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Assessment of biotransfer and bioaccumulation of cadmium, lead and zinc from fly ash amended soil in mustard–aphid-beetle food chain.

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ABSTRACT

The present study investigates the extent of biotransfer and bioaccumulation of cadmium (Cd), lead (Pb) and zinc (Zn) from fly ash amended soil in mustard (*Brassica juncea*)-aphid (*Lipaphis erysimi*)- beetle (*Coccinella septempunctata*) food chain and its subsequent implications for the beetle. The soil was amended with fly ash at the rates of 0, 5, 10, 20 and 40% (w/w). Our results showed that the uptake of Cd, Pb and Zn from soil to mustard root increased with the increase in fly ash application rates, but their root to shoot translocation was relatively restricted. Increase in chlorophyll content and dry mass of mustard plant on treatments ≥ 20% even at elevated accumulation of Cd (1.67 mg kg^{-1}), Pb (18.25 mg kg^{-1}) and Zn (74.45 mg kg^{-1} dry weight) in its shoot showed relatively higher tolerance of selected mustard cultivar to heavy metal stress. The transfer coefficient (TC[1]) of Cd from mustard shoot to aphid was always > 1, indicating that Cd biomagnified in aphids at second trophic level. But, there was no biomagnification of Cd in adult beetles at third trophic level. Zinc accumulation was 2.06 to 2.40 times more in aphids than their corresponding host shoots and 1.26-1.35 times more in adult beetles than their prey (aphids) on which they fed. Lead was only metal whose TC was less than 1 at both second and third trophic levels. The elimination of Cd via honeydew of aphids was most efficient as the ratio of metal in honeydew to aphid (ranging from 0.21 to 0.26) was higher than the Pb (0.16 to 0.20) and Zn (0.07 to 0.09). The statistically consistent ($p > 0.05$) biomass and predation rate of predatory beetles indicated that all levels of soil amendments with fly ash did not have any lethal or sub-lethal effects on beetles.

Key words: Biotransfer; bioaccumulation; heavy metal; food chain; mustard; beetle.

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[1] Transfer coefficient

1. Introduction

The combustion of pulverized coal in the furnaces of Thermal Power Plants results in generation of large amounts of fly ash as a byproduct (Ram and Masto, 2014), which must be disposed of in an environmentally sustainable manner. The agricultural use of fly ash is endorsed due to the presence of many nutrient elements essential for plant growth (Singh et al., 2010). However, fly ash also contains a variety of potentially hazardous heavy metals like Cd, Pb and Zn (Gallardo et al., 2006). Although, the utilization of fly ash as a fertilizer in the Indian agriculture sector is only 3% (total 57.63% in all sectors of the total fly ash generation), it has enormously increased (2100%) over the last one and half decades (Central Electricity Authority CEA[2], 2014). Increased use of fly ash in agriculture results in the accumulation of toxic metals in the soil, which remain for long periods. The long-time persistence of these toxic metals within soil and their accumulation in plant tissues at sub-phytotoxic levels may pose a grave risk to higher strata in food chains (Zhuang et al., 2009; Gall et al., 2015). In contrast to heavy metal concentration based guidelines for the use of sewage sludge in agriculture, guidelines for the agricultural use of fly ash are limited, only found in New South Wales and Australia (Yunusa et al., 2012) and no such recommended standards are available in India.

Despite the ecological and economic importance of the above ground arthropods in agroecosystems, they have been neglected while forming the regulations for the agricultural use of solid wastes. Aphids are serious insect pests (Rehman et al., 2014), but a group of predatory insects play a significant ecological role for the control of these pests, thereby minimizing the economic loss in infested crop (Winder et al., 1999).

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[2] Central Electricity Authority

Moreover, biological control of crop pests by means of predatory insects is of great economic importance due to the high costs of pesticides (Dar et al., 2015a). Through the consumption of heavy metal contaminated prey, the health and fitness of these predatory arthropods may be negatively affected, resulting in a proportionate reduction in the effectiveness of pest control (Merrington et al., 2001). Thus, protection of biocontrol agents is imperative for safe and sustainable disposal of industrial wastes like fly ash in agricultural soil.

Previous research has shown that Cd, Pb and Zn derived from sewage sludge amended soil contrast in the points of the food chain where transfer is enhanced or restricted (Dar et al., 2015a). Cadmium transferred most readily through the system, but showed the least tendency to transfer between aphids and predatory beetles. By contrast, although Zn showed the greatest tendency to transfer through the food chain, particularly from aphids to beetles, there was evidence that Zn transfer was regulated in higher trophic levels. Of particular concern was the relatively high transfer of Pb from aphids to beetle larvae and the apparent increasing retention of Pb in beetle tissues during pupation as Pb exposure increased.

There is currently a lack of research into the effect that differing sources of metal have on the transfer of metals through multi-trophic food chains. Fly ash provides an interesting comparison to sewage sludge as a metal source. Whilst sewage sludge contains many organic (and inorganic) binding sites and tends to be slightly acidic, the combustion process removes virtually all organic matter from fly ash, which is also usually alkaline. Fly ash amendment tends to increase soil metal sorption to a much greater extent than sewage sludge (Tsailas et al., 2009; Shaheen and Tsadilas, 2010), limiting plant uptake of metals. However, Gupta and Sinha (2009) found that fly ash increased Pb concentration in the shoots of *Vigna radiata* by both increasing the uptake of Pb and, crucially, increasing translocation from root to shoot. Root to shoot transfer was found to be the principle restriction on the transfer of Pb through the food chain by Dar et al. (2015a). Thus, fly ash may potentially enhance Pb transfer from contaminated soil.

The aim of the present study was to compare the extent of biotransfer of Cd, Pb and Zn from fly ash amended soil across the mustard-aphid-predatory beetle food chain previously used by Dar et al. (2015a) to investigate transfer of metals derived from sewage sludge. The present study also investigated the potential effects on predatory beetle, particularly their consumption of aphids. The Cd, Pb and Zn were selected as test metals because they are among the most dangerous heavy metals discharged into environment (Nica et al., 2012). Indian mustard (*Brassica juncea*) was selected as a model plant for the primary trophic level because of its widespread geographic cultivation, large biomass and its high tolerance to the accumulation of number of heavy metals without showing any visible symptoms (Dar et al., 2015b). The aphid *Lipaphis erysimi* is a harmful insect pest of Indian mustard, which causes huge crop loss (Rehman et al., 2014), whilst *Coccinella septempunctata* commonly known as seven spotted ladybird, was selected as a predator at third trophic level because its adults and larvae are voracious feeders of aphids.

