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Research article

The first evidence of accumulation and avoidance behavior of macroinvertebrates in a forest soil spiked with human-made iron nanoparticles: A field experiment

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ABSTRACT

Both earthworms and terrestrial isopods have been used to evaluate the quality of contaminated soil by NPs. However, most experiments have been conducted in the laboratory and under greenhouse conditions. Besides, little is known of Fe accumulation in earthworms from iron NPs (Fe NPs) under natural conditions. Therefore, the objective of this research was to evaluate the effect of manufactured NPs on the accumulation of Fe in macroinvertebrates from forest soil. Our results revealed that earthworms consume low amounts of Fe in a concentration of 1000 mg Fe NPs kg^{-1} of dry soil, with a behavior constant over time. Besides, we observed that earthworms could not detect Fe at low concentrations (1 or 10 mg Fe NPs kg^{-1}), so they do not limit soil consumption, which translates into high amounts of Fe in their bodies. By contrast, the content of Fe in organisms is inversely proportional to increasing concentrations in the soil (R^2 = -0.41, $p < 0.05$). Therefore, although studies are needed, in addition to considering environmental factors and the physicochemical properties of the soil, endogenous worms in the evaluated area could, under natural conditions, be useful to inform us of contamination of NP manufactured from Faith. Besides, for future research, a novel methodology should be considered to demonstrate more realistic avoidance behavior under field conditions.

1. Introduction

Nanotechnological innovations have been used during the last years in different knowledge areas (Verma et al., 2019). So that, the most nano-sized (1–100 nm) materials will be delivered to the environment (soil, water, air, or landfill) after they have fulfilled the function for which they were synthesized or manufactured (Terekhova et al., 2017). Therefore, despite the great benefits of nanomaterials (NMs), adverse effects on the environment have gradually emerged (Chen et al., 2017). As the global production and use of nanoparticles (NPs) increases, projected to grow to over half a million tons by 2020 (Robichaud et al., 2009). Keller and Lazareva (2013) and Keller et al. (2013) stated that in 2010 were released to the environment around 310,000 metric tons per year, with landfills receiving most of the waste, while around 50,000 metric tons per year were delivered to the soil, corresponding to 16% of the total released. Consequently, due to the increased of NMs, the release into environmental systems is inevitable, representing a concern for scientists, technologists since it could be an emerging ecological contaminant (Avila-Arias et al., 2019). Besides, ordinary people have also shown concerns regarding the unregulated use of engineering nano-sized materials or devices (Pérez-Moreno et al., 2019; Pérez-Hernández et al., 2020).

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The soil is the basis of multiple ecosystem services, such as human nutrition, the nutrient cycle, among others (Pachapur et al., 2016). Soil macroinvertebrates include a variety of organisms, such as earthworms, insects, isopods, among others, which are classified by their largest size (>2 mm). They are known as essential regulators of many ecosystem processes and, their high sensitivity to disturbances makes them good indicators of human alterations in ecosystems (Lavelle and Spain, 2001). Besides, macroinvertebrates have been used in the evaluation of different uses and management of agricultural and forest soils (Huerta et al., 2008; Zerbino, 2010; Mesa-Pérez et al., 2016). It is well known that soil contains abundant quantities of natural nano-sized minerals (Buzea and Pacheco, 2017), and plants and soil organisms have evolved along with natural nano-sized particles but not with engineering nanoparticles.

Fe NPs are potentially used and recommended for the remediation of contaminated soils. Indeed, the NMs commonly used in the remediation of heavy metal is nanometric ferric tetroxide (n-Fe_3O_4) and zero-valent iron (nZVI) (Gil-Díaz et al., 2019; Černíková et al., 2020). On the other hand, these NMs have been used and proposed as fertilizers in the agronomic management of crops to increase the physiological and biochemical characteristics of plants (Al-Amri et al., 2020). Nonetheless, few studies have addressed the damage that these NMs can cause soil organisms in natural conditions. In addition to the above, some experiments have carried out under laboratory and greenhouse. Specifically, with artificial soils and Petri dishes with filter paper, using standard OECD methods no. 207 (OECD, 1984), which are conditions very different under comparison to the ground natural in the field.

Several studies have evaluated the effect of different NP_S on soil macroinvertebrates out in conditions controlled. For instance, Romero-Freire et al. (2017), Jesmer et al. (2017), and Schlich et al. (2012) shown that in earthworms *Eisenia andrei* Savigny, the mortality, weight change, and reproduction were affected by the NP_S of ZnO and $ZnCl_2$, Ag, and $AgNO_3$, respectively. However, the NPs mentioned above have significantly different potential effects than the Fe NPs. In fact, investigations have documented that iron NPs exhibit toxic properties compared to conventional iron and iron oxides due to unique physical and chemical features that affect their absorption, biodistribution, and elimination (Paunovic et al., 2020). Recently, Liu et al. (2020), demonstrated that the consumption of NP of n-Fe_3O_4 by *E. fetida* caused the production of reactive oxygen species (ROS). In another study, Liang et al. (2018) reported that Fe accumulation in *E. fetida* was higher at high concentrations (500 and 1000 mg nZVI kg^{-1} dry soil) compared to the concentration lower (100 mg nZVI kg^{-1}). In the experiment of Novak et al. (2013), showed that in terrestrial isopods, the accumulation of Co^{2+} ions occurred in the hepatopancreas, but the Fe^{n+} ions accumulation not observed in the organism's body coming from the $CoFe_2O_4$ NPs. Furthermore, the Co^{2+} ions were more toxic than the Fe ions. For the above, it is suggested that the adverse effects depend on the type of organisms, NPs application methods, concentrations, and the physical and chemical properties of the soil.

On the other hand, Suthar et al. (2008) and Brami et al. (2017), argue that epigeic species do not inhabit the soil and have limited distribution so that they have limited ecological relevance when assessing soil quality. By contrast, that endogeic species are more susceptible to soil contaminants, in such a way that, the use of this species can give real information about the effects that NPs could cause in natural conditions. Moreover, to date, no research has been reported in which NPs are evaluated on endogenous soil organisms under natural conditions in forest land. Therefore, more information is needed to demonstrate the effect of NPs on soil macroinvertebrate communities under natural conditions (de Santiago-Martín et al., 2016). In such a way, experiments on natural soil, vegetation, and environmental conditions could help to understand the real effect of NPs on organisms, which play a role important in ecosystems, called 'ecosystem engineers'. The present study aimed to determine the impact of manufactured Fe NPs over time and under natural conditions on the accumulation of Fe in soil and macroinvertebrates from forest soil. We hypothesized that within the macroinvertebrate classes, will be an increase in the concentration of Fe inside the earthworm bodies, a decrease in biodiversity, an increase in the level of Fe in soil, pH and variation of soil pH (delta pH) at 2 h, 30, and 60 days of exposure of Fe NPs.

2. Materials and methods

2.1. Study site

The experiment was carried out in a forest in the 'Ejido Santa Rita', municipality of Arteaga Coahuila (25°14′ N, 100°29′ W). The soil is a sandy loam (92% sand, 6.0% silt, and 2.0% clay) of the Feozem series, with an electrolytic conductivity (EC) of 446.13 ìS cm^{-1}, pH in water of 7.5 (determined on a 1:2.5 soil/distilled water), pH in KCl 1N of 6.55 (determined on a 1:2.5 soil/KCl solution), delta pH of -0.65 ($\Delta pH = pH_{KCl}$-pH_{water}), 3.06 % organic matter (determined by loss on ignition at 400 °C during 4 h).

