Assessment of bioaccumulation of Cu and Pb in experimentally exposed Spiders, *Lycosa terrestris* and *Pardosa birmanica* using different exposure routes

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Abstract

There are major concerns regarding the environmental and human health risks caused by exposure to heavy metals. Spiders are often used as a model in ecotoxicological studies to assess soil pollution. In present study, we measured the bioaccumulation of copper (Cu) and lead (Pb) in spiders, *Lycosa terrestris* and *Pardosa birmanica* by ICP-MS. We determined whether Cu and Pb accumulation differed in (i) different spider species, (ii) single compared to combined exposure, and (iii) routes of exposure. Spiders were exposed to 10mM $CuSO_4$ and 10mM $PbCl_2$ solutions separately or combined (10mM+10mM) through different exposure routes i.e. spiked soil and food for 6 weeks. Effect of metals on survival and body mass of exposed and unexposed (control) spiders was determined. In both spider species, accumulation of metals increased with exposure time. In single metal exposure, Cu accumulation from food was higher than soil exposure in both spiders species, whereas the opposite was observed for Pb. In combined treatment, the uptake of both metals significantly decreased from similar accumulation routes. *L. terrestris* accumulated higher concentration of metals than *P. birmanica* during soil exposure. Metal exposure via contaminated food caused higher mortality compared to soil exposure. Body mass of both spider species was significantly decreased and negatively correlated with metal's concentration. These results suggest that bioaccumulation efficiency of Cu and Pb differs significantly in spiders exposed to metal's mixture compared to a single metal exposure, and is dependent on exposure route, type of metal and spider species. More understanding of the effect of exposure to metal mixture and exposure routes may help to improve risk assessment and ecological monitoring programs.

Keywords: Spiders, Environmental pollution, Metals, Bioaccumulation, Body mass change (BMC), Mortality.

Introduction

Industrial activities and agriculture practices have resulted in release of large amounts of metals into the environment, posing risks to human and environmental health (Wongsasuluk et al. 2014, Shulman et al. 2017). Copper (Cu) and lead (Pb) are common metals in soil ecosystems and are often found together in high concentrations especially near industrial areas and in agricultural fields irrigated with urban and industrial wastewaters (Iqbal and Khera 2015; Ahmad et al. 2018). The intensive use of fertilizers, insecticides and herbicides in agroecosystem also increase the level of these metals in soil (Chiroma et al. 2007). Together, Cu and Pb are a cause of concern because of their short- and long-term harmful effects on living organisms (Komjarova and Blust 2008; Cooper et al. 2009; Xu et al. 2011; Rouchon and Phillips 2017). Cu is a crucial element for normal physiological functions in organisms, but can be toxic at high concentrations and cause oxidative damage in cells by generating free radicals (Gaetke et al. 2014; Zeeshan et al. 2016). Also, free Cu ions bind with thiol group of membrane protein and disrupt its structure and function (Holmstrup et al. 1998; Huang et al. 2012). Pb represents a risk for terrestrial organisms due its high toxicity and long-term retention time in the environment (Kumar et al. 1995; Peterson et al. 2017). Pb is a redox inactive metal which produce oxidative stress indirectly by binding with sulfhydryl group of proteins and cause depletion of glutathione, alteration of Ca hemostasis, DNA damage and Lipid peroxidation (Stohs and Bagchi, 1993)

Spiders can accumulate large amount of metals and are generally considered as macro-concentrators (Dallinger 1993) and bioindicator of environmental pollution (Wilczek et al. 2004; Jung et al. 2007). Toxic effect of metals on organisms relies on exposure routes (Yang et al. 2016). In spiders along with other terrestrial invertebrates, food is considered a major route for the uptake of metals (Hopkin 1989; Zidar et al. 2009; Wilzeck et al. 2018). However, dermal uptake via contact with soil or soil water cannot be ignored (Kammenga et al. 2000). For instance, in earthworms being a soft-bodied organism, dermal contact is the main exposure route for metal's accumulation (Saxe et al. 2001; Vijver et al. 2003). In terrestrial isopods oral uptake of contaminated food or soil is the primary route for metal's exposure (Koster et al. 2005; Viverj et al. 2006). For Collembolan species, Cu assimilation via soil pore water is higher compared to other route i.e., water or food (Pedersen et al. 2000). So, in terrestrial invertebrates' multiple exposure routes of metals identified which are species-specific and depend on the feeding behavior of the organisms. At present, there is a lack of studies dealing with the effect of different exposure routes on the bioaccumulation of metals in spiders. Therefore, an integrated study of metals uptake routes and their impact on the exposed species is necessary to better understand the harmful effect of metals on spiders.

Previous studies have reported that spiders inhabiting metal-contaminated environment allocated a large portion of their energy in the regulation or detoxification of excessive amount of metal, which adversely impact on their survival, growth and development (Hendrickx et al. 2003; Ramirez et al. 2011). Thus, monitoring of different biological parameters in spiders inhabit near metal contaminated environment can enable assessment of the potential effects of these metals on their energy budget. Spiders of family Lycosidae are abundantly found in the agricultural fields of Punjab, Pakistan (Tahir and Butt 2008). Previous work showed that lycosids are good models for eco-toxicological studies because they can accumulate significantly high concentration of metals into their bodies and inhabit metal polluted areas in high densities (Chen et al. 2011, Bednarek et al. 2016). Also, metals, such as Cu and Pb have high background values in the agricultural soil of Pakistan due to crop irrigation with untreated industrial or municipal waste water and intensive use of fertilizer and pesticides (Jamal et al. 2013; Ahmad et al. 2018). Organisms inhabit these areas such as ground spiders may accumulate these metals directly via soil or indirectly through diet, which ultimately affect their biological functions.

