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Fluoroquinolone-contaminated poultry litter strongly affects earthworms as verified through lethal and sub-lethal evaluations

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ABSTRACT

Poultry litter is one of the main sources of fluoroquinolones (FQs) in agricultural soils. In this study, our main goal was to investigate FQ-contaminated poultry litter effects on *Eisenia andrei* earthworms. To achieve this, acute and chronic tests covered several endpoints, such as avoidance, biomass, lethality, reproduction and changes to immune cells. FQs (enrofloxacin and ciprofloxacin) were determined in a poultry litter sample through high performance liquid chromatography with a fluorescence detector. The avoidance test indicates that poultry litter strongly repels earthworms, even at the lowest concentration (50 g kg^{-1}). In the acute test, the lethal concentration of poultry litter to 50% of the earthworms (LC_{50}), was estimated at 28.5 g kg^{-1} and a significant biomass loss ($p < 0.05$) occurred at 40 g kg^{-1}. In the chronic test, a significant reproduction effect was observed at 20 g kg^{-1}. Cell typing, density and feasibility indicated significant effects ranging from 5 to 20 g kg^{-1}. A high risk quotient was estimated based on recommended poultry litter applications in field studies. Although FQ contamination in poultry litter and soils has been widely reported in previous studies, this is, to the best of our knowledge, the first toxicological assessment concerning earthworms exposed to FQ-contaminated poultry litter.

1. Introduction

Global poultry production has increased sharply in recent decades, from 9.0 to over 100 million tons between 1961 and 2019 (FAO, 2020; USDA, 2020). In 2018, the United States, Brazil and China, were responsible for 46% of the global poultry meat production (ABPA, 2019). Among top producer countries, Brazil is noteworthy as the largest exporter (4.1 million tons in 2018), with an estimated production increase of almost 30% from 2018 to 2028 (ABPA, 2019; MAPA, 2018).

With the growing demand for low-cost animal protein, the production of chicken eggs and poultry meat tends to increase, contributing to increases in poultry litter production, the main poultry farming waste (Chadwick et al., 2015; Miranda et al., 2015). Poultry litter is widely applied as agricultural fertilizer due to its high micro and macronutrient contents and the fact that it improves soil attributes, such as pH, water holding capacity and organic matter (Bolan et al., 2010; Vollú et al., 2018). Recycling nutrients from agricultural waste, such as poultry litter, is crucial for sustainable food production and for increased resilience of agricultural systems, advocated by the United Nations - Sustainable Development Goals (United Nations, 2015).

On the other hand, poultry litter can also be a source of contaminants for agricultural environments, including veterinary drugs, mainly antibiotics used as growth promoters and for prophylactic and therapeutic purposes (Ho et al., 2014; Leal et al., 2012). The continuous application

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of poultry litter as a soil fertilizer is associated with the contamination of food crops and the occurrence of antibiotic-resistant genes in soils (Chung et al., 2017; Parente et al., 2019a; Tasho and Cho, 2016). In addition, previous studies have reported bioaccumulation and negative effects on non-target soil organisms associated to contaminants commonly found in poultry litter and animal manure, such as pesticides and veterinary antibiotics (Li et al., 2015a,b; Zortéa et al., 2015). Among soil organisms, earthworms are key soil quality indicators, as they play an important role in organic matter mineralization and in the maintenance of soil porosity and structure through burrowing (Blouin et al., 2013; Lavelle et al., 2006). Li et al. (2016) observed decreased burrowing activity and respiration in *Eisenia fetida* earthworms exposed to 10 mg kg^{-1} of enrofloxacin (ENR), an antibiotic commonly applied in poultry farming. Using more sensitive endpoints in assays with the same species, Dong et al. (2012) observed DNA damage and oxidative stress through increased activity of enzyme biomarkers (superoxide dismutase and catalase) induced by tetracyclines at 3.0 mg kg^{-1}. Ciprofloxacin (CIP) alters antioxidants enzymes (Wang et al., 2018; Yang et al., 2020) and promotes DNA damage in *E. fetida* (Yang et al., 2020). A CIP concentration of 10 mg kg^{-1} was able to induce stress in earthworms due to upregulated SOD mRNA levels after exposure (Yang et al., 2020). In addition, CIP caused increases in carbonylated proteins and upregulated proteases from 6.4 to 12.8 mg kg^{-1} CIP (Wang et al., 2018). Previous studies have also performed toxicity and bioaccumulation assays in earthworms exposed to binary mixtures of antibiotics and metal ions (Gao et al., 2015, 2014; Li et al., 2016; Wen et al., 2011). However, considering that millions of tons of poultry litter are released annually in agricultural soils, assessments regarding earthworm exposure to this complex matrix are required. In addition, several recent studies have associated poultry litter to fluoroquinolone (FQ) soil contamination due to the recurrent administration of this class of antibiotics in poultry farming (Leal et al., 2012; Li et al., 2014; Mu et al., 2015; Parente et al., 2019a; Sun et al., 2017; Wu et al., 2014; Zhang et al., 2016). Therefore, the aim of the present study was to evaluate the potential toxicity of FQ-contaminated poultry litter in *Eisenia andrei* earthworms. To achieve this, acute and chronic assays were carried out with poultry litter samples collected from a poultry shed after treatment with fluoroquinolone enrofloxacin. The tests covered a variety of parameters, from both behavior and lethality in acute tests, to more subtle assessments, such as reproduction and immune system cell changes (cell density, feasibility and typing, e.g. amoebocytes and eleocytes) after chronic exposure.

