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Biomarkers response and population biological parameters in the earthworm *Eisenia fetida* after short term exposure to atrazine herbicide

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ABSTRACT

Atrazine is one of the most used herbicides and its over-application in fields can affect the soils and their associated biota. In this work, earthworms (*Eisenia fetida*) were exposed to different concentrations of atrazine to assess possible sublethal harmful effects. In the search for reliable biomarkers of these effects, acetylcholinesterase (AChE), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities were measured as well as behaviour and population biological parameters such as biomass, growth and production of cocoons. It was found that biomass changes were not significantly affected by atrazine presence and after 28 days the increment was around 18.3–27.3%. Protein content in earthworms was reduced in the presence of atrazine after 28 days. The lower atrazine concentration (2 mg Kg^{-1}) affected in the shortest term (7 days) the rate of cocoon production and increased LDH activity and, especially, the AChE activity in around 40%. The presence of a higher concentration (10 mg Kg^{-1}) of the herbicide reduced one third of the number of total cocoons, affecting the reproduction. ALP was the least sensitive biomarker to atrazine exposure. When exposed to up to 10 mg Kg^{-1} of atrazine, *E. fetida* showed a tendency to rebalance and maintained its general health.

1. Introduction

Atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) has become one of the most used herbicide in the world for controlling broadleaf and grassy weeds, especially in the United States, China and developing countries (Singh et al., 2018; Yue et al., 2017). In Argentina, the use of atrazine during 2013–2014 agricultural season was estimated in 10–15 million Kg or L of formulations (Alonso et al., 2018). However, atrazine is acknowledged as a threat, affecting endocrine, central nervous and immune systems (Sánchez et al., 2017), demasculinizing frogs (Hayes et al., 2010) and inhibiting non-target plant development by enhancing reactive oxygen species (Baxter et al., 2015). Some of the main ecotoxicological effects of this herbicide on humans, plants, animals and microorganisms are reviewed in the work of Singh et al. (2018). Even though atrazine is banned in the European Union, it is still detectable in groundwater after many years (Vonberg et al., 2014). A long half-life, moderate aqueous solubility and low adsorption in soil confer the herbicide high mobility (Barchanska et al., 2017; Huang et al., 2020). As a result, not only is it relatively persistent in groundwater but also it can be detected in freshwater nearby croplands (García et al., 2019). The adverse effects of long-term and over-application of persistent pesticides such as atrazine comprises also the pollution of the soils and thereby of their related biota, being earthworms a large fraction of the soil living biomass.

Earthworms are an important biological component in soil and are usually defined as “ecosystem engineers”, playing an important role in improving the structure and fertility of the soil (Jouquet et al., 2006; Römbke et al., 2005). Because they are easily collected, identified and breed, their ecological importance and their sensitivity to environmental pollution, they have been chosen as suitable sentinel organisms for ecotoxicological studies of pesticide residues in terrestrial ecosystems (Owagboriaye et al., 2020). Particularly, the species *Eisenia fetida* and *Eisenia andrei* have been largely used as standard test organisms for risk evaluation of pesticides and protocols have been extensively applied to assess their sensitivity to chemical pollution (Rico et al., 2016). These tests have been mainly used to evaluate acute lethal effects and biomass changes for a large range of pesticides (Wang et al., 2012).

In addition, due to their capabilities, earthworms are used as

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"vermiremediators" to amend soil contaminated with pesticides, as they help the degradation of these compounds through their metabolic and physical activities and contributing to the enrichment, propagation and stimulating of microbial work (Morillo and Villaverde, 2017). They are also engaged in soil aeration, water infiltration and mixture of soil horizons (Pelosi et al., 2014). According to Sanchez-Hernandez et al. (2019), as earthworms can alter the dynamic of organic matter and lower the mobility of pesticides, the possibility to reduce their runoff and leaching arises. Lin et al. (2019) observed that without the presence of earthworms, humus-fixed atrazine increased throughout time and both soil-bounded and available atrazine decreased more slowly than when these organisms were added. They contribute to atrazine remediation not by accumulating the herbicide but rather enriching the soil with intestinal atrazine-degrading flora or promoting the indigenous microorganisms which mineralize the herbicide. Although there have been several studies regarding the role of earthworms in degrading atrazine, most of them have focused on the herbicide removal, the soil properties or the microbial communities (Lin et al., 2018; Morillo and Villaverde, 2017; Neuwirthová et al., 2019).

Whether earthworms are used as sentinels for risk assessment or for the remediation of fields, the evaluation of their wellbeing becomes a priority. Changes due to the presence of a pesticide can be measured at different levels. In particular, the biochemical responses constitute a complementary approach to standard toxicity tests in the valuation of sub-lethal effects of pollutants in earthworms, offering information about the stress responses in the organism and the toxic mode of action of the assessed chemicals (Rico et al., 2016). Enzymatic activities have been considered as useful biomarkers of forthcoming damage and an early warning tool, but the number of studies evaluating enzymatic responses from atrazine in earthworms is rather limited.

