Applied Soil Ecology 209 (2025) 106038

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Effects of bituminous coal dust exposure on reproduction of *Sinella curviseta* (Collembola) and *Eisenia fetida* (Clitellata)

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ARTICLE INFO

Keywords:
Soil macroinvertebrates
Reproduction
Ecotoxicology
Environmental pollution

ABSTRACT

Although coal extraction is an important source of income for several countries, including Colombia, there are problems associated with mining activities, especially in open-pit coal mines. One of these is the deposition of air-borne dust in areas surrounding open pit mines, the accumulation of which can result in changes to the biogeochemistry of surrounding soils, as well as potential impacts on soil organisms, which have an important role in the maintenance of soil physicochemical properties, and other functional attributes of soils.

This study aimed to determine the ecotoxicological impact of sub-chronic exposure to bituminous coal dust (diameter up to 38 μm) in two soil invertebrate model organisms - an earthworm: *Eisenia fetida* (Savigny, 1826); and a collembolan: *Sinella curviseta* Brook 1882 - in soil with relatively low concentrations of coal dust. A total of four concentrations (1 to 4 % w/w) and one negative control were tested in artificial soil. Organisms were assessed after 28 and 60 days. Results from *E. fetida* showed that despite the low mortality of adult earthworms, a significant reduction in the number of cocoons and juveniles was observed in treatments ≥2 %, after 28 days and 60 days. In addition, the average number of cocoons observed was significantly reduced even at the lowest concentration of coal dust (1 %) relative to controls.

Similarly, a substantial reduction in the number of *S. curviseta* adults was observed in all concentrations after 28 and 60 days, respectively. Our results suggest that both organisms are highly sensitive bioindicators of soil disturbance. These findings further suggest that soil organisms and foodwebs in natural substrates in the vicinity of open pit coal mines may be at risk of impacts due to exposure to coal dust with potential consequences for soil system function.

1. Introduction

Coal dust is a by-product of coal mining activity, and is produced in large quantities by crushing, pulverizing, handling, and transporting raw coal, and is particularly problematic as an environmental hazard in open pit mining (Tirado-Ballestas et al., 2020; De Souza et al., 2021). Coal dust has the same physicochemical features as the material extracted for energy production, but due to its smaller particle size, it has potential to be more dangerous to flora and fauna in the environment (Zazouli et al., 2021; Kamanzi et al., 2024). This is because it has a greater ability to permeate into smaller spaces, including into tissue and at cellular levels. The particle causes inflammation and blockage and contains traces of heavy metals and metalloids such as mercury, lead, arsenic, and cadmium, as well as other pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Tirado-Ballestas et al., 2020; Mondal and Singh, 2021). Particulate matter (PM) smaller than 40 μm is known for its strong tendency to become entrained in the atmosphere when disturbed (e.g., as "fugitive" or escaped dust from mechanical processes), as well as the ability to get inside deep tissues and internal organs in flora and fauna. The management of coal dust is considered a primary concern because its emissions are poorly controlled in mining areas. Air quality is affected, and the mobility of these particles allows them to travel long distances on air currents (Tirado-Ballestas et al., 2020).

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https://doi.org/10.1016/j.apsoil.2025.106038
Received 26 August 2024; Received in revised form 13 March 2025; Accepted 14 March 2025
Available online 21 March 2025
0929-1393/Published by Elsevier B.V.

Soil invertebrates have been widely used as bioindicators of soil pollution, and investigating the response of different organisms is a key step to evaluate risk to the community structure, and by extension overall soil function (Robidoux et al., 2000; Nahmani and Rossi, 2003; Xu et al., 2009; Schlich et al., 2013; Liu et al., 2018). Earthworms and springtails are soil organisms used for detection of environmental issues that affect soil, and both have been well developed as ecotoxicological models with standard toxicity tests for both survival (acute toxicity) and reproduction (chronic toxicity) (Liu et al., 2018; Wurst et al., 2018). Earthworms are ubiquitous organisms that are important to soil processes and impact soil texture, humidity, pH, bulk density, microorganisms, and the availability of nutrients, (Sanchez-Hernandez, 2006; Tirado-Ballestas et al., 2020). Their utility as model organisms in the toxicology context is enhanced by several factors including the fact that they have intimate contact with the environmental medium (both through ingestion and dermal exposure), their biochemistry and behavior have been well studied, and their anatomy and reproductive cycle are well characterized (Tirado-Ballestas et al., 2020; Ezeokoli et al., 2021; Shi et al., 2021).

Similarly, the use of Collembola as indicators of soil pollution is well known and widely accepted (e.g., Xu et al., 2009). These primitive hexapods are also ubiquitous, occurring on all continents, in various habitats. Collembola have high population density and are understood to be indirectly involved in organic matter decomposition, mainly through their feeding on fungi (Eaton et al., 2004; Gao et al., 2017; Coleman et al., 2018). Collembola differ from earthworms in the ecotoxicology context in that they do not directly ingest soil particles, and so their responses to toxicants in soil may be mediated through different modes of exposure like dermal contact, respiratory contact, or via trophic transfer (Van Gestel, 2012).

