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Influence of ZnO Nanoparticles and a Non-Nano ZnO on Survival and Reproduction of Earthworm and Springtail in Tropical Natural Soil

Micheli Lucia Alves[(1)], Luís Carlos Iuñes de Oliveira Filho[(2)], Patrícia Nogueira[(3)], André Junior Ogliari[(1)], Márcio Antônio Fiori[(1)], Dilmar Baretta[(4)] and Carolina Riviera Duarte Maluche Baretta[(1)*]

[(1)] Universidade Comunitária da Região de Chapecó, Departamento de Ciências Exatas e Ambientais, Programa de Pós-Graduação em Ciências Ambientais, Chapecó, Santa Catarina, Brasil.
[(2)] Universidade do Estado de Santa Catarina, Centro de Ciências Agroveterinárias, Departamento de Solos e Recursos Naturais, Programa de Pós-Graduação em Ciência do Solo, Lages, Santa Catarina, Brasil.
[(3)] Universidade de Passo Fundo, Departamento de Agronomia, Programa de Pós-Graduação em Agronomia, Passo Fundo, Rio Grande do Sul, Brasil.
[(4)] Universidade do Estado de Santa Catarina, Centro de Educação Superior do Oeste, Departamento de Zootecnia, Chapecó, Santa Catarina, Brasil.

*** Corresponding author:**
E-mail: carolmaluche@unochapeco.edu.br

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ABSTRACT: In recent years, various studies and development using nanoparticles (NPs) have been carried out in the most diverse areas of knowledge. Although nanomaterials are widely employed by many sectors and some may have a fertilizing potential, little is known about their effects on the environment. This study aimed to evaluate the effect of applying, in tropical natural soil, different contents of nanoparticles of zinc oxide (NPs-ZnO) and non-nano zinc oxide (ZnO) on soil pH and on the survival and reproduction rates of earthworms (*Eisenia andrei*) and springtails (*Folsomia candida*) through standardized ecotoxicological tests. The tests used a tropical soil representative of Brazil, classified as Entisol (*Neossolo Quartzarênico órtico típico*) with no history of agricultural use, collected in the 0.00-0.20 m layer, previously sieved (2-mm mesh) and defaunated. The experimental design was completely randomized, and treatments consisted of two forms of zinc (Zn), NPs-ZnO and ZnO, at the following doses: 0, 50, 100, 200, 400, 800, 2,000, and 4,000 mg kg^{-1}. Standardized ecotoxicological tests showed no toxicity of NPs-ZnO in terms of lethality of *E. andrei* and *F. candida*. In *E. andrei* reproduction tests, NPs-ZnO were toxic at doses higher than 400 mg kg^{-1} (EC_{50} of 1,021 mg kg^{-1}). Tests with *F. candida* demonstrated that its reproduction rate was significantly affected by NPs-ZnO at a rate of 4,000 mg kg^{-1} (EC_{50} of 3,636 mg kg^{-1}). When used in Entisol, the NPs-ZnO inhibit the reproduction of earthworms and springtails; earthworms are more sensitive to such an effect, it being demonstrate at lower contents than those found for springtails.

Keywords: nanotoxicity, ecotoxicological tests, soil fauna, Entisol.

INTRODUCTION

Nanotechnology refers to technological applications of objects and devices with at least one of their physical dimensions between 1 and 100 nm (Lêdo et al., 2007; Batley et al., 2013), with a wide range of opportunities and possibilities for utilization. Although nanotechnology is widely employed by many segments, including the industry of pharmaceuticals, electronics, computers, automobiles, and more than 1,800 consumables (Bour et al., 2015), a better understanding of its potential of release to the environment is still sought.

In agriculture, nanomaterials started to be used approximately one decade ago (Gogos et al., 2012; Buzea and Pacheco, 2017), using as fertilizers, plant protection products, and for soil improvement, water purification, and pollutant remediation (Parisi et al., 2015), among other possibilities of application. Nanoparticles (NPs) are mainly applied in the form of an aerosol or as fertilizer directly to the soil (Sturikova et al., 2018), and seeds are soaked in aqueous NP suspension (Lin and Xing, 2007; Segatto et al., 2018). However, after release into soils, little is known about the dissociation behavior, toxicity, and risk of NPs to organisms in natural soil. Features that make NPs interesting from the technological application point of view may be undesirable when they are released into the environment because their small size facilitates diffusion and transport in the soil (Quina, 2004).

Without legislation on regulation of the use of NPs in agriculture, Brazil, as one of the largest grain producers in the world and with its economy significantly represented by the agro-industry, offers a wide range of opportunities for research and innovation using nanomaterials. Zinc oxide (ZnO) is one of the most used types of metal-based NPs, with the third largest annual production in volume (Merdzan et al., 2014; Romero-Freire et al., 2017) and a wide range of application, from antibacterial agent (Ma et al., 2013) to fertilizer (Parisi et al., 2015; Segatto et al., 2018).

Zinc is an essential element for organisms and plants, acting as cofactor for a variety of macromolecules, including enzymes, transcription factors, and cell signaling proteins, besides playing an important role in the stabilization and protection of biological membranes against oxidative stress and promoting the structural stability of various cell proteins (Borkert et al., 1998; Malavolta, 2006). Mobility, bioavailability, and distribution of Zn in soils are controlled by physicochemical properties including soil pH, redox potential, and surface charge of colloids (Donner et al., 2010; Romero-Freire et al., 2017), which influence the interactions between NPs and the soil matrix, modifying their availability and toxicity potential (Pan and Xing, 2012; García-Gómez et al., 2014).

Soil contains a wide diversity of edaphic organisms responsible for maintaining the biological processes underlying the ecosystem services provided by it. The monitoring of anthropic practices, such as utilization of nanomaterials, should also consider biological parameters as a fundamental indicator measured in studies that use natural soils capable of demonstrating, more realistically, the effect of using NPs and their effect on soil organisms.

