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Heavy metal biomagnification and genotoxic damage in two trophic levels exposed to mine tailings: a network theory approach

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Abstract

Background: The analysis of the negative effects of environmental metal pollution is complex and difficult to assess, because the great number of variables and levels of biological organization involved. Therefore, an integral interpretation of the structure of ecological interactions from the multifactorial toxicological vision can be achieved by the use of new analysis tools, such as the complex network theory analysis (CNT).

Results: Our results demonstrated that the trophic level has an effect on metal enrichment, being the detritivores who presented the highest bioaccumulation levels in comparison to plants, as well as higher biomagnification levels in the soil-plant-detritivores relationship. Also, *Vachellia farnesiana* displayed greater sensitivity to genotoxic damage than *Eisenia fetida*. Finally, the analysis of complex networks showed that detritivores are the key link in this dynamics, on which the interactions between heavy metals, plant and detritivores depend.

Conclusions: This study shows that there is an effect of the study site on heavy metal bioaccumulation and DNA damage induction, and that these responses are particular to each species and to each bioaccumulated metal, which in turn reveals specific sensitivity for each trophic level. Moreover, the application of CNT methodology allowed us to clarify in this particular system, the interaction types and the principal components of the trophic structure.

Keywords: Bioaccumulation, Detritivorous, Genotoxic damage, Graph, Primary producer

Background

Recently, ecosystem studies have focused on elucidating the relationship that exists between their biotic and abiotic elements, beyond an isolated interpretation of the population dynamics of the different species that compose them [1]. A new frontier in the trophic interaction studies is the analysis of complex ecological networks, which combine qualitative and quantitative characters, in order to identify, from a holistic approach, those ecological properties that are not evident through direct observation or not even through the sum of its parts [1, 2].

Complex Network Theory (CNT) refers to a set of interactions that has one or all of the properties of: (a) self-organization, (b) self-similarity, (c) attractor, (d) small world and (e) free scale, present in a group of variables directly and indirectly related [3]. The Complex Theory refers to systems composed by many parts, each having its own internal structure, and these in turn are in charge of carrying out a specific function that influences the structure of the interactions at different network levels [4]. Therefore, what modifies a part of the network can affect, in a highly non-linear way, the whole system [5].

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Application of the CNT has been increasing since the twentieth century, with extensive studies in social sciences [6], computer science [7], transport [8], and biological sciences [9]. In particular, the CNT has been increasingly accepted in ecological studies over the last 17 years, and its development is fast and consistent [10], being a robust tool for ecosystem stability analysis, with possible applications in biodiversity and conservation [11], and as an indicator of the ecosystem health [1]. This high diversity of applications shows its universality and makes possible the comparison between structural differences between networks, even between different knowledge areas and different variables that because of their different origin, they cannot be analyzed by conventional methods, a fact that is well recognized as one of the advantages of this methodology [5].

In environments impacted by mining activity, the analysis of the interactions between the exposed organisms and the ecosystem health, has become an issue of international relevance [12]. In particular, metals derived from mine wastes, which are called tailings, can be accumulated in living organisms, either by consumption, inhalation, or cutaneous absorption, which induces their transfer along the trophic chain involved, endangering the dynamics of the ecosystem, including humans [13, 14].

To understand the ecological impact caused by heavy metal exposure, we should analyze not only the metal mixture involved and their concentrations in the abiotic and biotic components, but also their relative mobility and their bioaccumulation potential within and among different trophic levels [15–17].

In ecotoxicological studies, the risk analysis from a multispecies approach is essential. The difference between the sensitivity of populations exposed to the same pollutant is a tool that allows the application of strategies for environmental management [18]. Research on heavy metal transfer through the food chain, as well as its genotoxic effects at these levels, is important and urgently required. However, because of the complex interactions within metal mixtures, the great number of exposed species and the biological responses, studies to date haven't been performed from an integrative approach [19]. Within terrestrial ecosystems, primary producers and detritivores are key links of food webs, for being in the base of the chain, and for its role as decomposers of organic matter, respectively [20]. In particular, *Vachelia farnesiana* and *Eisenia fetida* are good study system because: (a) they are in close contact with the pollutants, (b) they have the ability to bioacumulate heavy metals, (c) they play a key role in the ecosystem in function of their biomass and density, (d) *V. farnesiana* is considered as a heavy metal hiperaccumulator species, (e) *E. faetida* is considered as a bioindicator species. Hence, the aims of the present study are A) to analyze heavy metal transfer along the trophic chain [soil, primary producers (*V. farnesiana* (L.) Wight & Arn 1834), and detritivores (*E. fetida* Savigny 1826)] in Santa Rosa, Taxco de Alarcón, Guerrero State, Mexico, B) to evaluate in both species the influence of the heavy metal bioaccumulation on genetic integrity, and C) to characterize from the CNT perspective, the structure of the relationship between heavy metal bioaccumulation, genetic damage and the analyzed species (primary producers and detritivores) in Santa Rosa mine tailings.

Methods

Study area

The mine tailing "El Fraile" is located at 12 km southwest of Taxco city, Guerrero State, Mexico, between Santa Rosa and El Fraile towns, where the mining activity was suspended in 1970 [21]. This mine was considered as the most productive for the Taxco mining district, particularly for the exploitation of the mineral like galena, sphalerite and pyrite [21]. As a consequence of mining activities, two tailings were deposited in Santa Rosa, which were selected for this study, located at 18° 30′00" N and 99°40′00" W (Fig. 1). Tailing 1 is 470 m long, 170 m wide and 60 m height [22]. The second one is 470 m long, 170 m wide, with an average thickness of 30 m [22], representing a total of 7,191,000 m^3 of mining waste. Bioavailability of heavy metals reported for both tailings show the following concentration pattern: Zn > Pb > Mn > V > Cu > As > Ni > Cd [23–25]. Both study sites have a pH between 2.44 and 5.28 in 79% of their structure, and a variable granulometry, equivalent to fine and thick sand and sludges [23, 24]. As control sites, two populations of *V. farnesiana* both located at 8 km from the exposed site were selected.