Previous studies on spiders have examined the toxic effect of metals, such as Pb and Cu singly, through either metal-contaminated food or water (Jung et al. 2005; Babczynska et al. 2011b; Wilczek et al. 2018). The exposure to multiple metals may induce synergistic, antagonistic or additive toxicological effects which may be different from single metal exposure (Flouty and Khalaf 2015; Wijayawardena et al. 2018). However, very limited information is available on the toxic effects of metal mixture in spiders under laboratory conditions (Jung et al. 2005). In other invertebrates such as in terrestrial isopod *Porcellio scaber*, high accumulation of Zn was inversely related to the concentration of Cd (Zidar et al. 2009). For glochidia (bivalve), the effect of Cu and Pb mixture was time-dependent and was additive at 24 hours of exposure, but after 48 hours interaction became synergistic (Kovats et al. 2010). Therefore, assessment of the combined effect of metals via different exposure routes is necessary in order to further use of lycosids in eco-toxicological studies of environmental pollution.

In this study, the effect of exposure of two spider species of family Lycosidae, *Lycosa terrestris* and *Pardosa birmanica* to Cu and Pb, separately or combined was investigated via two different exposure routes (soil *versus* food) for 6 weeks. The effect of metal's exposure on the survival and body mass change (BMC) in both spider species was investigated in order to determine the biological consequences of metal uptake and bioaccumulation. The main aims of the present study where (i) to identify the difference in metal bioaccumulation capacity of spiders during single and combined exposure of Cu and Pb (ii) to explore the significance of metal exposure routes (soil *versus* food) on metal accumulation rate (iii) to compare the difference in Cu and Pb accumulation pattern between studied species (iii) to evaluate the toxic potential of Cu and Pb using mortality and BMC as potential markers of metal stress in spiders. We anticipate, the results can be helpful in evaluating the efficacy of spider species as bioindicator of metal pollution in the environment.

Materials and Methods

Collection and maintenance of Spiders

Female *L. terrestris* and *P. birmanica* spiders with egg sacs were collected by visual search method from unpolluted fallow land of University of the Punjab, Lahore, Pakistan, from April to June, 2017. Spiders were placed individually in transparent plastic containers (7.5cm L ×5.5cm W × 6cm H) with a perforated lid and a layer of moist soil (2cm thick) at the bottom of the container to maintain ventilation and humidity, respectively. Spiders were maintained in the laboratory (temperature: 33°C, at day/ 25°C, at night, relative humidity: 60 ±10% and photoperiod 14L:10D h) and fed with Drosophila adult flies as per requirement. After hatching of egg sac, each spiderling that left their mother's body were transferred in a separate plastic container and reared in lab conditions as described above. Each spiderling was fed with artificial food prepared by mixing 100ml whole milk, 100ml soya milk (non-dairy beverage) and one chicken egg yolk as described previously (Amalin et al. 2001). Food was supplied daily through cotton balls soaked in the artificial food. The containers of spiders were cleaned regularly in order to avoid fungal and bacterial infections. Fourth instar spiderling of both species were used for all tests performed in this study.

Experimental groups

Seven experimental groups (*n* = 150 spiders/group), including six treatments (three metal treatment conidtions × two metal uptake routes) and a control were established for each species. For each treatment, fourth-instar spiderlings of both species were randomly selected from their laboratory-reared populations. Metal solutions of Cu and Pb were prepared by dissolving of 250g of $CuSO_4.5H_2O$ and 270g of $PbCl_2$ per liter of distilled water. Spiders were exposed to 10mM of $CuSO_4$ and10mM of $PbCl_2$ separately or in combination (i.e. 10mM $CuSO_4$ +10mM $PbCl_2$) using two treatment routes (i.e. food and soil) (Fig. S1). The exposure concentrations of Cu and Pb were selected according to studies conducted by Babczyńska et al. (2011b) on *Agelena labyrinthica* and Jung et al. (2005) on *Pardosa astrigera,* respectively.

Soil Exposure

Artificial soil was spiked with metals (Cu, Pb or their mixture), according to Organization for economic cooperation and development (OECD) standard guide for testing of chemicals. Briefly, dried industrial sand (70%) was mixed with kaolin clay (20%) and sphagnum peat (10% which is an organic matter), and air dried. The pH of the soil was checked using Van Ranst et al. (1999) method and maintained at 6.0 ± 0.5 by addition of calcium carbonate ($CaCO_3$). The moisture content of the soil was maintained at 50% of its water holding capacity (WHC), as described in ISO (2008) guideline. Then, $CuSO_4.5H_2O$ was added in uncontaminated soil at 635mg Cu/Kg, $PbCl_2$ at 2000mg Pb/Kg and mixture of both metals at 635mg Cu/Kg and 2000mg Pb/Kg. The aqueous solution of $CuSO_4.5H_2O$ or $PbCl_2$ was mixed with artificial soil to obtain the required concentration of respective metals and maintain the moisture at 50% of water holding capacity. The spiked soil was thoroughly mixed for 15-20 minutes, 50g were weighed and placed in transparent plastic container (7.5cm L ×5.5cm W × 6cm H). Control spider group received no metal, but water was mixed at the same rate. Soil was equilibrated for 2 days before use in the experiment and loss in water contents was frequently examined by measuring the weight of containers and replenished by adding distilled water. Spiders were released in the containers of contaminated soil and retained there for 6 weeks. The containers were cleaned regularly and soil was replaced weekly. Artificial food was supplied on a daily basis during the entire duration of the experiment. At each week, 10 spiders from each treatment were randomly selected for metals analysis and stored at -20°C. Metal concentrations from each spider were measured separately.

