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# **Evaluation of Earthworms Present on Natural and Agricultural-Livestock Soils of the Center Northern Litoral Santafesino, República Argentina**

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/46228>

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## **1. Introduction**

For a long time in Argentina, farmers had concentrated on a mixed system of livestock and crops, mainly wheat and corn. Soybean was not a traditional crop [1].

During the 1990's (20<sup>th</sup> century) the agricultural frontier reached a planted surface that rose 11.000.000ha with the adoption of technologies that includes transgenic seeds, limited tillage and application of pesticides and fertilizers [2, 3]. The main crops are soybean monocultures and soybean/wheat soybean double crops corn. This two crop sequences have different C dynamics response to management such as tillage or fertilization [4]. In the 2000-2009's pesticide market had triplicate [5] being the most popular endosulfan, lambda-cyhalothrin, cypermethrin, chlorpyrifos, metamidophos and the herbicides glyphosate, atrazine and 2,4D [6]. Importance of edaphic fauna to the soil fertility is well known, especially with oligochaeta that are being used as bioindicators of the soil health [7-13].

Earthworms spent their whole life cycle in the soil horizons and because of their feeding and burrowing behaviour they directly or indirectly help to improve every physical, chemical and biological process of the soil. Earthworms participate in the mixing of organic and inorganic fractions of soil, formation of stable clusters, dynamic and recycling of nutrients from the decomposition of organic matter, their burrows help to the aeration, infiltration and drainage of soil [14]. Earthworms represent over the 80% of the biomass invertebrate of the soil, therefore they are an useful group to evaluate the effect of pesticides either on field as on laboratory tests. The ecotoxicological impact of Argentina's current production system

is not well known and evaluated and requires the realization of deeper studies using fast and reliable bioindicators in order to understand the biological processes related with the anthropogenic alterations of the environment [15-28]. Therefore we performed ecotoxicological bioassays under laboratory conditions and field evaluations of parameters such as biomass, richness and density of earthworms.

## 2. Materials and methods

### 2.1. Field experiences

#### 2.1.1. Study site description

In order to evaluate the influence of different production systems over the oligochaetofauna, five sites from different areas of the north and centre of Santa Fe province were sampled (Fig.1, Table 1).



**Figure 1.** Map of the sampling sites.

1. *Livestock in woodland (Ganadería en Monte Nativo: GMN)* located in Naré, is a native woodland, used for bovine livestock alternated with fallow periods. Characterized by the presence of *Prosopis nigra*, *Eucaliptus spp.*, *Acacia acaven*, *Erythrina crista-galli*, *Enterolobium contortisiliquum*, *Cynodon dactylon*, *Cynodon rotundus*, *Digitaria sanguinalis*.
2. *Fallow field (Lote en Descanso: LD)* located in Sarmiento, used 3 years ago as paddock for bovine livestock. At present without activities. Characterized by the presence of *Cynodon dactylon*, *Cirsium vulgare*, *Solanum sisymbriifolium*, *Digitaria sanguinalis*, *Melia azedarach* L.
3. *Non Tillage (Agrícola con Siembra Directa: ASD)* located in Isleta Norte, with over 30 years of agricultural practices (last 15 years with minimum tillage). Crops consisted in sunflower, soybean, corn and sorghum. Pesticides applied were glyphosate-coadyuvants ( $4.5 \text{ l.ha}^{-1}$ ), atrazine ( $3 \text{ l.ha}^{-1}$ ) and superphosphate triple calcium fertilizer ( $65 \text{ kg.ha}^{-1}$ ).
4. *Non Tillage with added organic amendments (Agrícola con Siembra Directa y Abono Orgánico: ASDAO)* located in Ocampo Norte, with over 20 years of agricultural practices with minimum tillage. Crops consisted in soybean, sunflower, cotton, corn and oat. Synthetic fertilizers superphosphate triple calcium ( $65 \text{ kg.ha}^{-1}$ ) and organic fertilizers (cow dung), glyphosate ( $4 \text{ l.ha}^{-1}$ ) and clorimuron ( $60\text{g.ha}^{-1}$ ) are applied.
5. *Livestock in grassland (Ganadería en Pastizal Natural: GPN)* located in El Sombrerito, is a natural grassland used for the feeding of ovine cattle, rotating with fallow periods. Agrochemicals and mechanic weed control are not used. Vegetation consist of *Erythrina crista-galli*, *Enterolobium contortisiliquum*, *Brachiaria platyphilla*, *Digitaria sanguinalis*, *Paspalum quadrifarium*, *Cynodon dactylon*, *Sorghum halepense*, and others.