In one example of how earthworm reproduction has been used successfully as an endpoint for a toxicity assay, Schlich et al. (2013) demonstrated that silver particulates in soil reduced reproduction rates by 46.4–75.2 %, whereas assays of toxicity in terms of survival or biomass loss in earthworms exposed to these particulates were not conclusive. Other authors have also confirmed that earthworm reproduction is a valid endpoint for the determination of soil ecotoxicology, suggesting a strong correlation between cocoon production and the number of juveniles in microcosms contaminated with explosives residue (Robidoux et al., 2000). Although not as well studied as earthworm ecotoxicology, there have been numerous studies showing that reproduction in Collembola serves as a highly sensitive endpoint which is more efficient and less complex than measures of growth response. In addition, the use of reproduction and mortality tests has led researchers to infer possible damage to invertebrate populations in soils impacted by air pollution compounds, and to recommend further studies evaluating more pollutants (Nakamura et al., 1992; Xu et al., 2009; Liu et al., 2018; De Lima e Silva et al., 2021; Zhang et al., 2021).

To investigate the environmental impact of bituminous coal dust from the El Cerrejón open pit mine in Colombia, we used standardized ecotoxicological tests in soil, using *Eisenia fetida* (Savigny, 1826) and *Sinella curviseta* Brook 1882. Our previous work in this area has shown that high concentrations of coal dust, in acute toxicity tests, can have significant impacts on earthworms in terms of survival and on several morphological and physiological measures including weight loss, increased mucus production, peeling of the epidermis, etc. (Tirado-Ballestas et al., 2020). The results from these acute tests motivated the present study to examine effects of chronic coal dust exposure in reproduction tests, expanding on the previous work with earthworms, and adding a second organism (Collembola) allowing an examination of alternate exposure pathways. The test organisms were selected due to their routine use in ecotoxicology tests, which is related to the ease of maintaining cultures in laboratory conditions, their high reproduction rates, short life spans, and high sensitivity to pollutant exposure and physicochemical changes in their habitats (Waldorf, 1971; Xu et al., 2009). We hypothesized that chronic exposure to bituminous coal dust, characterized by trace elements including heavy metals and polycyclic aromatic hydrocarbons, would induce concentration- and time-dependent alterations in the reproduction of soil invertebrates. Specifically, we expected to see reductions in reproductive output in terms of the number of cocoons and juvenile earthworms in experimental units with higher concentrations of coal dust. Likewise, we expected lower reproductive output from adult collembolans exposed to higher concentrations of coal dust. The study aims to elucidate the ecotoxicological impact of these trace elements on earthworm and springtail reproduction, providing a foundation for risk assessment and the formulation of guidelines for permissible coal dust concentrations in the environment (Smit and Van Gestel, 1996).

2. Materials and methods

2.1. Coal dust

The coal sample was obtained from an open-pit coal mine, called El Cerrejón near La Guajira, Colombia. Particulate matter was prepared according to Caballero-Gallardo and Olivero-Verbel (2016). The bulk mineral was crushed using a Planetary BallMill PS + E, ND 04 L. A Standard Sieve No. 200, NTC 32 ASTM 11–87, 00029″ was used to obtain dust with a diameter not >38 μm. Samples were stored in sterile polypropylene bags at 20 °C until the material was used in the tests. A characterization of heavy metal concentrations and hydrocarbon composition was performed. Those were reported elsewhere (Tirado-Ballestas et al., 2020, 2022).

2.2. Experimental design

2.2.1. Substrate

Artificial soil consisting of 70 % industrial sand, 20 % kaolin and 10 % peat, sieved at 2 mm, was prepared for the bioassays. The substrate was washed and dried at 105 °C/12 h (OECD, 1984; OECD, 2016a, 2016b). The coal dust was mixed with the substrate in concentrations of 0, 1, 2, 3 and 4 % w/w. These concentrations were chosen because they match with concentrations previously examined in other work for different animal groups and/or different end point response variables. Further justification for use of these concentrations is that they match those observable in soils collected from arable fields in the vicinity of the open pit mine under study (Caballero-Gallardo and Olivero-Verbel, 2016; Tirado-Ballestas et al., 2020). Substrate with no coal dust added was used as a negative control.

2.2.2. Earthworm (Eisenia fetida) tests

Earthworms used in the tests were obtained from Lombriabonos, a certified hatchery in Sucre (Colombia). Laboratory cultures were kept under stable temperature, moisture and light conditions (21 ± 2 °C, 70–75 % water content, 12:12 photoperiod) in plastic containers with artificial substrate (20 % kaolin, 10 % peat, and 70 % industrial sand, with pH adjusted to ~7.0 using calcium carbonate), and earthworms were fed on organic wastes free of pesticides and antibiotics.

The earthworms used in the experiment were adults with a developed clitellum and a weight range of 0.28–0.29 g, under laboratory constant conditions (21 ± 2°C, ≥70 % water content, 12:12 photoperiod). Calcium carbonate was used to maintain a pH range of 7.0 ± 0.5, according to Tirado-Ballestas et al. (2020).

For each test container (hereafter, experimental unit), 10 pre-weighed individuals were added to sterile glass flasks (10 × 12 cm) covered with a plastic mesh, containing 300 g of the substrate. Although the OECD standard for earthworm reproduction tests recommends 500 g of substrate, we elected to use 300 g substrate in our tests, primarily due to the size of test vessels that were available to us. We conducted pilot studies to ensure that these conditions would be suitable for conducting the trials, with acceptable results. The experimental trial consisted of five replicates per concentration, including the negative control (for a total of 25 experimental units). Because we wanted to ensure that the results were accurate and that the experiment was replicable, we conducted three separate trials of the experiment described above, using new earthworms, new substrate, and new test containers for each trial.