Food Exposure

Spiders food was spiked with metals by mixing $CuSO_4.5H_2O$ and $PbCl_2$ solutions in artificial food mixture (as described above) to obtain final metal concentrations of 635mg Cu/L and 2000mg Pb/L, and their mixture (635mg Cu/L and 2000mg Pb/L). Cotton balls were soaked in artificial food and offered to spiders on a small piece of wax paper in a transparent plastic container, in which uncontaminated moist artificial soil was already present. Both spider species (in treated and control groups) received food on a daily basis and water content of soil was replenished regularly (as mentioned above). Spiders in the control group were offered diet without metal solutions. At each week, 10 spiders of each species were separated for metal analysis and stored at -20°C.

Mortality and Body mass change (BMC)

The mortality rate of all examined spiders was recorded daily and percent cumulative mortality was calculated after every week, using the formula: % Cumulative Mortality = dead /total individuals × 100, as described previously (Mleiki et al. 2016). Spiders were considered dead when no movement was noticed with naked eye or under binocular microscope after stimulation with camel hair brush. To assess loss in body mass, the weight of 10 spiders from each treatment was measured on individual basis at a weekly interval using an electronic balance (AY120 type, Shimadzu Corporation, Japan). Body mass change (BMC) was calculated using the formula:

$$\mathrm{BMC} = \frac{\mathrm{BM(i)} - \mathrm{BM(f)}}{\mathrm{BM(f)}}$$

Where $BM_{(i)}$ is the initial mass of spiders and $BM_{(f)}$ is the mass of the same spider after every week, as described previously (Bednarska et al. 2015).

Sample preparation for elemental analysis

Spiders were freeze-dried for 24 hours, weighed and digested in 5ml mixture of 70% HNO_3, 30% H_2O_2 and 3ml deionized water for an hour at ambient temperature. These samples were placed for 45 minutes in a microwave. The solution obtained after digestion was diluted by adding 6ml distilled water (Goulle et al. 2005; Joy et al. 2015). Metal concentrations in each spider were then measured by Inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific, USA). The accuracy of experimental procedure was evaluated using bovine liver 1577c CRM (a certified reference material) bought from "National Institute of Standards and Technology" (NIST), Gaithersburg, USA). The percentage recovery was 94% for Pb and 99% for Cu. Total metal concentrations were shown as mg/kg dry matter (DM). Same procedure was used for quantification of actual metals concentration in contaminated soil and food.

Statistical analysis

Normality of the data was checked using Kolmogorov Smirnov test. Generalized linear model (GLM, $p < 0.05$) was applied to compare the metals body burden in spider species considering exposure routes and time as factors. Linear regression analysis was used to examine the correlation between metal concentration and mortality, and also between metal concentration and body mass change (BMC) over time in both species, separately. The difference in metal accumulation in both species via soil and food was determined by independent *t* test. All statistical analysis was carried out in Minitab 18.

Results

Metal Concentrations

The actual concentrations of Cu and Pb in artificial soil and food measured by ICP-MS were > 90% of the nominal concentrations (Table 1). Metals values are listed after subtracting from control groups.

Single Metal Exposure

The concentration of Cu and Pb was significantly increased in both spider species with the increase of exposure time in both exposure routes (Table S1). Accumulation of Cu in *L. terrestris* via food was significantly higher as compared to via soil over 3 weeks but appeared to be similar after 6 weeks (Fig. 1A, Table S1). Conversely, *P. birmanica* accumulated 2.5 times higher Cu contents from contaminated food within 6 weeks of exposure as compare to soil (Fig. 1E). Both species accumulated significantly higher amount of Pb from soil as compared to food (Fig.1B, F). After 6 weeks, Pb accumulation through soil was approximately four-fold higher in *L. terrestris* and two-fold higher in *P. birmanica* than food (Fig.1B, F).

Exposure to Metal Mixture

In metals mixture experiment, the uptake of Cu from contaminated food was remarkably low in both species after 6 weeks ($t = 8.02$, $P = 0.00$ for *L.t*; $t = 66.92$, $P = 0.00$ for *P.b*) (Fig.1 C, G). However, a modest increase in Cu content via soil exposure was observed, which was ~ 6-7.5% in *L. terrestris* and 10-16 % in *P. birmanica.* The uptake of Pb from spiked soil was decreased down to 40% over 6 weeks in the presence of Cu in both species (t = 13.60, P = 0.00 *L.t*; t = 12.79, P = 0.00 *P.b*). Conversely, Pb accumulation from spiked food increased slightly in *P. birmanica* and declined in *L. terrestris* in combined treatment (Fig. 1D, H). Thus, food exposure appeared to be an important route for accumulation of Cu, whereas for Pb soil exposure played a significant role.

Species comparison

Both species, accumulated roughly comparable quantities of Cu and Pb from contaminated food after a single metal exposure (Fig.1A, E, B, F). However, accumulation of both metals through soil was significantly different in both species (t = 5.37, P = 0.000 for Cu; t = 4.50, P = 0.000 for Pb). Likewise, during exposure to both metals, *L. terrestris* accumulated significantly higher amount of Cu and Pb during soil exposure experiment (t = 4.38, P = 0.000 for Cu; t = 2.96, P = 0.004 for Pb), whereas accumulation of metals through contaminated food was almost similar in both species (Fig.1C, G, D, H).