Sites	Geographical Coordinates		Location	Year/ Season
	South Latitude	West Longitude		
1. Livestock in woodland (GMN)	30° 53' 12.09"	60° 27' 01.92"	Naré (San Justo)	2009/Spring
2. Fallow field (LD)	31° 04' 25.11"	61° 10' 32.30"	Sarmiento (Las Colonias)	2011/Spring
3. Non tillage (ASD)	28° 27' 34.18"	59° 18' 57.17"	Isleta Norte (Gral. Obligado)	2011/Summer
4. Non tillage with added organic amendments (ASDAO)	28° 27' 21.84"	59° 22' 26.93"	Ocampo Norte (Gral. Obligado)	2011/ Summer
5. Livestock in grassland (GPN)	28° 38' 00.43"	59° 27' 59.79"	El Sombrerito (Gral. Obligado)	2011/ Summer

**Table 1.** Geographical coordinates of the sampling sites and sampling season.

### 2.1.2. Field sampling design

Twenty samples were taken for each site using a zigzag transect of random direction according to the TSBF standard method [29]. Each sample consisted of a soil block of 0,30 x 0,30 x 0,30 m with a distance between samples of 15 m, to assess the independence of data from each block. The collect of earthworms: adult, juveniles and cocoons took place at laboratory. Sexually mature earthworms (clitellated) were anesthetiated according to methodology described by Moreno & Borges [30]. The identification of earthworms was performed using a binocular stereoscopic microscope and the species diagnosis was performed according to Mischis taxonomy [31]. Abundance (total number of organisms on sampled site), species richness (number species on sampled site (S)) and density (org/m<sup>2</sup>) were recorded.

After the extraction and counting of earthworms, a soil sample of each site was taken to perform physical and chemical analysis at a soil specialized laboratory (IDICYT-Universidad Católica de Santa Fe). Pesticide residues determination were performed at Laboratorio de Medio Ambiente (INTEC–Universidad Nacional del Litoral). Insecticide residues were determinate by gas chromatography (GC) and herbicide residues by high performance liquid chromatography (HPLC) with specific detectors. Statistical analysis of the mean was performed using one-way ANOVA and Tukey's multiple test ( $p < 0.05$ ).

## 2.2. Bioassay characterization

Our main goal was to assess under laboratory conditions, the effects of the pesticides endosulfan, glyphosate and lambda-cyhalothrin on the earthworms *Eisenia fetida* and *Aporrectodea trapezoides* (standard bioassay species and common species in the sampled sites respectively) from the family Lumbricidae (Oligochaeta). In all the experiences, the laboratory tests were validated according to ISO 11268-1 Guideline [32], with a room temperature of 23± 2°C and photoperiod of 16:8 (intensity~800 lux). Acute and chronic tests were performed in OECD artificial soil or reference soil according to the OECD protocol [33]. Due the arthropod-specific action of pyrethroids their acute toxicity to earthworms is low and is more suitable to perform an avoidance test as an alternative to rapid toxicity assessment based on behavioural responses [34].

Earthworm adults (clitellated) *Eisenia fetida* or *Aporrectodea trapezoides*, obtained from our laboratory stock culture were used. Before starting the bioassays, earthworms fasted 24 hours to clean their guts. Transparent polypropylene boxes of 20x10x15cm with perforated lids were filled with 500g of dry substrate. The moisture content was kept using distilled water for the control groups and pesticide solutions with the proper concentration for each treatment. Hydrophobic pesticides as lambda-cyhalothrin and endosulfan were mixed with hexane to obtain the desired concentrations and placed under hood for 24 hours to evaporate the solvent. For *E. fetida* bioassays organisms were fed with dry, triturated and sieved cow dung on a 7 days frequency. For the *A. trapezoides* bioassays organisms were fed with a mixture (1:3) of cow dung (same as described before) and domestic organic residues on a 20 day basis. Survival (number of living organisms), moist weight (expressed on

grams), cocoon production and juveniles number was recorded weekly or monthly for *E. fetida* and *A. trapezoides* bioassays respectively.

Statistical approximation of effective concentration (EC<sub>50</sub>) was obtained graphically and lethal concentration (LC<sub>50</sub>) by Probit analysis. For the biomass and reproduction parameters one-way Anova followed by a post hoc Dunnet Multiple Comparison test were performed.