In this sense, Ismail et al. (1997) studied the glutamic oxaloacetic transaminase and glutamic pyruvic transaminase activities related to atrazine residues in earthworms tissues and they found a significant elevation of the activity of both transaminases, with peaks after 14 days of exposure but a decrease at the end of the experiment (56 days). Song et al. (2009) evaluated the effect of atrazine on the activity of antioxidative enzymes, namely superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD), as well as the DNA damage induced. The authors showed that atrazine induced oxidative stress and DNA damage in earthworms but although there were general tendencies, i.e. inhibition of SOD and stimulation of CAT and POD, there was an unclear dependency with time and dose of the herbicide. Finally, El-Aswad and Badawy (2015) investigated only the in vitro kinetic characteristic of alkaline phosphatase (ALP) isolated from earthworms. This is a metalloenzyme involved in several metabolic processes such as permeability, growth and cell differentiation, protein synthesis, absorption and transport of nutrients and gonadal maturation (Jiang et al., 2012). Their results showed a dose-dependent inhibition of the ALP activity with increasing concentrations of atrazine.

In addition to these enzymes there are other relevant ones which have been surveyed in earthworms exposed to other pesticides. Oxidative related enzymes such as SOD, CAT and glutathione S-transferase as well as acetylcholinesterase (AChE) are the most frequent ones (Pelosi et al., 2014). AChE plays a main role in neurotransmission and muscular activities (Hackenberger et al., 2018; Wang et al., 2015) and it has been particularly tested when earthworms were exposed to organophosphorus (OP) and carbamate compounds. Another enzyme which has been suggested as an enzymatic biomarker of stress exposure in earthworms is lactate dehydrogenase (LDH), an intracellular enzyme involved in anaerobic glycolysis (Owagboriaye et al., 2020). This enzyme has been tested for exposure to the pesticides phorate (Tripathi et al., 2009) and glyphosate-based herbicides (Owagboriaye et al., 2020; Samal et al., 2019).

The aims of the present work were to quantify the response of *E. fetida* at the sub-organism and organism levels when exposed to atrazine and to identify useful biomarkers to assess the effects of the herbicide. In order to do so, toxicity tests were performed, focusing on the measurement of the enzymes AChE, LDH and ALP activities, population biological parameters such as biomass, growth and production of cocoons, and borrowing and casting activities.

2. Materials and methods

All the experiments were carried out within the laboratories and the pilot plant at INTEC (Instituto de Desarrollo Tecnológico para la Industria Química) installations, located in Santa Fe, Argentina (31° 38′ 23.2″ S, 60° 39′ 59.8″ W).

2.1. Chemicals, soil and earthworms

Acetylthiocholine iodide (AcSChI) and 5,5′dithiobis-2 nitrobenzoic acid (DTNB) (Sigma-Aldrich), NaH_2PO_4, Na_2HPO_4 were of analytical grade. Commercial kits from Wiener lab® were used for total proteins and two of the enzymatic determinations. A commercial formulation of atrazine (5 g L^{-1}, Zamba®) was employed and subsequent solutions of the herbicide were prepared by diluting with distilled water. The commercial formulation was chosen to resemble field conditions.

The soil utilized for the assay was obtained from the vicinity of the Institute, from the first 10 cm of the profile. It was cleaned of all superficial vegetation, macroscopic organisms and debris particles. Then, it was sun dried and screen-sieved (<5 mm). This soil has no background of productive agro-activities, being used as control soil for the bioassays. Its main physicochemical characteristics are shown in Table 1. Organic matter content was obtained gravimetrically, as the difference between the original dry weight and the ashes (Sharma and Garg, 2018). Organic C was determined by ignition at 550 °C (Wright et al., 2008) and total N was obtained from elemental analysis (LECO CHN628 Series Elemental Analyzers). The pH was determined in a mixture of air-dried soil and deionized water (1:2.5 w/v) and measured with pH meters (HACH®HQd Field Case) and the texture, by the method of Bouyocus.

The species used for this research was *Eisenia fetida*, currently utilized for ecotoxicological assessment of substances in soil, and recommended as test species by the Organization for Economic Cooperation and Development (OECD) and International Standardization Organization (ISO). Adult, clitellated earthworms (mean body weight 300 ± 25 mg) were provided by the INTEC terrestrial oligochetes bioterium. Culture conditions were the following: 25 ± 2 °C, constant artificial light, 50 ± 10% moisture dry mass, fed weekly with dried milled cow manure and domestic vegetable waste (1:3 w/w) following the methodology detailed in Masin and Rodríguez (2012) and considering the guidelines of OECD 207 (1984). Prior to any exposure test, selected earthworms were placed in the dark within aerated recipients (glass boxes with perforated plastic films to assure gaseous exchange) for 24 h, to empty their gut content. Individuals were periodically cleaned and carefully moistened with sprayed distilled water.