Mortality

Percent cumulative mortality in the control groups after 6 weeks was 2.7% in *L. terrestris* and 6.8% in *P. birmanica*. In treated groups, mortality varied from 9.7 % to 26.8 % in *L. terrestris* and 11.5% to 24.6 % in *P. birmanica* (Fig. 2). In all experimental groups, mortality rate increased with metals exposure time (GLM, with P = 0.00) and differed significantly from control groups (Fig. 2). The cumulative mortality was higher in spiders exposed to metals through food as compared to soil, however the difference was not statistically significant (Fig. 2). In *L. terrestris* cumulative mortality was significantly different between single (Cu and Pb) and combined metal exposure via both routes (Soil, F = 9.94, P = 0.012; Food, F = 5.70, P = 0.041). The mortality of *P. birmanica* was only different between single (Cu and Pb) and combined treatments when exposed through soil (Soil, F = 6.23, P = 0.034; Food, F = 4.49, P = 0.064). Also, a significant positive correlation between mortality and quantity of accumulated metals (Cu or Pb) was observed in both species ($P \leq 0.007$) (Fig. S2).

Body mass Change (BMC)

Body mass change (BMC) was examined to quantify alterations in weight per week in both spiders species treated via different exposure routes (Fig. 4). Significant difference in BMC was recorded in control and treated spiders after 6 weeks ($P \leq 0.001$ *L. terrestris;* $P \leq 0.001$ *P. birmanica*). BMC of both species was gradually decreased and exhibited unsteady fluctuations (more prominent during food exposure) up to 6 weeks when exposed to Cu only and Pb only treatment. However, more reduction in BMC was observed in metals mixture (Cu + Pb) dietary exposure assay after 2 weeks in *L. terrestris* and after 4 weeks of exposure in *P. birmanica,* even though BMC indices were negative at 5 weeks, but spiders were able to gain some weight back at 6 weeks after exposure (Fig. 4C, F). However, change in body mass of both species after 6 weeks was not significantly different between single and mixture treatments (Soil, F = 1.66, P = 0.210; Food, F = 2.04, P = 0.105 for *L. terrestris*) (Food, F = 0.02 P = 0.987 for *P. birmanica)* except in *P. birmanica* during soil exposure route (F = 4.87, P = 0.016). Linear regression revealed significant negative ($P \leq 0.044$) relationship of BMC with metal quantity over 6 weeks in all experimental groups (Fig. S3).

Discussion

The use of spiders as a model for eco-toxicological studies due to their voracity, polyphagy, species diversity and wide geographic distribution, has achieved growing attention (Walters et al. 2009, Yang et al. 2016, Wilczek 2017). The present study investigated the effect of exposure of spider species, *Lycosa terrestris* and *Pardosa birmanica*, to Cu and Pb on their survival and body mass change (BMC). Our results showed that bioaccumulation of Cu and Pb differed significantly in spiders exposed to combined metals compared to a single metal exposure, and was dependent on exposure route, type of metal and spider species.

Single Metal Exposure

Spiders being macro-concentrator and polyphagous (Dallinger and Rainbow 1993), generally assimilate high quantity of metals either from their diet or surrounding environment (Kammenga et al. 2000; Jung et al. 2005). These metals are then sequestered into different parts of their body specifically in midgut glands in inactive form (Hopkin 1989). Comparative studies of arachnocenoses have shown that metal's load and its storage duration in spiders is mainly associated with feeding behavior (i.e. liquid feeder spiders suck out metals along with their food from soft tissues of insects), hunting strategy, habitat type, metal type, exposure route, and low rate of metabolism in metal storage organ (hepatopancreas or midgut glands) (Wilczek et al. 2008; Babczynska et al. 2011a). In our study, bioaccumulation capacity of Cu and Pb clearly indicate that both spider species have relatively high capacity of metals' accumulation. In Cu-treated spiders, the Cu concentration increased up to 153.78mg/Kg in *L. terrestris* and 175mg/Kg in *P. birmanica*. This concentration is very high and has been recorded in other spider species. For example, Babczynska et al. (2011b) revealed that *Agelena labyrinthica* collected from previously contaminated sites significantly accumulated higher amount of Cu compared with control when fed with metal contaminated diet in the laboratory. Also, bioaccumulation of Cu in *Steatoda grossa* fed with metal administrated prey were more than two times higher compared with control in a four weeks period (Wilczek et al. 2018).

The concentration of Pb in our study also increased with number of days and fell within range of 77 to 149 mg/Kg, which is much higher than that recorded in other spider species. Jung et al. (2005) observed that *Pardosa astrigera* accumulated Pb up to saturation level within two weeks and sustained nearly the same concentration over 8 weeks period without elimination despite of continuous metal exposure. Furthermore, our Pb uptake rate was similar with that recorded in other invertebrates, such as in green garden snail, *Cantareus apertus*, which accumulates 60-180 µg/g of Pb (Van Ginneken et al. 2015) and *Enchytraeus crypticus* with 244 mg/Kg of Pb at steady state phase (Zhang and Van Gestel 2017). This high accumulation capacity may be due to negligible affinity of Pb towards metallothionein (Vasak 1991).