The texture was determined following the protocols of the Official Mexican Standard (SEMARNAT, NOM 021-RECNAT-2000). The pH_{water} and pH_{KCl} values were obtained by a desktop pH detector (Thermo Scientific™, Orion Star A211, EUA). The delta pH was calculated as the difference between pH_{KCl} and pH_{water}. This parameter was determined to know the variation of the pH caused by the Fe NPs. Besides, the value indicates the predominance of positive, negative, or neutral charges in the soil (Mekaru and Uehara, 1972). The EC was determined by a portable EC detector (Thermo Scientific™, Orion Star A222, EUA). The OM was determined according to the methodology of Schulte and Hopkins (1996). Soil temperature measured with a thermometer (ECO brand).

2.2. Iron oxide nanoparticles

Fe NPs were obtained from the Investigation and Development of Nanomaterials (ID-nano), S.A de C.V., San Luis Potosí, Mexico. The NPs were supplied as dry powders with a purity of 99%, the particle size of 63.9 ± 16.9 nm with form semi-spherical (Figure 1A and 1B).

A sample of the Fe NPs was washed with deionized water five times to remove traces of formaldehyde and air-dried to remove moisture content. For characterization by x-ray powder diffraction (XRD), the powder was screened using a No. 100 mesh (150 μm). The samples were fixed on a carbon tape and were observed using an automated electron backscatter diffraction (EBSD). The observations were made at the Center for Research and Advanced Studies of the National Polytechnic Institute (Cinvestav), Saltillo, Mexico. The magnetite and hematite phases were identified (Figure 1C). The size and shape were confirmed with a field emission scanning electron microscope (FE-SEM), model JSM-7800F PRIME.

2.3. Experimental design, treatments and sampling method

In a secondary forest with uniform tree composition, soil type, mulch type, and slope, a 48 × 50 m plot was established. The plot was divided into three subplots: 16 x 50 m. In each subplot, experimental units (20 cm × 10 cm × 10 cm, length, width, and depth) were randomly placed with three replicates (Figure 2). The treatments were 0 (control), 1, 10, 100, and 1000 mg Fe NPs kg^{-1} dry soil. For the effective application of the NPs, an open metallic frame (20 cm × 10 cm × 10 cm, length, width, and depth) was used, corresponding to the same dimensions as the experimental unit. Previously, the NPs were placed in test tubes with lid and dispersed in 10 mL deionized water and sonicated for 10 min using an ultrasonic frequency of 40 kHz and adjusting to a weak power (AS2060B ultrasonic cleaner, Automatic Science Instrument, Co., Ltd, China). Subsequently, the NPs were placed in a flask, adjusted with deionized water to reach 1000 ml, and finally added to the soil (each experimental unit).

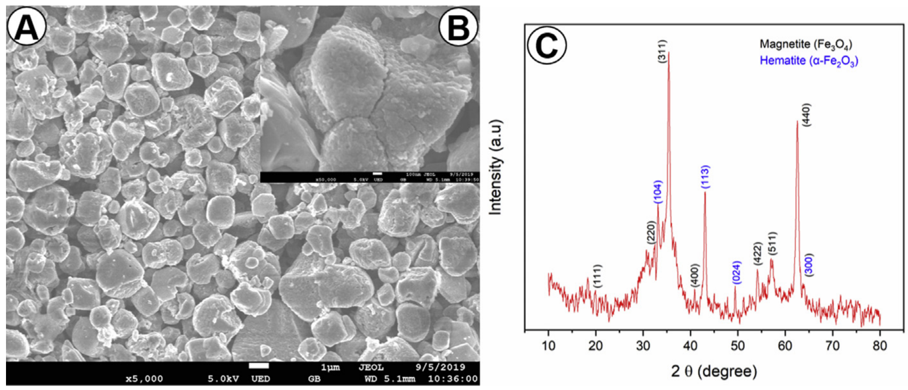

Figure 1. (A) micrograph of nano Fe at an amplified of x5000, (B) located where EDX analysis was performed at x50000, and (C) results of EDX analysis.

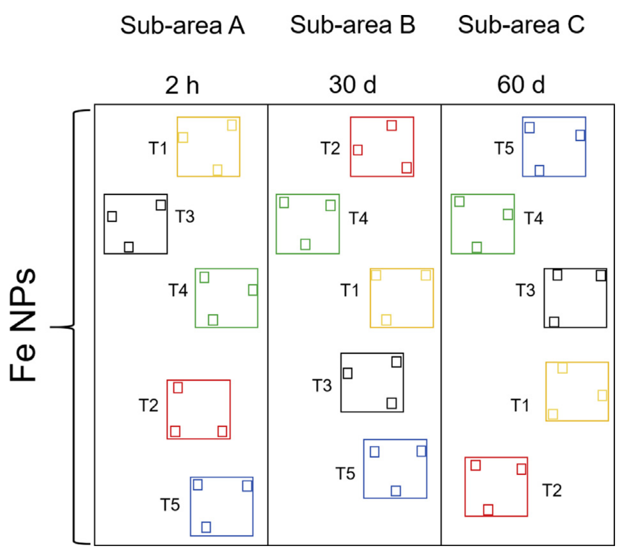

Figure 2. Design of the experimental space with an area of 48 x 50 m, subdivided into three areas of 16 × 50 m corresponding to the spaces destined for the sampling of the macrofauna and soil at 2 h, 30, and 60 days after exposure. Each sub-area contains 5 treatments (T1 = 1 mg kg^{-1}, T2 = 10 mg kg^{-1}, T3 = 100 mg kg^{-1}, T4 = 1000 mg kg^{-1} of dry soil and T5 = 0 or control [without nanoparticles]), with 3 repetitions each (45 experimental units [3 areas -sampling dates- × 5 treatments × 3 replications].

In each sub-area, at 2 h after exposure (HAE), 30 and 60 days after exposure (DAE), three samples (each experimental unit) were obtained for each treatment., i.e., 15 samples for each time. The distance of treatments between one and another subplot varied between 3 and 5 m, while the separation between experimental units varies between 1 m and 1.5 m. The proposed sampling method (modified) was according to Nahmani et al. (2006) and Ponge et al. (2015), in which open metallic frames were used to obtain macroinvertebrate samples from contaminated and disturbed soils. In this experiment, monoliths of 20 × 10 × 10 cm (length, width, and depth) were considered (the dimensions of the monolith correspond to the open metal frame). For this field experiment, the use of open metal frames it is based on three important aspects: 1) several experiments show that NPs are toxic to plants, macroinvertebrates, and considered soil contaminants, therefore, to avoid environmental damage, we decided dimensions of 10 cm × 20 cm (width and length). 2) before the start of the experimentation, ten soil samples were collected in order to know the maximum depth of the arable soil layer. In this sense, 10 cm was the average depth. Besides, below 10 cm depth, in most of the experimental sites, rocks between 4 and 6 cm of diameter were found. Therefore, we decided a height of 10 cm for the open metal frames. 3) one of the objectives of using the metal structure is to place the corresponding concentration of NPs and to ensure that the solution infiltrates the specific area. The frame was placed in each experimental unit, buried, and finally, the NPs were added. For sampling, the frame was buried to extract the soil. Figure 3 shows a diagram of the framework and its use during NPs application and soil and organism sampling.

Macroinvertebrates greater than 2 mm were searched manually. After that, they were counted and classified (class) with the help of a stereoscope. Finally, all the arthropods were concentrated in plastic jars with 70% alcohol and the earthworms, with 4% formaldehyde. The organisms were preserved until the laboratory analysis for adsorption and concentration in the tissue of their bodies. From the same collected samples during the organisms sampling, soil samples were obtained for each experimental unit. In the laboratory, the macroinvertebrates at the class level were identified, and we obtained the number of organisms and biomass per class, and we carried the calculation of the diversity indexes of Berger Parker and Chao 1 out.