A growing number of studies on the toxicity of NPs to soil organisms has been published in recent years, assessing the effects of short-term NP exposure on the earthworms *Eisenia fetida* (Heggelund et al., 2014; Yausheva et al., 2016), *E. andrei* (Velicogna et al., 2016; Romero-Freire et al., 2017) and *Lumbricus rubellus* (Lapied et al., 2011), the springtail *Folsomia candida* (Kool et al., 2011; Waalewijn-Kool et al., 2012, 2013, 2014; Velicogna et al., 2016), the isopods *Porcellio scaber* (Drobne et al., 2009) and *Porcellionides pruinosus* (Tourinho et al., 2013), the nematode *Caenorhabditis elegans* (Wang et al., 2009), the plants *Elymus lanceolatus*, *Trifolium pratense* (Velicogna et al., 2016), and *Zea mays* (Zhao et al., 2013), and soil microorganisms (Collins et al., 2012; Schlich and Hund-Rinke, 2015). These studies point to NPs being toxic to living organisms in soil; however, their effects are still unknown for Brazilian soils. In addition, more research is needed to provide insight into the ecotoxicological effects of exposure to NPs on organisms living in soil and to establish sound risk assessment for this class of substances (Waalewijn-Kool et al., 2012).

Soil-quality bioindicator organisms, such as earthworms, have been constantly used in tests with nanomaterials as a representative group in the soil (Kwak and An, 2015; Romero-Freire et al., 2017), being more sensitive to pollution by metals compared with other fauna (Spurgeon and Hopkin, 1996).

Hence, springtails represent an important group of invertebrates that inhabit the soil in different terrestrial ecosystems, are involved in organic matter decomposition and act as a stimulus to microbiological activity and nutrient cycling (Faber, 1991); they are also used in studies with NPs (Kool et al., 2011; Waalewijn-Kool et al., 2013; Lopes et al., 2017).

Due to the complex behavior of NPs in the soil, achieving realistic exposure in ecotoxicity testing poses major challenges (Kool et al., 2011). The hypothesis is that, in natural soil, nanoparticles of zinc oxide (NPs-ZnO) are more toxic than ZnO, directly affecting the survival and reproduction of soil organisms. The present study aimed to evaluate the effect of applying, in tropical natural soil, different contents of NPs-ZnO and a non-nano zinc oxide (ZnO) on the survival and reproduction rates of earthworms (*E. andrei*) and springtails (*F. candida*) through standardized ecotoxicological tests (ISO).

MATERIALS AND METHODS

Test materials

In the present study, NPs-ZnO with a mean size of 20 nm were synthesized at the Laboratory of Multifunctional Materials of the Universidade Comunitária da Região de Chapecó (Unochapecó), Chapecó, Brazil, in collaboration with the Laboratory of Materials and Corrosion of the Chemical Engineering Graduate Program of the Universidade Federal de Santa Catarina (UFSC), Florianópolis, Brazil.

Non-nano ZnO was used as a control compound (Sigma-Aldrich - 99.9 % purity). The shape and size of NPs-ZnO were evaluated by field-emission gun-scanning electron microscopy - FEG-SEM (Figure 1a), whereas their chemical composition was evaluated by energy dispersive spectroscopy - EDS (Figure 1b). Nanoparticles prepared in the form of powder had the shape of a rod with varied dimensions, a crystalline structure corresponding to wurtzite ZnO, and a high purity level, with a crystallite size of 1.99 nm calculated using the equation of Scherrer (1918), and thermal stability up to temperatures of 900 °C.

Test soil

The soil used in the test was a *Neossolo Quartzarênico órtico típico* (Santos et al., 2013), which corresponds to an Entisol (Soil Survey Staff, 2014), with a silty loam texture (further designated by Entisol), collected in the municipality of Araranguá (29° 00’ 19.98” S, 49° 31’ 02.84” W), Southern Santa Catarina State, Brazil. The soil was collected in the 0.00-0.20 m layer, in a forest area with no history of agricultural use, previously sieved (2-mm mesh) and defaunated (two freezing-thawing cycles, 24/24 h). Ecotoxicological tests were validated using a control (Entisol without NPs-ZnO and a ZnO) and tropical artificial soil (TAS) (to control photoperiod and temperature conditions), which consists of a mixture of 70 % industrial fine sand, 20 % kaolinitic clay, and 10 % coconut fiber, dried and sieved (Garcia, 2004). For the tests, moisture content in the soil and TAS was adjusted to 60 % of the maximum water retention capacity (WRC) (ISO, 1998a).

Prior to the tests, chemical properties were determined in the natural soil: pH(KCl) (ratio of 1:2.5 v/v) = 5.5; organic matter = 0.90 %; cation exchange capacity (CEC) at pH 7.0 = 4.92 $cmol_c\ dm^{-3}$; P = 6.7 mg dm^{-3}; K^+ = 34.0 mg dm-^{3}; Ca^{2+} = 2.0 $cmol_c\ dm^{-3}$; Mg^{2+} = 0.83 $cmol_c\ dm^{-3}$; Al^{3+} = 0.0 $cmol_c\ dm^{-3}$; H+Al = 2.0 $cmol_c\ dm^{-3}$; Cu = 1.5 $cmol_c\ dm^{-3}$;