### 2.2.1. Experiences with endosulfan

Even when their application and manufacture is banned or restricted in several countries, endosulfan (Class II) [35] is one of the most used organoclorated insecticides and acaricides in Argentina, especially in Santa Fe province. Nevertheless the endosulfan importation is going to be banned in July 2012 and their formulation and use will be banned in July 2013 [36]. Endosulfan is applicated to control several pests as *Rachiplusia un*, *Nezara viridula*, *Piezodorus guildinii*, *Spodoptera frugiperda*, *Colias lesbia*, *Heliothis zea*, *Spilosoma virginica* and *Anticarsia gemmatilis*. Due their high biocide action is applicated both on extensive crops (soybean, corn, wheat, alfalfa, cotton, fruit crops and tea crops) as on intensive crops (floricultural and horticultural). Their physicochemical characteristics, frequency and application doses made endosulfan a highly available pesticide for earthworms who are either responsible of organic matter degradation and humification processes in the soil than an important prey for many predators (20; 10). Prior to the bioassay the percent of active ingredient of commercial endosulfan (Atanor® 35%) was determined by GC using VARIAN 3700 with electronic capture detector. Acute toxicity test consisted in a range of 5 concentrations and a control group. They were set in 4 replicates containing OECD soil and 10 *Eisenia fetida* earthworms clitellated and with a mean weight of 300mg (ea) per box. Exposure time was 14 days and at the end of the test the number of dead organisms was recorded. For the chronic toxicity test range was 2; 3; 4; 7; 10 mg.kg<sup>-1</sup>dw, with an exposure time of 56 days to test survival and biomass. Cocoon and juveniles production was recorded at 56 and 84 days respectively and they were kept in boxes with the corresponding treatments until control juveniles developed a clitellum.

In order to perform degradation in soil analyses, samples of 10g of soil from the 2; 4; 10 mg.kg<sup>-1</sup>dw treatments were taken and sent to the gas chromatography laboratory for further analysis. Samples were analyzed by triplicate according to Miller & Miller [37]. Detection limits were 0.003; 0.003 and 0.005 mg.kg<sup>-1</sup> for endosulfan α, β and endosulfan sulfate respectively.

In order to assess the kinetics of degradation of biocide studied, an exponential regression analysis was performed for each data group: concentration vs. time of exposure. The velocity constant was determined for each case through the equation:

$$C_t = C_0 * e^{-kt} \quad (1)$$

Where C<sub>t</sub>=pesticide concentration at time *t* (mg.kg<sup>-1</sup>); C<sub>0</sub>=initial concentration (mg.kg<sup>-1</sup>); *k*=days<sup>-1</sup>; half-life time, *t*<sub>1/2</sub> (days), was determined by Equation (1) replacing C<sub>t</sub> by C<sub>0</sub>/2 resulting in: *t*<sub>1/2</sub> =ln 2/*k*.

### 2.2.2. Experiences with glyphosate

Transgenic soybean crops require the utilization of the herbicide glyphosate (Class III) [35]. Their persistence in the soil matrix could vary from days to months and depends of multiple edaphic and climatic factors. Prior to the bioassay the percent of active ingredient of commercial glyphosate was determined by HPLC with post column derivatization and Millenium<sup>32</sup> data acquisition system.

Chronic bioassays at the sublethal concentration range: 7; 11; 18; 30; 50 mg.kg<sup>-1</sup>dw of commercial glyphosate (Round up Monsanto® 48%) were performed. Each treatment and the untreated control group were set in 4 replicates containing reference soil and 10 adult clitellated *E. fetida* with a mean weight of 450mg (ea) per box. Glyphosate was mixed with distilled water in order to reach a 50% moist content. The same moist content was used for control groups by applying distilled water. Exposure time was 28 days. Detection limits were 0.05 µg.kg<sup>-1</sup> for glyphosate and their metabolite AMPA. Soil physicochemical parameters: humidity, C, N, C/N, texture, pH, CIC, P and soluble K were analyzed.

### 2.2.3. Experiences with Lambda-cyhalothrin

Lambda-cyhalothrin (Class II) [35] is a 4<sup>th</sup> generation pyrethroid insecticide widely used in Santa Fe province. It is highly active against a broad spectrum of pests in public and animal health and is also used in agriculture to control several pests such as hemiptera and lepidoptera in both extensive and intensive crops [38, 39]. Pyrethroids interfere with the normal function of nervous system of invertebrates. Their toxicity on non-target soil organisms is observed even at concentrations lower than the agricultural application rates [20, 21, 26, 40, 41].