2.4. Chemical analysis

The Liang et al. (2018) method was used to determine the amount of Fe in soil and in organisms, for acid digestion, with slight modifications. Besides, this method has already been reported by Wang et al. (2016) with the same purpose as ours. Firstly, soil samples were air-dried. Afterward, samples were ground (150 μm), later, 1 g of dry soil was weighed and transferred to a 250 ml beaker. Then, they were added 10 mL of concentrated HNO_3 (69 %, Jalmek brand), 5 mL of H_2O_2 (30%, JT Baker brand), and 3 mL of HCl (36 %, Jalmek brand). The reagents and brands used complied with the specifications of the American Chemical Society (ACS). The glasses were covered with glass and heated on a grill in constant agitation at a temperature of 180 °C for two hours. Finally, were added 25 ml of deionized water, and the solution was filtered with Whatman # 41 paper and brought to a volume equal to 100 mL. All samples were digested in triplicate and analyzed by plasma atomic emission spectrometry (ICP) using a Perkin Elmer Mod. Optima 8300 equipment. For the analysis of the organisms, all the individuals of each replicate were examined together. The only variant, in this case, is that for digestion, was used 5 mL of HNO_3 at 30% and 5 mL of H_2O_2 (Elga, Model PURELAB OptionQ). The digestions were performed in the chemical laboratory of Cinvestav, Saltillo, Mexico.

One earthworm was used per treatment and time. The organism was chosen at random. Furthermore, from our point of view, one earthworm for the area evaluated in the present experiment is sufficient to demonstrate that individuals can come into contact with Fe from Fe NPs. Furthermore, our intention was not to make a statistical comparison. The worms, after being preserved in 4% formaldehyde, were washed with deionized water. Each individual was placed on a stainless steel plate secured with double-sided conductive carbon tape. Subsequently, the earthworms were sprayed with gold-palladium using the sputtering

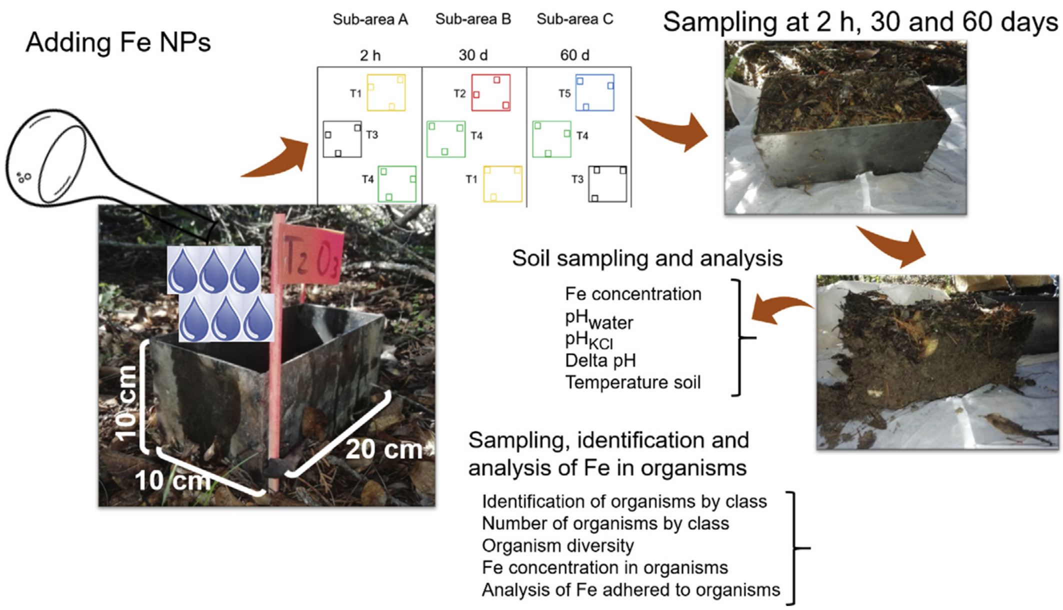

Figure 3. Diagram of the use of the metal frames for the addition of Fe NPs. Collection of soil samples, and organisms.

technique for 1 min (Murtey and Ramasamy, 2016). Later, the samples were analyzed with the scanning electron microscopy (SEM) equipped with energy-dispersive X-ray spectroscopy (EDX) to determine the elemental composition from the outer surface of earthworms, mainly, the presence of Fe (Antisari et al., 2015).

2.5. Data analysis

To assess the effect of the Fe NPs on the concentration of Fe in organisms and soil was determined using a two-way analysis of variance (ANOVA) with the general linear model procedure. When the analysis show differences, it was applied the Fisher's least significant difference procedure ($p < 0.05$) at the 95% confidence level. The normality and homoscedasticity were checked through the Kolmogorov-Smirnov and Barttlet test, respectively, ($p < 0.05$). The data were analyzed using Minitab software (version 18.0). The non-metric multidimensional scaling (NMDs) applying the metric of Canberra, which is the dissimilarity metric used to compare between individuals or objects (Emran and Ye, 2001). It is given by:

$$d(\mathbf{p},\mathbf{q}) = \sum_{i=1}^{n} \frac{|pi - qi|}{|pi| + |qi|}$$

where pi and qi were the variables measured in the study. Therefore, we research the relationships of biological factor $qi =$ number, biomass, class of organisms, and concentrations of Fe in organisms between the factors $pi =$ concentrations of Fe NPs, pHwater, pHKCl, delta pH and soil temperature. Statistical analyses were performed with R software (Version 3.0 http://folk.uio.no/ohammer/past/).

The Berger Parker index is a tool for monitoring biodiversity impairment linked to environmental conditions and by anthropogenic disturbance. This index explains the predominance of the most abundant species over the total abundance of all species on the whole (Caruso et al., 2007), while Chao 1 index is utilized to estimating the number of species in a community. Principally, the index is based on that rare species infer the most information about the number of missing species (Kim et al., 2017). The Berger Parker and Chao 1 index was determined using the PAST software package to know the diversity for each treatment over time (Hammer et al., 2001).

On the other hand, we determined correlation analysis using Spearman's rank correlation coefficient with a threshold value of $p < 0.05$ to investigate the relationship between Fe concentrations in organisms and concentrations in the soil as well as the link with biomass.

3. Results

3.1. The concentration of Fe in the soil and changes in pH

Results revealed a significant difference between treatments and the exposure time of the Fe NPs regarding the presence of Fe in the soil (Figure 4). In the case of exposure time, a decrease in Fe concentrations was observed in the 60 days, but much less at 30 days ($F_{c\ time} = 21.49$; d.f. $= 2$; *p-value* $= 0.000$). As for the treatments, there was not a significant difference observed between the concentrations evaluated at 2 h after exposure. However, the concentration of Fe in the soil showed an increase with the addition of 1000 mg kg^{-1} for the 30 DAE while the increase at 60 DAE, was observed with 100 and 1000 mg kg^{-1} of Fe NPs ($F_{c\ treatment} = 10.71$; d.f. $= 4$; *p-value* $= 0.000$).

On the other hand, the pH measured in water after the application of Fe NPs in the soil, indicated a significant difference between times and treatments ($F_{c\ time} = 38.54$; d.f. $= 2$; *p-value* $= 0.000$ and $F_{c\ treatment} = 8.02$; d.f. $= 4$; *p-value* $= 0.000$). The pH measured in KCl shows significant difference between times and treatments ($F_{c\ time} = 16.03$; d.f. $= 2$; *p-value* $= 0.000$ and $F_{c\ treatment} = 2.84$; d.f. $= 4$; *p-value* $= 0.037$). For Delta pH, the results not showed significant differences between treatment and time ($F_{c\ time} = 1.8$; d.f. $= 2$; *p-value* $= 0.182$ and $F_{c\ treatment} = 1.10$; d.f. $= 4$; *p-value* $= 0.374$).