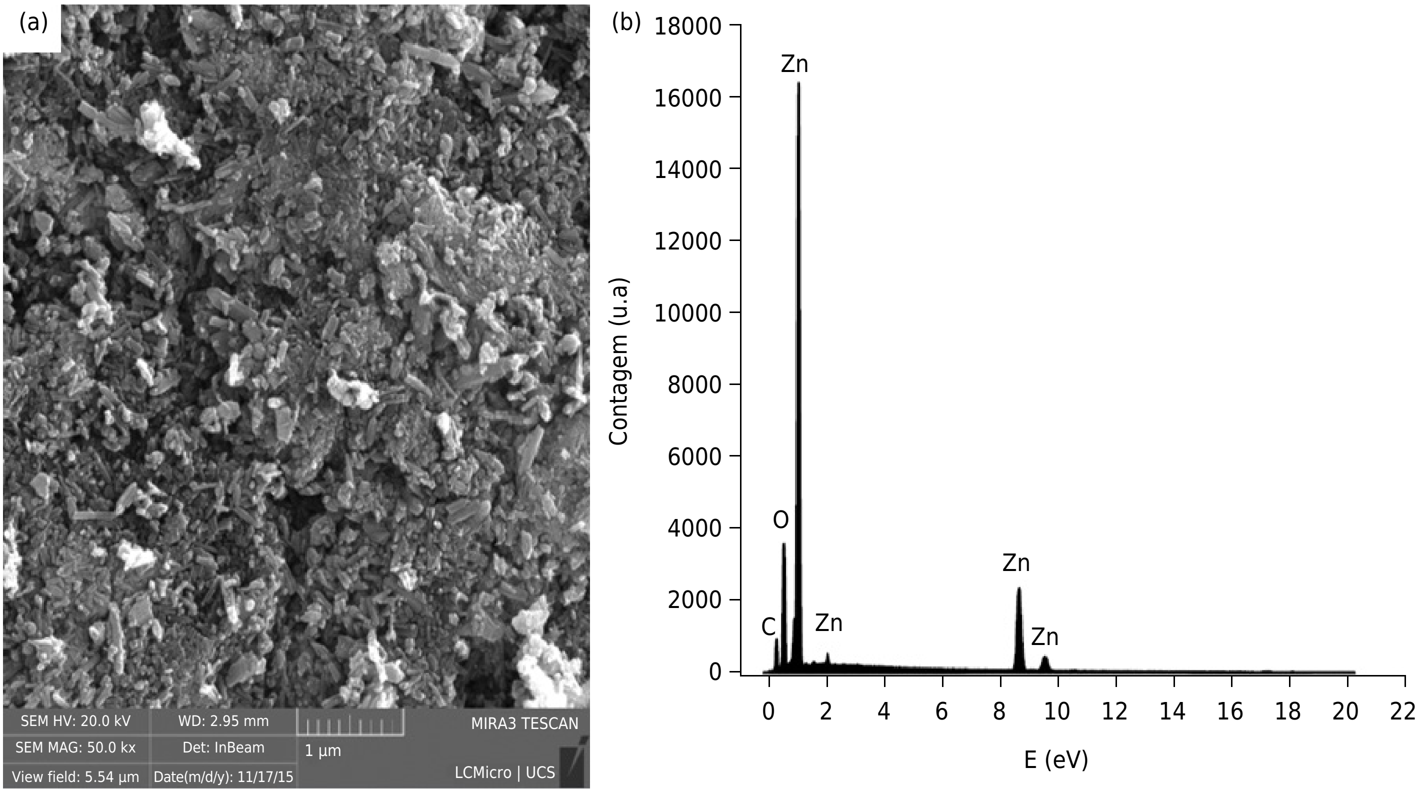

Figure 1. Micrographs obtained by Field Emission Gun-Scanning Electron Microscopy (FEG-SEM) of NPs-ZnO (a) and Energy Dispersive Spectroscopy (EDS) obtained from NPs-ZnO (b).

Zn = 1.0 $cmol_c$ dm^{-3}; Fe= 72.5 $cmol_c$ dm^{-3}; Mn = 2.10 $cmol_c$ dm^{-3}, according to methodology described by Tedesco et al. (1995). Soil granulometry (sand = 37.0 %; loam = 59.0 %; clay = 4.0 %) was determined following the methodology proposed by Donagema et al. (2011).

Lethality and reproduction tests were set in soil with pH adjusted to 6.0±0.5 by adding calcium carbonate ($CaCO_3$), characterizing the average pH of agricultural soils, for which the use of the tested NPs is proposed. Test organisms were evaluated for their adaptation to soil with natural pH (5.5) and to soil with adjusted pH (6.3), in order to rule out the effect of this chemical variable on them.

Evaluation of soil pH

Literature mentions a probable effect of NPs-ZnO and ZnO on the promotion of an increase in soil pH during the incubation period (Zhao et al., 2013; García-Gómez et al., 2014). A test was conducted to evaluate soil pH at the different contents of NPs-ZnO and ZnO tested, measured at the beginning of the test (day zero) and every 7 days during the entire period of the tests (56 days). Such a procedure aimed to monitor the variation of this property, and pH was measured in KCl (ISO, 2005). The test was carried out in a plastic pot (diameter: 14 cm; height: 9 cm), filled with 0.5 kg of fresh soil, with controlled temperature, moisture and photoperiod, and without organisms.

Test organisms

The organisms used in the tests came from the culture already established at the Unochapecó Soil Laboratory. The cultures were maintained according to the guidelines established by ISO 11268-2 (ISO, 1998b) and ISO 112687 (ISO, 1999), with adaptations for the species.

E. andrei (Oligochaeta: Lumbricidae) was the earthworm species used, maintained in plastic boxes containing 1 kg of dried substrate composed of two parts of dried, sieved

(2 mm) horse manure, one part of coconut fiber powder, and 10 % of the weight of these first two components of fine sand (90/100 granulometry). Deionized water was added to the substrates, and the organisms were fed weekly with a cooked mixture of coarse oat flakes and deionized water at 2:1 proportion (v/v). *F. candida* (Collembola: Isotomidae) was the springtail species used in the test, grown in a substrate of gypsum and activated charcoal (11:1) and fed weekly with instant dry yeast (*Saccharomyces cerevisiae*). Moisture in the culture medium was corrected by adding deionized water.

