Biological endpoints in earthworms (*Amynthas gracilis*) as tools for the ecotoxicity assessment of soils from livestock production systems

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Abstract

Due to the intensification and modernization of livestock farming practices, large amounts of trace metals, veterinary pharmaceuticals and pesticide residues are released to the soil along with animal feces. Hence, there is an increasing concern about the effects of pollutants derived from livestock activities on soil organisms. The objective of this study is to assess the ecotoxicity of soils from livestock production systems using a set of validated tissue and cellular biomarkers of non-native earthworms (*Amynthas gracilis*) exposed *ex situ* to real contaminated livestock soils.

Overall the results showed that livestock pollutants present clear environmental risks, since the exposure during 14 days to soils from livestock systems triggered significant sub-lethal effects in *A. gracilis*, revealed by the increase of acetylcholinesterase activity in earthworms' tissues (from 34.15 ± 0.79 to 62.74 ± 2.10 nmol of acetylthiocholine hydrolyzed min^{-1} mg^{-1} of protein), the decrease of antioxidant defense associated enzymes (superoxide dismutase activity, from 2.76 ± 0.11 to 1.90 ± 0.04 U mg^{-1} of protein) and of lysosomal integrity (neutral red uptake, from 113.00 ± 4.81 to 83.73 ± 2.25 %). Moreover, coelomocytes of earthworms exposed to the livestock soil displayed significantly higher DNA damage values (comet assay, from 126.67 ± 8.67 to 199.67 ± 23.15 GDI).

This study validates the applicability of the tested biomarkers as early warning tools to assess sub-lethal toxicity to organisms inhabiting soil impacted by livestock pollutants. This study also highlights the relevance of *A. gracilis* as a suitable sentinel species to provide an integrative and more ecologically relevant response of soil ecosystem health in livestock production systems.

Keywords: Livestock pollutants; Soil ecotoxicity; Earthworms; Biomarkers; Cytotoxicity; DNA damage.

1. Introduction

One of the most pressing environmental risks associated with modern livestock practices derives from the amount of manure produced in these systems. Despite the major efforts developed to focus the positive effect of soil amendments with manure to enhance soil fertility, little attention has been paid to their toxicological effects on soil organisms (Ajorlo et al., 2010). Due to the relatively high cost of transportation, the bulk amounts of manure produced in livestock production systems are frequently spread on farm soils, bringing not only organic matter and nutrients to the ecosystem, but also potentially toxic substances such as trace metals, veterinary pharmaceuticals and pesticide residues (Moscuzza and Fernández-Cirelli, 2009; Solliec et al., 2016).

Earthworms have been considered highly appropriate biological indicators to assess the bioavailability and ecotoxicity of many soil pollutants (Asensio et al., 2013; Calisi et al., 2013). These organisms are naturally in contact with the solid, aqueous and gaseous soil phases and, consequently, are directly exposed to soil contaminants (Schreck et al., 2012). Traditionally standard toxicity tests using earthworms evaluate endpoints related to acute toxicity (e.g., mortality, growth and reproduction), underestimating the effects at the molecular, biochemical and cellular levels that can function as early-biomarkers of toxicity (Lourenço et al., 2011).

Over the past few years, increasing emphasis has been placed on the use of biomarkers as early-warning tools to monitor environment quality. The neutral red uptake (NRU) assay indicates cell integrity impairment and it has been applied in a variety of organisms to determine the relative cytotoxicity of a wide spectrum of pollutants (Gómez-Mendikute and Cajaraville, 2003). The acetylcholinesterase activity (AChE) is a biomarker of exposure to neurotoxic compounds, widely used as an earthworm biomarker for neurotoxic effects (Calisi et al., 2013). The superoxide dismutase (SOD) is an essential enzyme in the antioxidant defense system of organisms, playing an active role scavenging reactive oxygen species (ROS) produced during exposure to various environmental stressors and protecting cells from damage during biological oxidation (Lesser, 2006). The SOD activity is regarded as a fast and reliable biomarker of exposure to environmental pollutants and oxidative stress effect (Łaszczyca et al., 2004). The comet assay (CA) is effective in measuring the DNA damage caused by various genotoxins in earthworm coelomocytes (Lourenço et al., 2011; Markad et al., 2012, 2015). However, despite the great efforts to assess the

genotoxic effects of environmental pollutants, little is known about the risk of DNA damage associated with exposure to livestock pollutants.

The novelty of the present study relies on the use of a non-native epi-endogeic earthworm (*Amynthas gracilis*) as a potential sentinel organism to determine the resulting ecotoxicity of soils from livestock production systems. Even though most of the studies about earthworm biomarkers have been conducted on *Eisenia spp.*, the ecological relevance of this epigeic ecological group to soil ecotoxicity assessment has been questioned (Sanchez-Hernandez, 2006). As litter-dwelling species, *Eisenia spp.* inhabits the soil surface, forming no permanent burrows and feeding on decaying organic matter. Considering that soil contaminants can occur at different depths, the suitability of epigeic earthworms as bioindicators for ecotoxicity assessment in soil ecosystems can be questionable. On the other hand, the use of species belonging to the epi-endogeic group, such as *A. gracilis*, will provide a more realistic ecological response since these earthworms not only inhabit the litter-soil interface, but are also able to incorporate the fresh litter into the upper mineral layer. By displaying this behavior, epi-endogeic earthworms are exposed to soil pollutants from both soil surface and the deeper topsoil layers.