3.2. Macroinvertebrates diversity found in the different treatment plots

For this experiment, we did not find a significant effect caused by the concentrations of Fe NPs on changes in diversity and richness of organisms (Figures 5 and 6). On the other hand, within the macroinvertebrates, the class clitellata was the most abundant at 30 DAE (Figure 7 and Table 1) and represented the most significant number of biomass, compared with the rest of the classes. According to the results of the total biomass in the experiment (29.0 g)., i.e., 83.26% corresponds to the biomass represented by the clitellata class (24.17 g). In comparison, 16.73% (4.85 g) corresponds to the rest of the classes found.

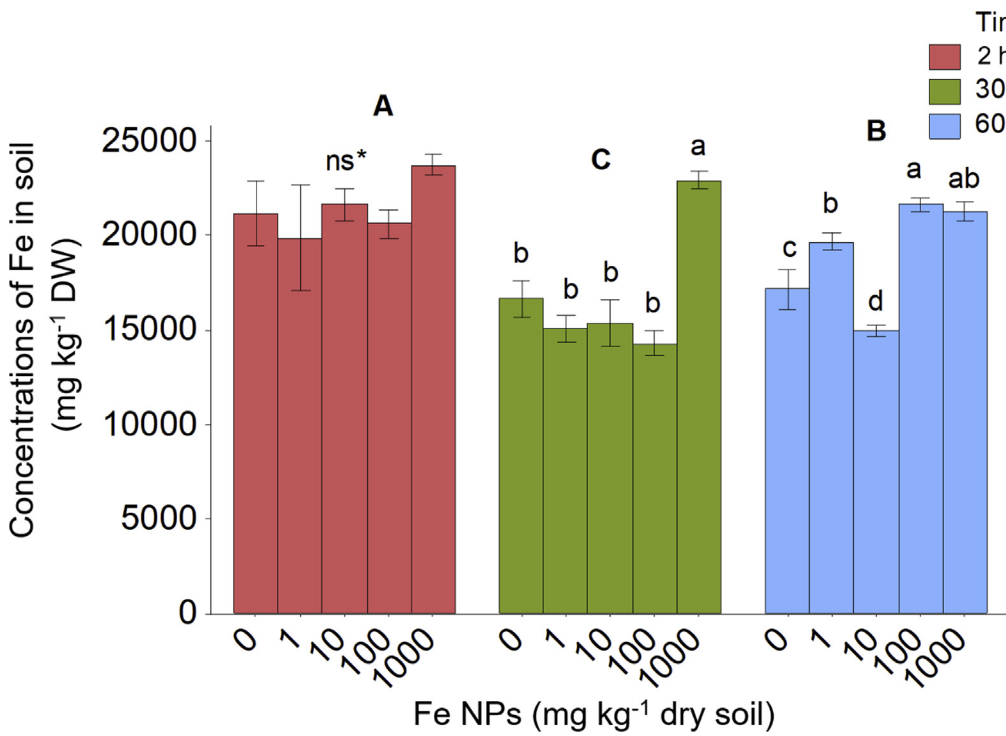

Figure 4. Fe concentration in forest soil. Different lowercase letters indicate significant differences between each treatment (0,1,10, 100, and 1000 mg kg^{-1} dry soil of Fe NPs), and different capital letters indicate significant differences between times (2 h, 30, and 60 days after exposure). Two-way analysis and LSD test, $p < 0.05$. Data points in each vertical bar are presented as means (± standard error; n=3).

3.3. The concentration of Fe on soil macroinvertebrates

The data obtained from the ICP-MS analysis for macroinvertebrates showed significant differences in the concentrations of Fe in organisms between times of sampling and treatments ($F_{c\ time} = 9.68$; d.f. = 2; *p-value* = 0.000 and $F_{c\ treatment} = 12.80$; d.f. = 4; *p-value* = 0.000, Figure 8). Firstly, at 2 h after exposure, the Fe concentration in organisms was high at the treatment with 100 mg kg^{-1}, followed by the 1 mg kg^{-1} treatment,

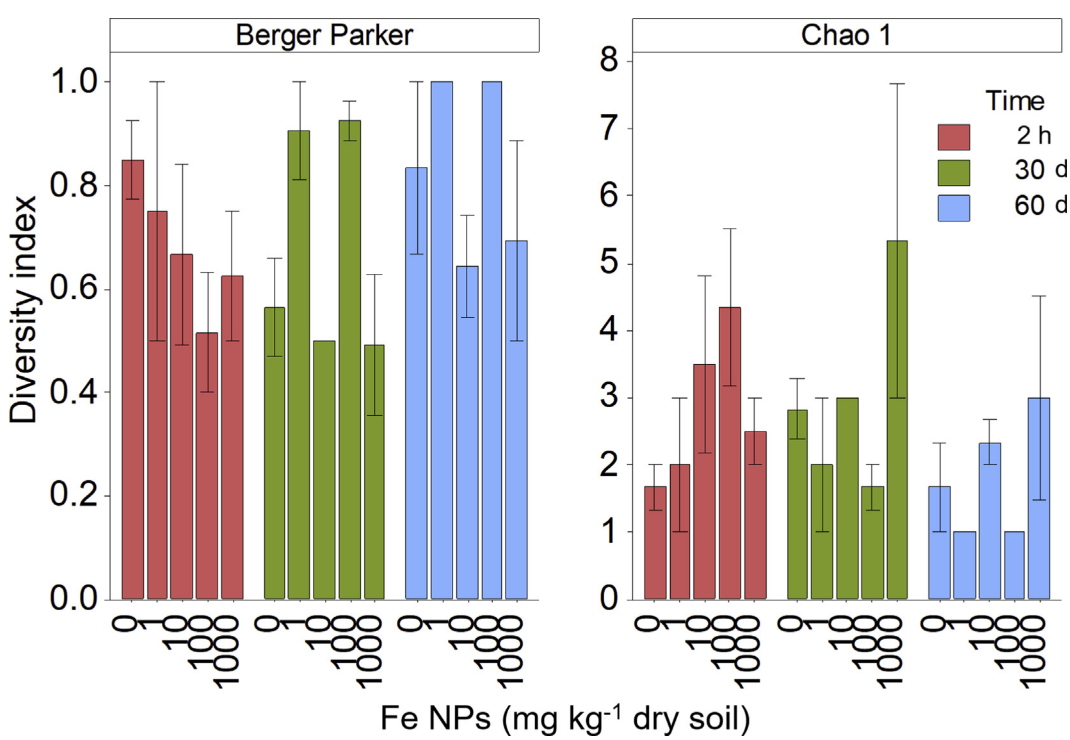

Figure 5. Average Berger Parker and Chao 1 diversity index at 2 h, 30, and 60 days after exposure to Fe NPs (0, 1, 10, 100, and 1000 mg kg-1 dry soil of Fe NPs). Bars without letters indicate that no significant differences (LSD test, $p < 0.05$) between times and treatments by two-way variance analysis (ANOVA), respectively. Data points in each vertical bar are presented as means (± standard error; n=3).