2. Materials and Methods

The soil used in the present study was collected from Aligarh Muslim University (AMU) agriculture farm fields and was air dried before mixing with fly ash. The fly ash was collected from Kasimpur Thermal Power Plant, Aligarh (UP, India) and was also air dried, finely powdered and sieved to 2 mm mesh size before being used for soil amendments. The mixing of soil and fly ash was conducted thoroughly before filling 23 cm diameter pots. The various amendments of soil with fly ash were 0, 5, 10, 20 and 40% (w/w). Treatments were replicated four times and designated as *T0* for the control, *T1* for 5%, *T2* for 10%, *T3* for 20% and *T4* for 40% soil amendments with fly ash, respectively. The soil mixtures in the pots were moistened to field capacity and incubated for three weeks to equilibrate under moist conditions as described by Dar et al. (2015a). No chemical fertilization was conducted before or during the experiment. Soil samples were taken from each pot for soil chemical and heavy metal analyses.

After three weeks of incubation of soil mixture, ten seeds of mustard (*B. juncea*) were sown in each pot to a depth of 0.5 cm. The pots were then kept in a fully randomised block design in a glasshouse (set to 25^{o}C and 16:8 h day–night regime) and were watered regularly with de-ionised water. Water retaining plastic plates were placed below each pot to collect and reuse the percolated water. Thinning of seedlings was done at the five to six leaves stage and three seedlings of equal growth and vigour were retained per pot.

To measure the effects of fly ash application on the growth of mustard, one plant from each pot was uprooted at 40 days after sowing for the analysis of chlorophyll content and dry mass. At this stage, 200 mustard aphids (*L. erysimi*) taken from laboratory culture were transferred to the remaining plants in each pot. Individual pots were subsequently kept under sleeve cages to prevent the movement of aphids between treatments. After three weeks, aphids were collected from each pot by following the method of Dar et al. (2015a). Collected aphid samples were divided into two groups. One group from each treatment was used for quantification of heavy metals and other was kept at -18 ^{o}C in deep freezer (DW- 40L626, Haier Bio-medical, China) before being used for feeding predatory beetles.

Plants were also sampled for the heavy metal analysis at this time. Heavy metal contents were also analysed in honeydew of aphids by following the method of Crawford et al. (1995). The feeding experiment of predatory beetles was conducted as described by Dar et al. (2015a). Twenty larvae (4th instar) of the predatory beetle were divided into five equal treatment groups. Each individual larva was reared separately in 9 cm diameter petri dishes containing filter paper moistened with distilled water and was fed fresh frozen aphids collected from one of the pot cultures. Individual petri dishes were covered by fine nettings secured with rubber band. Petri dishes containing larvae were then placed in a controlled environment cabinet (set to 25^{o}C and 16:8 h day–night regime). Consumption of aphids by individual larva was observed after every 24 hours and fresh (frozen) aphids were added to the petri dishes. The larvae of predatory beetles were fed until pupation. After pupation, newly emerged adult beetles were weighed and frozen at -18 ^{o}C before analysis of Cd, Pb and Zn.

2.1. Analysis for soil properties

The pH of the soil at different treatments was measured in a soil: water suspension (1:2.5 w/v) with a digital pH meter (181, India). Soil organic-carbon content was determined as described by Jackson (1958). Total nitrogen content in the soil sample was determined by the Micro- Kjeldahl method.

2.2. Heavy metal analysis

Soil samples, collected in triplicate from each pot, were air dried and ground gently before passing through a 2 mm mesh sieve. Subsequently, 1.0 g of each soil sample was digested in 20 ml of triacid mixture (HNO_3/H_2SO_4/$HClO_4$, 5:1:1) at 80 °C (Allen et al., 1986). After complete digestion, the solution was cooled and filtered through Whatman No. 42 filter paper and diluted to 50 ml with double distilled water (DDW) before analysis for total concentrations of Cd, Pb and Zn through Atomic Absorption Spectrophotometer (GBC, 932 plus; GBC Scientific Instruments, Braeside, Australia). For obtaining the extractable (bioavailable) fraction of heavy metals in soil, 15 g of sample was mechanically shook with 40 ml of diethylene triamine pentaacetic acid (DTPA) extractant (0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M TEA buffered at pH 7.3) for 2 hours (Lindsay and Norvell, 1978).

Dried roots and shoots were powdered and sieved. 0.3 g of dried plant samples were digested in 10 ml of tri-acid mixture (HNO_3:H_2SO_4:$HClO_4$; 5:1:1) at 80 °C until black fumes turned white and solution became completely transparent (Allen et al., 1986). The digest was cooled before getting diluted with DDW and then filtered through Whatman No. 42 filter paper. The filtrate was diluted to 50 ml with DDW. Dried samples of aphids, predatory beetles and their exuviae (from each treatment) were also digested by following the method of Dar et al. (2015a). Amounts of Cd, Pb and Zn in all samples were quantified by an Atomic Absorption Spectrophotometer (GBC, 932 plus; GBC Scientific Instruments, Braeside, Australia). All chemicals and reagents used were of analytical grade and supplied by Sigma-Aldrich. Analytical quality was checked by the analysis of certified reference materials i.e., GBW 07402 for soil, NIM-GBW10048 (celery plant) for plants and GBW 08552 (pork muscle) for insects. Mean recoveries from these materials were 96.7, 98.7 and 95.6% for Cd, Pb and Zn, respectively. Reagent process blanks were also digested and run in triplicate to check for process contamination.

2.3. Data analysis and calculation

Data were analysed using SPSS software version 17 (SPSS, Chicago, IL, USA) to determine the significance at $p < 0.05$. The significance of differences among treatments was determined by Duncan's multiple range test (DMRT). Transfer coefficients for Cd, Pb and Zn in a food chain were calculated by determining the ratio of concentration of a metal at the receiving level to the concentration of this metal at the source level. Heavy metal concentrations in the pupa of beetles before adult emergence were determined by adding the metal contents of the newly emerged adult to that of the exuvia. The percent of metal burden lost through pupal exuvia was calculated as:

$$\text{Percent of metal burden lost via exuvia} = \frac{\text{Metal contents in Individual beetle}}{\text{Metal contents in individual pupa}} \times 100$$

3. Results

3.1. Effect of fly ash on soil properties

The fly ash used for the amendment of soil in present experiment was alkaline in nature (pH = 8.85 ± 0.04) with a relatively low content of nitrogen (0.43 ± 0.02) and organic carbon (0.02 ± 0.004) than in the unamended soil (Table 1). Addition of fly ash (≥ *T2*) increased the pH of resultant soil mixture significantly ($p < 0.05$) compared to the unamended soil (Table 1). The organic carbon content in all levels of amended soil decreased significantly ($p < 0.05$) as compared to the control (*T0*). Application of *T2* to *T4* fly ash rates reduced the total nitrogen content significantly ($p < 0.05$) in the resultant soil mixtures as compared to unamended soil (Table 1).