After 28 days, adult earthworms were removed from each container, weighed, and submitted to a gentle mechanical stimulation of the anterior region to confirm survival. Each week, the experimental units were maintained by providing water (to keep soil moisture content at 70 %) and food (18 g pre-dried of pesticide free horse manure) for the earthworms. After the removal of adult earthworms (at 28 days), juvenile earthworms and viable cocoons were counted and returned to the containers and further incubated until they reached 60 days (Helling et al., 2000). Fig. 1 provides a description and identification of cocoons. The identification of unhatched cocoons is shown in Supplemental Video 1. It is straightforward to confirm that there are embryos inside the cocoon. The cocoon's coloration is a sign of embryo maturation and the start of hatching. The release of young earthworms from a cocoon is depicted in Supplemental Video 2.

After 60 days, all cocoons and earthworms were removed from the experimental units and earthworms and viable cocoons were counted (Fig. S1). This process involved emptying the contents of each container, and manually picking through all the contents, breaking up aggregates and spreading substrate into a thin layer, allowing removal of individual earthworms and cocoons.

2.2.3. Collembola (*Sinella curviseta*) reproduction tests

Cultured organisms were obtained from the laboratory of the USDA-Forest Service, Center for Forest Disturbance Science, in Athens, Georgia, USA. This culture originated from specimens collected in Lawrence, Kansas, USA, and had been continuously maintained since 1951, by D.A. Crossley, University of Georgia, Department of Entomology, Athens, Georgia, USA (same continuous culture as that described in Gist et al., 1974). Although there are no specific OECD guidelines for *S. curviseta*, the species is readily culturable, and possesses many of the same attributes as those species used in OECD tests, and has been used by other researchers in ecotoxicological testing (Xu et al., 2009). We adopted these guidelines for conducting our Collembola tests, with an evaluation of collembolan reproduction at 28 days (per OECD standards) but we additionally extended the Collembola test for a further 4 weeks beyond the OECD test, in order to make this more comparable to the 60 day test for earthworm reproduction.

Cultures were maintained under laboratory conditions according to OECD (2016b) guidelines, including a constant temperature of 20 °C; in constant darkness. Feeding was done every 2 weeks using Whatman No. 1 filter papers as a base for the food (yeast) (OECD, 2016b; Gao et al., 2017), and water (distilled and deionized = DDI). Plastic vessels (15 cm tall, 300 mL volume) were used as containers for the cultures, with about 150 g plaster of Paris and activated charcoal (5:1 w:w) mixed in DDI water. The mix was poured into the vessels to a thickness of 1–2 cm and allowed to set.

Because Collembola of similar ages were required for the test, we introduced 180 adults, evenly distributed, into 7 newly constructed culture containers. After 48 h, adult Collembola were retrieved from the containers. Eggs hatched after ten days of incubation, and this age-synchronized generation was used for the experiment (Fig. S2).

For the reproduction test, 20 age-synchronized individuals (10–12 days old) were deposited into glass containers with 30 g of the artificial soil substrate. Five concentrations (0 [negative control], 1, 2, 3, and 4 % coal dust) and a positive control (boric acid, 100 ppm), were used for the *S. curviseta* bioassay, according to OECD (2016b). The positive control was included to ensure that collembolan reproduction would be affected by a known toxicant (i.e., an expected response). These positive controls are critical in experiments where the test organism does not actually ingest the substrate, but rather is exposed to the toxicant via other pathways. Test substrates had pH of 7 ± 0.5 and were maintained at 70 % soil water content, 20 ± 1 °C, and tests were conducted with a 12:12 h L:D photoperiod for four weeks. The experimental trial consisted of five replicates of each treatment resulting in 30 experimental units for each incubation period (28 or 60 days) for a grand total of 60 experimental units per trial. The separate sets of experimental units for each incubation period were necessary because the enumeration of individuals at the end of an incubation period required destructive sampling and extraction of collembolans into ethanol. As with the earthworm trials, the experimental trial was repeated three times, with new animals, new substrate, and new containers for each trial. Experimental units were provided with food and moisture every 14 days until