Animal and plant sampling

Lines of 100 m were traced in every study site (two lines in each exposed site and two lines in each control site). For plant sampling, the closest individual sampling technique was selected. An individual of *V. farnesiana* was selected every 10 m (between 2.5 and 3 m height) ($n = 80$). From each plant, two leaf samples were collected from leaves with no apparent damage. One of the samples was used for nuclei isolation, which were processed for the alkaline gel electrophoresis or comet assay. The other one was dried at room temperature, ground and stored in sterile plastic containers for heavy metal analysis by inductively coupled plasma mass spectrophotometry.

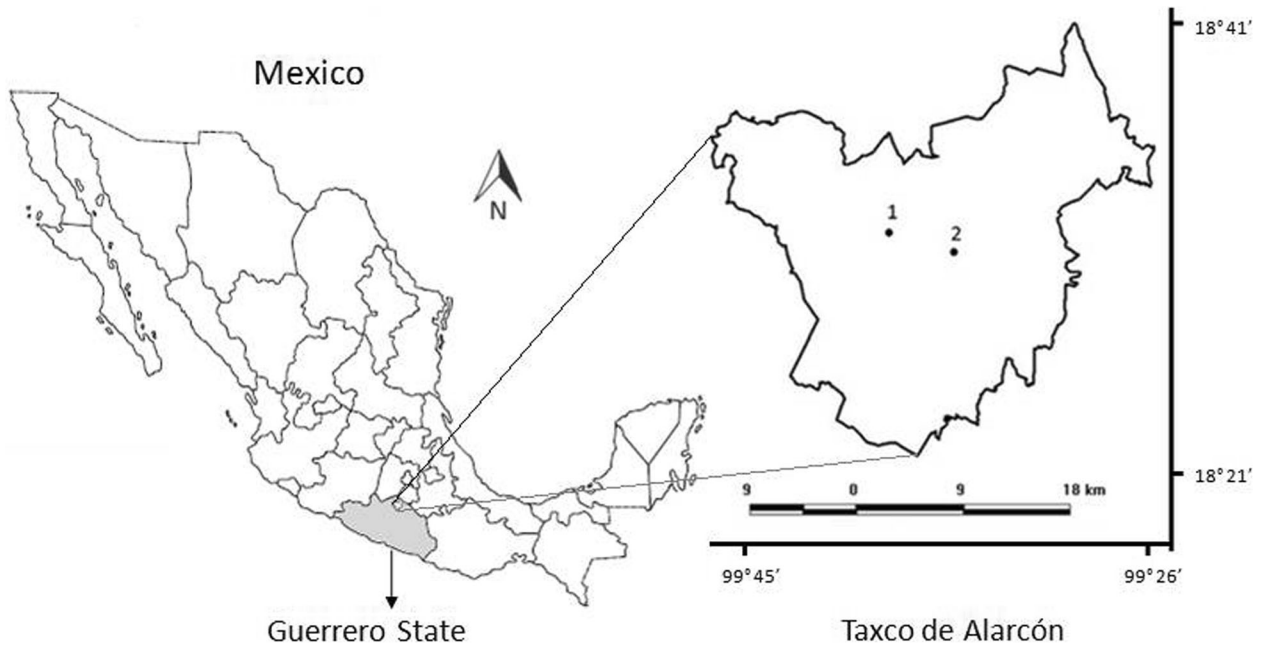

Fig. 1 Sampling localities for *Vachellia farnesiana* and and *Eisenia fetida* in Santa Rosa (1) and Taxco el Viejo (2), in the municipality of Taxco de Alarcón, Guerrero State, Mexico

For *E. fetida* sampling, lines of 100 m were traced as described before. Every 10 m, substrate was dug; and five to 10 worms with well-defined clitelum were captured. The sampled individuals were placed in dark containers (25 × 25 × 15 cm) with moistened paper towels, and covered with foil. The selected worms were purged for a period of 24 to 48 h in dark boxes with moistened paper towels, to eliminate both excess of mine tailing and soil from their digestive tract. Later, from each box, three individuals (30 per transect) were randomly selected. In general, these worms kept a reddish coloration, were reluctant to contact and showed an individual weight within the range of 300 to 600 mg. A total of 80 individuals (20 per line) were sampled for coelomocytes, for the alkaline comet assay analysis (pH 13). On the other hand, 20 individuals per site ($n = 80$) were dried at constant temperature (60 °C), thereafter, were ground and stored in sterile plastic containers for heavy metal analysis by inductively coupled plasma mass spectrophotometry.

Metal concentration analysis in *Vachellia farnesiana* and in *Eisenia fetida*

A total of 48 samples (24 for *V. farnesiana* and 24 for *E. fetida*) were evaluated for metal concentration analysis (As, Cd, Cu, Mn, Ni, Pb, V, and Zn). 250 mg of *V. farnesiana* leaf structure and 250 mg of *E. fetida* tissue were pulverized in previously washed HNO_3 containers. Samples were subjected to acid digestion using a Microwave Accelerated Reaction System (CEM® MARS-5) with a 4:1 mixture of HNO_3 65% and HCl 37% (JT Baker) in closed Teflon bombs. The samples were solubilized and dissolved in distilled water and filtered; this solution was diluted to a final volume of 50 mL until analysis. A sample without tissue was processed simultaneously which was used as a control. Thereafter, metals were analyzed by inductively coupled plasma mass spectrometry (MS Series ICP-MS Systems, Bruker, MA USA). The instrument was calibrated with standard solutions containing known concentrations of each element. Standard Reference Material of the National Institute of Technology and internal reference materials were used for precision, quality assurance and control for selected metal measurements.

Alkaline gel electrophoresis or comet assay

The plant samples were washed, gently dried and immersed in beakers with 20 mL of cold phosphate buffered saline (PBS 1X) for the nuclei isolation. The purged worms were placed in a glass petri dish with 3 mL of cold extrusion solution (1X PBS). Subsequently, 50 μL of the cell suspension was taken in both cases, and incorporated into an eppendorf tube with 50 μL of low melting point agarose (1% LMPA). After the suspension of plant or animal nuclei, 80 μL of the solution was taken

and placed on a slide with a preformed monolayer of normal fusion agarose (NMA 1.0% Gibco). Thereafter, covered with a coverslip and held on ice for 5 min. The coverslip was removed and a final layer of LMPA (0.5%) was placed at 4 °C for 5 min. From each sample two slides were prepared [26].