Higher fly ash application rates (*T3* and *T4*) increased the total concentrations of Cd, Pb and Zn significantly ($p < 0.05$) in the resultant soil mixtures as compared to the unamended soil (Table 1). The DTPA extractable fractions of Cd (0.19 mg kg^{-1} dry soil) and Pb (2.46 mg kg^{-1} dry soil) were significantly ($p < 0.05$) higher in soil mixture on *T4* application rate of fly ash and Zn on *T3* and *T4* application rates as compared to control. Among the three heavy metals, concentrations of Zn were highest followed by Pb and Cd in all amended and unamended soil samples. The treatment *T4* recorded the highest concentrations of all the three studied heavy metals (Table 1).

Table 1. Selected properties and heavy metal concentrations (total and extractable) in unamended soil and soil after amendments with fly ash (mean ± SE, *n* = 4). Values with different superscript letters in each group are significantly different from each other at $p < 0.05$.

Parameters	Fly ash	*T0*	*T1*	*T2*	*T3*	*T4*
pH	8.85±0.04	7.76±0.02^{a}	7.83±0.02^{a}	8.02±0.02^{b}	8.20±0.03^{c}	8.40±0.03^{d}
Organic C%	0.43±0.02	0.74±0.02^{a}	0.68±0.01^{b}	0.66±0.02^{b}	0.57±0.02^{c}	0.51±0.02^{d}
Total N (%)	0.02±0.004	0.16±0.014^{a}	0.15±0.007^{a}	0.12±0.011^{b}	0.11±0.004^{b}	0.09±0.011^{b}
Total heavy metals (mg kg^{-1})						
Cd	1.42±0.07	0.46±0.03^{a}	0.49±0.04^{a}	0.53±0.02^{a}	0.62±0.02^{b}	0.80±0.02^{c}
Pb	15.54±0.42	11.24±0.42^{a}	11.93±0.54^{ab}	12.15±0.31^{ab}	12.70±0.48^{bc}	13.54±0.37^{c}
Zn	86.73±3.19	35.9±2.20^{a}	37.87±2.50^{a}	40.74±1.90^{ab}	46.12±2.14^{b}	55.52±1.43^{c}
DTPA extractable heavy metals (mg kg^{-1})						
Cd	1.42±0.07	0.12±0.01^{a}	0.12±0.01^{a}	0.14±0.01^{a}	0.16±0.01^{ab}	0.19±0.01^{b}
Pb	15.54±0.42	2.11±0.09^{a}	2.16±0.08^{a}	2.22±0.06^{ab}	2.30±0.08^{ab}	2.46±0.07^{b}
Zn	15.54±0.42	3.22±0.11^{a}	3.16±0.08^{a}	3.52±0.11^{ab}	3.88±0.13^{b}	4.67±0.18^{c}

T0: unamended soil; *T1*: 5%; *T2*: 10%; *T3*: 20%; *T4*: 40% fly ash.

3.2. Effects on the mustard growth

The *T4* treatment of fly ash increased the chlorophyll- a content significantly ($p < 0.05$) as compared to control, whereas chlorophyll- b content was augmented ($p < 0.05$) on application of *T3* and *T4* levels of fly ash (Figure S1). The dry mass of mustard enhanced significantly ($p < 0.05$) on applications of *T3* and *T4* levels of fly ash in the soil. The maximum value of plant dry weight (3.56 g plant^{-1}) was recorded on applications of *T3* treatment (Figure S2).

3.3. Effect of fly ash application on translocation of cadmium in food chain

The Cd concentration in mustard roots increased significantly ($p < 0.05$) when grown on *T2* to *T4* fly ash amended soils, with maximum concentration (2.44 mg kg^{-1} dry roots) recorded on *T4* treatment (Figure 1). Transfer coefficients of Cd between the soil and mustard roots increased with levels of fly ash applications and ranged between 2.74 and 3.37 for total to root and 10.5 – 13.06 for extractable fraction to root (Table 2). The concentration of Cd in shoots also increased significantly ($p < 0.05$) on applications of 20% (*T3*) and 40% (*T4*) fly ash levels in soil, indicating Cd transfer to shoots in proportion to root uptake (Figure 1). The TCs of Cd between the root and shoot remained below one (Table 2).

The amounts of Cd in aphid bodies increased significantly ($p < 0.05$) with fly ash application rates of 10 - 40% (*T2* - *T4*) as compared to the control (Figure 1). Transfer coefficients from shoot to aphid were higher than one in all levels of fly ash amended soil including unamended soil (Table 2). The accumulation of Cd in newly emerged adult beetles increased significantly ($p < 0.05$) with 10 (*T2*) to 40% (*T4*) fly

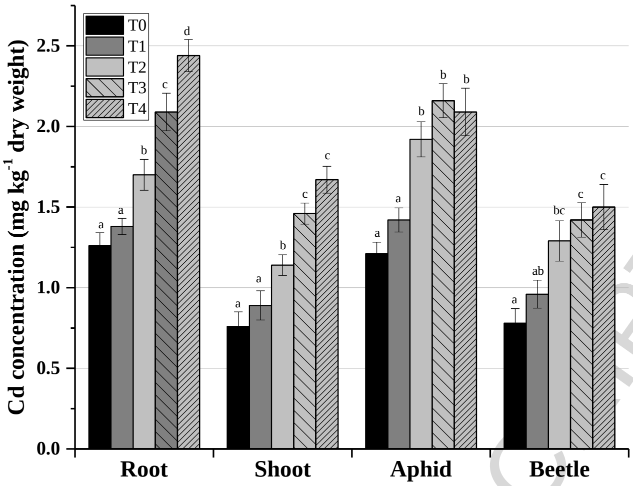

Fig. 1

Figure 1. Cadmium (Cd) concentrations (mg kg^{-1} dry weight; mean ± SE; *n* = 4) in roots, shoots, aphids and adult beetles on amendment of soil with varying levels of fly ash (*T0*: unamended soil; *T1*: 5%; *T2*: 10%; *T3*: 20%; *T4*: 40% fly ash). Bars with different letters in each group are significantly different from each other at $p < 0.05$.

Table 2. Transfer coefficients of Cd, Pb and Zn concentrations between various components of the soil – plant – aphid – beetle food chain on amendment of soil with various levels of fly ash.