Treatments

The experimental design was completely randomized with five replicates, using as treatments NPs-ZnO and ZnO applied to the natural soil, at the following Zn contents: 0, 50, 100, 200, 400, 800, 2,000, and 4,000 mg kg^{-1} (dry soil). Contents were determined based on the Zn content, according to CONAMA Resolution No. 420 of December 28, 2009 (Brasil, 2009), relative to the limits for contamination with micronutrients in the soil, which considers the values of 300, 400, 1000, and 2,000 mg kg^{-1} (dry soil) for prevention, agricultural use, residential use and commercial use, respectively; and Cetesb (2014), which establishes 60 mg kg^{-1} (dry soil) as minimum guiding values for soil and groundwater. Nanoparticles of zinc oxide and ZnO were added through a watery suspension, followed by homogenization. The suspension containing the NPs was used to correct soil moisture. Soil contamination followed the methodology proposed by Waalewijn-Kool et al. (2012) and Franklin et al. (2007), who demonstrated in previous studies that NPs-ZnO distribution in the soil is not influenced by addition as either dry powder or suspension.

Ecotoxicological evaluations

Tests with E. andrei earthworms

Lethality tests, with a duration of 28 days, and reproduction tests, with a duration of 56 days, were based on the protocol ISO 11268-2 (ISO, 1998b). Each replicate consisted of one plastic pot (diameter: 14 cm; height: 9 cm), filled with 500 g (fresh weight) of soil, with 10 adult earthworms (with noticeable clitellum) weighing between 250 and 600 mg. Earthworms were fed at the beginning of the test and every 7 days with 5 g of humid manure from horses with no history of use of medicines, and a diet based on pasture. At 28 days after the test started, adult organisms were removed and dead individuals were counted, considering as dead those that did not respond to mechanical stimulation of the anterior portion of the body. After removing adult individuals, the containers with contaminated soil and possible cocoons/juveniles remained for more 28 days. On the 56th day, we counted the number of individuals (juveniles) generated during the period in which the adults were present in the soil. Such a count was carried out by placing the containers in a water bath at 65 °C for 1 h, causing the juveniles to rise to the surface.

Tests with F. candida springtails

Lethality and reproduction tests with *F. candida* were based on the protocol ISO 112687 (ISO, 1999), with a duration of 28 days. Each replicate consisted of one plastic pot (diameter: 6.5 cm; height: 6 cm), filled with 30 g (fresh weight) of soil, with the contents tested. Each pot received 10 synchronized adult individuals (10-12 days of age) fed with instant dry yeast (*S. cerevisiae*) at the beginning of the test and at 14 days of incubation. The pots were opened weekly to promote aeration and for correction of moisture.

At 28 days after the test started, the content of each pot was transferred to another container, which received water and a few drops of black ink. After slight agitation, the surviving organisms floated, and the contrast of their color with the ink allowed counting. Living organisms found on the surface were photographed and counted using the software

ImageTool 3.0 (University of Texas Health Science Center at San Antonio, 2002). Adults and juveniles, separated by size, were independently counted.

Statistical analysis

Survival and reproduction data were tested for normality and homogeneity by the Kolmogorov-Smirnov and Cochran-Bartlett tests and then subjected to analysis of variance (One-way ANOVA), followed by Dunnett test ($p<0.05$), using the software Statistica 7.0 (Statsoft Inc., 2004). The LC_{50} values (estimated content expected to cause lethality in 50 % of a group) of the survival tests were obtained using the software PriProbit® 1.63 (Sakuma, 1998). The EC_{50} values (estimated content causing one or more specific effects capable of affecting 50 % of the organisms) were estimated through nonlinear regressions with a hormetic model using the software Statistica 7.0.

RESULTS

Effect of treatments on soil pH

Soil pH was affected by both forms of ZnO tested (NPs and non-nano), and its values increased during the tests (Table 1). After 28 days of the test, pH values in the soil contaminated with NPs varied from 6.20 for the lowest content tested (50 mg kg^{-1}) to 7.26 for the highest content (4,000 mg kg^{-1}). Such an effect was also observed using ZnO, and pH values were equal to 6.75 and 7.30 at the contents of 50 and 4,000 mg kg^{-1} soil, respectively. After 56 days of incubation for ZnO, pH values ranged from 6.40 to 7.10, respectively, for the lowest and highest contents, and from 6.00 to 7.10 in the soils

Table 1. Mean pH(KCl) readings obtained during *E. andrei* and *F. candida* reproduction tests in an Entisol contaminated with nanoparticles of zinc oxide (NPs-ZnO) and non-nano zinc oxide (ZnO)

Content	Day 0	Day 28	Day 56
		pH(KCl)	
mg kg^{-1}			
	NPs-ZnO		
Positive control*	5.58	5.50	5.30
0	6.30	6.33	6.30
50	6.60	6.20	6.00
100	6.49	6.17	6.00
200	6.43	6.65	6.70
400	6.50	6.02	6.10
800	7.00	6.75	6.70
2,000	7.20	6.96	6.90
4,000	7.49	7.26	7.10
	ZnO		
0	6.30	6.33	6.30
50	6.53	6.75	6.00
100	6.61	6.68	6.00
200	6.62	6.24	6.70
400	6.64	6.00	6.10
800	7.42	6.60	6.70
2,000	7.50	7.06	6.90
4,000	7.52	7.30	7.10

* Positive control = uncorrected natural soil. Other soil treatments corrected with $CaCO_3$ (test conduction standard).

contaminated by NPs-ZnO. In the control, no variations were observed in pH, which remained at 6.3 from the beginning to the end of the test (Table 1).