The gels were placed in a cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trisma-base pH 10) at 100 mL with 1% Triton-X and 10% Dimethyl Sulfoxide (DMSO) 10% in Kopling vessels at 4 °C for 1 h. Subsequently, the gels were placed in an electrophoresis chamber and covered with cold alkaline buffer [NaOH (300 mM) + 1 mM EDTA] at pH 13.0 for 20 min for the DNA unwinding process. The electrophoresis was performed at 300 mA and 25 V for 20 min for plant samples and 5 min for animal samples, under dark conditions.

Finally, the gels were washed three times with neutralizing buffer Tris (0.4 M pH = 7.5) for 5 min [26] and fixed with cold absolute ethanol for 10 min for later reading using Comet IV software integrated in the fluorescence microscope with excitation filters from 515 to 560 nm, and a 590 nm barrier filter.

Data analysis

In order to establish the bioaccumulation pattern of the trophic level mine tailings (soil) - plant and between the mine tailing - worm, as well as the biomagnification pattern in the trophic level plant-worm, the heavy metal enrichment values were determined. This was calculated as the quotient of each metal concentration detected at the trophic level evaluated (plant, earthworm), on the previous level (mine tailing, primary producer).

To analyze the effect of metal bioaccumulation on DNA damage in the exposed and control groups (tailing 1 vs. tailing 2 vs. control 1 vs. control 2) a two-way ANOVA analysis was performed [27] using the comet tail length value of 100 consecutive nuclei of each individual (50 nuclei per individual). Subsequently a Tukey post-hoc test of multiple comparisons was made to determine statistically significant differences between groups.

The specific sensitivity for each taxa, represented by an increase on genetic damage, was calculated, through the resulting quotient from the division of genotoxic damage (tail length) observed at the exposed sites, on the genotoxic damage values detected in control individuals. This was performed independently for each studied species [18]. Thereafter, the effect of the metal mixture on genetic damage levels (tail lenght) in *V. farnesiana* and *E. fetida* was estimated using a multiple regression approach. The software used for statistical analysis was STATISTICA 8.0.

Finally, to evaluate the relationship between distinct trophic levels, the CNT was applied [28]. A matrix interaction was constructed in order to identify the established links between: (a) the exposed sites (tailing 1 and 2), (b) the studied species (*V. farnesiana* and *E. fetida*), (c) the metal concentrations in tailings, (d) in plant and (e) animal tissues, as well as the (f) genotoxic damage in both species. The sites and the individuals of both species were considered as structural data sets in the network and as composition data the variables: metal concentration and genotoxic damage. Each variable in the network analysis was considered equal and a unimodal approach was established. To this end, and for computational efficiency, the scoring metrics used in this study [metal concentration (mg / kg) and genotoxicity (μm)] require that the data be discretized into the categories established as follows: Al, low [< 1000], medium [1000-5000] and high [> 5000]; As, low [< 50], medium [50-100] and high [> 100]; Ba, low [< 20], medium [20-30] and high [> 30]; Ca, low [< 1400], medium [1400-1500] and high [> 1500]; Cd, low [< 100], medium [100-200] and high [> 200]; Co, low [< 3], medium [3-9] and high [> 9]; Cu, low [< 10], medium [10-40] and high [> 40]; Fe, low [< 500], medium [500-5000] and high [> 5000]; Mg, low [< 1500], medium [1000-2000] and high [> 2000]; Mn, low [< 50], medium [50-100] and high [> 100]; Mo, low [< 1], medium [1-3] and high [> 3]; Ni, low [< 30], medium [30-60] and high [> 60]; Pb, low [< 100], medium [100-300 kg] and high [> 300]; V, low [< 5], medium [5-15] and high [> 15]; Zn, low [< 100], medium [100-500] and high [> 500]; and for DNA damage tail length [< 10], medium [10-40] and high [41-95].

Subsequently, the fitness subgrouping of nodes was established to two, three and four factions to determine the highest propensity to generate network links [29]. Thereafter, and in order to facilitate its visualization, the network was simplified according to the main connections established (48 nodes for graphic 1 and 52 nodes for graphic 2). Betweenness was calculated for the main nodes determination, acting as the bridge between other nodes along the network [30]. Closeness measure was used to determine the accessibility of a node to the rest of the network [31], and the eigenvector centrality value calculated for the identification of the node with greater influence [32]. The networks were performed using the NetDraw 2.084 program [33], under free scale model with infinite number of interactions for analysis, visualization, and contrast of the results [34]. Moreover, in order to validate the characteristics of the obtained networks we made a degree distribution graph for each one [5]. For detailed information about the CNT methodology, see Newman [5].

Results

Bioaccumulation and biomagnification of heavy metals

Seven heavy metals and a metalloid previously recorded in Taxco mine tailings were detected in tissue leaf samples of *V. farnesiana* and in the worm, *E. fetida*. In *V. farnesiana*, the accumulation pattern was: zinc (Zn) > vanadium (V) > copper (Cu) > nickel (Ni) > lead (Pb) > cadmium (Cd) > arsenic (As). In contrast, the worm bioaccumulation pattern was: Zn > Pb > Cd > Mn > As> Cu > Ni > V (Table 1).

No enrichment values higher than 1.0 were found in the trophic level mine tailing-plant, but for the mine tailing -worm relationship, we found enrichment values higher than 1.0. As (1.481 ± 1.28), Cd (11.489 ± 6.98) and Ni (1.285 ± 1.09), being Cd the element that showed highest bioaccumulation values in *E. fetida* (Table 1). Regarding the plant-worm trophic level, the biomagnification of six metallic elements was evidenced: As (803.712 ± 694.47), Cu (10.556 ± 3.17), Cd (1210.117 ± 735.31), Ni (8.975 ± 7.64), Pb (1276.846 ± 1167.91) and Zn: 20.045 ± 6.92) (Table 1).