Heavy metal	Amendments	Total soil-root	Extractable soil-root	Root-Shoot	Shoot-Aphid	Aphid-Adult beetle
Cd	*T0*	2.74	10.50	0.60	1.59	0.64
	T1	2.82	11.50	0.64	1.60	0.68
	T2	3.21	12.14	0.67	1.68	0.67
	T_3	3.37	13.06	0.70	1.48	0.66
	T_4	3.05	12.84	0.68	1.25	0.72
Pb	*T0*	1.39	7.39	0.62	0.85	0.65
	T1	1.54	8.51	0.57	0.90	0.63
	T2	1.82	9.96	0.56	0.91	0.69
	T3	2.12	11.71	0.56	1.03	0.71
	T4	2.15	11.85	0.63	0.84	0.66
Zn	*T0*	1.68	18.78	0.73	2.24	1.29
	T1	1.65	19.78	0.78	2.40	1.29
	T2	1.68	19.43	0.82	2.39	1.35
	T3	1.67	19.87	0.86	2.18	1.35
	T4	1.60	19.00	0.84	2.06	1.26

ash application rates as compared to control (Figure 1). Transfer coefficients of Cd between aphids and predatory beetles were always less than one in all soil-fly ash mixtures (Table 2).

3.4. Effect of fly ash application on translocation of lead in food chain

Concentration of Pb in roots increased significantly ($p < 0.05$) from 10% (*T2*) to 40% (*T4*) rates of fly ash amended soil as compared to control (Figure 2). The highest concentration of Pb (29.16 mg kg^{-1} dry weight) was found in roots of *T4* treated mustard plants. Like Cd, the TC between total Pb concentration in the soil and roots increased with fly ash application rate and ranged between 1.39 and 2.15 (Table 2). The TC between extractable Pb in varying soil-fly ash mixtures and mustard roots were very high and ranged between 7.39 and 11.85 (Table 2). Accumulation of Pb also increased in shoots of plants grown in *T3* and *T4* levels of fly ash as compared to control ($p < 0.05$). However, root to shoot TCs of Pb remained below one in all plant samples grown in fly ash amended soil (Table 2).

The Pb accumulation in aphids corresponded with levels of fly ash added in soil and quantity in the roots of mustard plants. The maximum amounts of Pb (15.58 mg kg^{-1} dry weight) accumulated in aphids reared on the *T3* treated mustard plants (Figure 2). The TCs of Pb from mustard shoot to aphids remained below one in most of the treatments (Table 2). The Pb concentration in newly emerged adult beetles increased significantly ($p < 0.05$) when fed on aphids nurtured on *T2* (10%) – *T4* (40%) treated plants (Figure 2). The TCs of Pb between aphid and beetles also remained below one irrespective of the fly ash treatment levels (Table 2).

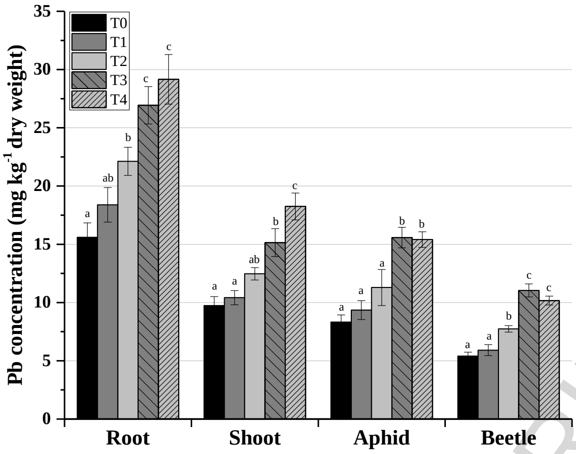

Fig. 2

Figure 2. Lead (Pb) concentrations (mg kg^{-1} dry weight; mean ± SE; n = 4) in roots, shoots, aphids and adult beetles on amendment of soil with varying levels of fly ash (*T0:* unamended soil; *T1:* 5%; *T2:* 10%; *T3:* 20%; *T4:* 40% fly ash). Bars with different letters in each group are significantly different from each other at $p < 0.05$.

3.5. Effect of fly ash application on translocation of zinc in food chain

Zinc concentration in roots increased significantly ($p < 0.05$) on 40% (*T4*) fly ash application in comparison to control (Figure 3). The roots of mustard grown in 40% (*T4*) fly ash amended soil accumulated 88.74 mg Zn kg^{-1} dry weight (Figure 3). Zinc accumulation in mustard roots was 1.60 – 1.68 times higher than the total concentration in the soil-fly ash mixtures and 19 - 20 times higher than the extractable fraction of Zn in the soil amended with varying levels of fly ash (Table 2). The amounts of Zn in shoots of mustard also increased significantly ($p < 0.05$) when grown in 20 (*T3*) and 40% (*T4*) fly ash amended soils. Highest Zn concentration recorded in shoots was 74.45 mg kg^{-1} dry weight on *T4* fly ash level in soil (Figure 3). Transfer coefficients of Zn between the roots and shoots increased with the rate of fly ash application in soil and remained below one in all treatments (Table 2).

Aphids accumulated higher amounts of Zn in their bodies and the concentration of Zn increased with the ratio of fly ash (≥ *T2*) as compared to control ($p < 0.05$). The highest Zn accumulation (153.31mg kg^{-1}) was found in aphids fed on *T4* treated mustard plants (Figure 3). Unlike Pb, Zn was biomagnified in aphids as TC of Zn between shoots and aphids were higher than two (Table 2). Zinc was transferred in newly emerged adult beetles when fed on aphids of mustard plants exposed to various levels of fly ash (Figure 3). The Zn concentration in predatory beetles increased significantly ($p < 0.05$) with increase in the quantities of fly ash (*T2* to *T4*) in soil as compared to control. The TCs of Zn from aphid to beetle were always observed to be greater than one (Table 2).

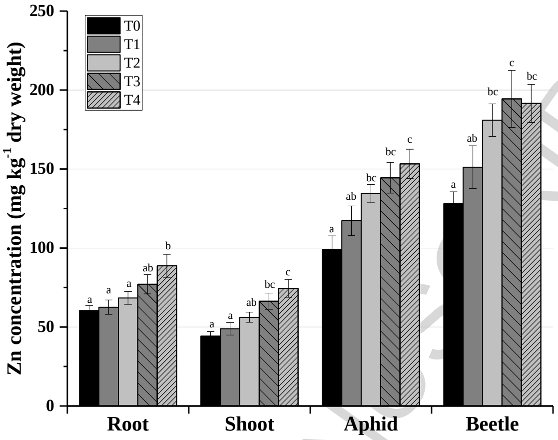

Fig. 3

Figure 3. Zinc (Zn) concentrations (mg kg^{-1} dry weight; mean ± SE; *n* = 4) in roots, shoots, aphids and adult beetles on amendment of soil with varying levels of fly ash (*T0:* unamended soil; *T1:* 5%; *T2:* 10%; *T3:* 20%; *T4:* 40% fly ash). Bars with different letters in each group are significantly different from each other at $p < 0.05$.