2. Material and methods

2.1. Standards and reagents

Enrofloxacin (ENR) and ciprofloxacin (CIP) standards, both ≥ 98% purity, were purchased from Sigma-Aldrich (Saint Louis, MO, U.S.). Acetonitrile, ethanol, methanol (Tedia® - USA) and all other chemicals - NH_4OH, $MgSO_4$, $Mg(NO_3)_2$, HNO_3, H_3PO_4, KH_2PO_4, NaCl, KCl, Na_2HPO_4 and $NaHCO_3$ were of analytical grade from Merck (Darmstadt, Germany) and Sigma-Aldrich (U.S.). An ultra-pure water Milli-Q® System (18.2 MΩ cm – high purity deionized water), Millipore/Merck (Darmstadt, Germany) with a 0.22 μm filter was used to prepare all aqueous solutions. FQs stock standard solutions (1000 mg L^{-1}) were prepared in acetonitrile with NH_4OH (2%) and stored at −18 °C.

2.2. Poultry litter sample, soil characteristics and test-organisms

The poultry litter pool sample, composed by 10 sub-samples, was collected from a poultry shed where ENR antibiotic was administered for therapeutic purposes during two production cycles. ENR administration was based on a Baytril® 10% Oral Solution (Bayer AG, Leverkusen, German) prescription, which recommends an ENR dose of 10 mg kg^{-1} body weight and a standard treatment period of five days. The shed area is 800 m^2, and 10,000 broilers were raised during each production cycle (50 days). An aliquot of each subsample was separated to compose a pooled sample to determine FQ concentrations. The sample, consisting mainly of *Eucalyptus* spp. sawdust and digested maize and soybean, was collected before its application as a fertilizer in agricultural soils.

The poultry farm (22°11′42.17″S; 42°55′32.39″O) is situated in São José do Vale do Rio Preto (SJVRP), in the upland region (220 km^2 and 615 m.a.s.l., mean elevation) of the Rio de Janeiro (RJ) state, in southeastern Brazil. SJVRP is the most important poultry pole in Rio de Janeiro, comprising about 100 poultry farms surrounded by agricultural areas of significant relevance in supplying fresh vegetables (e.g. tomatoes, chayote, zucchini, eggplant, cucumber) to the Rio de Janeiro metropolitan region.

The soil used in the tests was a Red-Yellow Ultisol according to the Brazilian System of Soil Classification (EMBRAPA, 2013). This soil is one of the most representative Brazilian soils and is composed of sand (605 g kg^{-1}), silt (107 g kg^{-1}) and clay (288 g kg^{-1}). No soil acidity adjustment was required, while moisture was corrected and maintained at 60% (OECD, 1984; USEPA, 1996).

The test-organisms, *Eisenia andrei* earthworms, were cultivated and maintained in the Toxicology Laboratory at the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil), at a relative humidity at 50% field capacity and optimal temperature of 25 ± 2 °C. Only adults were selected for the assays, with well-developed clitella and weight ranging from 300 to 600 mg (ISO, 2012). Before the tests, the earthworms were acclimated at 20 ± 2 °C for 24 h, individually washed (type 1 water) and weighed on an analytical balance (Sartorius MC1). Every three months, earthworms were exposed to chloroacetamide through an acute contact test to assess sensitivity by LC_{50} estimations. An LC_{50} of 5.1 ± 0.28 μg cm^{-2} for chloroacetamide was determined (OECD, 1984).

2.3. Analytical procedures with the poultry litter sample

2.3.1. ENR and CIP antibiotic measurements

The poultry litter (pooled sample) was frozen (−80 °C), freeze-dried, homogenized and stored, protected against sunlight and humidity. ENR and CIP analyses were conducted at the Radioisotope Laboratory (*Laboratório de Radioisótopos*) (Universidade Federal do Rio de Janeiro, Brazil). The FQ extraction method was adapted from Turiel et al. (2006). Briefly, 1.0 g of the sample was extracted with 8 mL of an aqueous $MgNO_3$ 50% (w/v) 4% NH_4OH solution in an ultrasonic bath. Extracts were cleaned-up by centrifugation (10 min with 3400 rpm) and the supernatants were filtered using Millipore™ 40 mm syringe filters (Darmstadt, Germany) (Leal et al., 2012). FQs were determined using a high-performance liquid chromatograph (HPLC) system (CBM-20 A) with a quaternary pump (LC-10ATVP) and a fluorescence detector (RF-10AXL) (Shimadzu Corp., Japan). The analytical columns used for the detections were a guard column C18, 10 × 4.0 mm, 5.0 μm and a C18 column, 250 × 4.6 mm, 5.0 μm, both from Kromasil® (Sweden). An isocratic elution was carried out with a flow rate of 1.0 mL min^{-1}. The mobile phase was composed of 0.02 M *o*-H_3PO_4:ACN (80:20) (Uslu et al., 2008). The excitation and emission fluorescence wavelengths were set at 280 and 450 nm, respectively. The extraction and analysis procedures were performed in duplicate. Recoveries and coefficients of variation were 81.7% (5.4) for ENR and 75.4% (3.4) for CIP. Limits of quantification were 171 μg kg^{-1} (ENR) and 271 μg kg^{-1} (CIP).