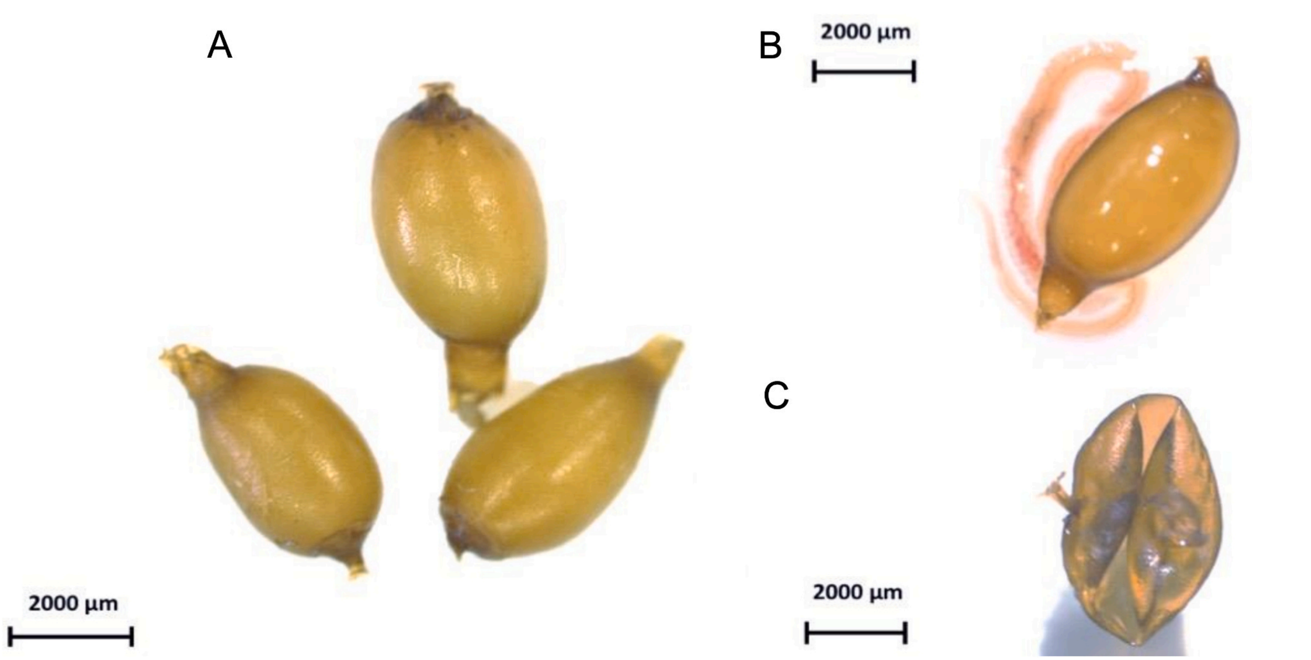

Fig. 1. Cocoons of *Eisenia fetida*. A. Unhatched. Each cocoon could contain several juveniles. B. Hatching after 20–30 days of incubation. C. Hatched or empty cocoons. No juveniles are inside, either due to hatching or lack of an embryo.

the end of the assay (Xu et al., 2009). The soil from each experimental unit was transferred into Tullgren funnels at the end of each time period for each trial (28 days or 60 days) until all the Collembola had been extracted by using an electric incandescent bulb suspended above the tray (light and heat extraction) forcing the animals downwards into the funnel into vials containing 70 % ethanol. Extractions continued for 5 days. Following extraction, a dissecting microscope was used to count adult and young springtails, according to Xu et al. (2009) (Fig. S2).

2.3. Statistical analysis

To analyze changes in earthworm weight throughout the incubation, we constructed a linear mixed effect model (all analyses conducted using R version 4.2.2; R Core Team, 2022), where the concentration of coal dust was a fixed effect and the experiment trial was a random intercept (lmer function in lme4 package; Bates et al., 2015). To analyze abundance changes for springtails, earthworm cocoons, and earthworm juveniles, respectively, we constructed generalized linear mixed effect models where coal dust concentration and incubation time (either 28 or 60 days) were treated as interacting fixed effects, and the experimental trial was a random intercept (glmer function in lme4 package; Bates et al., 2015). We added random intercept of observation numbers to these models to account for overdispersion as needed (Harrison, 2014). For each model, significance was assessed using the Wald chi-square test (car package; Fox and Weisberg, 2019). We performed posthoc pairwise comparisons of group means using the estimated marginal means with the Tukey adjustment for multiple comparisons (emmeans package; Lenth, 2022).

3. Results

3.1. *Eisenia fetida*

Results showed that there were significant impacts of coal dust concentration on *E. fetida* reproduction, with decreases observed in the number of cocoons produced (Fig. 2), as well as a decrease in the number of juvenile earthworms recovered from the experimental units (Fig. 3) after 28 and 60 days of exposure. Cumulative cocoon production showed a negative trend, being statistically different from the controls ($p < 0.001$). The number of cocoons ranged from 20 to 25 after 28 days and 15–20 after 60 days in the controls. Cocoon production decreased significantly by concentration, with cocoon numbers ranging between 0 and 5 at the 3 and 4 % concentrations.

Adult *E. fetida* used in the reproduction test lost weight in all four experimental coal dust concentrations, with 1 and 2 % coal dust-exposed individuals losing an average of 0.02 g per individual, and 3 and 4 % coal dust-exposed individuals losing >0.025 g per individual (Fig. 4).

3.2. *Sinella curviseta*

We found that after semi-chronic exposure to 1–4 % w/w bituminous coal dust, all treatments exhibited reproduction, with exposed populations having depressed reproduction rates, but there were slight differences between observations made after 28 days, and those made after 60 days of incubation (Fig. 5). After 28 days of incubation, all 4 concentrations of coal dust, and the boric acid control showed significantly less reproduction than the 0 % coal dust controls, with the 4 % and boric acid control treatments having similarly low reproduction relative to the other coal dust concentrations. However, after 60 days of