3.6. Heavy metal excretion with honeydew of aphids

Estimation of selected heavy metals (Cd, Pb and Zn) levels in aphid honeydew showed that concentrations of all the three metals increased in honeydew in proportion to the fly ash applied in the soil (Table 3). The ratio of metal in honeydew to metal in aphid bodies exhibited marked differences among the three selected heavy metals (Table 3). The elimination of Cd via honeydew was most efficient as the ratio of metal in honeydew to aphid was higher (ranging 0.21 to 0.26) than the Pb (ranging 0.16 to 0.20) and Zn (ranging 0.07 to 0.09; Table 3).

3.7. Heavy metals sequestered in pupal exuviae of beetles

The amounts of all three selected heavy metals (Cd, Pb and Zn) in pupal exuviae increased with fly ash application rate. Significant ($p < 0.05$) elimination of Pb via pupal exuviae of predatory beetles corresponded to the levels of fly ash application used in present experiment (Table 3). The percent (%) sequestration of metal burden in exuviae (Table 3, within parenthesis) indicated that Cd and Pb were sequestered almost with same efficiency. By contrast, Zn was most efficiently retained by the predatory adult beetles (Table 3).

3.8. Effect of fly ash on predation rate of beetle

The average aphid consumption rate (predation rate) by beetle larvae was not statistically affected ($p > 0.05$) when fed on aphids reared on fly ash treated mustard plants (Figure 4).

3.9. Effect of fly ash on biomass of aphids and predatory beetles

Amendments of soil with fly ash did not have statistically significant impact on the fresh and dry weight of aphids ($p > 0.05$). Soil amendments with fly ash also had no

Table 3. Heavy metal contents (mg kg^{-1} dry weight) eliminated with honeydew of aphids and in pupal exuviae of predatory beetles exposed to varying levels of fly ash (mean ± SE; *n* = 4). Values with different superscript letters in each group are significantly different from each other at $p < 0.05$.

Amendments	Cd (mg kg^{-1})		Pb (mg kg^{-1})		Zn (mg kg^{-1})	
	Honeydew	Pupal exuviae	Honeydew	Pupal exuviae	Honeydew	Pupal exuviae
T0	0.25±0.03^{a} (0.21)	0.50±0.06^{a} (8.74%)	1.33±0.11^{a} (0.16)	2.66±0.20^{a} (6.58%)	7.94±0.58^{a} (0.08)	51.51±5.09^{a} (5.43%)
T1	0.30±0.03^{ab} (0.21)	0.68±0.06^{a} (9.58%)	1.59±0.12^{a} (0.17)	3.84±0.23^{b} (8.85%)	8.21±0.72^{a} (0.07)	68.10±6.79^{ab} (6.62%)
T2	0.46±0.04^{abc} (0.24)	1.14±0.09^{b} (11.44%)	1.92±0.18^{a} (0.17)	5.53±0.43^{c} (9.41%)	9.42±0.41^{a} (0.07)	90.73±8.87^{bc} (6.80%)
T3	0.56±0.06^{c} (0.26)	1.39±0.12^{b} (12.76%)	3.07±0.27^{b} (0.20)	6.56±0.57^{c} (8.01%)	11.56±0.69^{b} (0.08)	98.82±9.06^{c} (7.00%)
T4	0.52±0.14^{bc} (0.25)	1.16±0.12^{b} (10.44%)	2.80±0.43^{b} (0.18)	8.20±0.36^{d} (10.58%)	13.80±0.82^{c} (0.09)	79.82±8.41^{bc} (5.76%)

T0: unamended soil; *T1:* 5%; *T2:* 10%; *T3:* 20%; *T4:* 40% fly ash.

Values within parenthesis in honeydew coloumns are the ratios of metal contents in honeydew vs metal contents in aphids.

Values within parethesis in exuviae coloumns are percentages of total metal burden lost via exuviae.

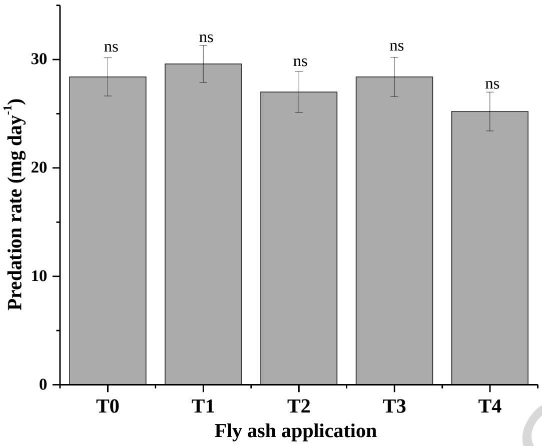

Fig. 4

Figure 4. Effect of various levels of fly ash application (*T0:* unamended soil; *T1:* 5%; *T2:* 10%; *T3:* 20%; *T4:* 40% fly ash) on the consumption of aphids by beetle larvae (mean ± SE; *n* = 4).

significant effect on the biomass (fresh and dry) of newly emerged adult beetles ($p > 0.05$; Table 4).

4. Discussion

The resulting higher pH of amended soil mixtures may be due to the higher (alkaline) pH of fly ash used in present experiment. The addition of CaO and MgO present in the fly ash may have modified the pH of amended soil accordingly (Su and Wong, 2003). The fly ash used in the present study contained higher amounts of Cd, Pb and Zn (Table 1) and eventually increased the concentrations of these metals when varying amounts were added to soil (Table 1). The DTPA extractable (phytoavailable) fractions of Cd, Pb and Zn in the resultant soil mixtures were not much impacted by the additions of fly ash except on treatment *T4* (Table 1). The phytoavailable or bioavailable fraction of heavy metals in soil is used for risk assessment as total heavy metals also involve the portions that are not easily available to the plants. Phytoavailability of metals is determined by the nature of metal species, their interaction with soil colloids, soil properties and duration of contact with the surface of these metals (Naidu et al., 2003). Moreover, it is reported that higher pH decreases the availability of heavy metals to root more effectively than lower pH (Adamczyk-Szabela et al., 2015). All these factors may have contributed to lowering the effect of fly ash additions on phytoavailable fraction of the heavy metals in resulting soil-fly ash mixtures. A comparison of the findings of the present study with that of Dar et al. (2015a), who investigated the transfer of Cd, Pb and Zn from sewage sludge to mustard plant, indicates that fly ash results in a lower phytoavailability of Cd and Zn than sludge at similar total metal loadings. However, this was not the case for Pb, which showed very similar transfer coefficients in both studies.