2.2. Toxicity tests: exposure of E. fetida to atrazine

2.2.1. Screening test: Contact filter paper test

A preliminary toxicity screening test was run with the earthworms,

Table 1
Soil physicochemical characteristics.

Parameter	Soil
Organic matter (%)	1.46
Organic C (%)	0.84
Total N (%)	0.088
C/N ratio	10
pH	6.0
Clay < 2μ (%)	20.5
Silt 2-50μ (%)	59.5
Sand > 50μ (%)	20.0

to identify potentially toxic concentration and therefore to choose sub-lethal concentrations that were also relevant for agricultural application (Song et al., 2009). The contact filter paper test was performed according to the OECD guideline (OECD 207, 1984). A piece of round filter paper was placed in a 9 cm Petri dish and moistened with 1 mL of atrazine solutions of three increasing concentrations (0.0255, 0.0637 and 0.1274 μg cm^{-2}, equivalent to 2, 5 and 10 mg L^{-1}, respectively) or distilled water (as control). The lowest dose corresponds to a typical concentration recommended for application onto the fields (Song et al., 2009) (Zamba®) and the higher is based on literature (Lin et al., 2019). For each concentration of atrazine and the control, 5 replicates were run, each containing one *E. fetida* earthworm per Petri dish. The test was run in darkness at 23 ± 2 °C for 72 h. After the exposure time, the earthworms were inspected for mortality. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end.

2.2.2. Sub-Chronic toxicity test

Based on the previous results, doses of 2 and 10 mg Kg^{-1} of atrazine were selected for this experiment. The toxicity test was performed according to ISO 11268-2 (2012). Each treatment (T1: 2 mg Kg^{-1}, T2: 10 mg Kg^{-1} and C: control) consisted of glass boxes (15 × 10 × 10 cm) with perforated plastic films as lid to allow gaseous exchange. Replicates of six boxes were assigned per treatment. Amounts of 400 g of dried soil were placed within, moistened to 25% of its water content by spraying with the atrazine solutions or distilled water, according to each treatment; they were mixed and left to equilibrate for 24 h. Then, an amount of 8 clitellated *E. fetida* earthworms (mean body weight 0.320 ± 0.050 g) was added per box. The boxes were subjected to a controlled photoperiod (16 h light/8 h darkness) and temperature was adjusted to 23 ± 2 °C. The earthworms were fed weekly, according to Masin and Rodríguez (2012). At 7 and 28 days of exposure, the following parameters were recorded according to ISO 11268-1 (2012) and OECD 207 (1984): survival (as number of living adults per total adults); biomass of adult earthworms (wet weight, g ind^{-1}, expressed as % biomass gain or loss in relation to initial weight); reproduction parameters including the number of clitellated individuals (expressed as relative clitellated individuals in relation to the number of individuals in each treatment) and the number of cocoons (in relation to the number of individuals in each treatment). The behavior of the earthworms in the substrate was also monitored (and recorded photographically) by observing burrows (mobility) and the presence of bioaggregates and spherical lumps of earth on the substrate surface. Also, samples of earthworms were randomly taken from the boxes at those days for enzymatic analysis.

2.3. Biomarkers response

The activities of alkaline phosphatase (ALP), lactate deshydrogenase (LDH) and acetylcholinesterase (AChE) were measured in earthworms after 7 and 28 days of exposure to atrazine and in the control group. Previously, randomly selected earthworms were separated and stored in the dark for 24 h to void their guts. Gut-cleaned earthworms (n = 6) were homogenized in phosphate buffer pH 7.2 (1:6 w/v) (Ultraturrax® T25 basic IKA labortechnik) at 24000 rpm for 1 min. Triplicates were run. The homogenates were centrifuged at 9000 g for 30 min at 4 °C to obtain the S9 fraction (microsomal and cytosolic fractions). After centrifugation, the supernatants were collected for further determinations. Procedures were carried out in ice bath.

Total Protein (TP) content in the S9 fraction was quantified with a commercial kit based on the Biuret colorimetric method. An amount of 0.05 mL of the homogenate and 2 mL of the reagent (13 mmol L^{-1} EDTA/Cu in NaOH and alkyl aryl polyether) were incubated at 37 °C for 15 min. Absorbance was read at 540 nm (UV/Vis Perkin Elmer Lambda 35) and protein content was determined based on a calibration curve with a standard albumin solution (Proti2 Wiener lab®).

Both LDH and ALP activities were also determined using commercial kits based on colorimetric kinetic determinations. For LDH, an amount of 0.04 mL of the homogenate was added to 2 mL of reagent (NADH in phosphate buffer solution pH 7.2, containing pyruvate and NaCl) and the absorbance was measured at 340 nm once per minute for 3 min. The specific enzymatic activity was expressed as nmol reduced pyruvate per min per mg of protein. For ALP, an amount of 0.02 mL of the homogenate was added to 2 mL of reagent (p-nitrophenyl phosphate (pNPP) in 1 mol L^{-1} diethanolamine solution pH 9.8, containing magnesium salts). The absorbance was measured at 405 nm once per minute for 3 min. The specific enzymatic activity was expressed as nmol of hydrolized p-NPP per min per mg of protein. Both LDH and ALP were determined at 37 °C.