#### 2.2.3.1. *Eisenia fetida* assays

Bioassays were performed using commercial lambda-cyhalothrin (Cilambda Ciagro® 5%). The moist content was 25% for avoidance test and 50% for chronic test. For the chromatographic determination on both soil and organisms, 98% lambda-cyhalothrin isomers mix, Chem. Service® was used. Concentration range for the 48 hours avoidance behaviour test was: 1.25; 7.5; 16.25; 32.5; 65 mg.kg<sup>-1</sup>dw, according to the ISO 17512-1 protocol [42]. Each treatment was set in 3 replicates. Plastic boxes were divided in two compartments using a piece of plastic fitted transversally in the box. One half of the box was filled with contaminated OECD soil and the other with OECD control soil. Then the separator was removed and 10 clitellated adults of *E. fetida* were placed in the separating line of each container test. The boxes were then covered with perforated plastic lids. At the end of the test period, the control and treatment soil were carefully separated and the number of earthworms in each section was determined. Individuals found between sections were considered as being in the soil as which the head was directed. Organisms found dead are considered as affected by the toxic [34, 38, 43].

For the statistical interpretation of avoidance test results the “Habitat Function” [44] was applied, considering toxic those soils where less than the 20% of the organisms were

found. The response of the organisms was measured using the equation proposed by Garcia [34]:

$$NR = ((C - T) / S) \times 100 \quad (2)$$

Where: NR=net response; C=sum of earthworms observed in control soil; T=sum of earthworms observed in treated soil; S=total number of earthworms per replicate

Concentration ranges for the chronic test were: 1; 2; 4; 8 mg.kg<sup>-1</sup>dw. Each treatment and the control were set with 4 replicates containing 10 clitellated adult *E. fetida* with a mean weight of 272 ± 15 mg (ea). Exposure time was 56 days to test survival, biomass and cocoon production. After the weekly register of cocoons, they were placed in a separate plastic box containing test or control soil and allowed to hatch. Number of juveniles was recorded weekly for an exposure time of 63 days and the results were fitted with a logistic regression model [34]:

$$Y = \frac{c}{1 + e^{(b \cdot (x - a))}} \quad (3)$$

where: Y=number of juveniles; a=natural logarithm (Ln) of EC<sub>50</sub> (mg.kg<sup>-1</sup>); b=slope; c=mean number of juveniles in control; x=Ln of concentration (mg.kg<sup>-1</sup>)

Surviving organisms from the concentrations 2, 4 and 8 mg.kg<sup>-1</sup> were aconditionated to assess the bioaccumulation at the end of test using the equation according ASTM [45] protocol:

$$BAF = \frac{C_B}{C_S} \quad (4)$$

where: BAF=bioaccumulation factor; C<sub>B</sub>=earthworm tissue concentration (mg.kg<sup>-1</sup>); C<sub>S</sub>=soil concentration (mg.kg<sup>-1</sup>)

In order to perform lambda-cyhalothrin degradation in soil analyses, samples of 10g from concentration 2; 4 and 8 mg.kg<sup>-1</sup>dw were taken at days 0, 56 and 86. Samples were dried at room temperature, extracted twice with proper solvent, cleaned-up, concentrated and analyzed by GC (d.l. 29ng.kg<sup>-1</sup>).

#### 2.2.3.2. *Aporrectodea trapezoides* assays

Concentration range for the 24 hours avoidance test were 1; 3; 9; 27 mg.kg<sup>-1</sup>dw. Each treatment were set in 3 replicates containing a reference soil with moist content of 30% and 6 clitellated adults of *A. trapezoides*. At the end of the test period, the control and treatment soil were carefully separated and the number of earthworms in each section was determined as described for *E. fetida*. The response of organisms was measured using Equation 2 and the EC<sub>50</sub> value was determined. Concentration range for the chronic test were 4.7; 6; 8 mg.kg<sup>-1</sup>. Each treatment and control were set with 3 replicates with a moist content of 35% and 5 clitellated adults *A. trapezoides* with a mean weight of 750±0,5 mg (ea). Exposure time was 70 days to test survival, behaviour and biomass.

3. Results and discussion

3.1. Field experiences

Sites ASD and ASDAO had the more acidophilus soils (Table 2). Those pH values could be explained by the exportation of bases that these soils suffers because of soybean crops and the application of nitrogenated fertilizers [46, 47]. Soils with pH between 6 and 7.5 as the GMN, LD and GPN from our research are consider optimums for the growth of the main cultures of this region [48].

Organic matter (OM) and the relation between Carbone (C) and Nitrogen (N) of the soil (C/N relation) are key indicators of the health and fertility of soil. Organic matter content was higher in LD site followed by GMN and GPN, this could be explained by the presence of weeds covering the soil which are incorporated as organic matter after the plant dies [49]. In the site ASDAO, even if the soil exploitation includes non tillage and addition of synthetic fertilizers and cow dung as organic fertilizer, the OM, C and N values were the lowest from all the tested sites (Table 2) indicating that the levels also depends on the intensity of the exploitation [48, 50-53].