The main objective of this study is to assess the sub-lethal toxicity of livestock soils to soil organisms, using earthworms (*A. gracilis*) as biological indicators. For that purpose, a set of validated tissue and cellular biomarkers was tested: neutral red uptake assay, neurotransmitter and antioxidant defense enzyme activities (AChE and SOD) and DNA damage (CA).

2. Material and methods

2.1. Study sites and soil sampling

In São Miguel Island (Azores archipelago, Portugal) the dairy cattle production has been traditionally extensive, using pastures as a source of animal nutrition. However, in the last decades, local farms have undergone significant transformations and, nowadays, the cattle are often maintained at a high density in livestock systems being fed in alternation with pasture and synthetic feeds.

To study the impacts of these livestock systems on soil ecotoxicity, soils from two selected sites were used in this study: a soil from a reference site (RF) and a soil from a pasture subjected to intensive dairy cattle production (LS) (Figure 1). The study sites are located in the same geological complex (Picos Fissural Volcanic System), ensuring the

same bedrock and pedological conditions, being soils only differentiated by the type of land use. The agricultural practices experienced in the selected LS are representative of the majority of livestock production systems in the island, where a variety of veterinary pharmaceuticals and agrochemicals are used. A more detailed description of the studied livestock system is presented in Supplementary Material – Table 1. The reference site corresponds to a forest reserve of centennial Japanese cedar (*Cryptomeria japonica*), an area with no historical records or evidence of farming activity.

Three composite soil samples (with three sub-samples each) from the top soil layer (0–20 cm) were taken from the selected pasture (from the cattle trajectory to the feeding operations) and reference site.

2.2. Soil physicochemical properties

Soil physicochemical properties [particle-size fractions, soil organic matter, pH (H_2O) and electric conductivity] were analyzed following nationally recommended procedures and the Portuguese official methods (LNEC, 1967 a,b).

2.3. Trace metal analysis

Soil trace metal (Li, Cr, Ni, Cu, Zn, Rb, Pb and Hg) contents were determined by inductively coupled plasma mass spectrometry (ICP/MS) and inductively coupled plasma optical emission spectrometry (ICP/OES; Activation Laboratories Ltd., Canada). Quality control was assured by the analysis of duplicate samples, blanks and reference materials (GXR-1, GXR-4, GXR-6 and USGS SAR-M).

2.4. Test organism

The earthworms used for the assay constitute a natural population from the reference site. A group of 69 healthy adult *A. gracilis* earthworms (Kinberg, 1867; Megascolecidae), with body mass above 1000 mg (fresh weight), was collected by digging and hand-sorting during July 2015. Prior to the start of the exposure to the soils, earthworms were allowed to acclimatize, during one week, to the experimental conditions (20 ± 1ºC, in darkness) in tanks containing the RF soil. This species was selected by virtue of ease of collection through digging and its easily-recognizable morphology. To minimize a differential response bias due to genetic diversity, specimens were initially identified by molecular methods using a DNA barcoding approach based on the mitochondrial cytochrome oxidase subunit II (COII) (Novo et al.,

158 2015). This assured that animals used in the exposure experiment belonged to the same
159 mitochondrial haplotype.
160
161 **2.5. Exposure procedure**
162 For each experimental soil and exposure time [day (T1), 7 days (T7) and 14 days
163 (T14)], 9 earthworms were exposed inside plastic boxes (37 x 37 x 30 cm) covered with
164 perforated lids (1 mm diameter holes) to ~30 L of each soil, the equivalent to ~19 kg of
165 RF soil and 28 kg of LS soil. Before the beginning of the exposure procedure (no
166 exposure time: T0), 9 earthworms were analyzed to set the baseline values for each
167 biomarker.
168 A total of 30 earthworms was transferred directly from the acclimatization tank to
169 each experimental soil, to avoid differences in stress condition related to earthworm
170 relocation. The experiment was carried out for 14 days at 20 ± 1ºC, in full darkness.
171 Earthworms were processed for further biomarker analyses, after 1 day, 7 days and 14
172 days of exposure to each experimental soil. The soil exposure experimental design is
173 represented in Supplementary Material – Figure 1.
174 After exposure to the soil, earthworms were kept on moist filter paper for 24 h to
175 allow gut clearance before biomarker analyses, except for the NRU assay that was
176 processed differently. In this study, 3 earthworms per experimental group (LF and RF
177 soils) and exposure time (T0, T24, T7 and T14) were pooled together to analyze the
178 enzymes activities (SOD and AChE); a similar approach was used to carry NRU assay.
179 In the CA each earthworm was processed individually, being analyzed 3 individuals per
180 experimental soil and exposure time.
181
182 **2.6. Earthworm sample preparation and enzyme activities (AChE and SOD)**
183 Three gut-cleaned specimens were placed into a pre-chilled mortar and ground under
184 ice-cold conditions in phosphate buffer (1:5 w/v, 100 mM, pH 7.2). The homogenate
185 was centrifuged at 10400 *g* for 30 min at 4ºC to obtain the post-mitochondrial fraction
186 (supernatant: S9). The sample (S9) was divided into several aliquots and stored at -80º
187 C for enzyme activity assays and determinations of protein concentration. The protein
188 concentration in S9 was determined using the dye-binding method according to
189 Bradford (1976), and bovine albumin was used as the standard.
190 The acetylcholinesterase (AChE: EC 3.1.1.7) activity was determined according to the
191 method of Ellman et al. (1961). The reaction medium included sodium phosphate buffer

