roots, as has been described for plant accumulation of other metals (Alford et al., 2010). Further tests on this subject could examine the benefits of dense plantings of one or more species to take advantage of the limited range of root effects on Pb uptake. However, above a certain density negative impacts on plant size due to competition are likely to occur.

While a population evenly split between two species was not shown to increase the effectiveness of Pb accumulation in sunflower and mustard, a more complex planting approach may yield some benefits for plant Pb accumulation and perhaps plant health. Since optimal growth of each individual plant is not the goal in phytoextraction plantings (as opposed to agricultural or horticultural settings), higher density plantings may be a viable method to increase the total biomass per unit area, and thus maximize the removal of Pb from soils. Plantings of more than two species may show even larger Pb accumulation gains, but further research on plant communities and Pb accumulation should be undertaken.

Since the mechanisms of Pb uptake in plants, and even high Pb accumulators, is still somewhat poorly understood, further studies into the mechanisms controlling Pb accumulation will allow for more effective utilization of species with high Pb accumulation capabilities. Further research into the root growth patterns of species useful for phytoextraction projects would also help guide design decisions, particularly how to arrange plantings to maximize above ground biomass production, and thus Pb extraction. However, since chelating agents have been shown to be so effective at improving Pb accumulation in plants, their careful and controlled use is highly likely to further improve the effectiveness of any phytoremediation effort.

Conclusion

No positive synergistic effect on growth or Pb accumulation in sunflower and mustard was evident in mixed plantings of these two species. This finding, when combined with current proposed mechanisms of Pb uptake in plants suggests that positive synergy between these two plant species does not occur. Total biomass appears to be the driving factor in total Pb accumulation in this study. Amendments to the whole soil have demonstrated greater improvements in increasing the Pb accumulation in plants, and this is likely to remain the case. Since sunflower root accumulation of Pb is much reduced in mixed plantings, mixed plantings of these two species would not be advisable if the whole plant is to be harvested. However, if only the shoot tissues of the plants are to be harvested, mixed plantings of these two plants could be useful to take advantage of other possible benefits of mixed plantings such as pest resistance.

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