2.3.2. Trace elements and chemical characterization

The elements calcium (Ca), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), lead (Pb) and zinc (Zn) were extracted from samples (poultry litter and soil) using 1.0 mL bidistilled nitric acid (HNO_3) followed by heating at 100 °C for 4 h. This method does not require hydride generation, as the heating is carried out in closed vessels and there is no loss of volatile elements, in contrast to open vessel methods (USP, 2013). After cooling, the samples were adequately diluted with ultra-pure water (Milli-Q® System). The analyses were

carried out on a NexIon 300x (Perkin Elmer-Sciex, USA) inductively coupled plasma mass spectrometry (ICP-MS) system. Equipment conditions were set as radiofrequency power 1100 W, plasma flow 17.0 L min $^{-1}$, auxiliary gas flow 1.2 L min $^{-1}$, carrier gas flow 0.98 L min $^{-1}$, skimmer and sample Pt, dwell time 70 ms per isotope, scanning mode peak hopping, resolution 0.7 u and five replicates. The certified materials from the National Institute of Standards and Technology (NIST SRM 2586 - Trace Elements in Soil) and the National Research Council of Canada (NRC DORM-4) were used for quality control. Recoveries ranged from 71 to 127%. These values meet the accuracy criteria of analyses performed by internal standard calibration, where recoveries should range between 70 and 130% (EPA, 2000). Limits of quantification ranged from 0.002 to 0.88 mg kg^{-1} for Cd and Fe, respectively. More details of soil and poultry litter chemical characterization and quality control are given in the Supplementary material.

2.4. Ecotoxicological assessments

2.4.1. Avoidance test

The avoidance test was performed according to protocol N^{o} 17512-1 from the International Organization for Standardization (ISO, 2011). The soil was mixed with poultry litter at the following concentrations: 50, 100, 150, 200, 250, 500, 750 and 1000 g kg^{-1}. A randomized experimental design was employed, consisting of five replicates for each treatment. Briefly, 200 g of soil were put in a container divided into two sections, the first containing soil and the second section containing soil added with poultry litter (different concentrations), both separated by a partition. Control groups without poultry litter on both sides were carried out with ultrapure water in two soil sections. The dividing line was removed and 10 earthworms were deposited in the container, allowing for free movement. The tests were conducted in at 20 ± 1 °C for 48 h. The containers were closed with micro perforated tissue, allowing oxygenation and preventing earthworm escape. The water content of the soil was maintained at 30%. The earthworms were not fed during the test. After 48 h of exposure, the organisms present in each soil section were counted. The avoidance response to different treatments was obtained through Equation (1) (ISO, 2011).

$$RL = \frac{C - T}{N}\ x\ 100 \quad (1)$$

where, RL = net avoidance response (%); C = total worms in the control soil; T = total worms in the treated soil; and N = total number of exposed worms.

A positive net response (RL) indicates “avoidance”, while a negative net response means “no answer” or “attraction” for the tested matrix. The avoidance effect is considered at a leakage between 20 and 80%, while >80% of avoidance indicates a habitat loss response.

2.5. Acute and chronic tests

Acute and chronic tests were performed as described below (USEPA, 1996b; ASTM, 2012; OECD, 2015). The experiments were performed under controlled temperature (20 °C), brightness (400 lx) and photoperiod (12 h:12 h) conditions. To avoid excess of contaminated waste generation, the poultry litter was added to 400 mL beakers containing 200 g of soil. The acute test evaluated 14 days of exposure at 20, 40, 60, 80 and 100 g kg^{-1} poultry litter concentrations. After homogenization, 10 worms were added to each becker, using five replications. Mortality was verified at 7 and 14 days, and biomass variations were determined at the beginning and at the end of the experiment. Earthworms were considered dead when no respond to a gentle mechanical stimulus. Based on the 14 d-LC_{50} of acute toxicity tests, three different concentrations of poultry litter were set to study the effect of cytotoxicity and reproduction of *E. andrei*. For the chronic test, 10 earthworms comprising six replicates were exposed to 5, 10 and 20 g kg^{-1} of poultry litter during 8 weeks. The soil received weekly humidity corrections kept in 30% and 5 g of unground dry cattle manure were applied to the surface as earthworm feed. The beakers were covered with microperforated paraffin to allow oxygenation and avoid earthworm escape. Biomass was measured once a week and reproduction effects were assessed by counting cocoons and juveniles by wet sieving the substrates through a 2.0- and 1.0-mm sieve system (Owojori et al., 2009). At day 28, cocoons were measured, and earthworms removed. After 4 weeks the numbers of juveniles hatched form the cocoons were counted. In order to present comparative data and validate the tests, the tests were accompanied by the control samples moistened with water. Every 7 days, cytotoxicity (cell density, feasibility and typing) evaluations were performed by coelomic fluid collection during the chronic tests.

2.5.1. Coelomic fluid collection and cytological characterization

Coelomic fluid collection (cell density, viability and typing of immune system cell) determinations were performed during the chronic soils experiments. Coelomic fluid collection was carried out using the extrusion method (Eyambe et al., 1991) in the chronic test. A total of 200 μL of the extrusion solution (5.0% ethanol in saline solution) were mixed with 2.5 mg mL^{-1} EDTA, 10 mg mL^{-1} of the mucolytic agent guaiacol glycerol ether, and adjusted to pH 7.3 (0.01 mol L^{-1} NaOH). After 3 min, 800 μL of the *Lumbricus Balanced Salt Solution* (NaCl – 71 mmol L^{-1}, KCl – 4.70 mmol L^{-1}, $MgSO_4.7H_2O$ – 1.09 mmol L^{-1}, KH_2PO_4 – 0.39 mmol L^{-1}, Na_2HPO_4 – 0.56 mmol L^{-1} e $NaHCO_3$ – 4.20 mmol L^{-1}) at pH 7.3 were added. The organisms were then removed, according to Stein et al. (1977). The solution was maintained at 4 °C for 30 min and the supernatants were discarded. Coelomocyte density and viability were adapted from Kirk (1975). A total of 30 μL of the sample were mixed with 30 μL of Trypan blue dye (0.4%). Subsequently, 20 μL of this mixture were transferred to a mirrored Neubauer chamber. Photographic recordings were performed for cell quantification under × 10 magnification using an Olympus C×31 microscope coupled to a digital camera (Olympus Camedia C- 5060 Wide Zoom). Total numbers of stained and unstained cells were counted to determine the density (Equation (2)) and cellular viability (Equation (3)) of organisms exposed to the poultry litter. For cellular typing, 20 μL of the coelomocyte fluid were pipetted onto a microscopic slide. After drying at room temperature for 24 h, the slides were submerged in methanol for 10 min, washed with ultrapure water and submerged in 5% Giemsa dye for 10 min (Pereira et al., 2017). The stained cells were analyzed using an Olimpux CX31 microscope at × 100 magnification to identify immune system cell ratios (% of amoebocytes and eleocytes). All procedures were performed in triplicate and analyzed in comparison to control samples.