192 (0.1M, pH 7.2), DTNB (0.67 mM), acetylthiocholine iodide (1 mM) and sample (S9).
193 Kinetics was recorded at 412 nm for 3 min at 25ºC, subtracting the absorbance increase
194 due to thiols present in each extract, quantified in a similar assay without
195 acetylthiocholine iodide. The enzymatic activity was expressed as nmol of
196 acetylthiocholine hydrolyzed per min per mg of protein and for calculations the
197 absorption coefficient of 13.6 x 10^3 M^{-1} cm^{-1} was used.
198 The superoxide dismutase (SOD, EC 1.15.1.1) activity was determined as described by
199 Misra and Fridovich (1972). The reaction medium included carbonate buffer (0.05 M,
200 pH 10.2), EDTA (0.1mM), adrenaline (0.6 mM), and sample (S9). The rate of
201 adrenaline autoxidation at 30ºC was monitored at 480 nm for 3 min. One unit of SOD
202 activity (U) was defined as the amount of enzyme required to cause 50% inhibition of
203 the oxidation of the epinephrine (SOD_{50}), and the result was expressed as U mg^{-1} of
204 protein.
205
206 **2.7. Cell integrity in coelomocytes (NRU assay)**
207 The specimens were cleaned with distilled water to remove any particle of soil
208 and gently massaged in Phosphate-buffered saline solution (PBS solution, pH 7.4) to
209 remove intestinal contents. Coelomocyte retrieval was performed using a non-invasive
210 technique (Engelmann et al., 2004). In order to allow extrusion of coelomocytes through
211 dorsal pores, each pool of earthworms was submerged in 3 ml of extrusion fluid (95%
212 PBS solution, 5% ethanol, 2.5 mg/ml EDTA, 10 mg/ml guaiacol glycerol ether) for 2
213 min. Coelomocyte suspension was cleaned by transferring it into tubes containing 5 ml
214 of cold PBS solution and then centrifuged (4ºC; 10 min; 264 *g*). Pellets were
215 resuspended in PBS solution and the Trypan Blue exclusion test was performed in order
216 to adjust the number of cells to 10^6 cells/ml. Different coelomocytes suspensions were
217 used for the NRU and CA assay.
218 The neutral red dye taken up by the coelomocytes was spectrophotometrically
219 measured in microplates according to Gómez-Mendikute and Cajaraville (2003) and
220 Homa et al. (2003). Briefly, 6 replicates of 200 µl of coelomocyte suspension from each
221 earthworm pool (see section 2.5.) were added to the wells of 96-well microliter test
222 plates to perform the NRU in vitro assay. Cells were incubated for 1 h to allow their
223 attachment to the chamber walls, the non-adherent cells were removed by centrifuging
224 the place for 10 min at 117 *g* and adherent cells were once again incubated for 1 h with
225 neutral red (freshly made 0.05% dilution in PBS solution) to allow dye uptake by living

cells. Wells without cell suspension were used as negative controls. Free neutral red and any remaining non-adherent cells were removed by washing the microplate several times until no color was visible in the negative control wells. Washing was performed by centrifuging the plate at 117 *g* for 5 min, subtracting the liquid with a vacuum pump and adding new PBS solution. Finally, neutral red was extracted from the cells with acetic acid–ethanol solution (1% acetic acid, 50% ethanol) and incubated in the solution for 20 min. Absorbance was determined at 540 nm in a microplate reader (BioRad model 680, USA). Values recorded in the negative controls were subtracted from sample values and results were expressed as percentage values relative to the control group (100%).