Validation of ecotoxicological tests

The tests of lethality and reproduction for *E. andrei* met the validation criteria based on the respective guideline ISO 11268-2 (ISO, 1998b). No lethality occurred in the TAS (100 % survival). In the reproduction test, the average number of juveniles was 101, with a coefficient of variation (CV) <30 % (8 %). Lethality rate did not exceed 10 % of the total number of individuals in

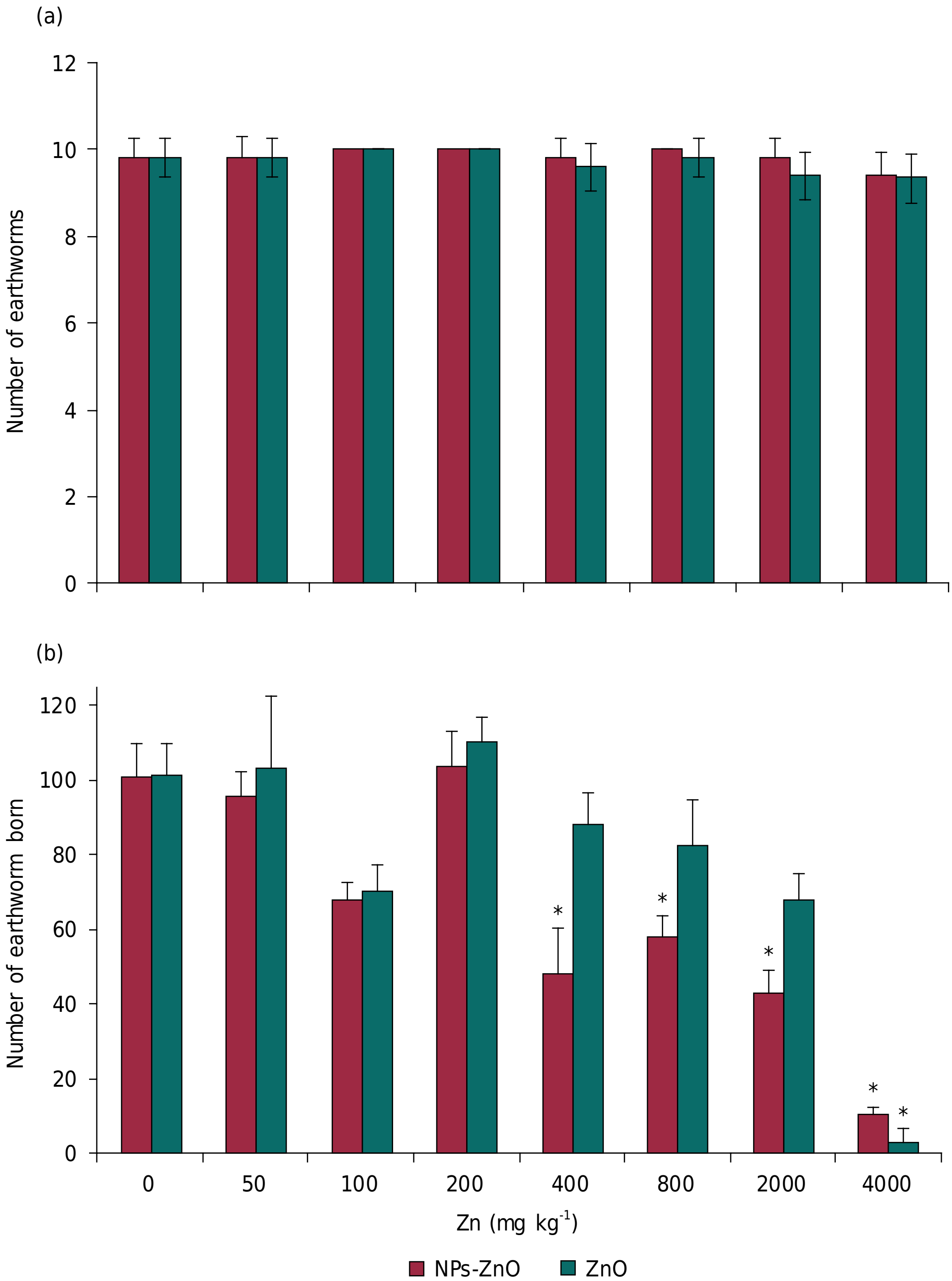

Figure 2. Mean number and standard deviation of live adults (a) and juveniles per treatment (b) *E. andrei*, in an Entisol contaminated with zinc oxide nanoparticles (NPs-ZnO) and non-nano zinc oxide (ZnO). * Significant reduction ($p<0.05$) for treatment (50, 100, 200, 400, 800, 2,000, and 4,000 mg kg^{-1}, dose-dependent effect) compared to the control (0 mg kg^{-1}).

the control (on average, 98 % survival), with CV <30 % (4.5 %). In the reproduction test, the average number of juveniles in the control was 101, with a CV of ± 8.6 %.

The tests of lethality and reproduction for *F. candida* met the validation criteria based on the guideline ISO 11267 (ISO, 1999). No lethality occurred in the TAS (100 % survival). In the reproduction test, the average number of juveniles was 225, with CV <30 % (20 %). Lethality rate did not exceed 20 % of the total number of juveniles in the control (on average, 95 % survival), with CV <30 % (6.1 %). In the reproduction test, the average number of juveniles in the control was 303, with a CV of ± 2.3 %.