$$D = \frac{\left[\left(\frac{\sum \text{viable cells}}{\text{n. Neubauer chamber area}}\right) \times \text{dilution factor}\right]}{\text{biomass}} = \left(\text{cells mL}^{-1}\right)\ mg^{-1} \quad (2)$$

$$VC = \left[\left(\frac{\sum \text{total cells} - \sum \text{stained cells}}{\sum \text{total cells}}\right) x\ 100\right] = (\%) \quad (3)$$

2.5.2. Risk assessment in soil

According to the Technical Guidance on Environmental Risk Assessment (European Commission, 2003), the risk quotient (RQ) is estimated from the ratio of predicted environmental concentration (*PEC*) to the predicted no-effect concentrations on non-target organisms (*PNEC*), through Equation 4: RQ = $PEC_{soil}/PNEC_{soil}$. The PEC_{soil} of the poultry litter was based on the recommended application after field experiments in different agricultural systems (forest system, forage system and food crops) (Supplementary material –Table S2). The $PNEC_{soil}$ was estimated based on the No Observed Effect Concentration (*NOEC*) by the chronic test (reproduction) divided by an assessment factor of 100 (European Commission, 2003).

2.6. Statistical analyses

Results are expressed as means and standard deviation. The avoidance test results were analyzed by Fisher's Exact test. The reproduction and biomass variation distributions detected in the chronic test and the coelomic fluid assessments were evaluated by the Shapiro-Wilk test for further comparison with control samples. Group comparisons were performed by applying the ANOVA test followed by Dunnett's post hoc test ($p < 0.05$) when data followed a normal distribution, and by the Kruskal-Wallis test followed by Dunn's post hoc test ($p < 0.05$) when data followed a non-normal distribution. All statistical tests were performed using the GraphPad Prism version 5 software.

3. Results and discussion

3.1. Poultry litter sample FQ antibiotic and chemical characterizations

High concentrations of ENR (23.6 mg kg^{-1}) and its metabolite CIP (6.74 mg kg^{-1}) were determined in the poultry litter sample used for the tests. These results are in the same range of maximum concentrations measured in poultry litter samples from São Paulo, Brazil - ENR 31.0 mg kg^{-1} and CIP 2.13 mg kg^{-1} (Leal et al., 2012) and in manure from Beijing, China - ENR 8.68 mg kg^{-1} and CIP 9.34 mg kg^{-1} (Li et al., 2015a,b). Both countries are global leaders in poultry production (USDA, 2020). In this context, soil organisms can be critically impacted by the continuous application of poultry litter, magnified by the fact that animal manure is the major source of antibiotic contamination in soils (Riaz et al., 2018; Tasho and Cho, 2016). Poultry litter exhibits chemical characteristics that reflect substrate composition, besides feed residues, manure and management during production (Table 1).

Concentrations of K, Ca and Mg were high compared to most other fertilizers and organic residues from agriculture activities (Adeniyan et al., 2011; Rogeri et al., 2015). The presence of high levels of crop macronutrients is the main reason why poultry litter is used as a fertilizer worldwide (Bolan et al., 2010). Mineral supplies such as manganese monoxide (MnO) and copper and zinc sulphate ($CuSO_4$ and $ZnSO_4$) are added to poultry feed to increase productivity, enriching poultry litter with these trace elements (Mn, Cu and Zn) (Bolan et al., 2010; Parente et al., 2019b). The Cr (0.05 mg kg^{-1}) and Ni (0.54 mg kg^{-1}) concentrations in the poultry litter sample used herein were much lower compared to the means in poultry litter from Brazil (Cr 2.21 mg kg^{-1}) (Parente et al., 2019b) and in manure from China (Ni 17.5 mg kg^{-1}) (Cang et al., 2004). The Pb concentration in poultry litter (0.22 mg kg^{-1}) was similar to the means determined in chicken manure, of 0.16 mg kg^{-1}, from poultry farms in the United States (US) (Antonious et al., 2012). The concentrations of elements in the soil used in the tests are well below the limit values established as reference for soil quality by the Brazilian legislation (Conama, 2009).

Table 1
Chemical parameters of the poultry litter and soil samples used in the toxicological tests.

Elements (mg kg^{-1})	Poultry litter		Soil[a]	
	Means	SD[b]	Means	SD[b]
K	14,970	294	116	68.9
Ca	3004	813	378	111
Mg	597	141	304	115
Na	438	132	13	5.97
Fe	145	34.2	16,375	4732
Mn	46.7	10.4	167	97.9
Zn	86.7	23.0	47.2	27.5
Cu	42.0	8.50	7.29	1.42
Cr	0.05	0.08	13.8	4.76
Ni	0.54	0.14	3.40	1.03
Cd	< LQ[c]	0.00	0.36	0.01
Pb	0.22	0.04	6.15	0.50
pH	8.7		5.7	
CEC[d]	22.0		9.0	
OM[e]	640		8.30	

a: Red-Yellow Ultisol - Brazilian System of Soil Classification (EMBRAPA, 2013); b: standard deviation; c: below the limit of quantification (0.002 mg kg^{-1}); d: total cation exchange capacity ($Cmol_c$ dm^{-3}); e: organic matter (g kg^{-1}).