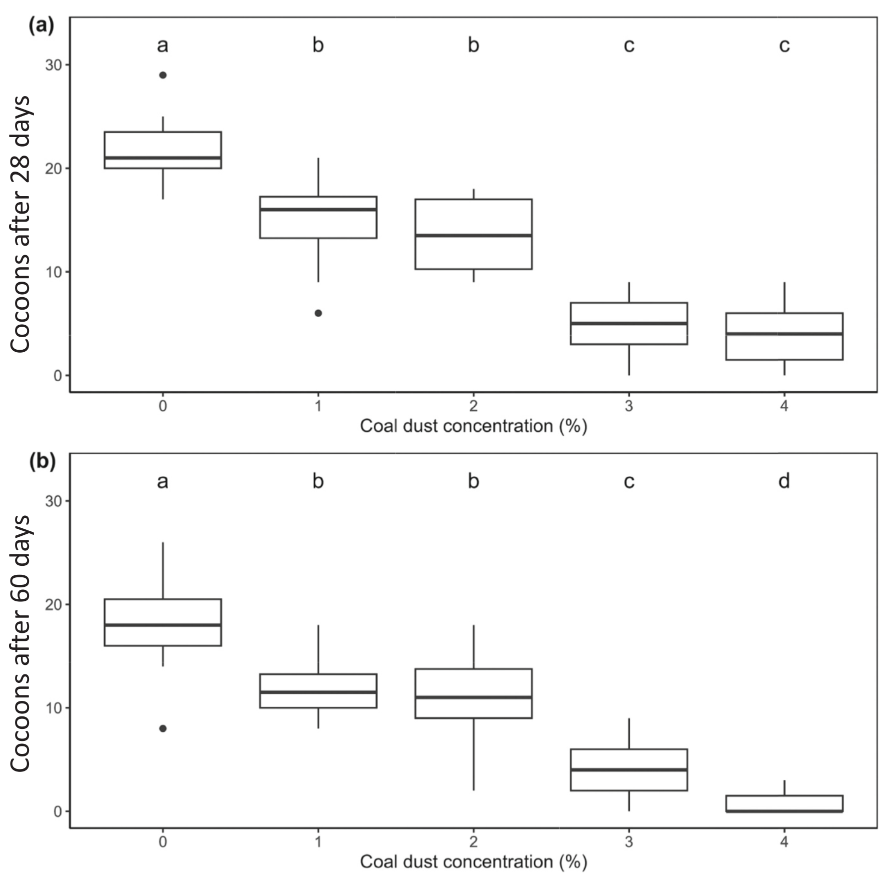

Fig. 2. Number of cocoons produced by *Eisenia fetida* after 28 (a) and 60 (b) days of exposure to 0, 1, 2, 3, and 4 % coal dust in artificial soil. Boxes represent the interquartile range (IQR), horizontal lines within boxes are median values, and whiskers represent points that fall within 1.5 * IQR. Points outside of that range are plotted individually. Letters above boxes indicate statistically significant differences (p </= 0.05).

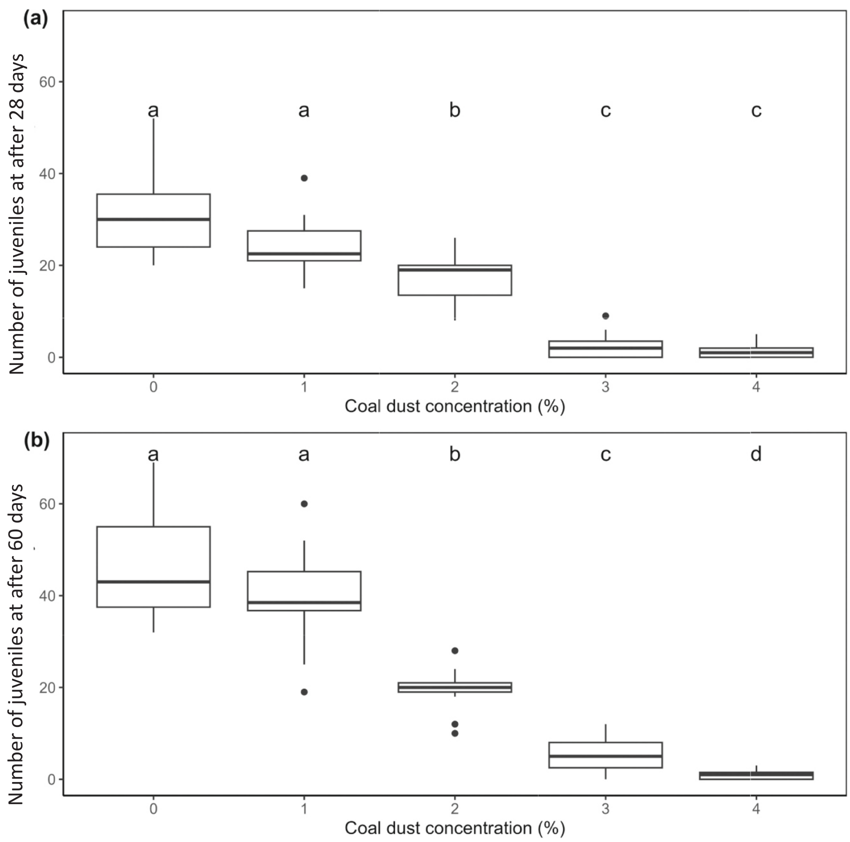

Fig. 3. Boxplot of number of juvenile *Eisenia fetida* after exposure to 0, 1, 2, 3, and 4 % coal dust in artificial soil (w/w) after 28 (a) and 60 (b) days. Boxes represent the interquartile range (IQR), horizontal lines within boxes are median values, and whiskers represent points that fall within 1.5 * IQR. Points outside of that range are plotted individually. Letters above boxes indicate statistically significant differences (p </= 0.05).