The AChE activity was determined according to the method of Ellman et al. (1961). The reaction medium consisted of 50 μl of the homogenate, phosphate buffer pH 8, 0.01 M DTNB and 0.075 M AcSChI as substrate. Kinetics was recorded spectrophotometrically at 412 nm. The specific enzymatic activity was expressed as nmol of acetylthiocholine hydrolysed per min per mg of protein, calculated with a molar extinction coefficient of 13.6 mM^{-1} cm^{-1}.

2.4. Statistical analysis

Biomass and enzymatic results are expressed as means ± standard deviations (SD). ANOVA was performed to biomass data to evaluate statistical significant differences, followed by Dunnett's post hoc test to compare both treatment groups with the control group. Unidirectional Kruskal-Wallis non-parametric analysis of variance was performed on clitellated individuals and cocoons production data. Independent-samples t-tests were performed on the enzymatic results. Minitab® (version 17.1.0) was employed and a significance level of $p < 0.05$ was set in all cases.

3. Results and discussion

3.1. Contact filter paper test

In the 72 h-test performed for the three atrazine concentrations of 0.0255, 0.0637 and 0.1274 μg cm^{-2}, no mortality of earthworms was evidenced, showing active and moving individuals without any observation of external damage in their body. For a 48 h filter paper test with *E. fetida*, Wang et al. (2016) informed a LC_{50} value of 4.93 μg cm^{-2}, which is >38 times more concentrated than the highest concentration tested in this work. This is consistent with the results obtained, considering as well that atrazine LD_{50} for earthworms was established as high as 78 mg Kg^{-1} (Lin et al., 2019). Other authors have reported lower toxicity of atrazine when compared to other pesticides tested on earthworm (Mosleh et al., 2003a). Based on this result, further investigation of the sub-lethal effects derived from the exposure to the atrazine concentrations tested were run on soil, since it mimics the natural environment of earthworms (Udovic and Lestan, 2010).

3.2. Effects on survival, biomass and reproduction

In agreement with the results obtained in Section 3.1, no mortality was recorded during this experiment for any of the treatments. The biomass of the earthworms was monitored throughout the experiment and the results are shown in Fig. 1. No significant difference was noticed between treatments ($P > 0.05$) although variations with time were observed and both control and treatments showed a similar behaviour. After 7 days, a slight decrease in this parameter was recorded for the control (3.6%) and T1 (3.3%), while for T2 the decrease was higher (7.5%). This could be partially related the limited available food during the first week (earthworms were fed every seven days). Also, according to Ismail et al. (1997), the accumulation of atrazine within earthworms exposed to this herbicide is the highest during the first week, therefore some feeding inhibition could also have occurred as a strategy of the earthworms to minimize poisoning by pesticides and preserve the energy for vital processes (Mosleh et al., 2003b; Ribeiro et al., 2001). After

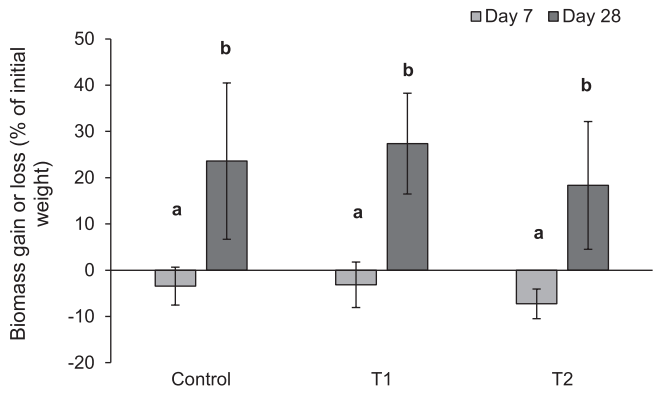

Fig. 1. Change in the biomass of *E. fetida* exposed to different atrazine concentrations (T1: 2 mg Kg^{-1}; T2:10 mg Kg^{-1}) after 7 and 28 days. Control: without atrazine. Different bold letters indicate significant difference ($p < 0.05$) between exposure times.

28 days, mean biomass of the earthworms significantly increased ($P < 0.05$), not only for the control (23.7%, 0.412 ± 0.06 g/ind.) but also for T1 (27.3%, 0.424 ± 0.04 g/ind.) and T2 (18.3%, 0.394 ± 0.05 g/ind.). This could be mainly related to the reproduction behaviour, discussed below, but also could reflect an adaptation of the earthworms to the soil and regular feeding.