2.8. DNA damage in coelomocytes (CA)

The conventional alkaline version of the CA was performed using the method described by Collins (2004), with minor modifications. Two gel replicates, each containing approximately 2×10^4 cells (coelomocytes suspension in PBS, the methodology described in 2.7) in 70 µL of 1% low-melting-point agarose in PBS, were placed on a glass microscope slide, pre-coated with 1% normal melting-point agarose. The gels were covered with glass coverslips and left for ± 5 min at 4 ºC to let the agarose solidify, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 ºC, for one hour. Then, slides were gently placed in the electrophoresis tank, immersed in electrophoresis solution (±20 min, 0.3 M NaOH, 1 mM EDTA, pH > 13) for alkaline treatment. Electrophoresis was performed at a fixed voltage of 25 V and a current of 300 mA, which results in 0.7 V cm^{-1} (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with SYBR® Safe (10 µL/ml). One slide with two gels each (50 nucleoids per gel) was observed per earthworm for each experimental group, with a Leica DMLS fluorescence microscope (400× magnification). DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in the tail), as described by Garcia et al. (2004). The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to the formula:

$$\text{GDI} = [(\%\ \text{nucleoids class } 0) \times 0] + [(\%\ \text{nucleoids class } 1) \times 1] + [(\%\ \text{nucleoids class } 2) \times 2] + [(\%\ \text{nucleoids class } 3) \times 3] + [(\%\ \text{nucleoids class } 4) \times 4]$$

GDI results were expressed as arbitrary units on a scale of 0–400 per 100 scored nucleoids (as an average value for the two gels). As positive controls, coelomocytes were treated with 50 mM hydrogen peroxide for 5 min, according to Collins et al. (1995), and the respective GDI values were scored.

2.9. Statistical analysis

Soil trace metal contents from both experimental soils (LS and RF) and DNA damage class, were compared by Mann–Whitney *U*-tests. For each biomarker, the effect of soil type (LS and RF) at each exposure time (T0, T1, T7 and T14) was compared using Student's *t*-tests ($p < 0.05$). Normality and homogeneity of the variances were tested before Student's *t*-tests and Mann–Whitney *U*-tests analysis, using the Shapiro-Wilk and Levene tests, respectively. All statistical analyses were conducted using SPSS 21.0 for Windows.

3. Results and Discussion

In this study, the soil from the livestock system was characterized by a higher electrical conductivity and clay-silt content, and a lower organic matter content (Table 1). These soil properties promote a higher bioavailability of some soil pollutants, particularly of trace metals (Sauvé et al., 2000; Seifi et al., 2010), increasing the environmental risks associated with livestock production activities to the resident biota. The tested soils (RF and LS) have a volcanic nature (classified as Andosols) (Parelho et al., 2014). Under undisturbed conditions, these soils preserve their andic properties, particularly their high organic matter content [on average 13% (Parelho et al., 2014; Tadashi and Shoji, 2002)]. The results of this study indicate that, when Andosols are intensively managed for agricultural purposes, the organic matter soil content declines, even when subjected to animal effluents inputs. Paul et al. (1996), in a study using agricultural soils from North America, also observed that the soil carbon content declined with long-term agricultural land-use and intensive tillage practices.

The majority of the analyzed metals (Li, Cr, Ni, Cu, Zn, Rb) was found in significantly higher concentrations in soil from the LS (Table 1). These metals are

known as key potential toxic elements occurring in agricultural systems in the study area (Parelho et al., 2014) being associated with the input of livestock pollutants (Bolan et al., 2004). The major sources of metals in intensively managed pasture soils are mineral lick blocks, natural and synthetic supplementary feed, animal manure, slurry application, pesticides and mineral fertilizers (Franco-Uría et al., 2009; Qishlaqi and Moore, 2007). All of these agrochemicals are used in the selected LS (Supplementary Material – Table 1). Thus, the differences observed in soil trace metal loads between the two experimental soils, reveal that intensive livestock activities are significant sources of soil contamination with trace metals. Results also show that the soil from RF has significantly higher concentrations of Pb and Hg (Table 1). This apparently contradictory phenomenon has been also reported in unmanaged forest soils in the same study area (Parelho et al., 2014), although no biological effects were recorded in the resident soil microbial community and fauna (Parelho et al., 2016 a, b). Although the discrimination between geogenic and agricultural contribution to the trace metal contents in the experimental soils was outside the scope of this study, we hypothesize that the concentrations of Pb and Hg in the RF soil are naturally inherited from the volcanic parent rock. Due to particular RF soil properties, such as the elevated organic soil matter content (4.22-fold higher than in LS soil), those contaminants are strongly bound to the chemically reactive inner and outer soil surfaces, and thus a low bioavailability is expected under these conditions.

In general, the observed differences in the biological endpoints between earthworms exposed to the experimental soils (RF and LS), should be interpreted as the net result of the joint-action of complex agrochemicals mixtures in variable amounts, rather than the single effect of trace metal soil loads. In this sense, for this study, soil trace metal loads were used as an indirect and partial measure of animal's exposure to livestock pollutants in the studied pasture. This approach has been previously validated in recent studies for soils from agricultural volcanic areas (Parelho et al., 2014; Parelho et al., 2016 a, b).