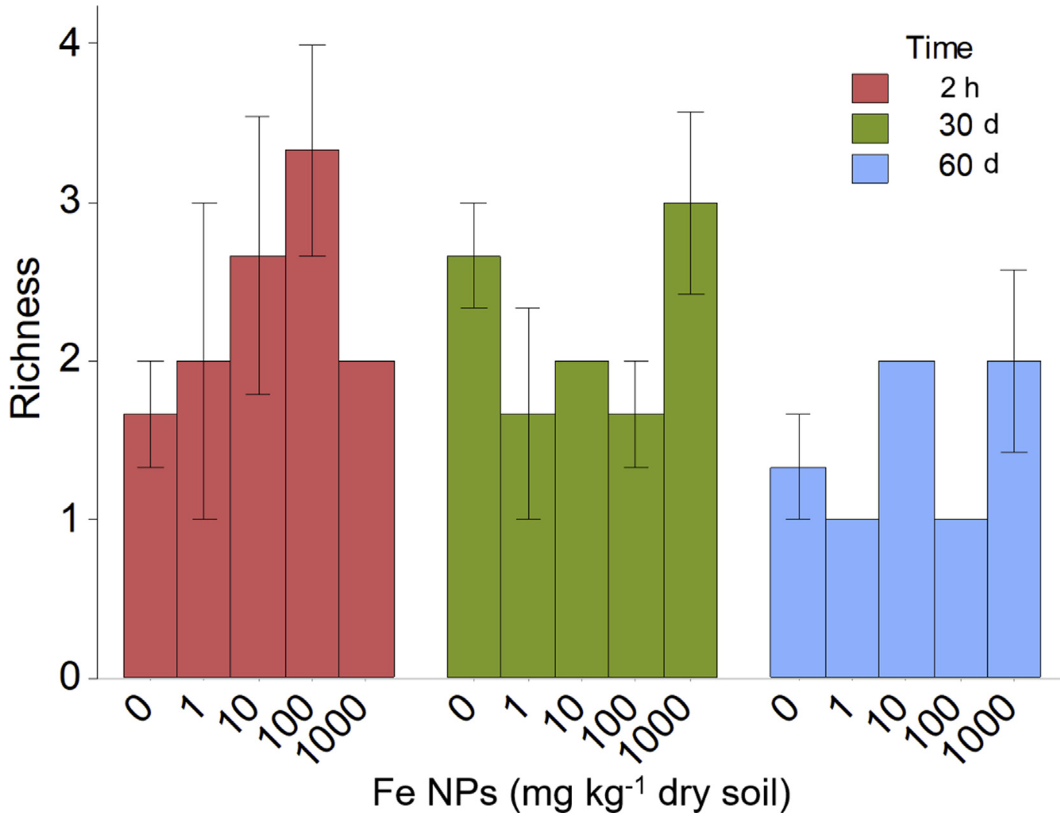

Figure 6. The average richness of organisms (classes) collected at 2 h, 30, and 60 days after exposure to Fe NPs (0, 1, 10, 100, and 1000 mg kg-1 dry soil of Fe NPs). Bars without letters indicate that no significant differences (LSD test, $p < 0.05$) between times and treatments by two-way variance analysis (ANOVA), respectively. Data points in each vertical bar are presented as means (± standard error; n=3).

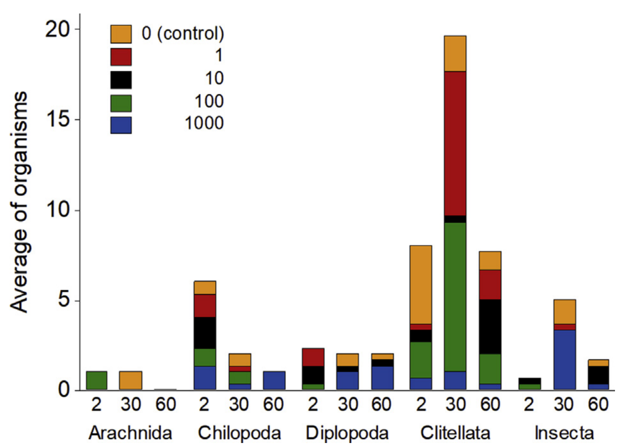

Figure 7. Average of organisms found in each of the treatments (0, 1, 10, 100, and 1000 mg kg^{-1} dry soil of Fe NPs) and for each time (2 h, 30, and 60 days after exposure to Fe NPs).

except for the rest of the treatments. For the 30 DAE, the concentrations of NPs were significantly higher in the 10 mg kg^{-1} treatment, followed by the 1 and 100 mg kg^{-1} treatment. However, there was no statistical difference compared to the control. Nevertheless, a significant difference was observed compared to 1000 mg kg^{-1} treatment, which presented a lower content of Fe in macroinvertebrates. After 60 days of exposure at 1 and 10 mg kg^{-1}, a higher concentration of Fe in organisms observed compared to control and in the 1000 mg kg^{-1} treatment. Besides, the concentrations of Fe in macroinvertebrates decreased throw the time at 1000 mg kg^{-1}, while at 1 and 10 mg kg^{-1} the concentration remained constant over time.

3.4. Relationship between concentrations of Fe, environmental factors, and biological parameters

Exposure to Fe in all concentrations and environmental factors did not relate to changes in the organism's number and pH soil for both measurement in water as well as KCl and Delta pH. Only was observed a relationship between the effects of NPs at 2 HAE and 60 DAE. The variable soil temperature and chilopoda class were related to 2 HAE, while the arachnida variable with time 60 DAE. Although were related to time 30 DAE, the variables biomass, class clitellata, and soil Fe concentration, the effects of that time were dispersed (Figure 9).

4. Discussion

4.1. The concentration of Fe in the soil and changes in pH

Regarding the concentration of Fe in the soil, the presence of Fe showed an increase with the concentration of 1000 mg kg^{-1} at 30 days, while the increase at 60 days after exposure, was observed in the concentrations of 100 and 1000 mg kg^{-1} of Fe NPs. Based on the results, we reveal that with the soil characteristics mentioned above, the Fe NPs remain in the soil for 60 days. Similarly, Liang et al. (2018) found that when evaluating different concentrations of Fe NPs, the iron content in the soil gradually increased with increasing levels of Fe NPs. Also, Yirsaw et al. (2016) found that in two types of soils similar in texture as in the present experiment, the concentration of iron in the soil suspension was constant at a concentration of 1500 mg nZVI kg of dry soil. The authors suggest that clay content greater than 10% may influence the binding of metal ions in the soil, as well as the presence of organic components.

It is essential to clarify that the transformation of Fe NPs was not determine at the end of the experiment. Still, the most common oxidation states are maghemite (γ-Fe_2O_3), magnetite (Fe_3O_4), and hematite

Table 1. Summary of macroinvertebrates class found by treatment (0, 1, 10, 100, and 1000 mg Fe NPs kg^{-1}) and time (2 h, 30, and 60 days after exposure) in the present experiment.

Time	Treatments	Arachnida	Chilopoda	Diplopoda	Clitellata	Insecta
2 h	0	0	2	0	13	0
	1	0	4	3	1	0
	10	0	5	3	2	1
	100	3	3	1	7	1
	1000	0	4	0	2	0
	Total	**3**	**18**	**7**	**25**	**2**
30 d	0	2	2	2	6	4
	1	1	1	0	24	1
	10	0	0	1	1	0
	100	2	2	0	25	0
	1000	1	1	3	3	10
	Total	**6**	**6**	**6**	**59**	**15**
60 d	0	0	0	1	3	1
	1	0	0	0	5	0
	10	0	0	1	9	3
	100	0	0	0	3	0
	1000	0	3	4	1	1
	Total	**0**	**3**	**6**	**21**	**5**