Table 4. Variation in fresh and dry weight (mg individual^{-1}) of aphids and newly emerged adult beetles exposed to various application rates of fly ash (mean ± SE; *n* = 4).

Amendments	Fresh mass (mg)		Dry mass (mg)	
	Aphid	Adult beetle	Aphid	Adult beetle
T0	0.21±0.02^{ns}	31.2±1.50^{ns}	0.029±0.001^{ns}	6.55±0.13^{ns}
T1	0.21±0.02^{ns}	30.65±1.64^{ns}	0.030±0.002^{ns}	6.43±0.13^{ns}
T2	0.20±0.01^{ns}	31.32±1.73^{ns}	0.035±0.002^{ns}	6.64±0.07^{ns}
T3	0.18±0.01^{ns}	30.32±1.21^{ns}	0.030±0.002^{ns}	6.45±0.10^{ns}
T4	0.18±0.01^{ns}	28.56±1.78^{ns}	0.03±0.002^{ns}	6.32±0.09^{ns}

T0: unamended soil; *T1:* 5%; *T2:* 10%; *T3:* 20%; *T4:* 40% fly ash.

4.2. Effect on mustard growth

Chlorophyll contents of leaves and plant biomass are reliably used for monitoring the effects of various environmental stresses on a plant (Dwivedi et al., 2007) as both parameters are linked to the visible symptoms and plant productivity. The increase in chlorophyll content in leaves of mustard plants on ≥ *T3* treatments of fly ash may be due to the accumulation of required amounts of the essential element magnesium (important constituent of chlorophyll molecule) present in fly ash. The enhancement in dry biomass of the mustard plants may be attributed to abundant availability of nutrients through the addition of nutrient rich fly ash (Dwivedi et al., 2007).

4.3. Heavy metal transfer from soil to mustard

Heavy metals taken up by roots from the soil are either stored in the root or transported to above ground tissues of the plants. The extent to which heavy metals are retained by roots and their upward translocation varies with the species of plant, nature of metal and environmental conditions. It has been reported that multiple transporter proteins are present in plant root cells, which help in the uptake of essential elements (Verbruggen et al., 2009; Gall and Rajakaruna, 2013). Zinc- Regulated Transporter1/Iron- Regulated Transporter1 (ZRT1/IRT1)-like proteins are well known transporters responsible for the uptake of Zn^{2+} by root cells (Kramer et al., 2007). Further movement of Zn from root to shoot is made possible by proteins of ZIP (Zinc- regulated transporter Iron- regulated transporter Proteins) and CDF (Cation Diffusion Facilitators) families (Verbruggen et al., 2009). Thus, in our study, the higher TCs of Zn from soil to root and from root to shoot (Table 2) can be described by the presence of specific uptake mechanisms for this essential metal, which are absent for non-essential elements (Dar et al., 2015a, 2015b).

The elements Cd and Zn possess similar chemical properties and therefore both have parallel interactions in biological systems, which results in Cd transport by the members of the ZIP family transporters (Kramer et al., 2007). It is reported that Ca^{2+} uptake channels in plant cells are also involved in Cd^{2+} transportation (Perfus-Barbeoch et al., 2002). Thus, a similar pattern of translocation of Zn and Cd from root to the shoot can also be explained on the basis of similar biochemical interactions between these two metals (Figure 1, 3, Table 2).

In the present study, Pb absorption by roots from the soil was less than that of the other two heavy metals as evident from their TCs (Table 2). Lead is a non-essential element and plants do not possess any specific transport mechanisms for its uptake. It is also not clear on how Pb enters the root cells of a plant. According to Monferan and Wunderlin (2013), Pb makes entry into roots passively, but Pourrut et al. (2013) suggested that families of ZIP/CDF or Natural resistance-associated macrophage proteins (Nramps) may help in Pb transport into roots. By whichever mechanism, plants have a limited potential for Pb uptake compared to both Cd and Zn. Further movement of Pb from mustard root to shoot was also less than for Cd and Zn (Table 2). Compared to the findings of Dar et al. (2015a), whether Pb was derived from sewage sludge or fly ash made no apparent difference in the extent to which Pb was translocated to shoots.

It is reported that maximum amounts of Pb accumulation occurs in roots of the plant, making the root an effective barrier against Pb translocation to the shoot (Karak et al., 2013). The greater affinity of Pb to carboxyl groups and pectins within the cell wall, leads to its retention in the roots (Qiao et al., 2015). Association with plasma membranes and middle lamellae, precipitation in intercellular spaces and sequestration in the vacuoles of root cells also contribute to Pb immobilization and retention in roots (Pourrut et al., 2013).

4.4. Shoot to aphid transfer

The Cd contents were high in the bodies of aphids feeding on shoots (stem and leaves) of fly ash treated mustard plants as compared to control (Figure 1). The accumulation of Cd in some aphid species fed on a variety of plants grown in sewage sludge contaminated soil has been reported (Winder et al., 1999; Green et al., 2003, 2010; Dar et al., 2015a), but, the level of Cd accumulation in most of these studies remained well below the uptake level of Cd we found. In the present study, the TCs of Cd between mustard shoot and aphid were greater than one in all fly ash soil amendments, indicating that Cd biomagnified in aphid bodies (Table 2). After entering the midgut epithelial cells of arthropods, heavy metals are turned metabolically inactive by binding with metallothioneins (Zhou et al., 2015). Cadmium is the metal which is mainly complexed with soluble proteins (metallothioneins), because Cd ions have a high affinity for the sulfydryl (SH) group in cysteine residues (Roosens et al., 2005). The biomagnification of Cd by mustard aphids in the present study suggests that they use this storage-detoxification mechanism. The Cd biomagnification reported by other workers (Crawford et al., 1995; Green et al., 2003; Alonso et al., 2009) also affirm that similar mechanisms also operate in other aphid species for this metal.

The higher concentrations of Cd, Pb and Zn in aphid honeydew in higher treatment appeared to be an important pathway for heavy metals elimination. In the present investigation, Cd was more efficiently eliminated with honeydew than Pb and Zn (Table 3), which agrees with the findings of Dar et al. (2015a). Efficient elimination of Cd with honeydew by mustard aphids might have proportionally contributed to the relatively low transfer of Cd to aphids, particularly at high levels of fly ash applications in soil. Thus, aphids may have regulated the Cd accumulation to some extent via eliminating this metal with honeydew (Dar et al., 2015a). In contrast, Crawford et al. (1995) found that aphid species *Aphis fabae* had no such evolved mechanism to eliminate Cd with honeydew. The findings of the present study and those of Crawford et al. (1995) suggest that regulation of Cd by this mechanism may vary with aphid species.