3.2. Earthworm toxicity assessments

3.2.1. Avoidance test

The avoidance test is a behavioral assessment that indicates the preference of the tested organisms after 48 h between a control and a contaminated soil. Among the different behavioral effects assessed in earthworms (e.g. avoidance behavior, biomass and burrowing), avoidance is considered one of the most efficient and sensitive endpoints (Saggioro et al., 2019a). Fig. 1 exhibits the avoidance behavior of earthworms exposed to natural soil containing 50–1000 g kg^{-1} of poultry litter. The red line indicates the 80% avoidance behavior represents a possible loss of habitat function (ISO, 2011).

The test revealed that poultry litter strongly repels earthworms from their habitat, as the end of the experiment, all organisms were in the section with control soil, even at the lowest concentration. Although this was not the main aim of the test, mortality of some organisms between poultry litter concentrations of 250–1000 g kg^{-1} was observed. The highest mortality rate (52% of the organisms) occurred at the maximum poultry litter concentration (1000 g kg^{-1}). In addition, strong morphological changes were observed in dead worms, such as swelling, partition and bottlenecks.

In complex matrices (e.g. poultry litter and soil), fluoroquinolones (FQs) bind to divalent and trivalent metal cations, which can affect their environmental dynamics and their effects on non-target organisms (Riaz et al., 2018). Some studies have evaluated the toxic effects of FQs in sandy media, which reduce possible interference from soil components, such as organic matter, clay minerals and metal ions (Huang et al., 2009; Li et al., 2015a,b). Using an artificial soil with 70% quartz sand, an avoidance response of *E. fetida* earthworms exposed to ENR at concentrations between 0.25 and 2.5 g kg^{-1} was observed (Li et al., 2015a,b). On the other hand, Segat et al. (2015) observed *E. andrei* preference for soil sections (Tropical Artificial Soil and Ultisol) containing swine manure in concentrations up to 100 m^3 ha^{-1} (equivalent to about 20 g kg^{-1}). Manure from other animal sources also does not seem to affect earthworm behavior, since it is common to use bovine and equine manure as a source of organic matter for worm maintenance in laboratory conditions (Tang et al., 2015; Zhiqun et al., 2017).

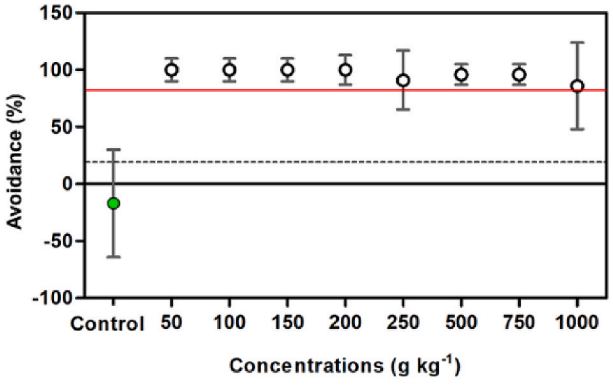

Fig. 1. Avoidance percentage (means and standard deviation) after earthworm exposure to 50, 100, 150, 200, 250, 500, 750 and 1000 g kg^{-1} of poultry litter and the control group (green). The dashed line indicates avoidance behavior by 20% of the tested organisms and the red line indicates the same behavior by 80% of the earthworms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.2. Acute tests: estimation of LC_{50} and biomass

Increased mortality was observed between 7 and 14 days, resulting in the death of all organisms exposed to 60 g kg^{-1} of poultry litter. After 7 days of exposure the estimated LC_{50} was of 37.6 g kg^{-1}, decreasing to 28.5 g kg^{-1} after 14 days. The LC_{50} (14 days) is equivalent to the application of 57 t ha^{-1} of poultry litter in an agricultural area. Li et al. (2015a,b) estimated an $LC_{50} = 11.01$ g kg^{-1} after 14 days of exposure in an experiment with *E. fetida* exposed to soil with ENR. The estimated LC_{50} based on ENR toxicity greatly exceeds the concentrations previously reported in soils (Leal et al., 2012; Parente et al., 2019a; Wu et al., 2014), suggesting that ENR does not affect worm survival under environmentally relevant conditions. On the other hand, considering a matrix-based approach, Bhering et al. (2020) recently reported the application of 600 g of poultry litter by seedling, or 42 t ha^{-1} of poultry litter application, based on crops with 70,000 seedlings per hectare. With this application pattern, the LC_{50} can be achieved in short crop cycles, where between four and six cycles are grown per year. Regarding biomass, a significant loss ($p < 0.05$) of 42% was observed after exposure to 40 g kg^{-1} of poultry litter. No biomass changes could be estimated at concentrations between 60 and 100 g kg^{-1}, since all exposed individuals died within 7 days of the experiment (Fig. 2).

Since the present assessment was performed with a complex matrix, the observed toxic responses can possible related to fluoroquinolone complexation with metal ions. Tests with binary mixtures of CIP and Cd or Cu result in tissue bioaccumulation pattern changes and toxic effects in earthworms (Huang et al., 2009; Li et al., 2016; Wen et al., 2011). Although the Cd concentration in the poultry litter sample was below the limit of quantification (<0.002 mg kg^{-1}), Cu exhibited a relevant concentration (42.0 ± 8.5 mg kg^{-1}), which may have influenced the observed toxic responses.