The results of this study reveal that the exposure during 1 and 14 days to the LS triggers an increase of AChE activity (Table 2). Even though the quantification of pesticide residues was not assessed in this study, organophosphate and carbamates pesticides are applied in the selected pasture and widely used in the local agricultural context; these compounds are known as specific inhibitors of AChE (Colovic et al., 2013). The use of these pollutants in the studied livestock production system pasture is

confirmed trough the higher soil loads of Li, a validated tracer in volcanic soils for carbamate pesticides inputs (Parelho et al., 2014). The results show a time-dependent increase of the AChE activity when earthworms are exposed during 1 and 14 days to the LS soil, compared to the results obtained for the same times of exposure to the RF soil (Table 2). Although most studies report a decrease in AChE activity as an acute effect of exposure to anti-AChE molecules, others refer to its recovery as a time-dependent effect, a consequence of the detoxification process that enables long-term recovery of the target nervous tissue. The recovery of AChE activity after long-term exposure to organophosphate and carbamates compounds was previously reported in other earthworm species (*Drawida willsi*, Panda and Sahu, 2004; *Eisenia andrei*, Velki and Hackenberger, 2013; *Eisenia fetida*, Gambi et al., 2007; *Lumbricus terrestris*, Vejares et al., 2010) and was attributed to the capacity of the animal to detoxify and eliminate the pesticide through an enhanced metabolism, such as an increased synthesis of carboxylesterase. It is assumed that carboxylesterase provides protection against pesticides intoxication via detoxification by hydrolysis of ester bonds and, by providing alternative sites for binding so that one molecule of pesticide is scavenged by stoichiometric phosphorylation, which reduces the amount of pesticide available for AChE inhibition (Jokanovic, 2001). From our results it may be hypothesized that the short-term exposure to livestock soils triggered parallel detoxification processes in *A. gracilis*, causing a decrease of neurotoxins in earthworm tissues and, ultimately leading to a recovery of the AChE catalytic activity after 14 days of exposure. Our results clearly reveal that exposure of earthworms to LS triggered a different pattern of response in comparison to the observed with RF soil, enlightening the presence of neurotoxic compounds in LS soil.

Within the cell, SOD activity constitutes the first line of defense against ROS (Alscher et al., 2002). Therefore, an increase in SOD activity would be expected in earthworms exposed to LS soil as a short-term response against oxidative stress. Yet, data from this study reveal that exposure to soils from livestock production systems induces a significant decrease of SOD activity in earthworms at T7 and T14, with activity values significantly lower (27–32%) to those observed in earthworms exposed to the RF (Table 2). The excess of ROS molecules is a potential threat to normal cellular function, disrupting the antioxidant defense mechanism that results in the decrease of SOD activity and, consequently, causing oxidative stress (Markad et al., 2012). Therefore, the observed decrease of SOD activity in *A. gracilis* exposed LS soil, can be

358 explained by the presence of additional stressors in this soil that are associated with
359 oxidative damaging effects.
360 Alterations in the coelomocytes, such as specific responses of the lysosomal
361 system, provide a first answer to pollutant exposure, since injurious lysosomal reactions
362 frequently precede cell and tissue pathology (Spurgeon et al., 2000). The most
363 investigated coelomocyte alteration is represented by lysosomal membrane integrity
364 (measured trough NRU assay), an indicator of xenobiotic exposure and its associated
365 biological effects (cytotoxicity). A significant reduction in NRU was observed after 1
366 day in earthworms exposed to LS compared to the group exposed to RF soils (Table 2).
367 Moreover, in LS, the endocytic activity of coelomocytes was also decreased in T14,
368 being the recorded values significantly lower (~26%) than those observed in
369 earthworms exposed to the RF (Table 2). These results reveal not only the presence and
370 bioavailability of pollutants in soils from the livestock systems, but also that
371 earthworms are suffering from general stress. Although the mechanism causing
372 alterations in membrane stability of lysosomes is still not well understood, the
373 lysosomal membrane stability is regarded as a potential biomarker of various
374 environmental pollutants, particularly of metals. A reduced NRU has often been
375 reported in coelomocytes of earthworms exposed to soils contaminated with a variety of
376 metals (Asensio et al., 2013; Calisi et al., 2013). For example, in a recent study, Markad
377 et al. (2015) observed that earthworm *Dichogaster curgensis* exposed during 14 days to
378 fly ash contaminated soils (polluted with Pb, Zn, Ni, Cd, Cr, and Cu) was associated
379 with coelomocytes lysosomal membrane destabilization. Other studies suggest that ROS
380 produced in the cellular system due to exposure to metals, plays a major role in metal-
381 induced cellular responses and affects various cellular organelles and their repair
382 systems (e.g. Vallyathan et al, 1998). In the present study, the decreased lysosomal
383 stability in earthworms exposed to LS soil could be attributed to its higher loads of Li,
384 Cr, Ni, Cu, Zn, Rb.
385 In the present study, the increased DNA damage in the coelomocytes of
386 earthworms exposed to the livestock soil was time-related, being significantly affected
387 after 14 days of exposure (Table 2). Considering the results regarding DNA damage
388 classes (Table 3), after the exposure of earthworms for 1 day (T1) to both experimental
389 soils, the predominant DNA damage classes were 1 and 2, similar to the observed at the
390 beginning of the experiment (T0). After 14 days of exposure to LS soil, the significant
391 increase of DNA damage was associated with an increase in the frequency of classes 2,

3 and 4. Meanwhile, for the earthworms exposed to the RF soil, both GDI values and DNA damage classes showed a time-independent pattern of response, remaining similar to the baseline values (T0). The level of DNA strand breaks in the organisms has been regarded as a sensitive biomarker for genotoxic effects of xenobiotics and is widely used for environmental biomonitoring and risk assessment (e.g. Lourenço et al., 2011). Considering that DNA damage assessed by the CA is a result of a balance between damage and repair activities, which are dependent on the type and concentration of genotoxic chemicals in the environment (Qiao et al., 2007), the observed increase of DNA damage in the coelomocytes of earthworms exposed to the LS, reveals not only the presence of genotoxins in soils from livestock production systems, but also the time dependent bioaccumulation of these pollutants and their genotoxic effects to soil organism. These results also validate the applicability of CA on *A. gracilis* for field assessment of soil genotoxicity.