In the present investigation, Pb was the only heavy metal which did not biomagnify in aphids at any level of soil amendment with fly ash (Table 2). These results are in line with the findings of Cowgill (1973), Devkota and Schimidt (2000), and Zhou et al. (2015), who also reported biominimization of Pb in aphid *Rhopalosiphum nymphaea*, grasshoppers and the silk worm *Bombyx mori*, respectively. Like Cd, the Pb excretion with honeydew of aphids was in proportion to the level of solid waste added in the soil (Table 3). This supports the suggestion of Dar et al. (2015a) that aphids regulated Pb concentrations in their bodies by eliminating this metal with honeydew.

In the present study, only Zn biomagnified by a factor > 2 in the selected species of aphid when nurtured on shoots of mustard plants in the fly ash treatments (Table 2). Zinc, being an essential micronutrient, is absorbed by the roots and conducted acropetally through the xylem channel (Rascio and Navari-Izzo, 2011). The Zn is then metabolised and unloaded into the phloem (Riesen and Feller, 2005). Since aphids feed directly on the phloem sap, the readily available Zn in phloem is directly consumed by aphids in high amounts. In earlier findings, excess Zn accumulation was also recorded in the aphid *Drepanosiphum platanoids* fed on shoots of *Acer pseudoplatanus* plants

(Sinnett et al., 2010) and also in *L. erysimi* fed mustard plants grown on sewage sludge amended soil (Dar et al., 2015a). Ingestion of Zn does not always induce metallothionein synthesis to bind with it and retain it localized within the gut. Sometimes, Zn is bound to smaller peptides and transported to other tissues of the bodies of arthropods (Sterenborg et al., 2003). The detoxification mechanism for Zn may therefore differ from Cd due to its status as an essential element. Thus, the aphids may have also evolved a long-term Zn accumulation mechanism in their bodies.

The excreted Zn concentration in honeydew did not conform with the Zn concentration levels within aphids (Table 3, Figure 3), suggesting that excretion of this metal with honeydew was not the only regulatory mechanism. The reduction in TC of Zn between mustard shoots and corresponding aphids may possibly be due to deterrence effect of aphids feeding on excessive heavy metal accumulating shoots of mustard plants when grown in higher levels of fly ash. Moreover, the combination of metals intensifies toxicity through joint effects and acts as defense arsenal against herbivory (Kazemi-Dinan et al., 2014). The amalgamation of Cd and Zn synergized the toxic effect even at the lowest applied concentrations of 2 mg Cd kg^{-1} and 100 mg Zn kg^{-1} soil (Kazemi-Dinan et al., 2014).

4.5. Aphid to beetle transfer

The TCs of Cd between aphids and newly emerged adult beetles were always less than one in all soil amendments (Table 2), suggesting that Cd biominimized in predatory beetles at third trophic level when fed on Cd accumulating aphids. Efficient regulation of Cd by predatory beetles, even at high soil amendments with fly ash, led them to maintain comparatively low Cd concentrations in their bodies (Scheifler et al., 2002;

Wang et al., 2016). These data reinforce previous studies (Laskowski and Maryanski, 1993), indicating that transfer of metals in food chains doesn't depend only on storage forms (i.e. bioavailability) of metals in prey, but the physiology of the predator also plays a vital role (Scheifler et al., 2002). The percent of Cd burden lost through the larval exuviae of predatory beetles increased with the fly ash application rates in soil (Table 3). It may be inferred that the loss of Cd through pupal exuviae was an effective mechanism to biominimize the Cd body burden at the pupation stage of predatory beetles.

The TCs of Pb at first two trophic levels (soil to plant and plant to aphid) were less than the TCs of Cd and Zn (Table 2), but, the TCs of Pb at the third trophic level (aphid to predatory beetle) were similar to Cd (Table 2). The percent sequestration of Pb in the pupal exuviae was lower relative to the percent sequestration of Cd. These findings clearly indicate that Pb would have accumulated in those tissues of the body which were not shed/lost during metamorphosis. The relatively high transfer of Pb from aphid to adult beetle may be due to the lower loss of Pb via pupal exuviae during metamorphosis, which resulted in the retention of Pb in the adult predatory beetles. Dar et al. (2015a) found that Pb derived from sewage sludge amended soil was similarly transferred to the third trophic level. Thus, the source of Pb appears to have little effect on its transfer to the secondary consumer level.

Zinc in adult beetles biomagnified (TC > 1) on all levels of fly ash applications (Table 2). Probably because of the essential metabolic requirement for Zn, beetles have evolved an ability to retain this metal to maintain homeostasis (Green and Walmsley, 2013). It has been suggested that essential metals are usually not biomagnified as their concentration in the tissues of arthropods are more strongly regulated than non-essential metals (Maryanski et al., 2002). On the contrary, Zn in the present study biomagnified in aphids and predatory beetles, whereas Pb (non-essential and toxic metal) did not biomagnify in aphids or predatory beetles. On the other hand, Cd- another non-essential and toxic metal did biomagnify in aphids, but at lower level than Zn. Elimination of Zn in the pupal exuviae seemed to be a poor mechanism to exclude Zn from adult beetle as the proportion of Zn lost in the exuviae did not differ among treatments (Table 3). This confirms an earlier study by Green et al. (2003), which showed that Zn sequestration in pupal exuviae had no significant effect on the Zn concentration retained in newly emerged adult beetles. Therefore, the Zn regulation might have occurred in the larval stage of predatory beetles prior to pupation, feasibly by excreting this metal with faeces (Dar et al., 2015a).

4.6. Effects on aphids and newly emerged adult beetles

Application of fly ash to soil had no statistically significant effect on the biomass (fresh and dry mass) of aphids ($p > 0.05$). Similarly, the phloem-feeding aphids (*Myzus persicae*) were unaffected when *B. juncea* plants were grown in soil contaminated with 30 mg Cd kg^{-1} soil (Konopka et al., 2013). Alonso et al. (2009) did not find any effect on the dry weight of aphid (*Rhopalosiphum padi*) even after the accumulation of Cd over 10 mg kg^{-1} dry weight in their bodies. No reduction in the fresh mass was found in aphid (*Sitobion avenae*) which accumulated far greater amounts of Zn (250 mg kg^{-1} dry weight) in their bodies (Green and Tibbett, 2001) than recorded in aphids in present investigation (Figure 3).