In a multi-species-soil-system with plants and invertebrates, Delgado et al. (2012) reported a similar reduction in biomass (41%) for *E. fetida* exposed to as application equivalent to 10 t ha^{-1} of sawdust poultry litter after 21 days of experiment. Compared to the present study, the significant biomass reduction was observed at an 8-fold lower concentration (equivalent to 5 g kg^{-1}). It is possible that the results were influenced by the soil used herein, since the authors reported a higher loss of biomass (48%) in the experiment only for the control soil.

3.2.3. Chronic effects

Biomass variation less than 30% and no mortality were observed in control groups. No significant difference in earthworm biomass compared to the control exposed to 5, 10 and 20 g kg^{-1} of poultry litter during the 8 weeks of the chronic test (56 days) was observed (Supplementary material -Fig. S1). On the other hand, a significant negative effect was observed in the reproduction test was observed, with a decreasing number of cocoons and juveniles at 20 g kg^{-1} (Table 2) of poultry litter (equivalent to 40 t ha^{-1}).

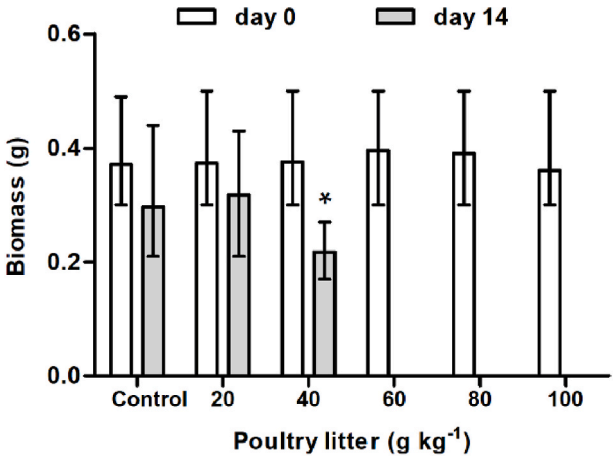

Fig. 2. Absolute variation of earthworm biomass (g) after acute exposure to 20, 40, 60, 80 and 100 g kg^{-1} poultry litter and a control group during 14 days of exposure; * Represents statistical differences compared to the control group (ANOVA; $p < 0.05$).

The results indicate the absence of visible negative effects in earthworms (biomass) but demonstrate that the poultry litter affects population maintenance through significant reproduction effects. Previous studies reported an increase in the number of juveniles with increasing organic matter addition in earthworm reproduction tests with swine (Segat et al., 2015) and cow (Domínguez-Crespo et al., 2012) manure. These studies suggest that other animal manure does not affect earthworm reproduction as much as observed with poultry litter under the present study conditions. In another approach, Li et al. (2015a,b) observed significant juvenile decreases after chronic exposure to 70% quartz sand soil containing 50 mg kg^{-1} ENR. Fluoroquinolones (FQs) are ionizable compounds (pK_a 6–8) and this class of antibiotics is expected to display high sorption rates influenced by cation-exchange capacity (CEC) in acid soils (pH < 6) (Leal et al., 2013; Vasudevan et al., 2009). In the present matrix-based approach, the high potential of poultry litter CEC (22 $Cmol_c$ dm^{-3}) in increasing soil CEC 9.0 $Cmol_c$ dm^{-3} is noted, resulting in higher ENR and CIP sorption. In field conditions, high FQs sorption can increase exposure and negative earthworm effects, since these organisms are exposed to soil contaminants through dermal contact and via the digestive tract, by feeding on organic matter and soil particles.

In earthworms, immune cellular functions are maintained by coelomocytes, cells of a mesodermal origin found in the coelomic cavity, which can be formed by two main cell-types based on morphological characteristics, namely amoebocytes and eleocytes (Genázio Pereira et al., 2017; Mácsik et al., 2015). Fig. 3 displays the earthworm coelomycete percentages (amoebocytes and eleocytes) after exposure to poultry litter during the chronic test.

A significant difference was observed (day 42) for the decreasing percentages of amoebocytes and increasing eleocytes at 10 and 20 g kg^{-1} poultry litter. The results corroborate other assessments that indicate that eleocytes are more sensitive than amoebocytes when exposed to contaminants (Pereira et al., 2019; Saggioro et al., 2019a). Eleocytes are responsible for contaminant and exogenous particle accumulation and detoxification (Bilej et al., 2010; Kurek et al., 2007). Their increase over the exposure period may indicate a detoxification mechanism related to xenobiotics. On the other hand, decreasing amounts of amoebocytes can be justified, since the primary cells for the elimination of potentially toxic substances are eleocytes. Amoebocytes are related to coagulation, wound healing, inflammations and graft rejection, among others (Adamowicz, 2005; Kurek et al., 2007). Irizar et al. (2015) observed similar results when establishing the toxicity of different coelomocyte cell-specific sensitivities subpopulations to a series of metals. The results revealed a continuous decrease in the number of amoebocytes with increasing Cd-exposure doses, while stabilized eleocytes gradually increased in number.

Studies on the recovery of coelomocytes and coelomocyte derived factors are still scarce. Concerning the few studies available, Santocki et al. (2016) evaluated the restoration of experimentally depleted coelomocytes in juvenile and adult composting earthworms *Eisenia andrei*,

Table 2
Reproduction parameters determined in *E. andrei* exposed to different poultry litter concentrations.

Poultry litter concentration (g kg^{-1})	Mean cocoons per individual	Mean hatchlings per cocoon
Control	0.89 ± 0.25	3.50 ± 0.71
5	0.65 ± 0.21	2.13 ± 0.75
10	0.59 ± 0.35	1.75 ± 0.35
20	0.34 ± 0.16[a]	1.23 ± 0.56[a]

[a] Statistical significance compared to the control group ($p \leq 0.05$).