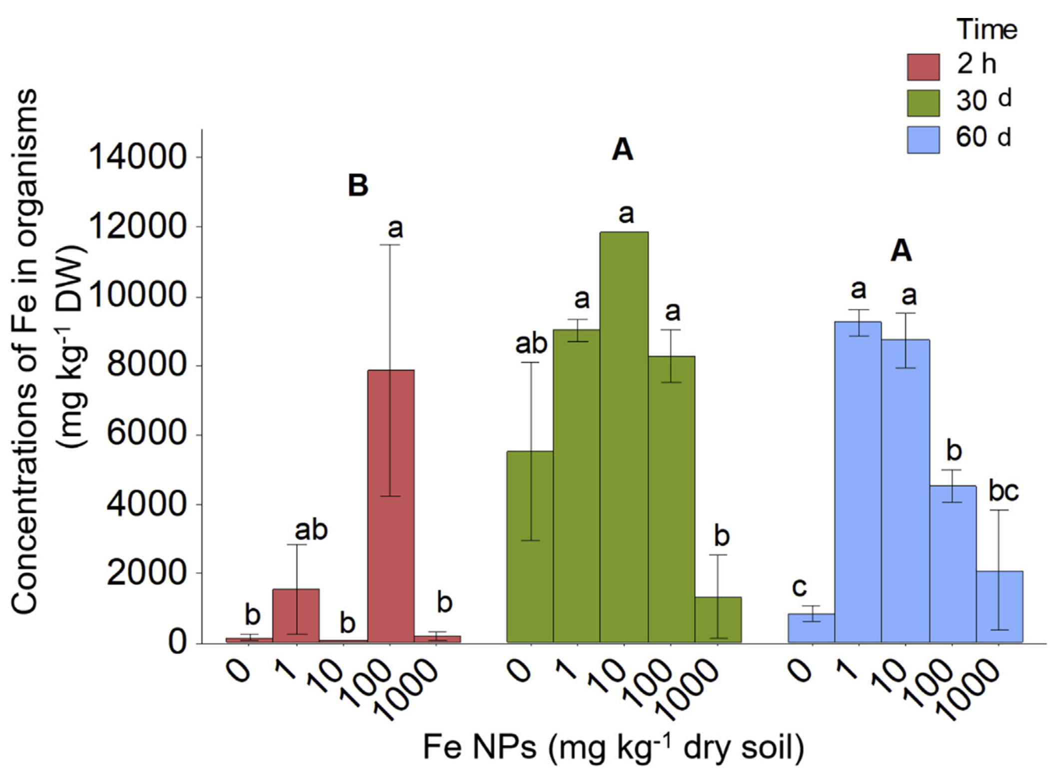

Figure 8. Fe concentration in macroinvertebrates from plots treated with different concentrations of Fe NP at 2 h, 30, and 60 days after exposure. Different lowercase letters indicate significant differences between each treatment (0, 1, 10, 100, and 1000 mg kg^{-1} dry soil of Fe NPs), and different capital letters indicate significant differences between times. Two-way analysis and LSD test, $p < 0.05$. Data points in each vertical bar are presented as means (± standard error; n=3).

(α-Fe_2O_3) (Taylor and Konhauser, 2011). However, iron is a reducing metal, which makes it difficult to predict its impact within an ecological system and to create a detailed model of its behavior. It is due to limited and incomplete studies (Wagner et al., 2014), particularly in a natural environment due to physical heterogeneity and chemical properties that affect the mobility and toxicity of metallic NPs to soil organisms (Klaine et al., 2008). In this regard, Thompson et al. (2006) indicate that due to the capacity of reduction-oxidation of iron, iron NPs can cause changes in the concentration of colloidal and dissolved material as well as changes in pH. For instance, in a study of remediation of soil Vítková et al. (2017) observed that the addition of nZVI in agricultural soil increased natural pH compared to control. By contrast, Hussain et al. (2019) reported that regardless of the concentration of iron NPs applied in an agricultural soil contaminated with wheat cultivated (*Triticum aestivum*), there was no change in soil pH. Likewise, Rizwan et al. (2019) found that the soil pH was not affected in all treatments of Fe NPs compared to the control when evaluating the plant growth in wheat. Possibly, the buffering capacity of the studies mentioned was what caused that there were no changes in the

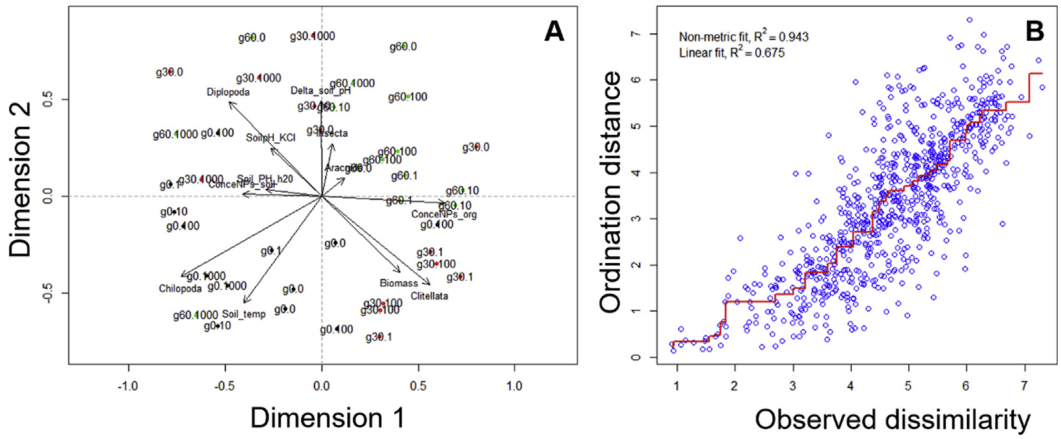

Figure 9. Non-metric multidimensional scaling (NMDS) ordinations in which represents (**A**) the relationships among the effect's concentrations Fe NPs in time with biological parameters (number, biomass, and class of organisms) and environmental data (pH soil [(pH measured in water and KCl while Delta pH = pH_{KCl}-pH_{water})] and soil temperature), (**B**) based on Canberra distance (non-metric fit R^2 = 0.943).Vectors representing Fe concentrations, biological parameters, and environmental variables while the points marked in black, red, and green indicate the time 2 h, 30, and 60 days after exposure, respectively.

soil pH. When Gil-Díaz et al. (2014) evaluated the addition of nZVI, observed that acidic soil showed a lower buffering capacity than calcareous soil. Thus, no changes were detected of pH in soil calcareous. In this experiment, pH measured in water and KCl shown changes both times and treatments. However, the difference occurred due to a value of 6.42 and 7.27 (pH water) in the concentration of 1 and 10 mg kg^{-1} dry soil at 30 DAE, respectively. For the 2 HAE and 60 DAE, the treatments showed no statistical difference. This same condition occurred when we obtained the pH value measured in KCl at 30 DAE. Therefore, we cannot explain these changes in pH at time 30 DAE, since all treatments at 2 HAE and 60 DAE showed no statistical differences. Nevertheless, pH values in water were in the range from 6.4 to 8.04, while that pH measured in KCl was in from 5.54 to 7.56. Possibly there are other factors involved in these changes. What we confirm is that, due to the type of soil, colloids have negative charges, which confirmed with a Delta pH value average of -0.65 (Mekaru and Uehara, 1972).

It is known that under environmental conditions, Fe_3O_4 NPs are not very stable and can be oxidized to Fe_2O_3 or dissolve in an acidic medium. Although there is a change in oxidation, the ionic forms will be present and possibly in the soil solution. Therefore, the main concern is that NPs can affect different soil horizons, altering physicochemical properties, and organisms (Pachapur et al., 2016). Still, above all, the bio/geo-transformation can be derived (Rajput et al., 2019). It is known that of all transformations, aggregation is the most important, which decides whether NMs behave as aggregates. The OM can prevent this condition, but the interaction of OM with clays and microorganisms makes it challenging to understand the state of aggregation in the soil, such as toxicity in organisms (Li et al., 2016). In this study, because at high concentrations of iron NPs (1000 mg kg^{-1}), they remained constant over time, possibly to the interaction of the OM, soil moisture, and is soil with negative charges, the Fe NPs are retained between 0 to 10 cm deep (Yirsaw et al., 2016). This leading to limited mobility where organisms can be exposed to low or higher concentrations of Fe NPs. Therefore, the results obtained in the laboratory and the greenhouse are different from those found in the field. The study of Wagner et al. (2014) discussed various aspects related to the process of agglomeration and mobility between natural and manufactured Fe NPs. This explains the contradictory toxicity results that were reported due to experimental conditions and the influence of transformations of iron NPs that would alter their toxicity. For instance, Yirsaw et al. (2016) found that in a soil alkaline (pH of 8.67), the Fe concentration final was low regardless of the concentration applied nZVI NPs. They argue that high pH values and organic components could affect the release of Fe (II).