Exposure to metal mixture

In order to evaluate combined toxicity and assimilation efficiency of heavy metals, spiders were exposed to binary mixture of Cu and Pb via different uptake routes for 6 weeks. In the presence of Pb, spider species assimilated lesser amount of Cu from metals mixture during food exposure in comparison to single Cu treatment. However, both spider species accumulated almost similar quantity of Cu through soil during single and combine exposures. A significant decrease of Pb uptake rate was observed in co-treatment with Cu through soil in both spider species. These results indicate that competitive inhibition between Cu and Pb may occur as both metals have partly similar uptake pathway i.e Cu compete with Na+ for uptake and Pb interfere at absorption sites of both Na+ and Ca+ ion simultaneously on the cell surface (Rogers et al. 2005). Reference studies on the combined effect of Pb and Cu uptake by spiders are not available as yet. However, analysis of the bioaccumulation of Pb in the presences of Hg and Cd in *Pardosa astrigera* showed that, biouptake of Pb and Hg in combined treatment was reduced, whereas Cd concentration continuously increased with time (Jung et al. 2005). This result has led the authors Jung et al. (2005) to assume that both metals (Pb and Hg) and Cd might interact with each other for uptake sites and have complex uptake pattern. Flouty and Estephane (2012) reported that in *Chlamydomonas reinhardtii* (a unicellular algae), the uptake of Pb was considerably decreased after introducing high level of Cu into its body. Likewise, joint action of Cu and Pb mixture was antagonistic on the sea urchin embryo *Strongylocentyotus intermedius* (Xu et al. 2011). Komjarova and Blust (2008) reported that in *Daphnia magna,* accumulation of Cu contents increased at a maximum concentration (0.25 µM) of Pb, but Cu in turn had no impact on the uptake rate of Pb. Likewise, Pb addition did not affect Cu accumulation (and vice versa) in the isopod *Asellus aquaticus* after exposure through water (Van Ginneken et al. 2015). These finding suggested that the interaction between Cu and Pb for uptake sites can be competitive or non-competitive depending on exposure concentration of metals, their uptake route and examined species.

Comparison between Exposure Routes

Food is considered to be a major route for uptake of metals in spiders (Jung et al. 2005). Metals can also be taken up from contaminated water (Liu et al. 2013; Li et al 2016). Therefore, it is possible that spiders can accumulate metals from soil water when they come in contact with water phase of the contaminated soil (Pedersen et al. 2000). In the present study, metal's bioaccumulation by exposure routes was comparable in both spider species depending on the type of metal and examined spider species. During single metal exposure, spiders accumulated the highest amount of Pb through soil, whereas Cu uptake rate was highest during food exposure. Pedersen et al. (2000) discussed the importance of different exposure pathways on the uptake and effect of copper in two different species of Collembola. Both species accumulated significantly high amount of Cu from spiked soil as compared to food (yeast) and additive response was observed when Cu was given via both exposure routes. However, isopods *Porcellio Scaber* accumulated equal amount of metal from food and soil, and simultaneous assimilation via both routes appeared to be additive (Vijver et al. 2006). Similarly, accumulation rate of Cd in shrimps *Palaemon varians* was more than additive when exposed simultaneously via water and food as compared to individual uptake route (Pavlaki et al. 2018). In Japanese medaka *Oryzias latipes*, accumulation of Cr was higher during dissolved Cr (VI) exposure as compared to dietary exposure (Chen et al. 2016). Thus, the finding of present and previous studies shows that bioavailability and accumulation of metals in different species is influenced by exposure routes. Therefore, the relative importance of different routes should be considered to obtain complete data on metal's contamination and their effects on the tested organisms.

Comparison between Species

In present study, metal's body burden of *L. terrestris* was relatively higher during soil exposure. On the other hand, both species accumulated approximately comparable quantity of metals from contaminated food. Previous investigations have shown that difference in accumulation of metals in spiders is determined by combined effect of various factors (i.e. exposure route, feeding activity and hunting strategy) that fluctuates between and among species (Wilczek et al. 2008; Yang et al. 2016). For example, Wilczek et al. (2008) showed that wolf spiders *Xerolycosa nemoralis* collected from metal polluted areas accumulated high concentration of metals as compared to web building spiders *Linyphia triangularis* and *Agelena labyrinthica* from same site probably due to the difference in their hunting strategy, diet composition and habitat type. Similarly, Simon (2016) reported that *P. oblongopunctatus* (generalist predator) accumulated a higher amount of metals than the specialized predator *C. violaceus* collected from same metal's polluted areas. Collembolan species *F. candida* accumulated more Cu than *F. fimetaria* from contaminated food as compared to soil, possibly due to difference in their growth rate and foraging strategy (Pedersen et al. 2000). In the present study, both species are generalist predator and accumulated significant amount of metals via both uptake routes. However, the difference in metal's uptake rate during soil exposure may be related to body size, behavior or activity of spider species.

Mortality and Body mass Change

In the present study, mortality rate in control group spiderlings that were kept in artificial laboratory conditions (artificial food and soil) was varied between 0-2.6 % for *L. terrestris* and 0-6.8 % for *P. birmanica*. In a reference study less than 10% mortality was observed after 4 weeks in *Pardosa pseudoannulata* spiderlings fed with uncontaminated Drosophila melanogaster and Tendipes sp (Li et al. 2016). Similarly, Wang et al. (2019) recorded less than 10% mortality in 4th instar spidering of *Pardosa pseudoannulata* which were fed with Drosophila melanogaster reared on corn meal without any metal solution. Comparative study of Sac spiders on natural and artificial diet by Amlin et al. (2001) revealed that survival rate of spiders was similar on either diet but higher percentage of spiders reached their adult stage which were reared on artificial diet as compared to natural diet (Drosophila melanogaster or citrus leafminer). So, Amlin et al. (2001) concludes that artificial diet was good enough to be used in mass rearing of spiders under laboratory conditions. The artificial soil used as a substrate in the present study greatly helped in maintaining the humidity requirement and hence survival of the studies species. Furthermore, in our study the body mass of spiders which were not exposed to metals was constantly increased with time. Based on these results we can conclude that the artificial food can be used in laboratory experiments as an alternative to natural diet to assess toxic effect of pollutant in these spiders.