The question of the identification and characterization of appropriate sentinel earthworm species to be used as field-collected organisms, in order to provide a quick assessment of soil pollutants effects, has already been raised by several authors (e.g. Sanchez-Hernandez, 2006). Overall, the results of this study validate the applicability of the studied biomarkers (AChE and SOD activities in earthworm tissues; NRU and CA in coelomocytes) on *A. gracilis* as a valuable early warning model to assess sub-lethal toxicity on organisms inhabiting soil impacted by livestock pollutants, encompassing evaluation of key processes (i.e. neurotransmission, antioxidant defense mechanisms, cytotoxicity and DNA integrity).

4. Conclusion

The results of this study reveal that soils from livestock production systems contain biological relevant doses of neurotoxic compounds (observed through the pattern of response of AChE), as well as other pollutants (revealed by SOD, NRU and CA biomarkers). Furthermore, earthworm exposure to soils impacted by livestock pollutants was associated to sub-lethal toxicity effects encompassing key processes, such as neurotransmission, oxidative stress, cytotoxicity and DNA damage.

This study also validates the use of the selected biomarkers on *A. gracilis* as a valuable early warning model to assess the sub-lethal toxicity of livestock pollutants to soil organisms. The use of this earthworm species provides a more realistic insight of the link between laboratory exposure results and the toxic effects on soil organisms,

since due to their epi-endogeic activity they are exposed to soil pollutants from both soil surface and the deeper topsoil layers, enabling an integrated overview of the biological effects of livestock soil pollutants to soil organisms.

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Figure
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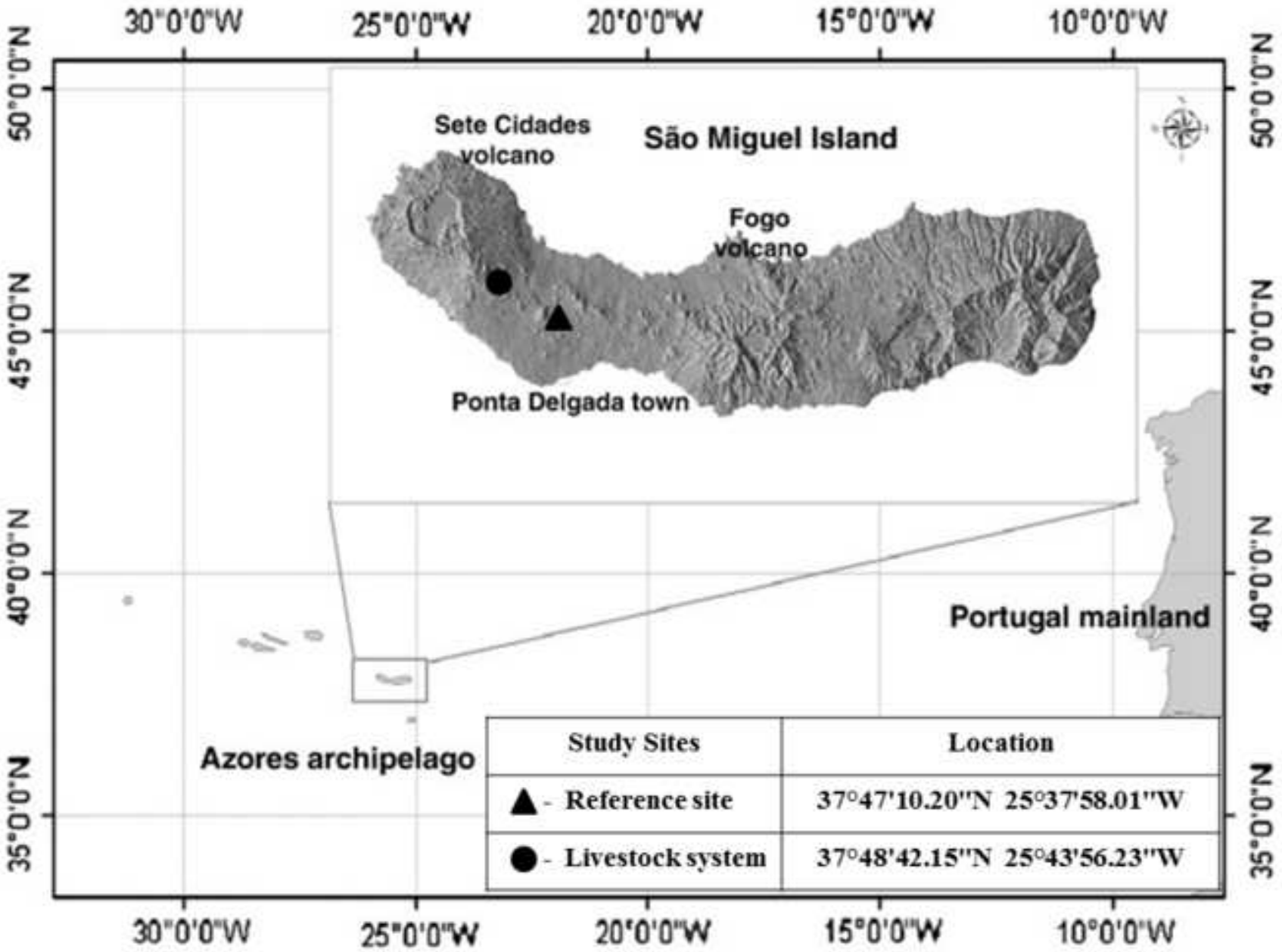