4.2. The concentration of Fe on soil macroinvertebrates

The study of evaluations of NPs has been investigated on macroinvertebrates of soil, principally with earthworms. However, recently most investigations have been carried in laboratory conditions utilizing epigeic earthworms (*E. Andrei* and *E. fetida*) with arguing of evaluating parameters such as mortality, reproduction, uptake, molecular response, avoidance behavior and in the evaluation of the physicochemical quality and integrity of the soil (Shoults-Wilson et al., 2011; Bouguerra et al., 2016; Velicogna et al., 2016; Romero-Freire et al., 2017; Liang et al., 2018; Valerio-Rodríguez et al., 2019). Besides, there have been utilized isopods terrestrial such as *Armadillidium vulgare, Porcelio scaber* (Novak et al., 2019). Even although these investigations have helped to understand the ecological and biological implications caused by NPs released to the environment, the debate for the use of macroinvertebrates (epigeic, endogeic, and anecic earthworms) as effective bioindicators of soil pollution is discussed (Suthar et al., 2008). In the present experiment, contrary to the raised hypothesis, we find evidence that earthworms prevent the consumption of Fe from NPs. Consequently, this study is the first evidence showing the accumulation of Fe in macroinvertebrates in forest soil conditions from the Fe NPs. In the 30 and 60 DAE, we found a clear decreased in the presence of Fe in the body of macroinvertebrates when organisms were exposed at concentrations of 100 and 1000 mg kg^{-1} dry soil of Fe NPs. Nevertheless, when the organisms were exposed to time 2 HAE at 10 mg kg^{-1}, higher concentrations of Fe were found in the organism's body, but no so in the treatment of 1000 mg kg, in which the organisms presented less Fe in the body. Therefore, we argue that the macroinvertebrates could not detect the low concentration of 1 and 10 mg kg^{-1} dry soil, both at 30 and 60 days. Consequently, the concentrations are higher in the body of organisms. In the Lourenço et al. (2011) experiment, they observed that at time 0, the *E. fetida* worms (n = 5) accumulated up to 1793 mg Fe kg^{-1} of dry weight, and after 56 days, the accumulated amount reduced to 1373 mg kg^{-1} of dry weight. Therefore, our results suggest that at 2 h after exposure, earthworms desperately under wet conditions consume amounts of soil and, in turn, accumulate high amounts of Fe. This behavior has been tentatively associated with ionic metal fractions that suddenly appear in the solution and soil pores (Shoults-Wilson et al., 2011).

On the same line, in Table 1 (summary), it is clearly observed that despite the existence of different classes of macroinvertebrates (chilopods, diplopods, Insecta, and arachnid), endogeic earthworms were found in all treatments and times of exposure; at least one or two individuals of organisms were sufficient to demonstrate Fe consumption. To further support our suggestions, earthworms (Clitellata) followed by centipedes (Chilopoda) and millipedes (Diplopoda) were the organisms that were found between 4 to 10 cm deep. In this sense, despite that no known protocol for the evaluation of accumulation of NPs on soil organisms under natural conditions, for our experiment (with the proposed methodology), were the endogeic earthworms that indicated contamination of the soil from Fe NPs under natural conditions. Some studies suggest that the concentration of metals in endogeic earthworms could be related to the organic fractions of ingested soil. The authors for this study demonstrated that Fe accumulation in agricultural land reaches a concentration close to 500 mg Fe kg^{-1} of dry soil (Suthar et al., 2008). Furthermore, the studies by Lourenço et al. (2011) confirm that, at 0 days, the *E. fetida* worms accumulate high amounts of Fe while at 56 days, the amount decreases. Therefore, it was observed clear evidence that earthworms are those that indicated high or low amounts of NPs in the environment compared to the rest of the organisms.

Our studies contrast with Diez-Ortiz et al. (2010), they found that in *E. andrei*, the absorption of molybdenum (Mo) NPs increases with its increasing concentration in soil. Still, factors of accumulation in organisms are lower at higher levels of exposure. Likewise, Liang et al. (2018), observed a linear correlation between Fe accumulations in earthworms and Fe NPs content in soil at 28 days. Nevertheless, the experiment was conducted in containers under controlled conditions. Therefore, we argue that worms in field conditions can be mobile slightly when detecting large amounts of Fe NP. Even so, it is tempting to speculate that avoidance behavior may have occurred in our experiment. Nonetheless, we are careful of these arguments since, when compared to avoidance studies according to the standard ISO guideline protocol (ISO, 2005), in these tests, there are used plastic containers with compartments that divide the control treatment and the treatment conditioned with NPs used. The test involves placing earthworms for allowing them to migrate or move in treated and untreated soils. After 24 h, the compartment is placed to divides the two treatments. Subsequently, the earthworms were counted in order to determine the number of migrated earthworms between the treatments with NPs. For more detail on this methodology, see the document by El-Temsah and Joner (2012).

As far as we know, this is the first study under field conditions that determines the adsorption of Fe on earthworms. However, it is difficult to know if the Fe adhered to the earthworms comes from the natural soil or the additions of Fe NPs. In this sense, when we analyzed the presence of Fe adhered in the organisms, found greater adherence of Fe in the body of the earthworms at the concentration 1000 followed by 100 and 10 mg Fe kg^{-1} dry soil at 30 DAE, but not at 2 HAE and 60 DAE as can be seen in Figure 10 and 11. Therefore, although field studies are needed, these results could be another indication that, although the organisms remain in contact with the NPs, they limit their consumption, that is, the dermal contact versus intestinal adsorption varies (Nannoni et al., 2011). In this sense, in the present experiment, the content of Fe in the organisms' body is inversely proportional to increasing concentrations in the soil (r^2 = -0.41, *p-value* = 0.000; see Figure 12). We agree with Yirsaw et al. (2016), regarding that the relationship between the total Fe content in the soil and its effect on terrestrial organisms may not necessarily be with a direct relation. Van Gestel et al. (2011) mentioned that exist other factors link to potentiate the effects of NPs and caused mortality in soil organisms. They found that regardless of the amount of molybdenum NPs in natural soils, it was the soil properties (particularly pH value and clay content) that had a specific effect on toxicity in 3 different species of soil invertebrates, including in this evaluation *E. andrei*.

As we have said before, the experiments were performed with epigeic earthworms, confined in Petri dishes, microcosm, and macrocosms and induced to consume metals. In our field observations, endogenous earthworms were the only organisms that were found inside the soil, so that their diet is mainly based on decomposed organic matter and ingest large amounts of soil. Different to endogeic earthworms, the rest of the organisms found have an epigeal habit, i.e., they live on the soil surface (leaf litter), to this group belong spiders, ants, centipedes, and some predatory beetles, among others (Huerta et al., 2008). The review by

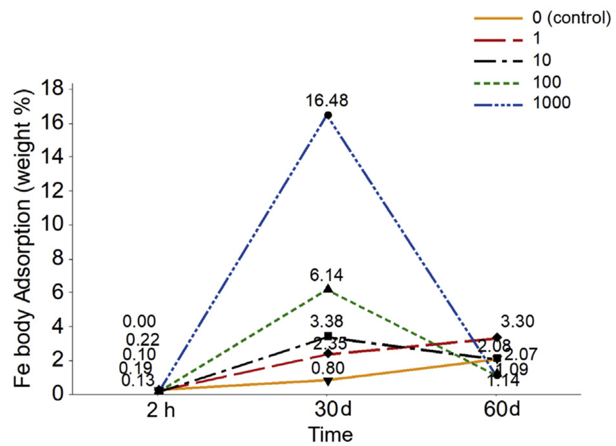

Figure 10. Amount Fe (weight %) adhered to the body of earthworms at 2 h, 30, and 60 days after exposure to Fe NPs (0, 1, 10, 100, and 1000 mg kg^{-1} dry soil of Fe NPs). The values were obtained from SEM and EDX analysis.