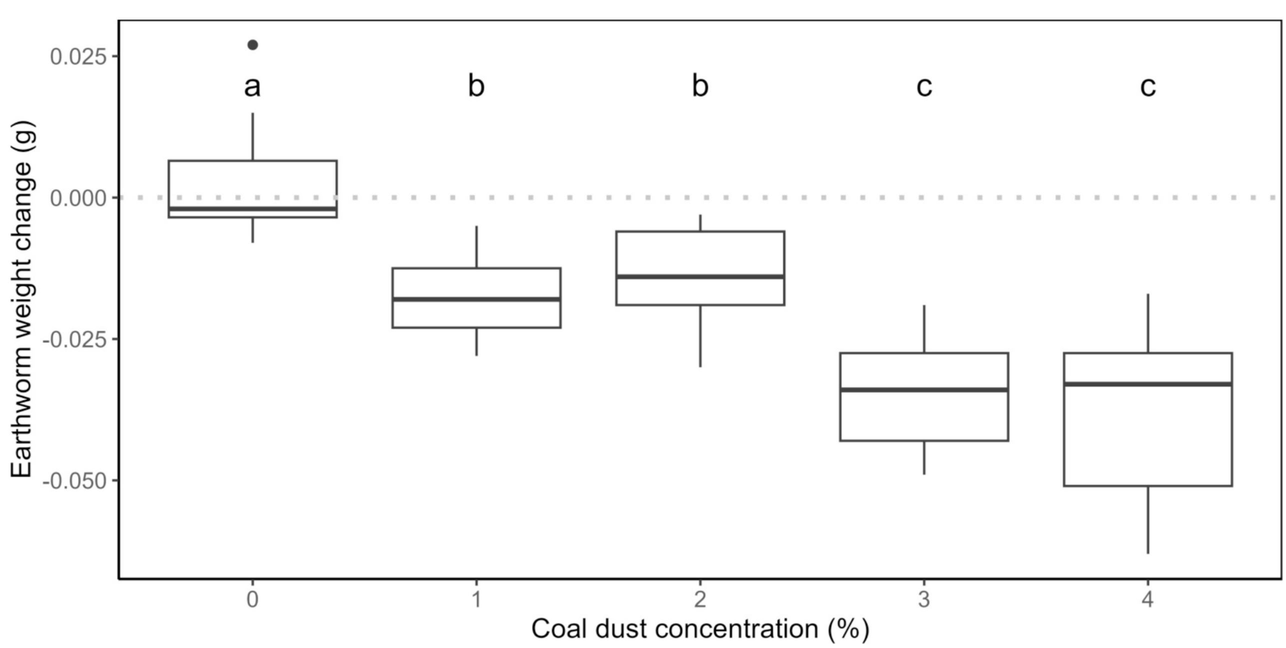

Fig. 4. Weight changes of the live adult earthworms after 28 days of exposure to 1, 2, 3 and 4 % coal dust. Boxes represent the interquartile range (IQR), horizontal lines within boxes are median values, and whiskers represent points that fall within 1.5 * IQR. Points outside of that range are plotted individually. Letters above boxes indicate statistically significant differences (p </= 0.05).

incubation, collembolan reproduction in the 1 % coal dust treatments was not different from the 0 % coal dust control. The 4 % coal dust and the boric acid controls were again similar and had significantly lower reproduction than the other treatments, and 2 and 3 % treatments had intermediate levels of reproduction relative to the positive and negative controls.

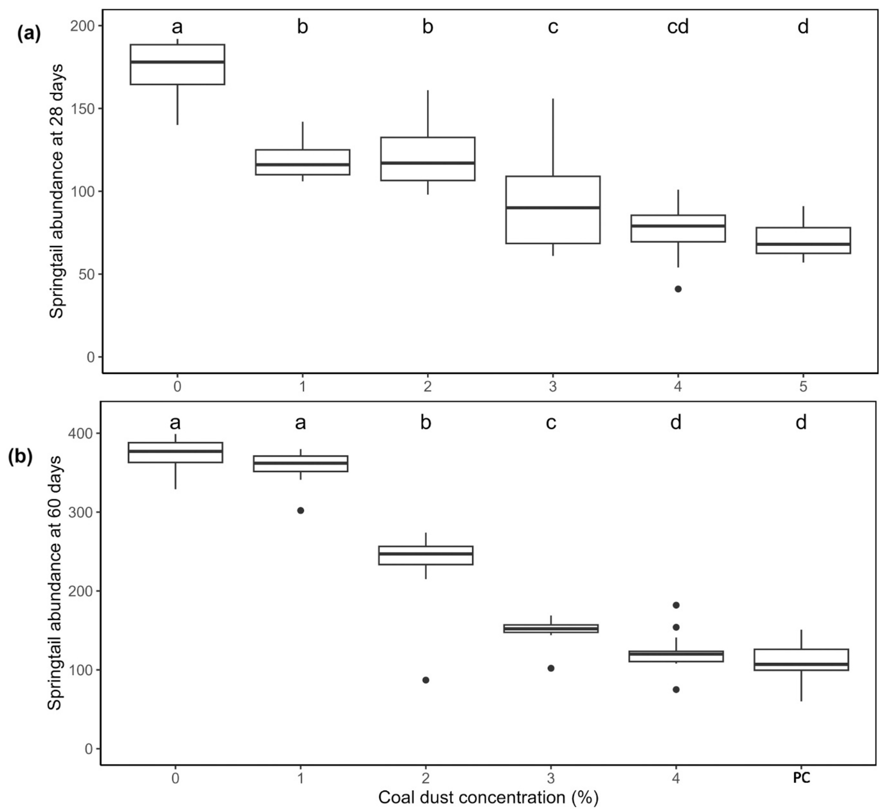

Fig. 5. Boxplot of the number of springtails per experimental unit of springtails exposed to 0, 1, 2, 3, and 4 % coal dust in artificial soil (w/w) after 28 (a) and 60 (b) days. PC: Positive Control. Boxes represent the interquartile range (IQR), horizontal lines within boxes are median values, and whiskers represent points that fall within 1.5 * IQR. Points outside of that range are plotted individually. Letters above boxes indicate statistically significant differences (p </= 0.05). Note differences in the y-axis scales between (a) and (b).

4. Discussion

4.1. Eisenia fetida mortality

Tirado-Ballestas et al. (2020), have previously reported mortality rates for earthworms exposed to coal dust, and the mortality data collected from the present study (data not shown) confirm that *E. fetida* have the capacity to survive sub chronic exposure to bituminous coal dust. However, in the previous (and current) studies, we observed that *E. fetida* did exhibit some mortality at the highest coal dust concentrations tested (3 and 4 %), and that these were statistically significant differences compared to the controls (4–6 % mortality at 3 % coal dust, and 10–12 % mortality at 4 % coal dust, after 28 days exposure). The findings of low mortality of earthworms (i.e., no acute response to exposure), were indeed partly the motivation to conduct a test specifically examining reproduction, as this would provide a complement of ecotoxicological tests needed to determine the net effect of coal dust contamination on soil animals.