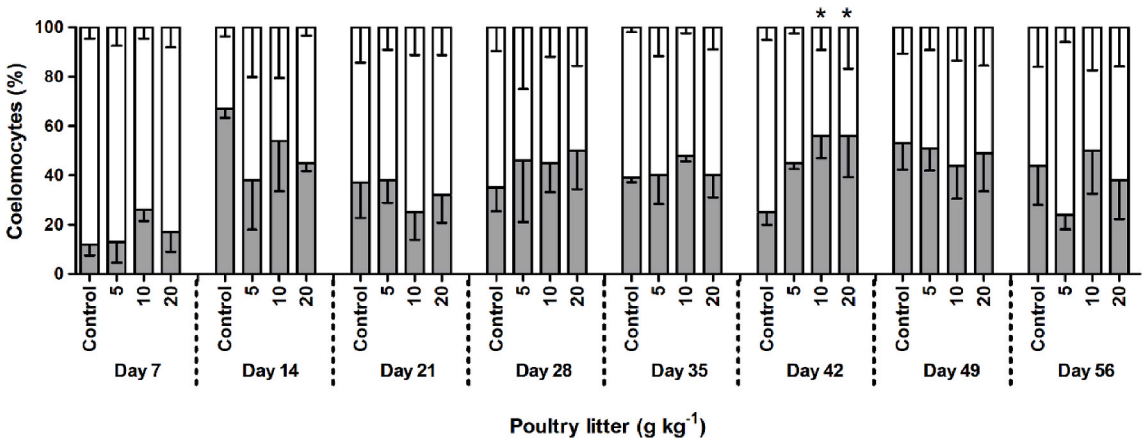

Fig. 3. Coelomycete percentages - amoebocytes (white) and eleocytes (gray) in coelomic fluid from earthworms exposed to poultry litter at 5, 10, and 20 g kg^{-1} in the chronic test. * Represents statistical differences compared to the control group (ANOVA; $p < 0.05$).

E. fetida and *Dendrobaena veneta*. According to the authors, amoebocytes and eleocytes presented gradual restoration, with amoebocytes exhibiting faster recovery. In addition, cells restoration was faster in juveniles than in adults and associated with accelerated body mass gain in the former. Senescent coelomocytes of adult earthworms easily undergo fragmentation, while this is seldom observed in the coelomocytes of juveniles and in newly formed coelomocytes replacing expelled cells in both adult and juvenile worms (Irizar et al., 2015; Plytycz et al., 2011; Saggioro et al., 2019b). According to this information, adult earthworms in the reproduction phase, such as those used herein, are more sensitive to contaminated poultry litter.

Regarding cell density, i.e. the number of cells, during chronic exposure, a reduction trend was observed on days 14, 21 and 28 (Fig. 4A). Significant differences ($p < 0.05$) were observed of decreased cells (day 28) at exposure to 10 g kg^{-1} poultry litter and increased cells (day 35) at exposure to 5 g kg^{-1}. Cell density reduction was observed only after a long exposure time (28 days). The initial decrease followed by fluctuations of these values indicate that the contaminants resulted in cytotoxic or cytostatic effects. Over time, the organism finds a new equilibrium and stimulates the production of new cells. This explains the relationship with the observed increase in the number of eleocytes and increased cell density at 42 days.

The percentage of live cells in comparison to control (feasibility %) remained mostly stable during the chronic test (Fig. 4B). Exceptions were noted on day 21, with increased viability at 10 and 20 g kg^{-1} and on day 28, where a reduction percentage was observed after exposure to