Pérez-Hernández et al. (2020) reported contradictory cases of effects of different metallics NPs on microfauna, mesofauna, macrofauna, and edible and inedible plants.

Svendsen et al. (2005) and Suthar et al. (2008), have debated the use and have proposed to endogeic earthworms as indicated to measure soil contamination by heavy metals. By contrast, the epigeic worms do not inhabit mineral soil, have a limited distribution associated with naturally occurring organic matter and are therefore considered to have limited ecological relevance (Lowe and Butt, 2007; Brami et al., 2017; Mariyadas et al., 2018). Besides, *E. fétida* and *E. andrei* species are considered cosmopolitan species, so they can resist disturbances compared to endemic species that are only restricted to certain natural conditions (Fragoso and Rojas, 2014). This condition can be that occurred in several experiments under laboratory conditions when reported contradictory cases., i.e., toxic effects and null effects of NPs on *E. andrei* and *E. fetida*. For our experiment, the earthworm is an endogeic species belonging to the Acanthodrilidae family and gender *Balanteodrilus*. Therefore, we consider this genus of earthworm as a potential organism to detect Fe in natural soil.

4.3. Relationship between concentrations of Fe, environmental factors, and biological parameters

Microelements are essential for metabolism in organisms. However, elements above allowed concentrations and exposure times are toxic, and they affect the abundance, diversity, and distribution of animals (Lukkari et al., 2004). Therefore, the excesses, the physicochemical properties of NMs, the properties of the soil, such as the pH, content of OM, soil temperature, the water content in the soil, among others, which alter the processes of transformation, aggregation, agglomeration, dissolution, and bioavailability of the NPs, dictate fate and toxicity in soil organisms (de Santiago-Martín et al., 2016). For our study, there was not an observed relationship between the evaluated concentrations and soil factors on the evaluated biological parameters. What we observed was a relationship among earthworms biomass, clitellata class, and Fe concentration in the soil. These results give us a new indication that earthworms are ideal organisms for determining the quality and integrity of soil metal-contamination. Nevertheless, as various authors have mentioned, the toxic or non-toxic effect on soil biota depends on several factors. However, in relation to the date, there is a lack of investigations that demonstrate the effects of Fe NPs on earthworms in forest areas.

From the ecological point of view, earthworms contribute more than 60% of the biomass of the macroinvertebrate group and are at the base of many food chains. Thus, it is not only necessary to understand the accumulation of NPs in natural scenarios, but also to understand the risks that can represent secondary populations. Therefore, if the NPs are taken

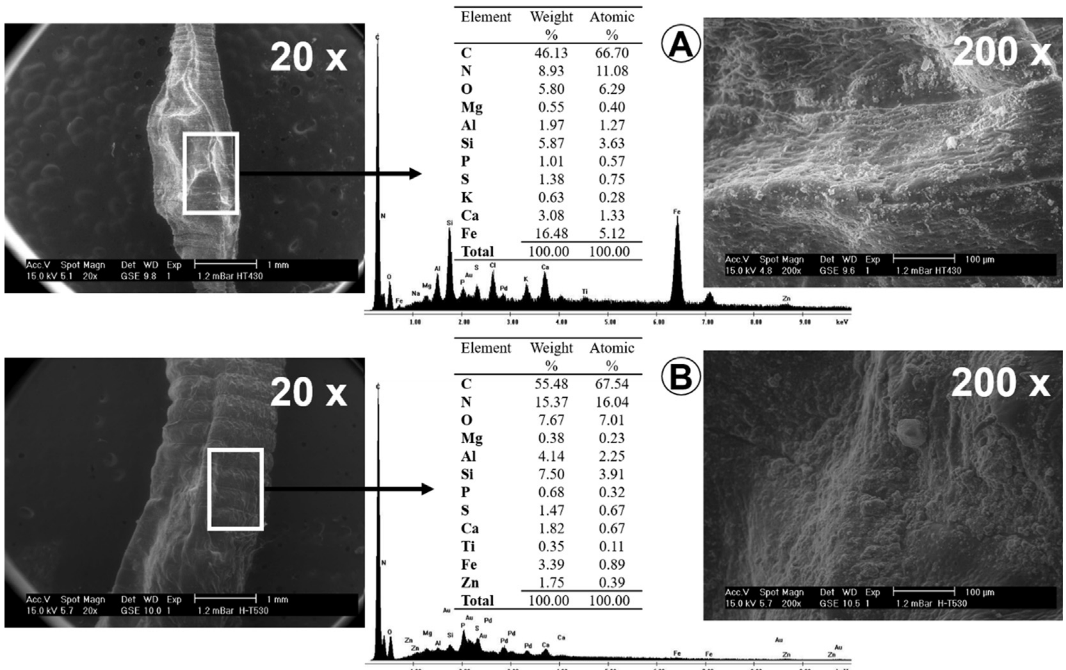

Figure 11. SEM and EDX analysis in earthworms (**A**) exposed to 1000 mg kg^{-1} of Fe NPs, and (**B**) the control treatment at 30 days after exposure. The EDX spectrum was measured at 15 keV.

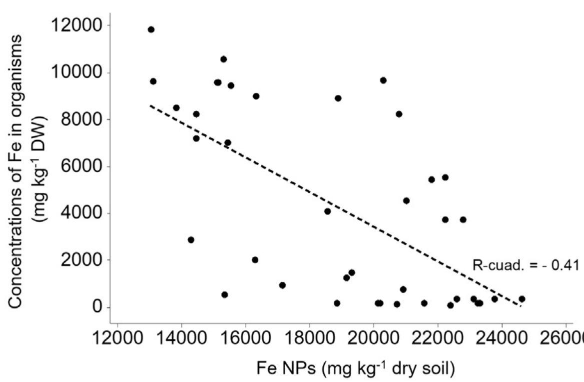

Figure 12. Relationship between Fe concentrations in organisms (mg kg^{-1} dry weight [DW]) and Fe concentrations in soil (mg kg^{-1} DW) at 60 days after exposure to Fe NPs.

up into the earthworms, they can facilitate the movement into the food web via bioaccumulation and bio-magnification processes (Shore et al., 2014). Nevertheless, the toxicity mechanisms of metal oxide NPs, included Fe NPs, are not precisely known. Therefore, for future research work under field conditions, an adequate methodology capable of efficiently demonstrating that earthworms can be potential organisms to determine low or high amounts of Fe NP must be implemented.

5. Conclusions

This study shows the avoidance behavior of earthworms at concentrations of 1000 mg kg^{-1} of dry soil of NPs of Fe under forest soil conditions. Besides, high concentrations of Fe oxide were detected in earthworms treated with low concentrations (1 and 10 mg kg^{-1}) of Fe NPs because they did not detect the metal and consumed the contaminated soil for 60 days. The level of NPs in the soil remains constant under natural conditions up to 60 days when the soil was treated with 1000 mg of NPs of Fe kg^{-1} of dry soil. Besides, the present experiment showed that at low concentrations of Fe NPs, earthworms indicate the presence of Fe in forest soil conditions. By contrast, the content of Fe in organisms is inversely proportional to increasing concentrations in the soil. Therefore, endogeic worms could be used as a bioindicator organism of the presence of Fe NPs. However, to implement evaluation methodologies in natural conditions, it should be considered that organisms avoid NPs at high concentrations. Therefore, for future field toxicology studies, it is suggested to include macrocosms in natural soil. It will allow organisms to remain in contact with NPs and thus be able to make comparisons with areas or experimental units in bare soil, as raised in the present experiment.

Declarations

Author contribution statement

Hermes Pérez-Hernández: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fabián Fernández-Luqueño: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Esperanza Huerta-Lwanga, Jorge Mendoza-Vega: Conceived and designed the experiments; Analyzed and interpreted the data.

José David Álvarez-Solís, Edilberto Hernández-Gutiérrez, Javier Francisco Valle-Mora, Marcos Pérez-Sato: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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