Genotoxic damage

Results of DNA damage analysis of *V. farnesiana* foliar tissue and *E. fetida* celomocytes, showed a significant effect of the site (control 1, control 2, tailing 1 and tailing 2) on the induction of single strand breaks independently of the evaluated species ($F_{3,76} = 769.35$, $P < 0.001$ and $F_{3,76} = 762.74$, $P < 0.001$, respectively). The organisms established in mine tailings (tailing 1 and tailing 2) presented the highest values of genotoxic damage in relation to the organisms established in the control sites, regardless of the species (Fig. 2). On the other hand, it was observed that *V. farnesiana* populations established in the mine tailings presented 13.16 times more genotoxic damage than the control populations (14.28 with respect to tailing 1 and 12.04 with tailing 2). In contrast, exposed populations of *E. fetida* showed 5.03 times more damage than the control populations (5.47 for tailing 1 and 4.60 for tailing 2). In both cases, it was observed that the highest genotoxicity values occur in the individuals established in tailing 1 with respect to the control sites, regardless of the species evaluated. Finally, we observed that for *V. farnesiana* the genotoxicity levels were statistically and positively related with Pb concentrations ($F_{1,13} = 6.784$, $P < 0.05$), but not with *E. fetida* where a statistically and positive relationship was observed for Co ($F_{1,13} = 9.678$, $P < 0.01$), Ni ($F_{1,13} = 4.973$, $P < 0.05$) and Zn ($F_{1,13} = 24.774$, $P < 0.001$).

Network analysis

The relationship between heavy metal concentration, species type and study site showed that the nodes subgrouping with the greater fitness is given to two factions (394.000), in which the differentiation between subgroups is delimited by the species (*V. farnesiana* and *E. fetida*) (Fig. 3). Also, the highest betweenness and eigenvector centrality values were given by *E. fetida* from tailing 1 (479.000 and 0.444 respectively), followed by the same species in tailing 2 (433.000 and 0.421 respectively), which identifies *E. fetida* as the main link between the network nodes and the most influential node (Appendix 1).

Likewise, the lowest measures of closeness were presented in *E. fetida* (123.000 in individuals from tailing 1 and 125.000 in individuals from tailing 2) (Appendix 1).While it is true that a bidirectional relationship between the nodes of worms and the nodes of the study plant for each site is observed, the high values of centrality of detritivorous showed greater

Table 1 Average values (± SD) of soluble heavy metal concentrations detected in mine tailing, *Vachellia farnesiana* (foliar tissue), and *Eisenia fetida* (earthworm), and their enrichment values in the throphic levels (tailing–plant, tailing-earthworm, and plant-earthworm) in Santa Rosa, Guerrero State, Mexico

Metal	Concentration (mg / kg)			Enrichment		
	Tailing[a]	Plant	Earthworm	Tailing-Plant	Tailing-Earthworm	Plant-Earthworm
As	38.001 ± 16.486	0.072 ± 0.009	56.260 ± 22.213	0.002 ± 0.002	1.481 ± 0.732	803.713 ± 402.075
Cu	108.549 ± 32.156	2.856 ± 0.193	30.189 ± 10.334	0.027 ± 0.008	0.278 ± 0.130	10.556 ± 3.451
Cd	9.478 ± 2.021	0.087 ± 0.027	108.911 ± 63.127	0.008 ± 0.002	11.488 ± 3.902	1210.118 ± 523.656
Mn	409.005 ± 187.101	99.002 ± 25.001	59.070 ± 11.567	0.237 ± 0.076	0.144 ± 0.056	0.597 ± 0.173
Ni	16.201 ± 4.002	2.323 ± 1.081	20.821 ± 7.862	0.141 ± 0.006	1.285 ± 0.245	8.975 ± 2.645
Pb	1108.807 ± 341.011	0.144 ± 0.120	178.758 ± 29.971	$10^{-4} \pm 10^{-5}$	0.161 ± 0.026	1276.846 ± 736.820
V	157.786 ± 47.231	4.844 ± 2.070	2.824 ± 0.711	0.031 ± 0.005	0.018 ± 0.007	0.582 ± 0.286
Zn	1426.193 ± 112.244	20.133 ± 17.201	403.495 ± 98.567	0.009 ± 0.001	0.283 ± 0.178	20.044 ± 2.938

[a]Average obtained from Talavera et al. [25], Galarza [23], Ruiz & Armienta [24]

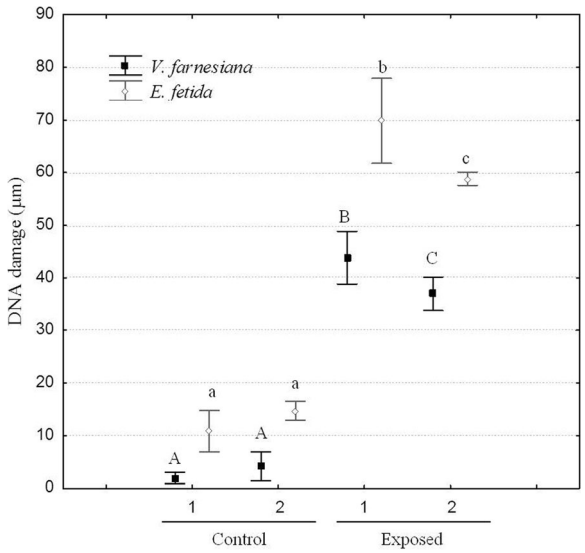

Fig. 2 Average value (± EE) of tail length (µm) of *Vachellia farnesiana* leaf cells, and *Eisenia fetida* celomocytes collected in the Santa Rosa mine tailing (exposed 1 and exposed 2), and control populations (control 1 and control 2). Capital and lowercase letters represent significant differences between sites for *Vachellia farnesiana* and *Eisenia fetida* respectively (Tukey test, $P < 0.05$)

influence on the established links in the network, including the primary producer (Fig. 3).

Additionally, in the degree distribution analysis for heavy metal bioaccumulation in *V. farnesiana* and in *E. fetida,* a structure like a free scale was detected, where the nodes (48) establish 166 interactions, being the vertex *"E. fetida* tailing 1" and "*E. fetida* tailing 2" which presented the highest degree of connectivity in the network (27 and 25 respectively) concentrating the 31.33% of the links which confirms its central role (Fig. 4).