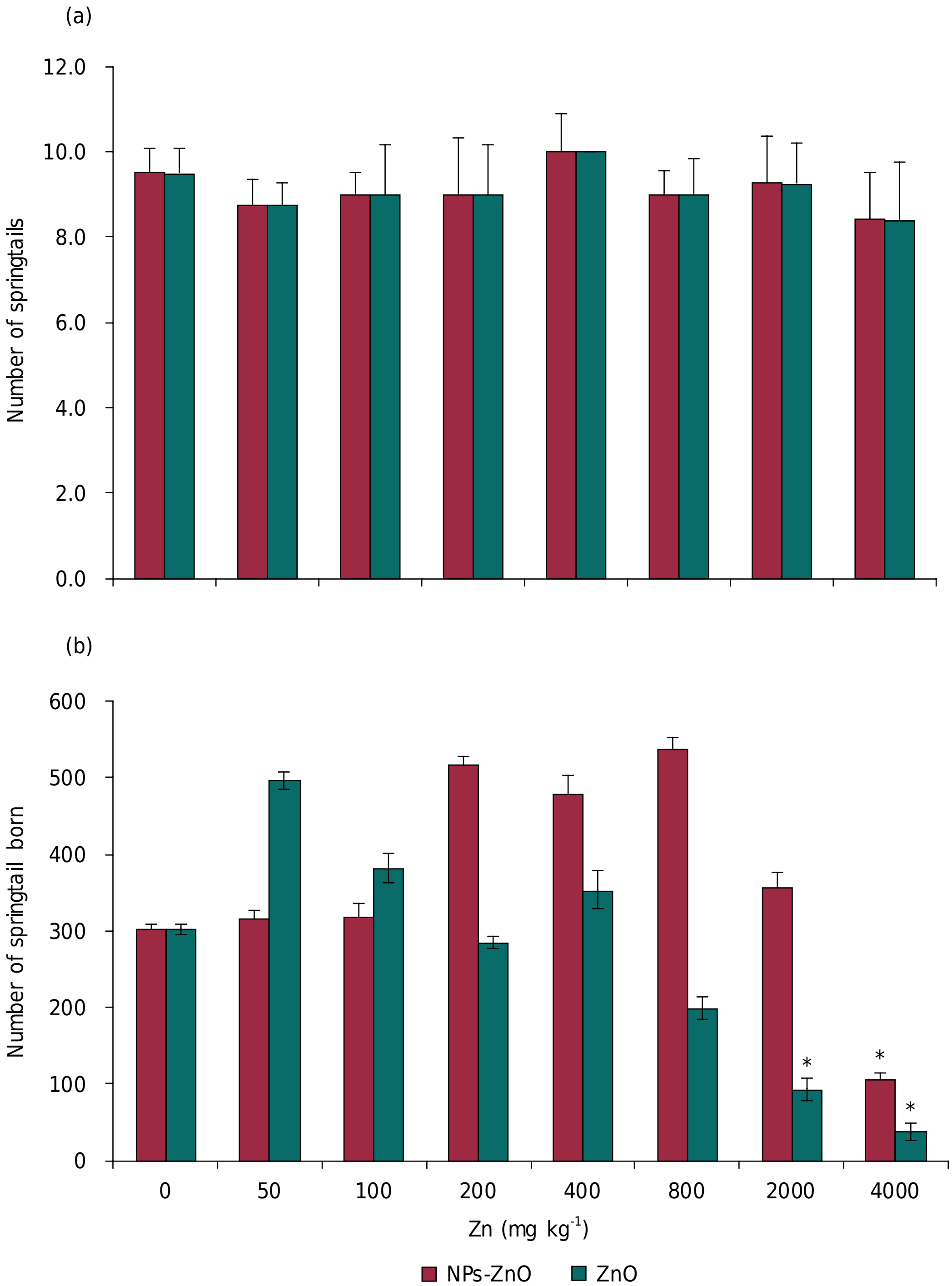

Figure 3. Mean number and standard deviation of mean live adults (a) and juveniles per treatment (b) *F. candida*, in an Entisol Typic Quartzipsamments contaminated with zinc oxide nanoparticles (NPs-ZnO) and non-nano zinc oxide (ZnO). * Significant reduction ($p<0.05$) for treatment (50, 100, 200, 400, 800, 2,000, and 4,000 mg kg^{-1}, dose-dependent effect) compared to the control (0 mg kg^{-1}).

Lethality and reproduction tests with earthworms

Nanoparticles of ZnO and ZnO did not affect earthworm survival (>90 % for all treatments) at any content tested, after 28 days of incubation in the Entisol (Figure 2a). In the present study, it was not possible to calculate the lethal content (LC_{50}), because it was higher than the highest dose tested (4,000 mg kg^{-1} soil).

Earthworm reproduction was significantly reduced ($p<0.05$) from the content of 400 mg kg^{-1} for NPs-ZnO, and was only affected at the highest content (4,000 mg kg^{-1}) for ZnO, compared with the control (Figure 2b). The EC_{50} values and their respective confidence intervals were 1,021 mg kg^{-1} (339-1,703 mg kg^{-1}) for NPs-ZnO and 2,050 mg kg^{-1} (1,283-2,817 mg kg^{-1}) for ZnO.

Lethality and reproduction tests with springtails

No reduction in the survival rate of adult springtails was caused by the contents of NPs-ZnO ($p>0.05$) and ZnO ($p>0.05$) after 28 days of incubation in the Entisol (Figure 3a). Springtail reproduction was significantly reduced at the highest content (4,000 mg kg^{-1}) for NPs-ZnO ($p<0.05$), and hampered at the content of 2,000 mg kg^{-1} for ZnO ($p<0.05$), in comparison to the control (Figure 3b). The EC_{50} values and their respective confidence intervals were 3,636 mg kg^{-1} (2,175-5,097 mg kg^{-1}) for NPs-ZnO and 2,572 mg kg^{-1} for ZnO (confidence interval could not be calculated).

DISCUSSION

Behavior of soil pH

The effect of NPs-ZnO and ZnO on an increase in soil pH has also been found in other studies (Kool et al., 2011; Zhao et al., 2013; García-Gómez et al., 2014; Romero-Freire et al., 2017). Romero-Freire et al. (2017) observed that Zn addition via NPs-ZnO caused an increase in the pH of three different natural soils (LUFA 2.2, NLGA, and SPCA), with different values of organic carbon (1.55, 3.44, and 5.43 %, respectively), CEC (8.19, 18.8, and 21.4 $cmol_c$ kg^{-1}, respectively) and clay content (80.27, 40.80, and 230.6 g kg^{-1}, respectively). These authors found that, over the period of NP exposure to the soil (1 to 168 days), the contents of Zn dissolved in the water contained in the pores (capillary/available water) increased proportionally, with low soil resistance to pH change (> LUFA 2.2). These results were also obtained by Kool et al. (2011) in the presence of NPs-ZnO and ZnO applied in natural soil (LUFA 2.2). An increase in the content of these forms led to increments in pH and Zn contents in the capillary water contained in the pores. Neither of these two studies explained the effect of NPs-ZnO and ZnO on the promotion of this increment.