Reproduction parameters were also examined. Fig. 2(a) shows the relative number of clitellated earthworms in relation to the initial ones (all clitellated). It can be observed that clitellated individuals decreased significantly ($P < 0.05$) with time to approximately 50% (between 23 and 26 individuals) in the control and both T1 and T2, during the first

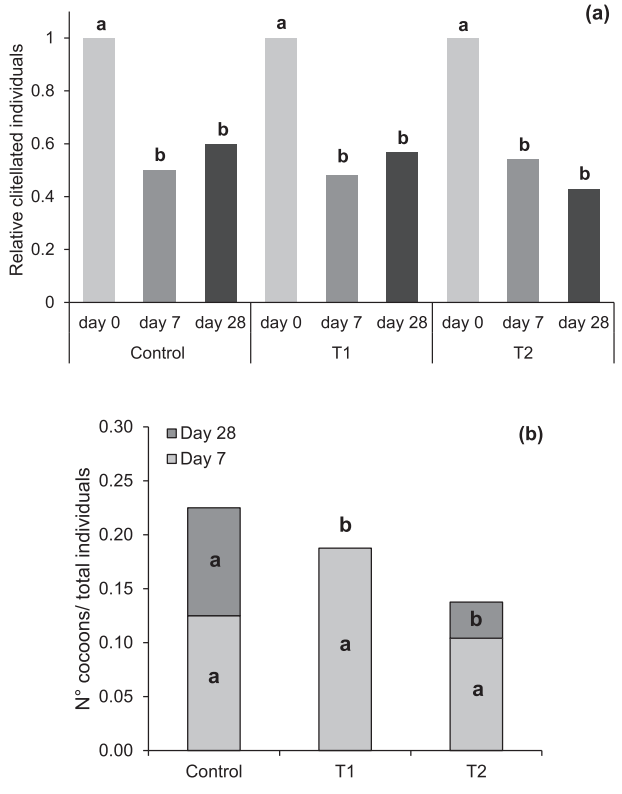

Fig. 2. (a) Relative clitellated individuals and (b) accumulated number of cocoons relative to the total earthworms *E. fetida* exposed to atrazine. T1: 2 mg Kg^{-1}; T2:10 mg Kg^{-1}. Control: without atrazine. Different bold letters indicate significant difference ($p < 0.05$) between exposure times.

week. The clitellium in the body of the earthworm indicates reproductive maturity and allows the organisms to produce cocoons. In this case, the momentary loss of clitelium (meaning that it was no longer conspicuous and very noticeable in the body of the earthworm) could be related to the energy required in the production of the cocoons as well as the stress derived from the exposure to the herbicide (Reinecke and Viljoen, 1990; Venter and Reinecke, 1988). Reproduction requires great energy expenditure from the individuals, which also affects their biomass. This can be associated with the decrease in biomass observed, presented in Fig. 1. After 28 days, C and T1 showed an increase of 10% of relative clitellated individuals while T2 showed a decrease of 11%, in accordance with the lower biomass increment (Fig. 1).

Likewise, Fig. 2(b) illustrates the accumulated number of cocoons relative to the amount of total earthworms for each treatment and sampling day. It can be appreciated that, during the first week, the lowest dose of atrazine (2 mg Kg^{-1}) accelerated the production of cocoons, when compared to the control; whereas, with 10 mg Kg^{-1}, the number of cocoons was similar to the control. This result could be explained in terms of a hormesis effect, where non-harmful levels of the pesticide may help the organism in an environment that is not optimal (Domínguez et al., 2016). Suthar (2014) found that at low doses of pesticides, the stress of the xenobiotic exposure can trigger the cocoon production in *E. fetida*, while activating also repair mechanisms of the body. Nevertheless, it should be mentioned that not all earthworm species may react in the same way. The differences in cocoon production rate during the first week, could be connected to these mechanisms by which the stress of exposure to ATZ generates the early release of the progeny, at the expenses of the biomass loss of individuals (Yasmin and D'Souza, 2010). Statistical significant differences were observed only after 28 days ($P < 0.05$) for both treatments. For T1, no production was observed after 28 days, even though the cumulative number of viable cocoons was the same as the control (9 cocoons). On the other hand, with 10 mg Kg^{-1} (T2), the earthworms produced a reduced amount of total cocoons (around 33% less). This result can be associated to the lower clitellated individuals observed in Fig. 2(a).

Earthworm burrowing and casting activities were very noticeable for all the treatments during the 28 days (Fig. 3) which evidenced the high activity of the earthworms in the substrate. As a consequence, there is an increase in porosity, oxygenation and moisture retention of the soil while also their mucus accelerate the decomposition of organic matter, allowing the availability of nutrients for microbial growth (Huang and Xia, 2018; Li et al., 2015). These activities are related to the improvement of the soil physicochemical properties and can contribute to the degradation of atrazine. This is supported by the work of Lin et al. (2019) who found that *E. fetida* accelerated the atrazine degradation during a 28-days vermiremediation by enhancing the indigenous microorganisms in the soil responsible for mineralizing the herbicide.