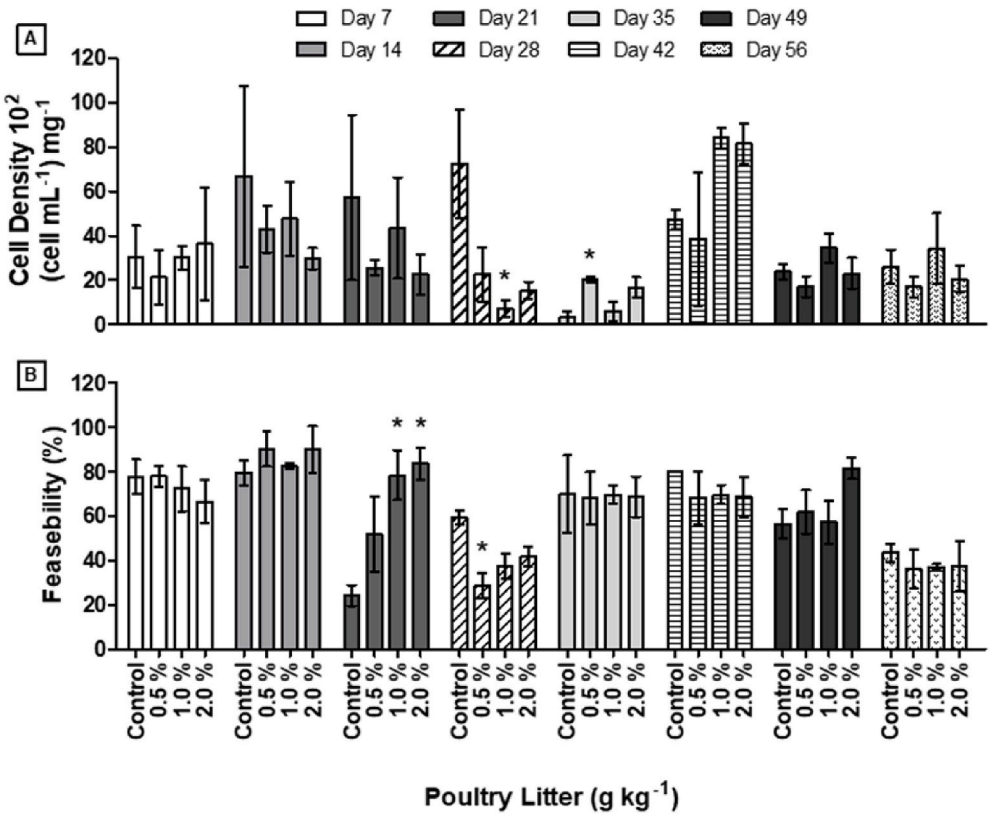

Fig. 4. (A) Coelomocyte density and; (B) Cellular viability in earthworms exposed to poultry litter at 5, 10, and 20 g kg^{-1} in the chronic test. * Represents statistical differences compared with the control group (ANOVA; $p < 0.05$).

5 g kg^{-1} poultry litter. The results indicate that earthworm exposure to the contaminated substrate did not result in continuous depletion of cell viability. As observed when evaluating the density values, earthworm effects were delayed, followed by a reestablishment of individual cytological conditions. Cellular cytotoxicity is widespread among invertebrates, although very limited knowledge how the exact mechanism is mediated is available. In a recent study, antioxidant enzyme alterations (e.g. superoxide dismutase, peroxidase and glutathione) were reported as promoting oxidative stress after earthworm exposure to CIP (Yang et al., 2020), while inhibition of catalase expression in intestinal tissues of worms exposed to ENR has also been described (Gao et al., 2008). CIP has also been associated to the generation of damaged proteins as a result of excessive production of reactive oxygen species in earthworms (Wang et al., 2018). Thus, it is clear that cytotoxic compounds can lyse extracellular, intracellular membranes, and DNA damage (Mácsik et al., 2015). The prolonged and repetitive cytotoxic effects with the application of new doses of poultry litter as organic fertilizer may cause cellular permeability damage and direct or indirect effects on the intraplasmic system, consequently leading to the observed density and viability decreases until organism death.

3.2.4. Risk assessment in fertilized soils

In the present study, the different PEC_{soil} were based on recommended poultry litter applications in different agricultural systems. The No Observed Effect Concentration (*NOEC*) based on the reproductive chronic test was of 10 g kg^{-1}. To estimate the $PNEC_{soil}$, the *NOEC* was divided by an assessment factor of 100. This assessment factor reflects the uncertainty in extrapolating data generated in experiments with a single species to the real condition of the environment (European Commission, 2003). Therefore, the estimated $PNEC_{soil}$ was of 0.10 g kg^{-1} of poultry litter, equivalent to the application of 200 kg ha^{-1} in a fertilized area. The estimated risk quotient (RQ) classifies environmental risk as low (<0.1), medium ($0.1 \leq RQ < 1$) and high (≥1) (European Commission, 2003), and is displayed in Fig. 5.

The estimated RQ was high (>1) for all scenarios based on the recommended poultry litter application, with the following ranges (RQ minimum – maximum): forest system (RQ 23–100), forage system (RQ 17–90) and food crops (RQ 16–129). Most studies applying the PEC_{soil} proposal are very cautious in relation to the disadvantages of using poultry litter, such as possible surface and groundwater contamination by nitrate and phosphorus (Friend et al., 2006; McGrath et al., 2010). However, it is difficult to predict all the impacts derived from the continuous application of such a complex matrix. In this sense, the fact that poultry litter is often associated with contamination by veterinary antibiotics is the main focus of this assessment. Previous studies estimated a high RQ (>1) for soils contaminated with FQs and other classes of antibiotics due to poultry litter and manure application (Parente et al., 2019a; Wu et al., 2014; Yang et al., 2016). These assessments suggest the occurrence of potential impacts on soil ecosystem function maintenance (e.g. supporting soil biodiversity, nutrient cycling and contaminant degradation), and are in agreement with the present assessment based on a poultry litter application approach.

4. Conclusions

The present toxicological assessment indicates that fluoroquinolones-contaminated poultry litter can critically affect earthworm populations. The avoidance test demonstrated strong habitat loss effects, and biomass reduction and lethality in the acute test were observed, with an 50% of lethal concentration for 14 days at 28.5 g kg^{-1} of poultry litter in environmentally relevant concentrations. In the chronic tests concerning reproduction and immune cell system endpoints, significant effects ($p < 0.05$) occurred at even the lower poultry litter concentrations, with the $PNEC_{soil}$ (based on reproduction) estimated at a value equivalent to 200 kg ha^{-1}. In addition, a high environmental risk (RQ) was estimated based on the poultry litter applications recommended by experimental field studies on forest, forage and food crops systems. This study thus demonstrates that matrix-based toxicological assessments can be important tools for a better understanding of soil organism impacts. In this sense, these studies can be used to identify critically affected areas in order to plan actions for environmentally safe management.

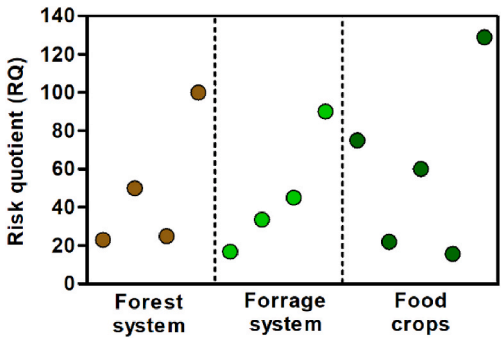

Fig. 5. Risk quotient based on the application of poultry litter recommended by experimental field studies in different agricultural systems.

CRediT author statement

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111305.

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