Metal's burden up to a certain level can disrupt physiological and life history parameters in spider (Hendrickx et al. 2003; Eraly et al. 2011). In present study, positive correlation between mortality and internal metal concentration in spider's species was detected in all treatment conditions. Mortality rate was also comparable in both species. Highest mortality was found in metal's mixture treatments during food exposure irrespective of metal's concentration. The possible reason of this contrasting result could be that high concentration of metals in food can disrupt midgut gland structure, which effect the food digestion and absorption, causing starvation and ultimately increased mortality rate. Wilczek et al. (2014) described that starvation increases the toxicant effect and as a result enhance the apoptotic and necrotic degenerative changes in midgut gland cells of spider *Xerolycosa nemoralis* relative to toxicant alone. By contrast, Jung et al. (2005) and Babczynska et al. (2011b) found no mortality when exposed to nearly similar concentrations of Pb and Cu. Liu et al. (2013) and Li et al. (2016) illustrated that mortality in *Agelena labyrinthica* and *Pardosa pseudoannulata* was increased on exposure to increasing concentration of Hg and Cd, respectively in separate experiments. These finding suggest that the high mortality rate in present study may be related to the high accumulation of metals with time.

Body mass of both spider species decreased in correlation with the increase in metal concentration. The lowest weight was observed in mixture treatments during food exposure in both species. This suggest that at high metal concentrations, body mass may possibly be reduced because of increased detoxification effort or reduction in food consumption. Ramirez et al. (2011) revealed that body mass of *Argiope trifasciata* was negatively correlated with internal metal concentration and presumed that this might be due to energy consumption for metal resistance rather than on foraging and growth. Similarly, Chen et al. (2011) showed that after exposure to sublethal concentration of Pb and Zn, growth was significantly reduced as a function of metal resistance. Additionally, Jung et al. (2005) reported significant reduction in growth in *Pardosa astrigera* spiderlings when exposed to Pb *via* food. Our study revealed that mortality rate and BMC are consistent with internal metals' concentration in spiders species and therefore can potentially be used as markers of metal contamination ecotoxicological studies studies.

Conclusion

The Present study demonstrated that *L. terrestris* and *Pardosa birmanica* has the ability to withstand high concentrations of metals (10mM Cu, 10mM Pb) and also have the capacity to accumulate significant amount of both metals into their bodies. During simultaneous exposure, Cu and Pb exhibited antagonistic effect on the uptake rate of one another depending on the exposure route. Therefore, knowledge about metal's interaction and their uptake routes should be taken into account during risk assessment studies in order to avoid overestimation of the level of metals contamination. Based on bioaccumulation rate, *L. terrestris* accumulated high concentration of metals from soil. Our results also demonstrated that the accumulation of metals significantly affected the survival and body mass of spiders, which were correlated with duration of exposure and level of metal contamination. Taken together, these findings present the both spider species as good bioindicator for Cu and Pb contamination and possibly for other metals.

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Table. 1 Actual Copper (Cu) and Lead (Pb) concentrations in artificial soil and food.

Treatments	Artificial Soil		Artificial Food	
	Cu mg/Kg DM	Pb mg/kg DM	Cu mg/kg DM	Pb mg/Kg DM
Control	1.12 ± 0.002	0.353 ± 0.005	1.41 ± 0.009	4.230 ± 0.542
Cu	629.85 ± 3.23	-	628.01 ± 5.44	-
Pb	-	1889.9 ± 4.84	-	1935.3 ± 3.21
Cu + Pb	628.33 ± 3.18	1882.5 ± 6.15	624.34 ± 3.36	1954.0 ± 4.73