The relationship between heavy metal concentration, DNA damage and species per site showed a similar pattern in the network (Fig. 3). In the nodes subgrouping, the analysis of two factions showed the highest fitness (450.000), establishing the differentiation of two groups, one for each species (*V. farnesiana* and *E. fetida*) (Fig. 5). The highest values of betweenness and eigenvector centrality were given by worms from mining tailing 1 (515.583 and 0.444 respectively), followed by the same species from the tailing 2 (483.083 and 0.413 respectively) (Appendix 2).

Finally, the lowest closeness measures were presented in *E. fetida* (132 individuals for tailing 1 and 133 in individuals from tailing 2) (Appendix 2).

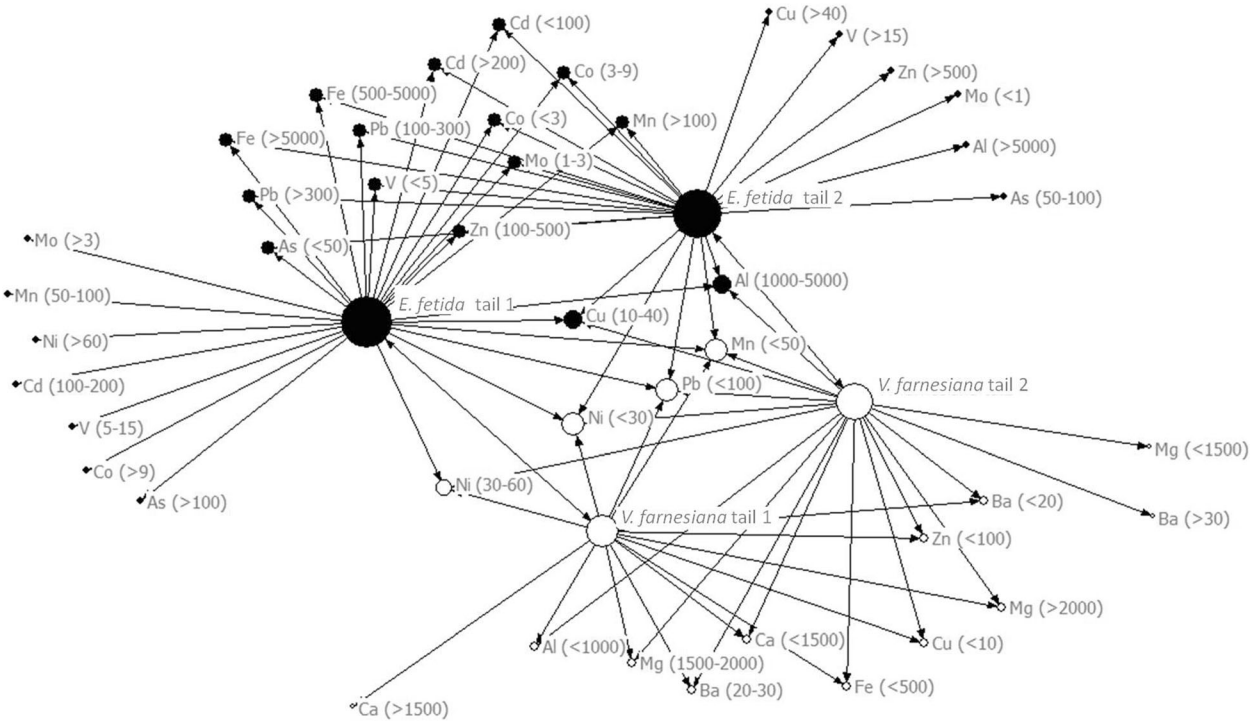

Fig. 3 Eigenvector centrality analysis in two factions of the network established by heavy metal concentrations (mg / kg) detected in *Vachellia farnesiana* and *Eisenia fetida* in Santa Rosa mine tailing 1 and 2. Color differences refer the factions analyzed (●: Faction 1, ○: Faction 2). Larger frame sizes indicate greater degree of influence of the node within the network

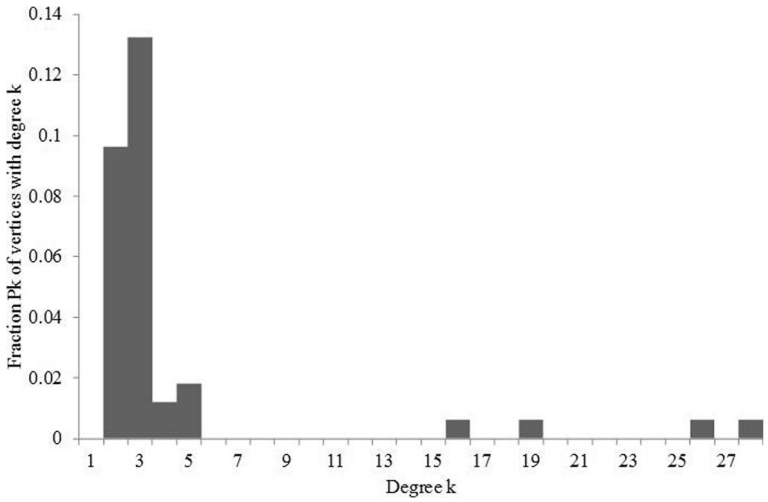

Fig. 4 Degree distribution graph of the network established by heavy metals concentrations (mg / kg) detected in *Vaquellia farnesiana* and *Eisenia fetida* in Santa Rosa mine tailings 1 and 2

Network analyses (Fig. 4) showed that both metal concentration and genotoxic damage are linked to the nodes of each species, being the detritivorous the most influenced by the major eigenvector centrality (Fig. 5).

In the degree distribution analysis for the relationship between heavy metal concentrations and genotoxic damage detected in *V. farnesiana* and in *E. fetida* a structure like a free scale was detected, where the nodes (52) established 178 interactions, being the vertex *"E. fetida* tailing 1" and "*E. fetida* tailing 2" which represent the highest degree of connectivity in the network (28 and 26 respectively), concentrating the 30.34% of the links which confirms again its central role (Fig. 6).