Increments in pH values in the order of 0.6 and 0.8 caused by NPs-ZnO and ZnO, respectively, compared with the control, were found by García-Gómez et al. (2014). As found in our study (Table 1), the differences in soil pH decreased over time, and the authors attributed this behavior to the slow increment of Zn^{2+} in solution through its release by the tested forms of ZnO (Table 1).

Zinc oxide solubility in water is highly dependent on soil pH, and the highest contents of dissolved Zn are usually found under lower pH conditions (Franklin et al., 2007; Ma et al., 2013; Heggelund et al., 2014; Lebedev et al., 2015; Romero-Freire et al., 2017). Under higher pH conditions, NPs tend to prevail over the form of particulates, normally forming clusters (Romero-Freire et al., 2017), which naturally reduces Zn release potential and, consequently, its toxicity (Pan and Xing, 2012; Ma et al., 2013).

Research data indicate that the solubility of NPs-ZnO and ZnO (<1 nm and >200 nm) is very similar (Tourinho et al., 2012), and soil properties (pH and OM content) are determinant to promote it (Ghosh et al., 2010; Bian et al., 2011). Soluble ionic forms of Zn [Zn^{2+} and $Zn(OH)^{+}$] released from ZnO prevail at a pH lower than 6.0, and the range of values from 6 to 9 is a condition for the formation of Zn precipitates and a reduction in Zn solubility. Besides pH itself, the solubility of NPs is conditioned by soil OM content, and their aggregation increases in soils with a lower OM content, due to the neutralization of charge by the adsorption of humic acids (Bian et al., 2011).

Based on the factors mentioned above, the soil in the present study is ideal to demonstrate the effect of contamination of natural soils in the presence of NPs, especially considering that the class of Entisol is the third most frequent class of soils, with relative distribution of 13.18 % in the Brazilian territory (Santos et al., 2013), besides being representative in Santa Catarina State. Entisol is characterized by a low degree of development, basically sandy texture, low capacity for adsorption of nutrients, and low OM content (Oliveira, 2008; Sales et al., 2010). These conditions allow it to have low buffering power, compared with other soil classes, maximizing the availability of nutrients and/or metals in the solution that could affect the community of edaphic organisms present in the soil.

Effect of soil pH on the tested species

Soil chemical properties can directly affect edaphic organisms and influence a higher or lower availability of contaminants in the soils (Natal-da-Luz et al., 2008). The increment in soil pH caused by the tested forms of ZnO may affect these organisms. However, authors such as Jänsch et al. (2005) reported that *E. andrei* earthworms are tolerant to a diversity of environments and can withstand a pH range from 4 to 9, but prefer neutral or slightly acid pH conditions (between 5 and 7) and soils with a high OM content.

Greater variability in the tolerance to different pH ranges is found for *F. candida*, with values from 3.2 to 7.6 (Jänsch et al., 2005). The pH values found in the different treatments evaluated are within the range of tolerance by earthworms and springtails according to the mentioned authors and do not directly affect the results obtained for the tested organisms.

Lethality and reproduction of *E. andrei*

The study has demonstrated that the use of NPs-ZnO and ZnO does not cause lethality in *E. andrei* earthworms. Similarly, previous studies evaluating the use of NPs-ZnO in natural soils did not find significant mortality of earthworms, confirming that their growth and mortality are not affected by NPs dispersed in the soil (García-Gómez et al., 2014; Kwak and An, 2015; Romero-Freire et al., 2017).

The absence of lethality does not necessarily mean that NPs do not cause damage to the organisms tested. This damage may not directly cause lethality, but lead to disorders and diseases in these organisms, which can be observed in tests such as the reproduction test (García-Gómez et al., 2014; Heggelund et al., 2014), as found in the present study.

A toxic effect of NPs-ZnO on the reproduction of different earthworm species (*E. andrei*, *E. fetida*, and *E. veneta*) has been reported in the literature (Cañas et al., 2011; Heggelund et al., 2014; Romero-Freire et al., 2017). In the present study, *E. andrei* reproduction was significantly affected by the use of NPs, compared with ZnO, and from the content of 400 mg kg^{-1} soil, the number of juveniles decreased by 52.5, 20.8, 57.6, and 89.7 % in comparison to the control (400, 800, 2,000, and 4,000 mg kg^{-1}) (Figure 2b). The reduction in reproduction for ZnO contamination was significantly affected at the highest content (4,000 mg kg^{-1}), and the number of juveniles decreased by 97.2 % compared with the control.

Similar results regarding the greater inhibition of earthworm reproduction by NPs-ZnO, in comparison to ZnO, were found by García-Gómez et al. (2014), comparing the use of NPs-ZnO, ZnO, and $ZnCl_2$ in natural soil contaminated with content equivalent to 1,000 mg kg^{-1} soil. These authors observed that, at the content tested, $ZnCl_2$ caused full inhibition of *E. fetida* fertility. While NPs caused a reduction in fertility of 72 % based on the number of juveniles per cocoon, compared with the control, ZnO led to an increment of 36 % in the number of juveniles. The authors attributed the negative effect of NPs-ZnO, compared with ZnO, to their different capacity to penetrate biological membranes and affect mechanisms of action. Authors such as Romero-Freire et al. (2017) also found a reduction in *E. andrei* reproduction rate using NPs-ZnO in different types of soil, during the incubation period. After 140 days of soil contamination, in the soil with lower capacity to retain elements (low CEC, low OM content, and low clay content), there was a significant reduction in earthworm reproduction compared with the control at the contents of 500 and 1,000 mg kg^{-1}.