3.3. Effects on the protein content and enzymatic activities in E. fetida

Fig. 4 shows the changes in the protein content in *E. fetida* after 7 and 28 days of exposure. Results are displayed as relative protein content in relation to the control. Comparing to the control boxes without atrazine, the amount of protein decreased after 28 days for both tested concentrations, however only T1 was statistically significant ($P < 0.05$). Numerous authors have reported the decrease in protein content as a key toxic effect of pesticides. Ismail et al. (1997) found a decrease in total protein content of earthworms *Aporrectodea caliginosa* exposed to atrazine, with the greatest reduction at 28 days, in consonance with the variation of atrazine concentration in the soil. Other authors also registered a reduction in the protein content in *E. fetida* exposed to the herbicide Siduron (Li et al., 2019); they suggested an increase in the energy required to detoxify and excrete the harmful compound leading to a detriment in the energy spent for the earthworm growth. This does not seem to be the case for atrazine at the concentrations tested here in *E. fetida*; as it was observed in Fig. 1, the biomass of the earthworms

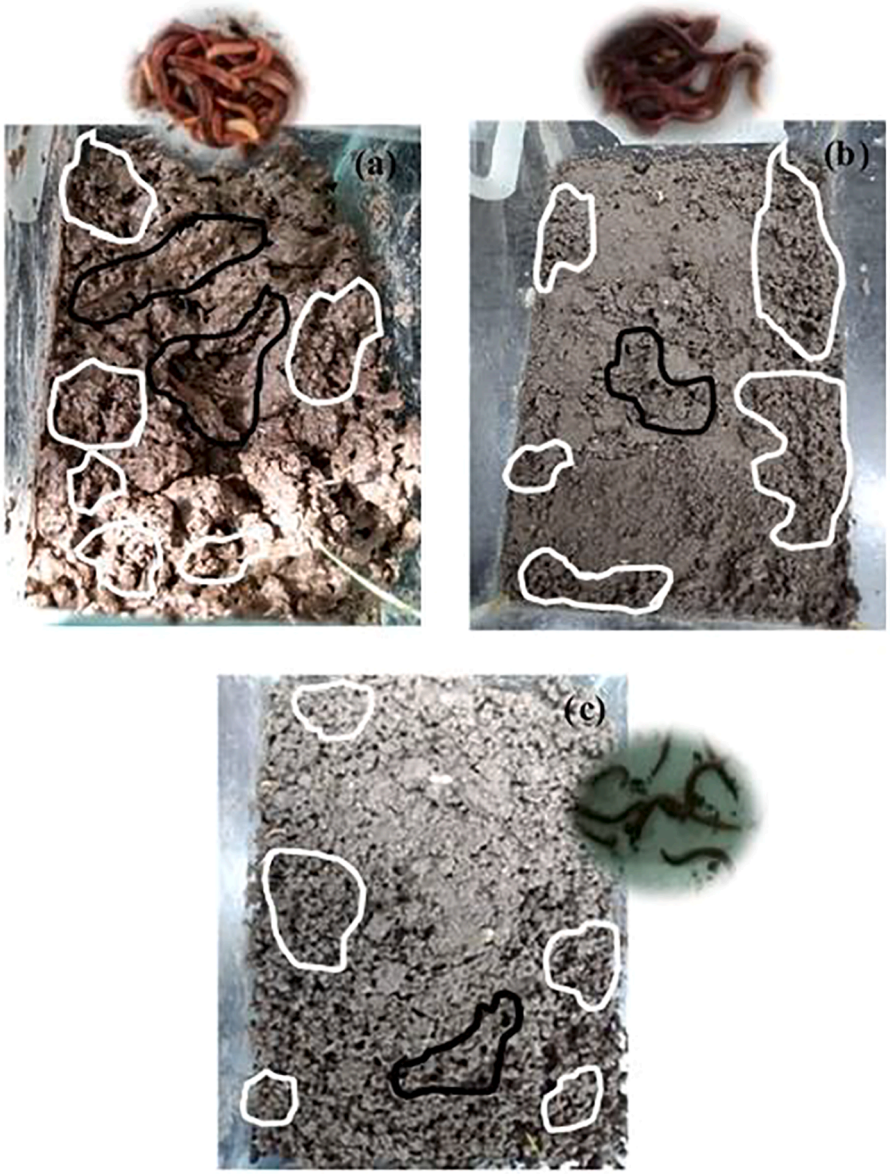

Fig. 3. Earthworm burrowing (marked with black lines) and casting activities (marked with white lines) in the Control (a), T1: 2 mg Kg^{-1} (b) and T2: 10 mg Kg^{-1} (c) after 28 days of bioassay.

increased after 28 days and growth was not affected. Then, it is possible that ATZ might have inhibited the protein synthesis or even enhanced their degradation. This was stated by Tripathi et al. (2009) who found a phorate-dose and time dependent fall in both supernatant and mitochondrial protein concentrations in three species of earthworms.