The number of juvenile earthworms was also impacted by the concentration of coal dust, with a marked decrease in abundance with increasing concentrations of coal dust, which is consistent with the results related to the presence of cocoons. The number of juvenile earthworms was not statistically different from controls in the 1 % coal dust treatments (after 28 or 60 days of incubation), but this population consistently decreased with increasing concentrations of coal dust, with a statistically significant decrease at 2 % coal dust, and the lowest numbers observed in the 3 and 4 % concentrations. Recruitment of juveniles (i.e., survival of new hatchlings) after 60 days of incubation was observed in 1 % coal dust treatments, but this was not observed in the higher coal dust concentrations after 60 days, suggesting that cocoons either did not hatch, or hatched juveniles did not survive well in these treatments.

In systems impacted by coal dust, the majority of diseases and pathologies in human populations manifest only after long-term exposure (i.e., there are few acute health impacts) (So et al., 2023; Din et al., 2024). Thus, the relatively low mortality rates we observed for earthworms in this study are perhaps not surprising, given that they are known to have adaptations that allow them to resist environmental stressors, and that they can survive long-term exposure to contaminants. This observation has been made many times, and for many different classes of pollutants (e.g., Daam et al., 2011; Shoults-Wilson et al., 2011; Yates et al., 2023;). The epidermis, cuticle and the presence of glandular cells that produce mucous and serous secretions are likely the most important among the earthworm's defense mechanisms (Roberts and Dorough, 1984; Tirado-Ballestas et al., 2020). In fact, earthworms used in toxicity tests are often robust in acute testing, and this observation led to alternatives like avoidance or reproduction tests being used for certain contaminants (e.g., Hund-Rinke and Wiechering, 2001; Van Gestel, 2012; Tirado-Ballestas et al., 2020). Notably, we did observe some mortality of earthworms under the two highest concentrations we tested, but there were always some adult earthworms that survived in each experimental unit. The presence of trace elements in the coal dust material used in our study, including metals Cu, Pb, As, Ba, and Sr, as well as hydrocarbons such as benzo(*a*)anthracene (Tirado-Ballestas et al., 2020, 2022), may be responsible for the observed mortality.

4.2. Eisenia fetida growth

Earthworm growth rate (or failure to grow, or individual mass loss) is another sensitive endpoint for evaluating the toxicity of different materials. In toxicity testing, this normally manifests as mass loss, which can come about from behavioral changes in earthworm activity or through biochemical stressors at the cellular level. For example, the metabolic costs of avoidance behavior (energy expenditure on increased movement or increased mucous production due to skin irritation) could result in mass loss. Likewise, disruption at the cellular level of the lysosomal membranes within coelomocytes has been demonstrated for certain toxicants (Tirado-Ballestas et al., 2020; Shi et al., 2021), causing imbalances in homeostasis, and possibly causing significant decrease in earthworm mass. We did observe significant mass loss for each of the coal dust concentrations tested in this study, relative to the no dose controls, and the 3 and 4 % coal dust treatments resulted in significantly more mass loss than the lower tested concentrations.

Individual mass is a key factor for *E. fetida* reproduction (Helling et al., 2000; Xu et al., 2009), with more massive individuals producing more offspring. This reproductive success of larger adult individuals is because cocoon production requires resources from progenitor like proteins, such as albumin (Siddique et al., 2005). If adult worms are induced to lose weight via either of the mechanisms mentioned above, it necessarily decreases the number of resources available for cocoon production, leading to decrease not only in the number of cocoons but the number of juveniles from the hatched cocoons.

4.3. *Eisenia fetida reproduction*

To date, we are unaware of any studies examining the potential effects of coal dust on the reproduction of earthworms in formal toxicological tests. However, several studies have demonstrated the environmental risks of coal dust and documented the impacts of this material on the concentrations of heavy metals in soils surrounding mining operations (e.g., Masto et al., 2017; Raj et al., 2019). Whereas, some studies have focused on human health risks (Mondal and Singh, 2021; Zazouli et al., 2021), there may also be environmental risks to other organisms responsible for ecosystem services (e.g. soil function, plant productivity, etc.), and this possibility was another motivation for our current study. We found that *E. fetida* produced significantly fewer cocoons in all the tested concentrations of coal dust relative to the control units, and these reductions were more pronounced when exposure extended from 28 to 60 days. These findings confirm that the reproduction test is sensitive and is useful for understanding sub-lethal effects of environmental contamination.

Although there have been few (if any) direct ecotoxicological tests of coal dust on earthworm reproduction, we can make suppositions about what the source of the negative effects of coal dust may have been on the earthworms in our study. Specifically, Zhang et al. (2021) and Zhang et al. (2022) reported an inhibition of normal development, as evidenced by the absence of reproduction, in earthworms exposed to nanoparticulate matter, iron, and hydrocarbons. Furthermore, Shi et al. (2021) suggested that the trace elements contained in biochar altered the physiology of the earthworms, including reproduction, even at low concentrations of the biochar material. These results are consistent with our findings, as the coal dust material used in our study is known to contain PAHs, heavy metals, and metalloids (Achten and Hofmann, 2009; Caballero-Gallardo and Olivero-Verbel, 2016; Tirado-Ballestas et al., 2020).