Data demonstrating that reproduction is a more sensitive parameter than the survival of earthworms exposed to ZnO were found by Heggelund et al. (2014). These authors compared NPs-ZnO and ZnO applied in natural soil at different contents (238, 381, 610, 976, 1,520, and 2,500 mg kg^{-1} for NP, and 381, 976, and 2,500 mg kg^{-1} for ZnO) and pH conditions (5.2, 6.4, and 8.2), and observed a reduction in *E. fetida* reproduction. In the present study, EC_{50} values were estimated at 1,020.66 mg kg^{-1} for NPs [close to those found by Heggelund et al. (2014)] and 2,049.83 mg kg^{-1} for ZnO. The difference between the tolerance contents found in the present study for ZnO and those found by Heggelund et al. (2014) may be associated with the type of soil used and its characteristics that influence the availability of the metal. In artificial soil, Lock and Janssen (2003) found EC_{50} values of 764 mg kg^{-1} (426-1,030 mg kg^{-1}) using ZnO. For *E. veneta*, Hooper et al. (2011) found a reduction in the reproduction of 50 % at contents of 764 mg kg^{-1} for NPs.

Various factors such as dimensions, content, and soil properties affect the bioavailability and bioaccumulation of metals (Hobbelen et al., 2006; Heggelund et al., 2014; Lebedev et al., 2015). The potential for dissociation over time and the mechanisms of exposure to contaminants of earthworms may affect bioaccumulation of metals in these organisms, affecting more relevant ecological parameters such as their reproduction capacity, as observed in the present study.

Bioaccumulation of NPs by earthworms has been described in the literature (Canesi and Procházková, 2014; Heggelund et al., 2014; Romero-Freire et al., 2017) and is related to the numerous mechanisms of contact these organisms may have with contaminants including NPs. Earthworms are in permanent contact with soil particles and soil microorganisms, either by contact with their skin, due to their large body area, or by their food habit, with daily ingestion of large amounts of soil (Jager et al., 2003; Drake and Horn, 2007; Roubalová et al., 2015); besides that, they are contaminated by both soil particles and capillary water (Tourinho et al., 2012).

When NPs are ingested, they may get stuck in the digestive tract and not be absorbed, but promote physiological changes that cause damage to the organism, such as a decrease in the absorption of nutrients (Bour et al., 2015). Hooper et al. (2011) raised the possibility that a fraction of Zn accumulated in *E. veneta* organisms through NPs is present in the nano-form, remaining intact inside the cell but still affecting its metabolism. Although NPs are intact inside the cells, their accumulation causes disorders in the cells, both in dissociated form and as clusters, causing toxicity through the formation of reactive oxygen species (ROS), as found by Dimkpa et al. (2011) and Heggelund et al. (2014).

Lethality and reproduction of *F. candida*

The effects of soil pH on *F. candida* were studied by Greenslade and Vaughan (2003), who found a reduction in survival and reproduction from pH 5.38 to 3.47. Additionally, according to Sandifer and Hopkin (1996), van Straalen and Verhoef (1997), and Greenslade and Vaughan (2003), *F. candida* reaches maximum reproduction in soils at pH 5.5, with reduction above or below this value. Tests with the evaluated organisms in control soil with corrected pH (6.3) did not have an effect on *F. candida* mortality and reproduction. Although this variable did not have a direct effect on the organisms, it may have affected the dissociation of NPs, with an effect on their toxicity.

There were no negative effects of NPs-ZnO and ZnO application on *F. candida* survival at 28 days at the maximum content tested (4,000 mg kg^{-1}). The same result was found by Kool et al. (2011), who evaluated the toxicity of NPs-ZnO (<200 nm) in natural soil (LUFA) with pH 5.5, and observed that *F. candida* survival at 28 days was not affected by NPs-ZnO and ZnO at contents up to 6,400 mg kg^{-1}. On the other hand, these authors observed a dose-dependent reduction in reproduction, with EC_{50} values at 28 days of 1,964 and 1,591 mg kg^{-1} for NPs-ZnO and ZnO, respectively (Kool et al., 2011). Despite that, in our study, EC_{50} values were higher than those found by these authors (3,636 mg kg^{-1} for NPs-ZnO and 2,572 mg kg^{-1} for ZnO). It is also worth highlighting that juvenile springtails are more sensitive than adults to the presence of contaminants in the soil solution (Scott-Fordsmand and Krogh, 2005), which may be related to the negative effects on reproduction since lethality was not significant compared with the control.

Another factor that may be related to the higher toxicity of Zn, compared with NPs-ZnO in our study, is the trend of NPs to form clusters at higher pH values (Pan and Xing, 2012; Ma et al., 2013; Romero-Freire et al., 2017). The behavior of NPs in soil is a complex process, due to their aggregation/agglomeration (Quik et al., 2010), and the most important soil properties determining the equilibrium partition of metals in the soils are the adsorption phases (clay, organic matter, and hydroxides), number of available sorption sites (CEC) and pH (Janssen et al., 1997).

Various studies (Tourinho et al., 2013; Heggelund et al., 2014; Waalewijn-Kool et al., 2014; Lebedev et al., 2015; Romero-Freire et al., 2017) have reported that toxicity of NPs-ZnO depends on soil properties, but that the main factor of their toxicity is related to pH, since at more basic pH NPs tend to prevail over the form of particulates (Romero-Freire et al., 2017), which reduces their toxicity. This fact is consistent with the results found in the present study because only at very high contents did the applied Zn forms compromise *F. candida* reproduction, substantially exceeding the levels expected in the environment.

CONCLUSIONS

It is concluded that in tropical natural soil (Entisol), the use of NPs-ZnO and ZnO promotes an increase in soil pH and does not affect the survival of *E. andrei* earthworms and *F. candida* springtails.

Effects on the reproduction of these organisms were observed, and earthworms were more sensitive to the toxicity caused by NPs-ZnO than springtails, probably due to their numerous routes of contamination (body surface and ingestion).

Despite the effects on *E. andrei* and *F. candida* reproduction, the contents causing an effect greatly exceed the expected levels of NPs-ZnO and ZnO in the environment.

Further studies should be conducted using other tropical soils, at different contents, also evaluating the dissociation behavior of these NPs in the soil, to increase the level of knowledge and improve understanding of their response to the environment.

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