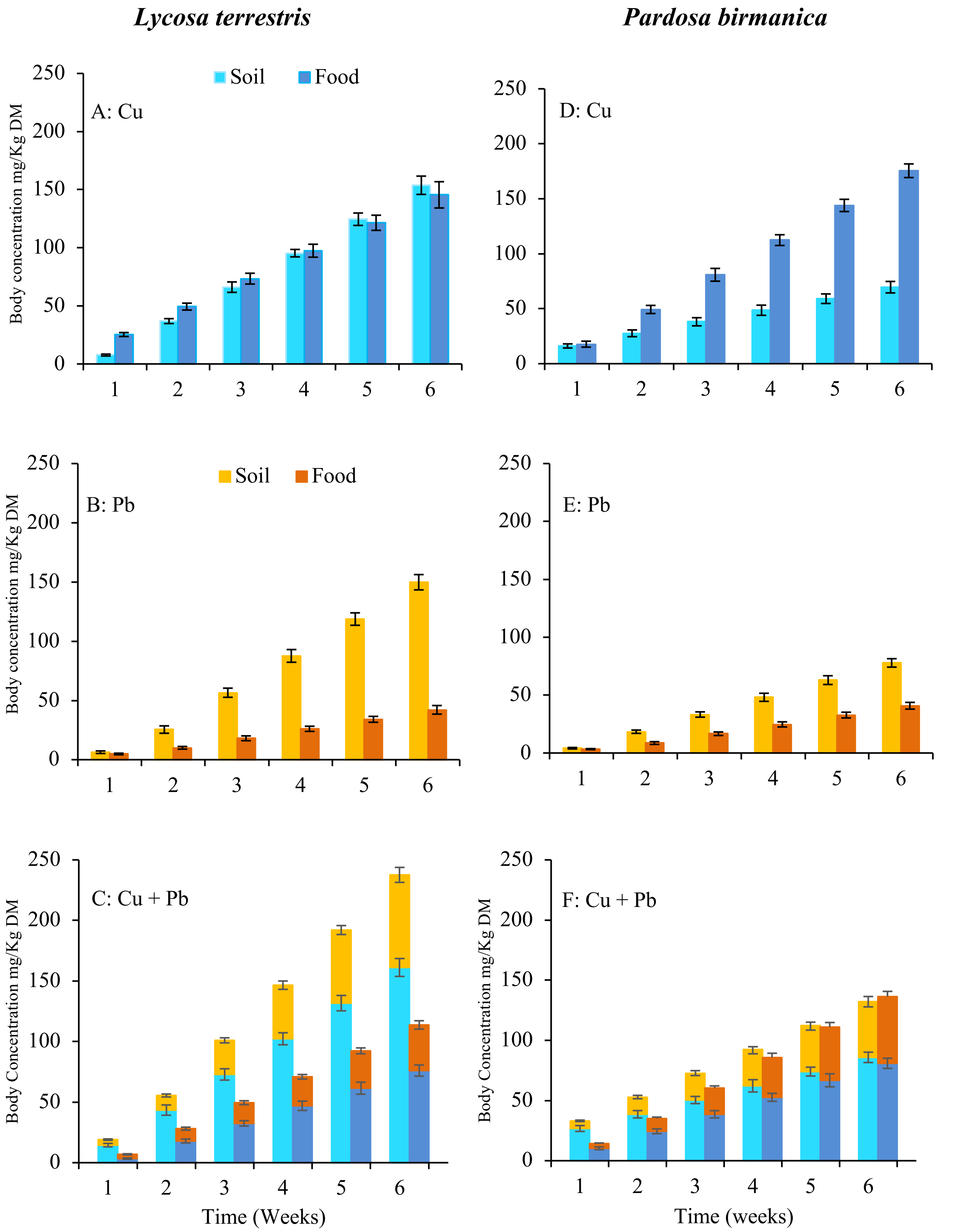

Fig.1 Total body concentrations of metals in *Lycosa terrestris* (A-C) and *Pardosa birmanica* (D-F) expressed as mg/Kg DM (Dry matter) upto 6 weeks treated with copper (Cu), lead (Pb) and their mixture (Cu + Pb) via different exposure routes (Soil, Food).