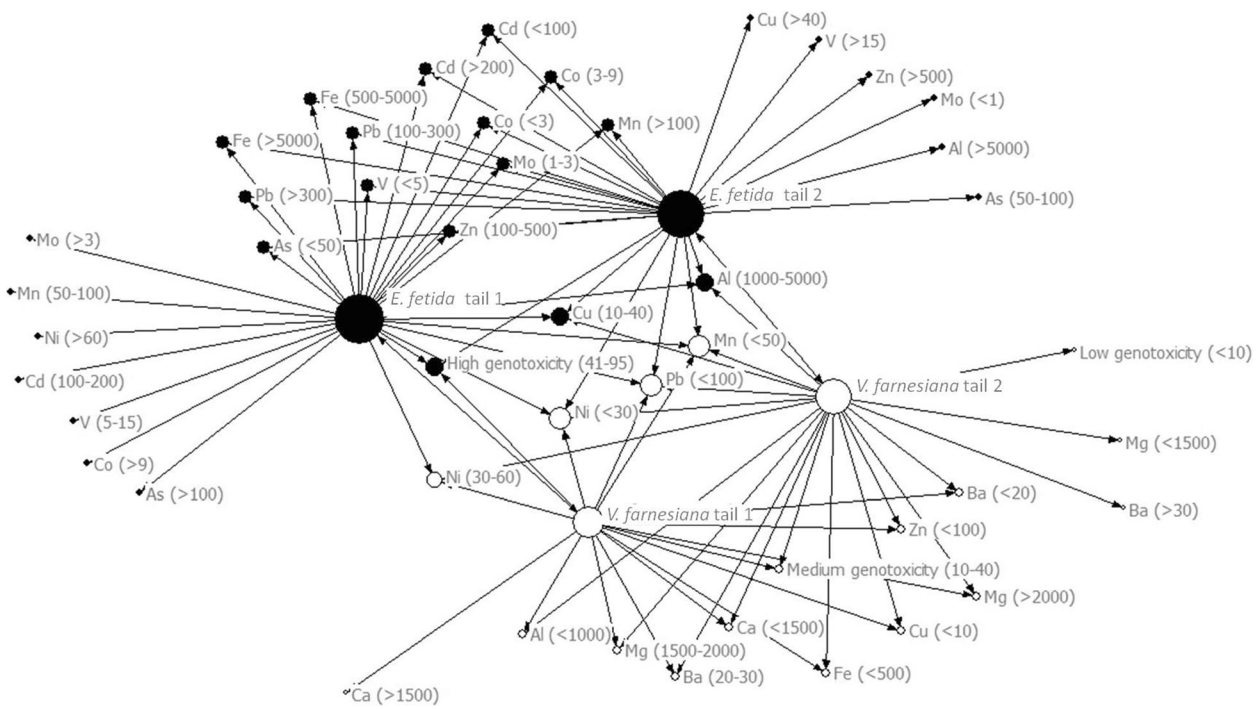

Fig. 5 Eigenvector centrality analysis in two factions of the network established by heavy metal concentrations (mg / kg) and DNA damage (μm) detected in *Vachellia farnesiana* and *Eisenia fetida* in Santa Rosa mine tailing 1 and 2. Color differences refer to the factions analyzed (●: Faction 1, ○: Faction 2). Larger frame sizes indicate greater degree of influence of the node within the network

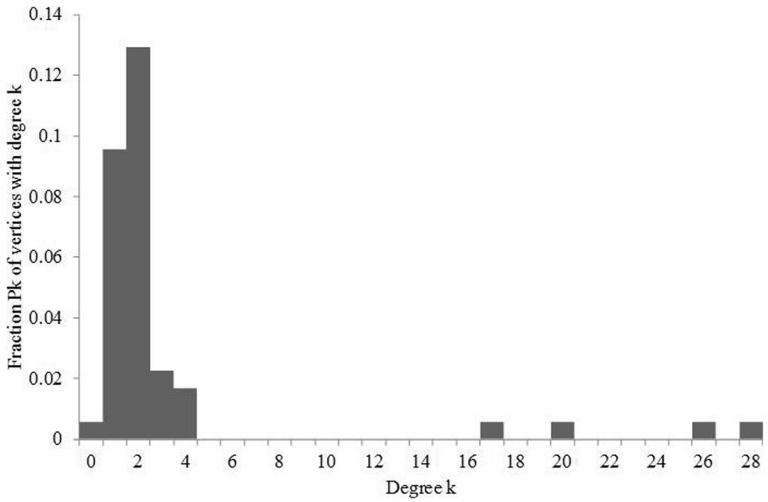

Fig. 6 Degree distribution graph of the network established by heavy metals concentrations (mg / kg) and DNA damage (μm) detected in *Vaquellia farnesiana* and *Eisenia fetida* in Santa Rosa mine tailings 1 and 2

Discussion

Bioaccumulation and biomagnification of heavy metals

The concentrations of bioavailable heavy metals reported for the "El Fraile" mine site in Santa Rosa recorded the following pattern: Zn > Pb > Mn > V > Cu > As > Ni > Cd [23–25]. This pattern is different to the observed bioaccumulation pattern in *V. farnesiana* foliar tissue. This may be explained by the fact that heavy metal transfer from soils to plants is carried out differently depending on edaphic characteristics and plant species [35, 36]. For example, it has been documented in plant species that the type of element and the accumulated amount depends primarily on the ability of the radical cells to concentrate nutrients, and exclude the toxic elements [37]. In addition, the detected concentration in foliar tissue will depend on the transportation of these elements from the root [38].