The activities of the three selected enzymes at sub-chronic exposure times are presented in Fig. 5(a–c). Compared to the control values, ALP activity (Fig. 5(a)) slightly decreased after 7 days for both concentrations T1 and T2, however this change was not statistically significant. ALP activity in *E. fetida* exposed to different pesticides has exhibited different responses. Organophosphate insecticides (trichlorfon and dimethoate) and fungicides (carbendazim and prochloraz) decreased ALP activity, but fungicide tebuconazole had no effect on the enzyme (Rico et al., 2016). Other authors reported that ALP in gastrointestinal tissue did not change after chlorpyrifos exposure but ALP in lumen did, so response can be localization-dependent for that insecticide (Sanchez-Hernandez et al., 2018). Particularly for atrazine, but in *in vitro* assays, higher concentrations (1.5 and 5 mM) inhibited ALP in *Aporrectodea caliginosa* (El-Aswad and Badawy, 2015). However, it is also worth noting that soil dwelling species (e.g. *Lumbricus terrestris*, *Aporrectodea caliginosa*) are usually more sensitive to pesticides than epigeic earthworms (*E. fetida*) which live on the soil surface (Pelosi et al., 2014).

Fig. 5(b) depicts the LDH levels in *E. fetida* during the experiment. After 7 days, no significant change was observed either for T1 or for T2, but a significant increase in LDH activity was recorded with T1 after 28 days, in comparison to the control ($P < 0.05$). LDH is a metabolic enzyme involved in anaerobic energy production, NADH recycling and gluconeogenesis (Tripathi et al., 2011); results might indicate that higher activity of this enzyme was needed to fulfil the increased energy requirements. Rico et al. (2016) found that LDH activity in *E. fetida* depended on the type of pesticide but also on the concentration used; for instance, tebuconazole increased LDH levels at lower concentrations but inhibited it at higher ones. Some authors have also found that LDH in other species of earthworms decreased with increasing doses of an insecticide (Tripathi et al., 2009). Also, a decrease in the control values of LDH was observed between the days 7 and 28. Differences in control values for other enzymes in *E. fetida* have also been reported by other authors (Owagboriaye et al., 2020; Song et al., 2009). This enzyme has

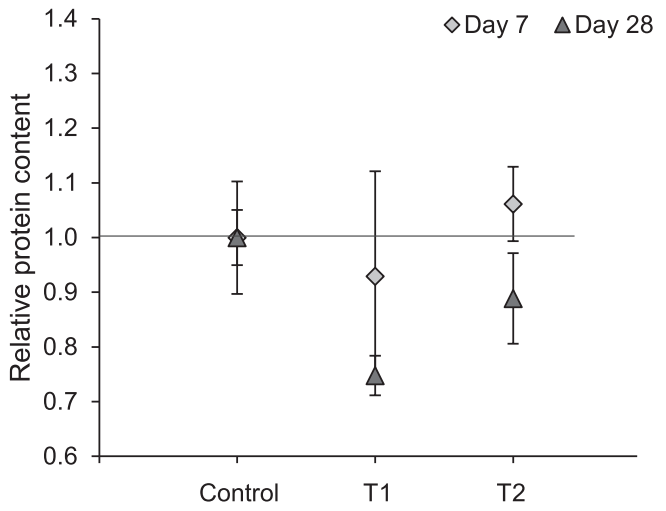

Fig. 4. Relative protein content in adult *E. fetida* exposed to atrazine. T1: 2 mg Kg^{-1}; T2:10 mg Kg^{-1}. Control: without atrazine.

been observed to vary with environment and feeding conditions (Diehl and Collier, 1991; Tripathi et al., 2011).

The most perceivable change was observed in AChE levels; the results are presented in Fig. 5(c). AChE activity values of the unexposed earthworms were found very similar to those reported by Bednarska et al. (2017) also for *E. fetida* and Velki and Hackenberger (2013) for *E. andrei*. Atrazine enhanced AChE activity at the lower concentration T1; these values were statistically significant after 7 and 28 days ($P < 0.05$). The induction of this enzyme has been linked to oxidative stress and alterations in the intracellular ion homeostasis as well as to apoptosis-induced substances (Barbosa Melo et al., 2003; Zhang et al., 2002). This behaviour has been reported for other enzymes. Antioxidative enzymes like catalases have also been stimulated at low atrazine concentrations and inhibited at high concentrations (Song et al., 2009). This hormetic response is suggested as an adaptive stress response and a mechanism against the toxicity of a compound (Owagboriaye et al., 2020).