Figure 1 – Location of the Azores archipelago in the North-Atlantic Ocean. Inset: São Miguel Island with study sites location. Adapted from Cordeiro et al., 2012.

Table 1 – Mean values (± SD) of soil physicochemical properties and concentration of trace metals in topsoil samples (0-20 cm) from the livestock production (LS) and reference site (RF). Means within each line followed by different letters are significantly different at $p < 0.05$ (Mann–Whitney *U*-tests).

	LS	**RF**
pH (H_2O)	6.80 ± 0.18	6.72 ± 0.35
Electric Conductivity (µS cm^{-1})	119.67 ± 2.08 a	101.00 ± 15.13 b
Organic Matter Content (%)	3,12 ± 0,30 b	13.18 ± 3.70 a
Clay-silt Content (%)	3.47 ± 1.72	2.16 ± 0.23
Trace Metals (mg kg^{-1}, d.w.)		
Cr	45.00 ± 3.24 a	17.67 ± 1.54 b
Cu	53.88 ± 2.65 a	19.04 ± 1.89 b
Hg	0.03 ± 0.00 b	0.11 ± 0.01 a
Li	3.42 ± 0.14 a	2.39 ± 0.05 b
Ni	44.88 ± 3.44 a	17.44 ± 1.52 b
Pb	6.88 ± 1.75 b	10.95 ± 0.44 a
Rb	15.98 ± 0.87 a	5.80 ± 0.58 b
Zn	112.33 ± 3.75 a	94.08 ± 1.77 b

Table

Table 2 - Biochemical responses [acetylcholinesterase (AChE) and superoxide dismutase (SOD) activities; neutral red uptake assay (NRU) and comet assay (CA)] of earthworm *Amynthas gracilis* exposed (T0, T1, T7 and T14 days) to the experimental soils [livestock soil (LS) and reference soil (RF)]. Means (±SE) within each line followed by different letters are significantly different at $p < 0.05$ (Student's t-tests).

Mesocosm	**CA**		**NRU**		**AChE**		**SOD**	
Time	(GDI)		(%)		(nmol of acetylthiocholine hydrolyzed min^{-1} mg^{-1} of protein)		($U\ mg^{-1}$ of protein)	
	LS	**RF**	**LS**	**RF**	**LS**	**RF**	**LS**	**RF**
T0 days	114.33 ± 16.60	114.33 ± 16.60	100 ± 0.00	100 ± 0.00	54.80 ± 1.38	54.80 ± 1.38	1.90 ± 0.13	1.90 ± 0.13
T1 day	140.00 ± 4.58	124.33 ± 13.35	12.40 ± 0.47 b	33.60 ± 3.06 a	33.35 ± 0.00 a	29.38 ± 0.79 b	1.69 ± 0.02	1.77 ± 0.11
T7 days	162.33 ± 12.45	127.67 ± 16.80	20.96 ± 3.12	33.70 ± 4.37	34.94 ± 2.10	34.94 ± 0.79	1.14 ± 0.06 b	1.51 ± 0.05 a
T14 days	199.67 ± 23.15 a	126.67 ± 8.67 b	83.73 ± 2.25 b	113.00 ± 4.81 a	62.74 ± 2.10 a	34.15 ± 0.79 b	1.90 ± 0.04 b	2.76 ± 0.11 a

Table

Table 3 - Mean frequency (%) (±SD) of DNA damage classes in coelomocytes of *A. gracilis* exposed (T0, T1, T7 and T14 days) to the experimental soils [livestock soil (LS) and reference soil (RF)]. Means within each exposure time and between each experimental soil, followed by different letters are significantly different at $p < 0.05$ (Mann–Whitney *U*-tests).