All levels of soil amendments with fly ash did not have any impact ($p > 0.05$) on the consumption of aphids by predatory beetle larvae (Figure 4). Thus, biocontrol of *L.*

erysimi by *C. septempunctata* would be unaffected by fly ash amendment. It could be inferred that the amounts of metal accumulated in aphid (*L. erysimi*) bodies were unable to produce any deterrence effect on their natural enemy (*C. septempunctata*), suggesting metals accumulation in aphids is not an evolutionary adaptation to deter predation. In general, accumulation of Cd and Pb and biomagnification of Zn in newly emerged adult beetles did not have any lethal or sublethal effect on them in present study. However, it is pertinent to mention here that the arthropods have a limited capacity to store or detoxify heavy metals and beyond this capacity, toxic effects can become evident and sub-lethal effects can turn into lethal ones (Crommentuijn et al., 1995).

4.7. Conclusion

Soil amendments with fly ash proved to be beneficial for the growth of the selected cultivar of mustard as its chlorophyll content and dry weight were enhanced upon higher application rates. Moreover, toxicity symptoms were not visible in mustard plants in the present study. The transfer of essential element Zn from soil to mustard roots was much greater than the transfer of non essential elements (Cd and Pb). In the plant – aphid – beetle food chain, Zn biomagnified in aphids at second trophic level and in predatory beetles at third trophic level. Fly ash appeared to reduce the availability of Cd and Zn compared to sewage sludge, but there was little apparent effect on Pb. Combined with the mobility of Pb in the third trophic level, this suggests that Pb may be a more problematic contaminant than has hither to been realised.

The statistically consistent predation rate and biomass of beetles indicated that all levels of soil amendments with fly ash did not have any lethal or sub-lethal effects on predatory beetles, but it should be noted that arthropods can detoxify and store heavy metals only to some threshold concentrations, above which the accumulated metal(s) become toxic. Therefore, a precautionary approach to soil metal loading rates is necessary while applying fly ash in soils, so that the biotransfer of toxic heavy metals to higher trophic levels, if it occurs, is kept at minimum. Moreover, information regarding the tri-trophic transfer of heavy metals along the soil-mustard-aphid-beetle food chain may be of significant importance for the sustainable phytoremediation projects using metal accumulating plants like *B. juncea* for contaminated soils.

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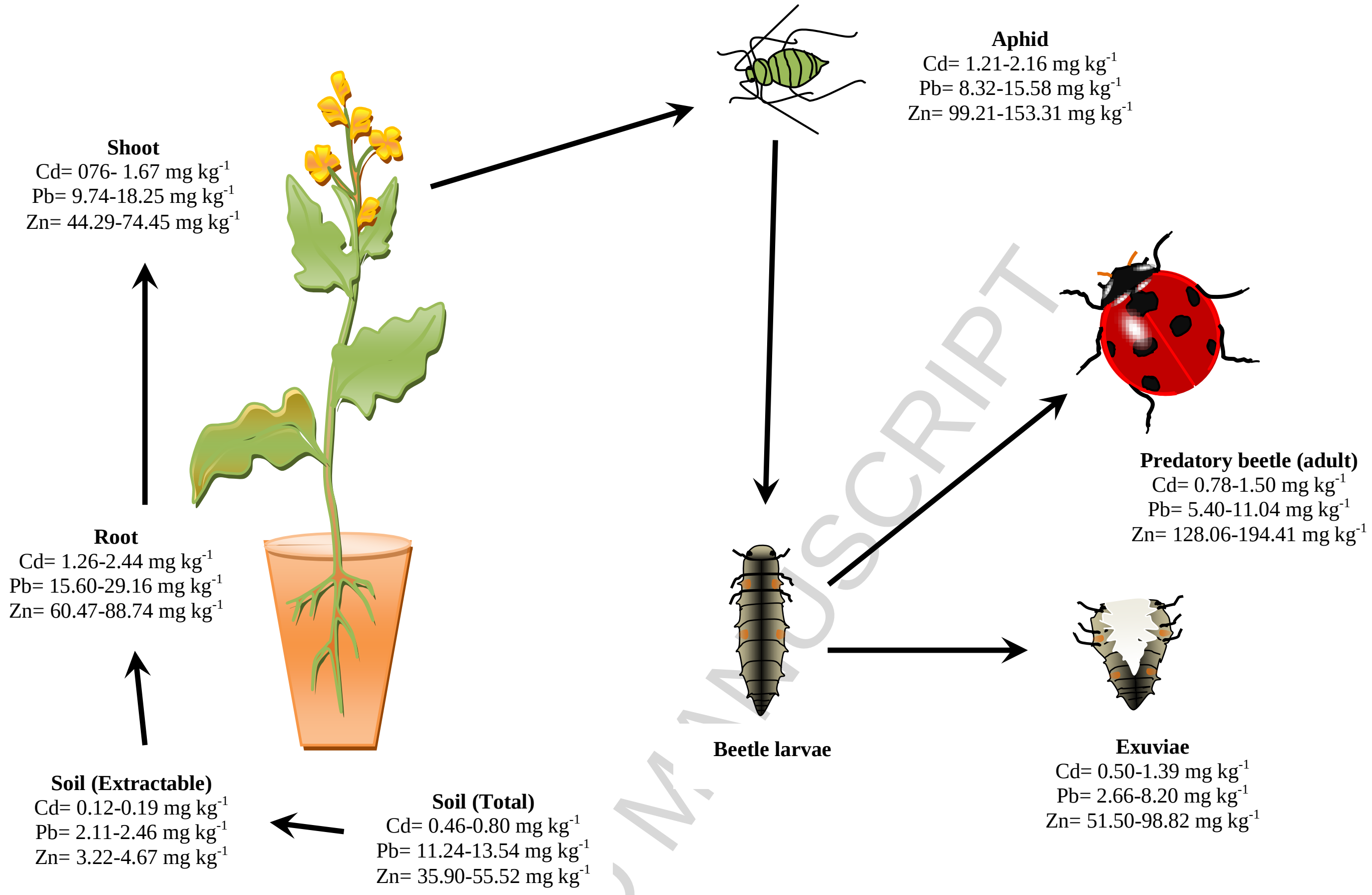
Graphical abstract



The range of cadmium, lead and zinc concentrations (mg kg^{-1} dry weight) in the components of the soil-mustard-aphid-beetle food chain exposed to varying rates of fly ash application (0 to 40%).

Highlights

- Biotransfer of Cd, Pb and Zn from fly ash amended soil in mustard (*Brassica juncea*)- aphid (*Lipaphis erysimi*)- beetle (*Coccinella septempunctata*) food chain was investigated.
- The Zn biomagnified in plants, aphids and predatory beetles.
- There was no biomagnification of Cd and Pb in adult beetles at third trophic level.
- All levels of soil amendments with fly ash did not have any lethal effects either on mustard or beetle.