4. Conclusions

The enzymes alkaline phosphatase, lactate deshydrogenase and acetylcholinesterase in *E. fetida* earthworms and some behaviour and biological parameters were surveyed in the search for a suitable biomarker to assess the effects of exposure to field-resembling concentrations of atrazine. Some biomarkers were more sensitive to atrazine exposure and the search for the appropriate ones is vital. AChE was the most sensitive enzyme to the herbicide. Although there were some signals of progeny production alterations, all adult earthworms maintained high burrowing and casting activities, indicating also the low impact of the atrazine concentrations assayed at short term. A longer surveillance of these parameters may help to reassure these findings. Based on the surveyed parameters, when exposed to these atrazine concentrations, *E. fetida* can be applied as potential vermiremediator without compromising their general health and reproduction. Future experiments, with higher concentrations of the herbicide, will define the limits of usage.

CRediT authorship contribution statement

All authors: Sofía Lammertyn, Carolina Elisabet Masín, Cristina Susana Zalazar and Maria Emilia Fernandez contributed to the Conceptualization, Methodology, Formal analysis, Investigation, Writing - review and editing. Writing the original draft was made by Maria Emilia Fernandez. Resources and Funding acquisition by Cristina

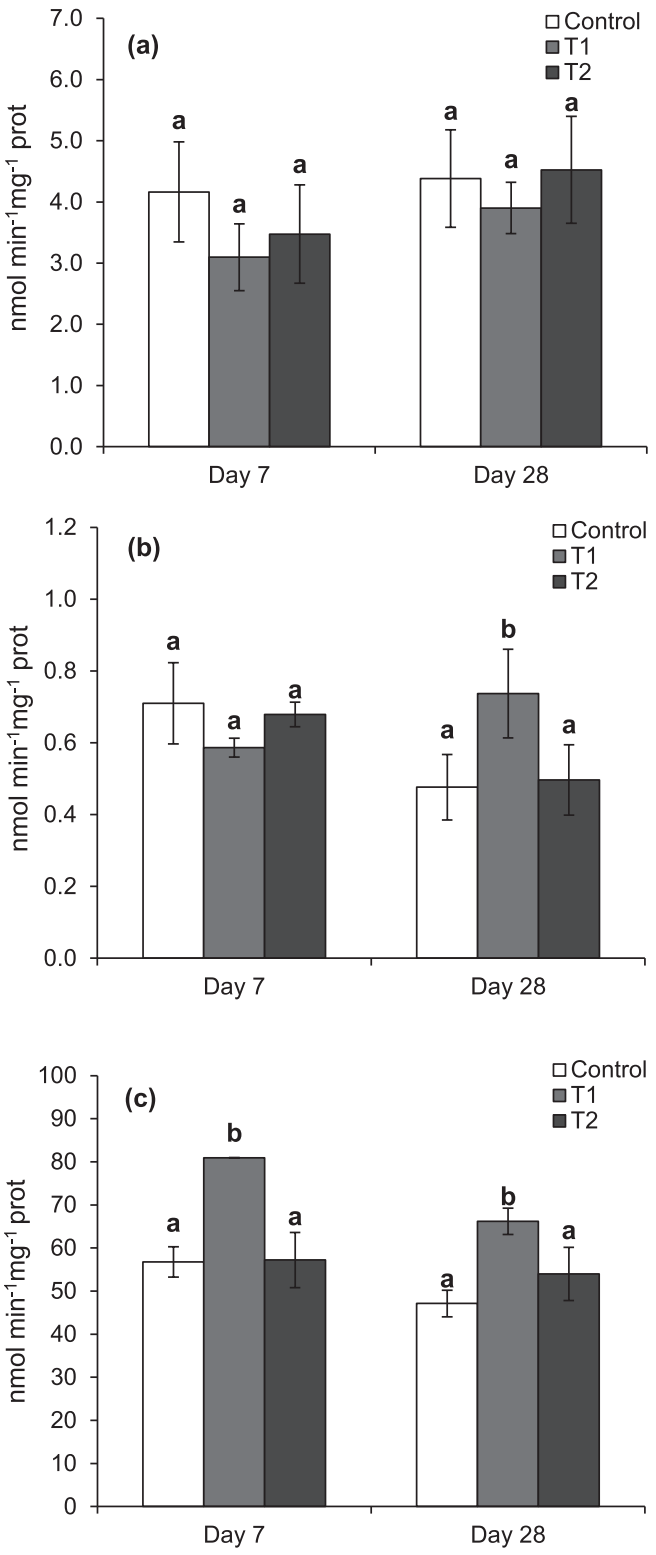

Fig. 5. Enzymatic activities of (a) alkaline phosphatase (b) lactate deshydrogenase and (c) acetylcholinesterase in adult *E. fetida* exposed to atrazine. T1: 2 mg Kg^{-1}; T2:10 mg Kg^{-1}. Control: without atrazine. Different bold letters indicate significant difference ($p < 0.05$). Enzymatic activities of (a) alkaline phosphatase (b) lactate deshydrogenase and (c) acetylcholinesterase in adult *E. fetida* exposed to atrazine. T1: 2 mg Kg^{-1}; T2:10 mg Kg^{-1}. Control: without atrazine. Different bold letters indicate significant difference ($p < 0.05$).

Susana Zalazar.

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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