Exposure time	**DNA damage classes***				
	0	**1**	**2**	**3**	**4**
T0	11.33 ± 10.69	65.67 ± 5.51	19.33 ± 13.20	3.33 ± 3.05	0.00 ± 0.00
T1 day					
LS	5.33 ± 3.79	56.33 ± 4.04	31.33 ± 6.03	7.00 ± 2.00	0.00 ± 0.00
RF	8.00 ± 6.25	64 ± 6.56	24.00 ± 8.54	3.67 ± 3.51	0.33 ± 0.58
T 7 days					
LS	0.33 ± 0.58 a	56.33 ± 8.33	26.67 ± 0.58	14.00 ± 4.00	2.66 ± 4.62
RF	9.00 ± 7.94 b	60.67 ± 12.01	24.00 ± 13.00	6.33 ± 5.03	0.00 ± 0.00
T14 days					
LS	1.33 ± 1.53	34.33 ± 18.01 a	36.00 ± 7.00 b	20.00 ± 9.17 b	8.33 ± 6.43 b
RF	2.67 ± 4.62	71.00 ± 3.61 b	23.33 ± 3.06 a	3.00 ± 5.20 a	0.00 ± 0.00 a

*DNA damage classes according to tail intensity and length, from 0 (no tail, less damage) to 4 (almost all DNA in tail, highest damage).

Supplementary Material

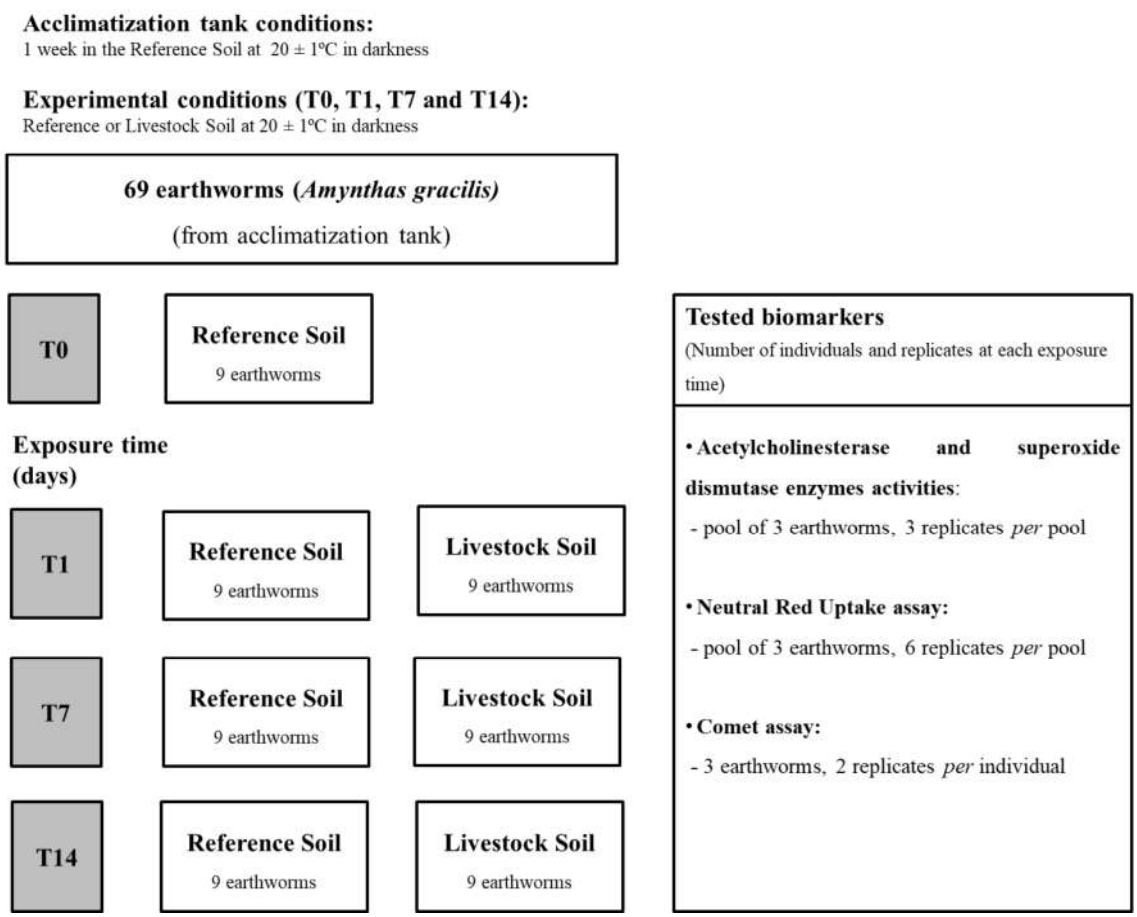

Figure 1 – Soil exposure experimental design.

Table 1 – Livestock system description with the specific veterinary pharmaceuticals applied to animals and agrochemicals applied to soil.

Farming System	Pasture size	Cattle heads	Agrochemicals					
			Inorganic Fertilizers	Veterinary Pharmaceuticals			Pesticides	
				Antibiotics	Anti-inflammatory	Others	Insecticides	Herbicides
Livestock System	3674 m^2	100	Yes	Ceftiofur Danofloxacin Penicillin and streptomycin Cloxacillin	Carprofen	Ornipural Veteglan	Yes	Yes