Lycosa terrestris ***Pardosa birmanica***

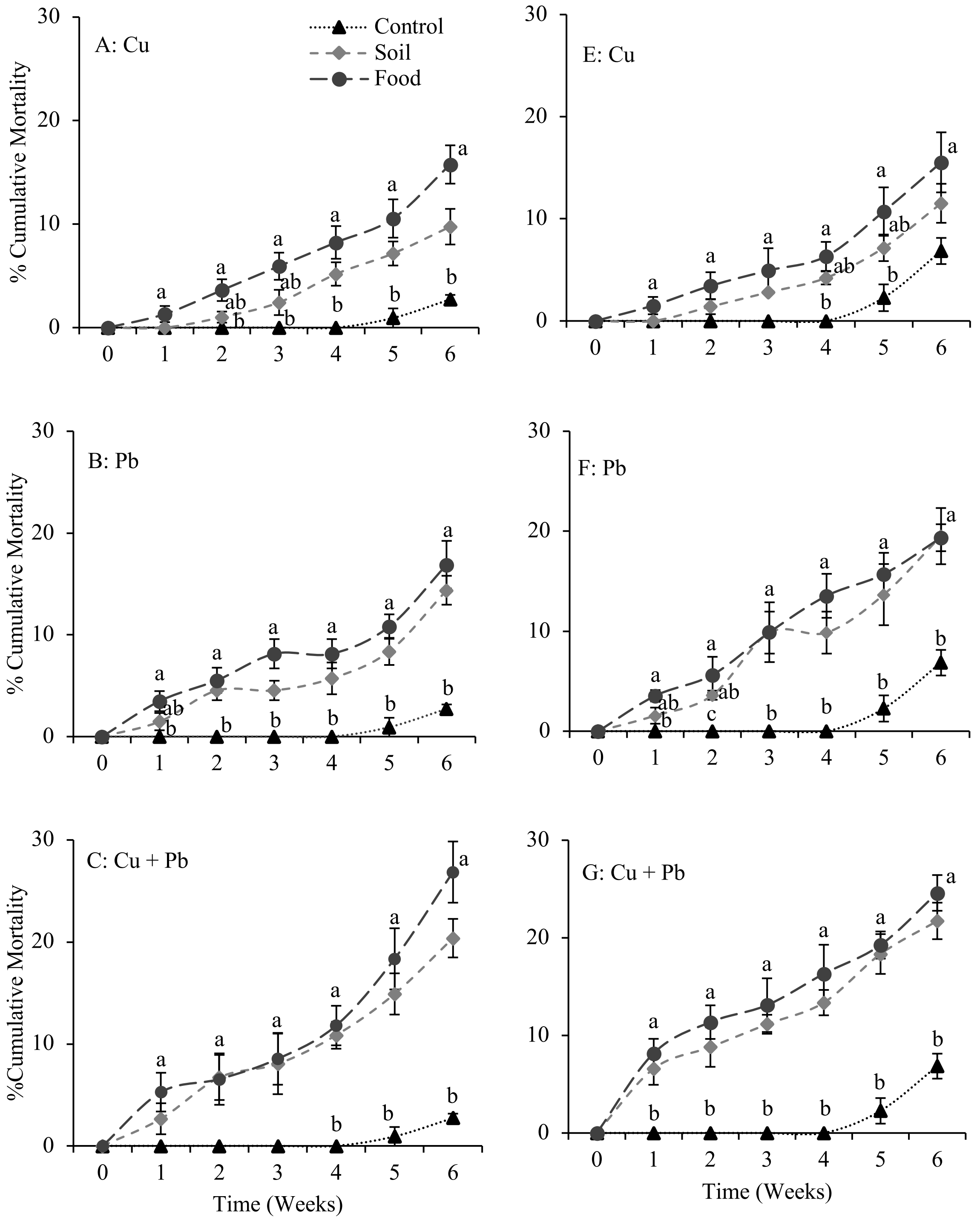

Fig. 2 Mortality within 6 weeks (0-6) in *Lycosa terrestris* (A-C) and *Pardosa birmanica* (D-F) exposed to Copper (Cu), Lead (Pb) and their combined mixture (Cu + Pb) via different routes of exposure. Different letters(a-c) indicate statistically significant difference between treatments ($P < 0.05$).

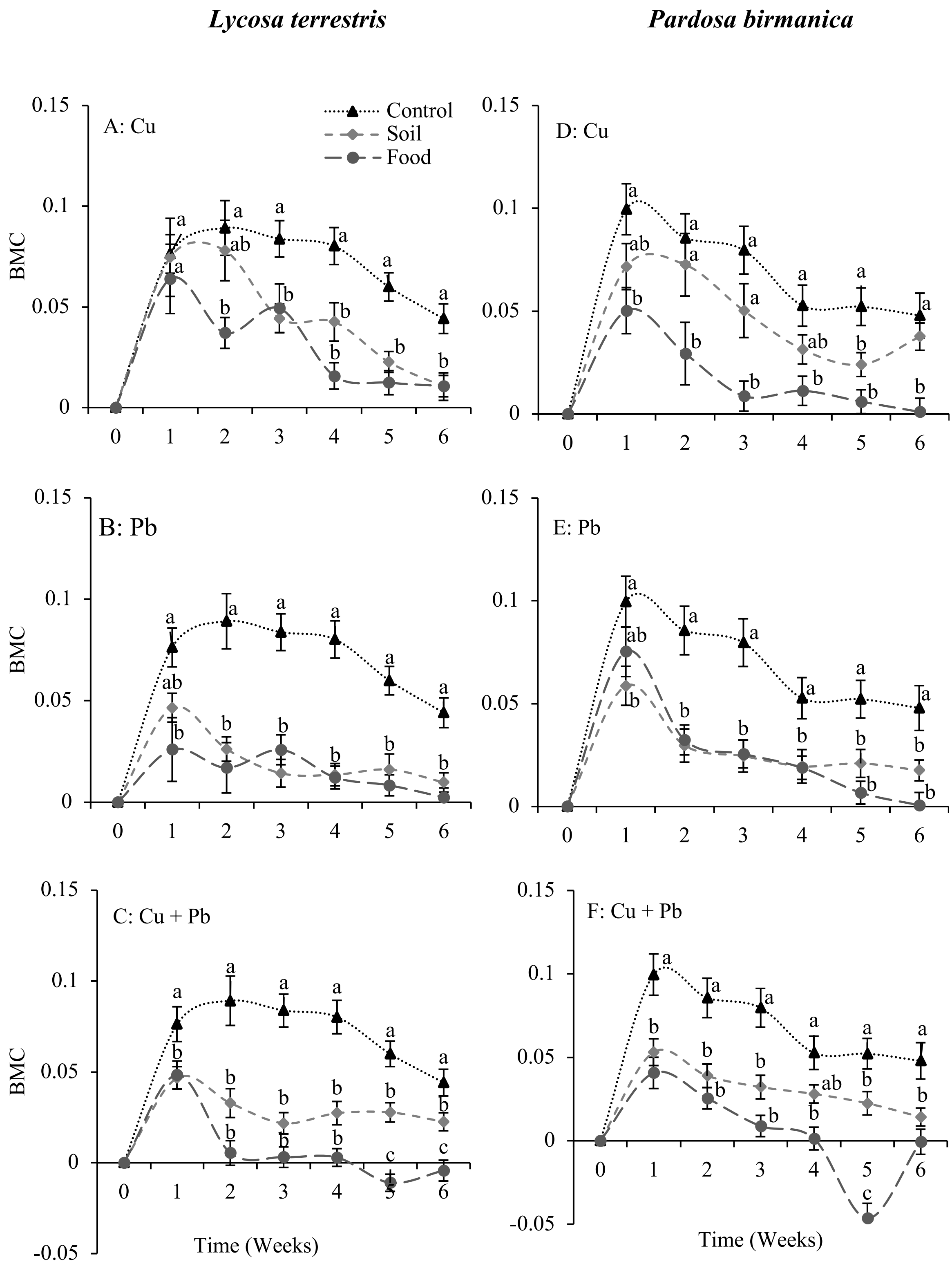

Fig. 4 Body mass change (BMC) in *Lycosa terrestris* (A-C) and *Pardosa birmanica* (D-F) exposed to Copper (Cu), lead (Pb) and their mixture (Cu + Pb) via different routes of exposure.