The highest concentrations of essential heavy metals detected in this study (Cu, Co, Fe, Mn and Zn) are due to the existence of specific protein transporters that facilitate their mobility trough the foliar tissue, in which process participates mainly the ZIP family of metal transporter proteins (ZRT / IRT proteins) [39]. On the contrary, Pb and As low concentrations detected can be attributed to the fact they are usually retained in the root symplast and later, in a lesser extent, translocated to the leaves through the same channels due to their electrochemical mimicry [38]. An interesting finding was that worms showed a similar bioaccumulation pattern of Zn and Pb with respect to the mine tailing metal content. In general, it has been documented that the heavy metal accumulation pattern in mine tailings is: Pb > Cd > Cu [40–44], which corresponds to the detected metals in worms from this study, placing *E. Fetida* as a consistent bioindicator species which reflects the metal content from soils. Also, contrasting results about heavy metal biomagnification patterns from mine tailings to plants and from plants to animals has been reported [45, 46]. In the present study, no relationship was found between metal bioavailability in mine tailings, and heavy metal bioaccumulation in plants. In contrast, the bioavailability of As, Cd and Ni in mine tailings showed an enrichment effect on the mine tailing-worm relationship. This result differs from Heikens et al. [11], who reported that worms exposed to Cd did not show biomagnification. The relationship found in this study between the primary producer and the detritivorous (*V. farnesiana-E. Fetida*), support the biomagnification pattern of As, Cu, Cd, Ni, Pb and Zn, being Pb, Cd and As the elements with higher enrichment values (1276.846, 1210.118 and 803.713 respectively). These findings are consistent with previous studies that identified Pb and Cd as the main non-essential elements transferred within a trophic chain [41, 47, 48]. In this system earthworms were the superior trophic bioreceptors with respect to the primary producers [49], which was corroborated by the biomagnification pattern of these elements.

Genotoxic damage

The results of the genotoxicity analysis in leaf tissue of *V. farnesiana* and *E. fetida* celomocytes showed a

statistically significant effect of the study site (tailings vs. controls) on DNA single strand break induction, being the exposed individuals from Santa Rosa, who presented the highest levels of genotoxic damage. This is consistent with the genotoxic evaluation studies of heavy metals on primary producers and detritivorous, which have demonstrated their sensitivity to environmental metal exposure [18, 50, 51]. In general, cellular and molecular responses of toxicity in plants established in a stress environment by heavy metals are unknown. However, several studies that evaluated phytotoxic and genotoxic effects of different heavy metals on plants, reported that in most cases, and independently of the life form of the studied plant species, a significant and positive relationship exists between heavy metal concentration and the amount of mitotic abnormalities [52], number of micronuclei [53], chromosomal aberrations [54] and DNA strand breaks [55], as well as a negative and significant relationship with the mitotic index [56, 57]. This increase in DNA damage has been linked to a significant decrease in the antioxidant enzymes activity, such as superoxide dismutase (SOD), glutathione peroxidase (GPOX) and ascorbate peroxidase (APX) [55, 57].

For annelids, the exposure of both celomocytes and whole animals to heavy metals, under different conditions (ie, substrate, humidity and temperature), increases their genetic damage, which is directly related to the heavy metal concentration [58]. In this study, *V. farnesiana* recorded a higher genotoxic sensitivity (sensitiveness) with respect to *E. fetida*, presenting 13.16 times more genotoxic damage in exposed individuals than control individuals. This can be partially attributed to the cellular response of each species to heavy metal exposure, which varies considerably between taxa [59, 60] and also between individuals [61, 62]. The lowest sensitivity detected in *E. fetida* worm with respect to the *V. farnesiana* plant, can be attributed to differences in the life history of both species (plant / animal), particularly, life cycle and number of offspring; which can determine the evolutionary response of populations to heavy metal stress, being the organisms with shorter life cycles and greater number of offspring, which have demonstrated faster adaptive responses [63, 64]. Particularly, *E. fetida* has been used successfully for lethal and sublethal analysis of different pollutants, concluding that long-term exposure in this species can lead to resistance responses to the evaluated pollutants [49, 65].

Network analysis

In addition to the analyses of metal bioaccumulation, biomagnification, and genotoxicity analyses, the application of CNT in this study proved to be a robust tool for the visualization of established trophic interactions between *V. farnesiana* and *E. fetida.* Firstly, it is observed that in both networks "site-species-metal concentration" and "site-species-metal concentration-genotoxic damage" the factions subgrouping of the nodes are established by species as the highest fitness. This indicates that the specificity given by each taxa is the factor that determines the metal concentration and genotoxicity levels observed, beyond the study site or type of metals detected. Previous studies have determined that the environmental characteristics such as pH, humidity and quantity of organic matter are the main factors that facilitate the introduction of heavy metals into trophic networks [66]. The present study suggests that in this system, the species characteristics determine the detected metal concentrations. This result supports the findings documented by Hossain et al. [67], and Gall & Rajakaruna [66] who demonstrated the ability of plants to evade heavy metal assimilation from the root, or to tolerate metal accumulation in other tissues [66, 68]. Hence, for most species, heavy metal concentrations in different tissues depend primarily on their physiology [12]. On the other hand, Mathews et al. [69] documented that the direct contact of earthworms with the contamination source, can delimit metal accumulation. Also, in a review study about the accumulation of Cu, Cd, Pb and Zn (from 1993 to 1998) in terrestrial invertebrates, Heikens et al. [41] mentioned that metal concentrations were highest in isopods, followed by lumbricids, and low in coleopteran, related to the edaphic habitat and food characteristics of each rate. Some showed cumulative preferences for some metals, such as worms that consistently exhibited high Cd bioaccumulation.

Secondly, it was identified that despite the bidirectional relationship established between both species, the nodes of *E. fetida* presented the highest values of intermediation and eigenvector centrality, as well as the lowest centrality values, identifying it as the major degree actor of linkage in the network and of greater influence. This reflects the strong impact of this species on the structure of both evaluated networks, as the main facilitators of metal flow and propagation, even above the primary producers. Actually, there are no studies with a complex system approach in trophic networks exposed to pollutants, which can serve to compare with the present study. Nevertheless, the analysis of the centrality measures obtained agrees with the vision of Jouquet et al. about earthworms as the ecosystemic engineers [29, 36], since they modulate the availability of resources and conditions for other species, both directly and

indirectly [70–72]. Moreover, this group is considered among the most important edaphic engineers, due to its ability to construct mineralorganic structures with specific chemical, physical and biological functions, while moving through the soil [73–75]. However, the bidirectional relationship between species reflects that the soil engineering depends on the feedback between the organisms that control and modify the environment, and the selective environments that other species provide [76], in this case *V. farnesiana*.