The number of juveniles produced and their survival are generally correlated with the number of cocoons produced. This was also observed in the present *E. fetida* reproduction test, with one interesting exception. We found, that in spite of the significantly lower number of cocoons produced in the 1 % coal dust treatment, the number of juveniles from this treatment was not different from the number recovered from the no-dose control treatment. Further, the number of juveniles in the 1 % coal dust treatment increased from 28 to 60 days, indicating that the lower level of contamination may be below the threshold for substantial negative effects on earthworm populations. It could be that 1 % is sparse enough that the worms can effectively avoid contact with the contaminants (i.e., a behavioral solution to contamination). Concentrations of dust higher than 1 % may make it impossible for the worms to avoid ingestion or contact (Tirado-Ballestas et al., 2020).

For all other concentrations, the number of juveniles was significantly lower when compared to the control, and their numbers decreased with time. In fact, the number of juveniles in the 4 % coal dust treatment nearly reached zero after 60 days, suggesting that concentrations of coal dust at or above this level might result in elimination of earthworms altogether. These findings support Reinecke and Venter (1987), who discuss in detail the changes in the growth and quantity of juveniles exposed from the cocoon stage to compost with unfavorable properties. Again, this finding is related to the number of cocoons produced (which was dramatically reduced at the high concentrations), but it is notable that not only were adults impacted at the higher concentrations, but juvenile survival was also affected at these concentrations.

4.4. *Sinella curviseta reproduction*

Similar to earthworm toxicity tests, there have been few, if any, tests examining the responses of springtails to coal dust exposure. Our study perhaps represents the first time such tests have been conducted. We found that *S. curviseta* populations were significantly negatively impacted by all tested concentrations, and this suggests that coal dust exposure, even to organisms that likely do not directly ingest the particulate material can be affected. These findings suggest that contaminants in the coal dust either decrease the production of the preferred food of the collembola (yeasts and fungal hyphae), or that the fungi are mobilizing the contaminants in coal dust and making them bioavailable to the collembolans. It is known that heavy metal-containing particulate matter can have negative effects on collembolan reproduction and populations, as demonstrated by Xu et al. (2009), although the concentrations used were much higher than those applied in the form of coal dust in our study.

It is also notable that the outcome of toxicity tests using collembolans can be dependent upon the environmental conditions under which the tests were conducted. For example, a study conducted by Bandow et al. (2014) demonstrated that the collembolans, *S. curviseta* and *F. candida*, were sensitive indicators when exposed to pesticides, but the relative sensitivity of the organisms depended on soil moisture and temperature conditions. Although outside the objective of our present study, which was to establish baseline reproductive toxicity information for coal dust contamination of soil, testing this material for reproductive effects at different moisture/temperature conditions might translate into useful information for real world conditions, or information that could be predictive under various climate change scenarios.

In any case, our study demonstrates that the collembolan model organism, *S. curviseta*, under standard conditions proved to be sensitive to coal dust contamination at all tested concentrations (Fig. S3). This result suggests that coal dust contamination of natural soils in the vicinity of open-pit coal mines (specifically in Colombia, but likely elsewhere) may be impaired with respect to soil fauna composition, along with associated soil functions.

5. Conclusions

This study at the nexus of environmental ecology and ecotoxicology has significantly advanced our understanding of the consequences associated with chronic exposure to bituminous coal dust in soil ecosystems. The systematic investigation into the impact of coal dust, a by-product of mining activities, on key soil-dwelling organisms such as earthworms and Collembola (in this case, the ecotoxicological model organisms *E. fetida* and *S. curviseta*) has unveiled complex dynamics that extend beyond surface-level assessments. The integration of standardized ecotoxicological tests and a meticulous experimental design offers insights into the time- and concentration-dependent effects of coal dust exposure on critical ecological endpoints. Notably, the observed alterations in reproduction, mortality, and weight changes in these sentinel organisms underscore the nuanced ecological ramifications of coal dust pollution.

The findings of this study hold particular significance in elucidating the broader environmental risks associated with coal mining activities. The presence of heavy metals, polycyclic aromatic hydrocarbons, and other trace elements within coal dust has been revealed as a key contributor to the observed ecological disturbances. Furthermore, the concentration-dependent impacts on both earthworms and springtails underscore the sensitivity of these organisms to varying levels of coal dust exposure. Such insights not only contribute to the scientific discourse on soil pollution but also have far-reaching implications for shaping effective environmental management strategies and policies, especially in regions characterized by extensive coal extraction. This research paves the way for continued investigations and the development of targeted mitigation measures to alleviate the ecological burden imposed by coal mining activities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106038.

CRediT authorship contribution statement

I.P. Tirado-Ballestas: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M.K. Taylor:** Writing – review & editing, Visualization, Formal analysis, Data curation, Conceptualization. **K. Caballero-Gallardo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **J. Olivero-Verbel:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **M.A. Callaham Jr.:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Conceptualization.

Funding sources

This work was supported by the National Program for Doctoral Formation (MINCIENCIAS) grants 567-2012 and 647-2014, Vice-Presidency for Research of the University of Cartagena grant N119/2018, and the Fulbright Colombia Visitant Researcher grant 2018.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank the National Program for Doctoral Formation (MINCIENCIAS) grants 567-2012 and 647-2014, the Vice-Presidency for Research of the University of Cartagena grant N119/2018, the Fulbright Colombia Visiting Researcher grant 2018, and Roberto Carrera-Martínez for supporting the data analysis. This work was made possible, in part, by the USDA Forest Service's International Programs Office, and Southern Research Station, Center for Forest Disturbance Science.

Data availability

Data will be made available on request.

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