Conclusion

Our results show that there is en effect of the study site on heavy metal bioaccumulation and DNA damage induction, and that these responses are particular to each species and to each bioaccumulated metal, which in turn reveals specific sensitivity for each trophic level. Moreover, the application of CNT methodology allowed us to clarify in this particular system, the interaction types and the principal components of the trophic web. Also, we showed that despite the bidirectional trophic relationship established between *E. fetida* and *V. farnesiana*, *E. fetida* presents the major degree of linkage in the evaluated networks, as the actor with the greater influence. Our study also confirms the importance of the application of CNT as a holistic tool for the visualization of established trophic interactions in polluted areas, as a possible indicator of the ecosystem health.

Appendix 1

Table 2 Centrality measures of the nodes corresponding to the network established by the relationship between sites (tailing 1, tailing 2), species (*Vachellia farnesiana*, *Eisenia fetida*), and heavy metals (mg / kg)

Node ID	Betweenness	Eigenvector centrality	Closeness
E. fetida tailing1	479.000	0.444	123
E. fetida tailing2	433.000	0.421	125
V. farnesiana tailing1	278.583	0.305	132
V. farnesiana tailing2	214.000	0.258	135
Mn (< 50)	12.239	0.195	138
Ni (< 30)	12.239	0.195	138
Pb (< 100)	12.239	0.195	138
Al (1000-5000)	7.111	0.159	140
Cu (10-40)	7.000	0.159	140
Ni (30-60)	4.462	0.137	145
As (< 50)	3.111	0.118	152
Cd (< 100)	3.000	0.118	152
Cd (> 200)	3.000	0.118	152
Co (3-9)	3.000	0.118	152
Co (< 3)	3.000	0.118	152
Fe (500-5000)	3.000	0.118	152
Fe (> 5000)	3.000	0.118	152
Mn (> 100)	3.111	0.118	152
Mo (1-3)	3.111	0.118	152
Pb (100-300)	3.111	0.118	152
Pb (> 300)	3.111	0.118	152
V(< 5)	3.111	0.118	152
Zn (100-500)	3.111	0.118	152
Al (< 1000)	0.462	0.077	166
Ba (20-30)	0.462	0.077	166
Ba (< 20)	0.462	0.077	166
Ca (< 1500)	0.462	0.077	166
Cu (< 10)	0.000	0.077	166
Fe (< 500)	0.000	0.077	166
Mg (1500-2000)	0.000	0.077	166
Mg (> 2000)	0.000	0.077	166
Zn (< 100)	0.462	0.077	166
As (> 100)	0.000	0.061	169
Cd (100-200)	0.000	0.061	169
Co (> 9)	0.000	0.061	169
Mn (50-100)	0.000	0.061	169
Mo (> 3)	0.000	0.061	169
Ni (> 60)	0.000	0.061	169
V (5-15)	0.000	0.061	169
Al (> 5000)	0.000	0.057	171
As (50-100)	0.000	0.057	171
Cu (> 40)	0.000	0.057	171
Mo (< 1)	0.000	0.057	171
V (> 15)	0.000	0.057	171
Zn (> 500)	0.000	0.057	171
Ba (> 30)	0.000	0.042	178
Mg (< 1500)	0.000	0.042	178
Ca (> 1500)	0.000	0.035	181

Appendix 2

Table 3 Centrality measures of the nodes corresponding to the network established by the relationship between sites (tailing 1, tailing 2), species (*Vachellia farnesiana*, *Eisenia fetida*), heavy metals (mg / kg), and DNA damage [μm]

Node ID	Betweenness	Eigenvector centrality	Closeness
E. fetida tailing1	515.583	0.437	132
E. fetida tailing2	483.083	0.413	133
V. farnesiana tailing2	353.583	0.307	139
V. farnesiana tailing1	249.750	0.278	143
Mn (< 50)	12.352	0.190	147
Ni (< 30)	12.352	0.190	147
Pb (< 100)	12.352	0.190	147
Al (1000-5000)	8.281	0.153	149
Cu (10-40)	8.281	0.153	149
High genotoxicity [41-95]	6.447	0.149	151
Ni (30-60)	5.905	0.135	154
As (< 50)	2.947	0.112	163
Cd (< 100)	2.947	0.112	163
Cd (> 200)	2.947	0.112	163
Co (3-9)	2.947	0.112	163
Co (< 3)	2.947	0.112	163
Fe (500-5000)	2.947	0.112	163
Fe (> 5000)	2.947	0.112	163
Mn (> 100)	2.947	0.112	163
Mo (1-3)	2.947	0.112	163
Pb (100-300)	2.947	0.112	163
Pb (> 300)	2.947	0.112	163
V (< 5)	2.947	0.112	163
Zn (100-500)	2.947	0.112	163
Al (< 1000)	0.571	0.077	175
Ba (20-30)	0.571	0.077	175
Ba (< 20)	0.571	0.077	175
Ca (< 1500)	0.571	0.077	175
Cu (< 10)	0.571	0.077	175
Medium genotoxicity [10-40]	0.571	0.077	175
Fe (< 500)	0.571	0.077	175
Mg (1500-2000)	0.571	0.077	175
Mg (> 2000)	0.571	0.077	175
Zn (< 100)	0.571	0.077	175
Al (> 5000)	0.000	0.055	182
As (50-100)	0.000	0.055	182
As (> 100)	0.000	0.058	181
Ba (> 30)	0.000	0.041	188
Ca (> 1500)	0.000	0.037	192
Cd (100-200)	0.000	0.058	181
Co (> 9)	0.000	0.058	181
Cu (> 40)	0.000	0.055	182
Low genotoxicity [< 10]	0.000	0.041	188
Mg (< 1500)	0.000	0.041	188
Mn (50-100)	0.000	0.058	181
Mo (< 1)	0.000	0.055	182
Mo (> 3)	0.000	0.058	181
Ni (> 60)	0.000	0.058	181
V (5-15)	0.000	0.058	181
V (> 15)	0.000	0.055	182
Zn (> 500)	0.000	0.055	182

Abbreviation
CNT: Complex network theory

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Authors' contributions
Conceived and designed the experiments: LTCR, ETS. Performed the experiments: LTCR. Analyzed the data: LTCR, MRL, ETS. Contributed reagents/materials/analysis tools: PMG, MLOH, ESS. Wrote the paper: LTCR, MRL, ETS, PMG. Critically evaluated the manuscript: MRLP, PMG, ETS. All authors read and approved the final manuscript.

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