	Site				
	GMN	LD	ASD	ASDAO	GPN
pH	6.05	6.10	5.80	5.80	6.00
OM	2.60	4.04	1.87	1.56	2.06
C (%g)	1.51	2.34	1.08	0.90	1.19
N (%g)	0.14	0.23	0.10	0.09	0.11
C/N (%G)	11.25	10.00	12.00	11.00	11.00
Conductivity (mmhos/cm)	0.86	0.89	0.24	0.327	0.31
Ca++ (me/100g)	9.30	6.60	11.00	7.80	8.80
Mg++ (me/100g)	1.15	1.00	0.40	0.40	1.00
Na+ (me/100g)	1.04	1.07	0.62	0.50	0.57
K+ (me/100g)	0.80	4.06	0.44	0.50	0.30
Species richness	<i>A.trapezoides</i>	<i>A.trapezoides</i>	<i>A.trapezoides</i>	<i>A.trapezoides</i>	<i>A.trapezoides</i>
	<i>A. rosea</i>	<i>A. rosea</i>		<i>A. rosea</i>	<i>A. rosea</i>
	<i>M. dubius</i>				<i>O. tyrtaeum</i>
Density (org./m²)	54	42	9	87	31

Table 2. Physicochemical and biological characteristics of the sampled sites.

When crops alternate within a short time period as in soybean crops, the amount of weeds and stubbles is low and leads to a progressive loss of fertility. About C/N relation, soils from the all sampled sites showed values between 10 and 12 which indicate an equilibrium between mineralization and humification process. According to López [49] the soil is considered as fertile when the relation C/N is near to 10 as found in LD soil.

Conductivity was high in the LD and GMN soils and the lowest value was for ASD (Table 2). Cationic exchange capacity (C.I.C.) is the ability of the soil to retain and exchange different ion ( $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ) and it is influenced by organic matter and mineralization. Site ASD showed high values of  $\text{Ca}^{++}$  which can be explained by the use of synthetic fertilizers [47]. Sites GMN and LD showed high values of  $\text{Mg}^{++}$ ,  $\text{Na}^+$  y  $\text{K}^+$  (Table 2) due to the non intensive exploitation [54-56]. Soils were analyzed to determinate the presence of organochlorated, organophosphorated, pyrethroids and herbicides (atrazine and phenoxiacetics) not finding residues over the detection limits (between 5 and 45  $\text{ng.g}^{-1}$  for the first three groups; 0.05 $\mu\text{g.g}^{-1}$  for atrazine and metabolites and 0.5 $\mu\text{g.g}^{-1}$  for phenoxiacetics); glyphosate residues could not be determined. Sites were sampled at seasons where precipitations were scarce and temperatures moderate to high, factors that influence bioavailability and water solubility of pesticides. According to Caffarini & Della Penna [57] pesticides impact over non target invertebrates not only by immediate or long term exposition but also affecting factors like the susceptibility and recovering (biological factors). In the last two decades, a significant increase in earthworm casting activity has been observed across the world suggesting that highly toxic and persistent pesticides have been supplanted by less toxic and easily degradable pesticides [58].

Oligochateofauna varied significantly between sites ( $F 8,09$   $p < 0.05$ ), having the ASDAO the higher density followed by sites GMN, LD, GPN and least ASD (Table 2). Agricultural soils with non tillage had effects over the properties of the edaphic environment inducing changes that makes it less favorable for earthworms [59, 60]. Organisms collected from sites ASD and ASDAO were mainly juveniles that could be associated to the presence of pesticides [61-63]. However the highest earthworm density was found at ASDAO, this could be due to the presence of herbicides and organic supplies (cow dung) stimulating the development of microorganisms that are part of the earthworms diet [64]. This compensates the physical and chemical disturbances generated by the agricultural practices [65]. Sites GMN and GPN showed the highest species richness with 3 different species each (Table 2). *Microxcoles dubius* and *Octolasion tyrtaeum* are species from soils rich in OM and with low perturbation levels [9, 66, 67] as the sites as they were found. The genus *Aporrectodea* is generally found in sites with medium to deteriorated fertility [8-10, 68-74]. Even if ASDAO site showed the highest density, general results shows that species richness decrease in soils with higher exploitation.

## 3.2. Laboratory tests

### 3.2.1. Endosulfan

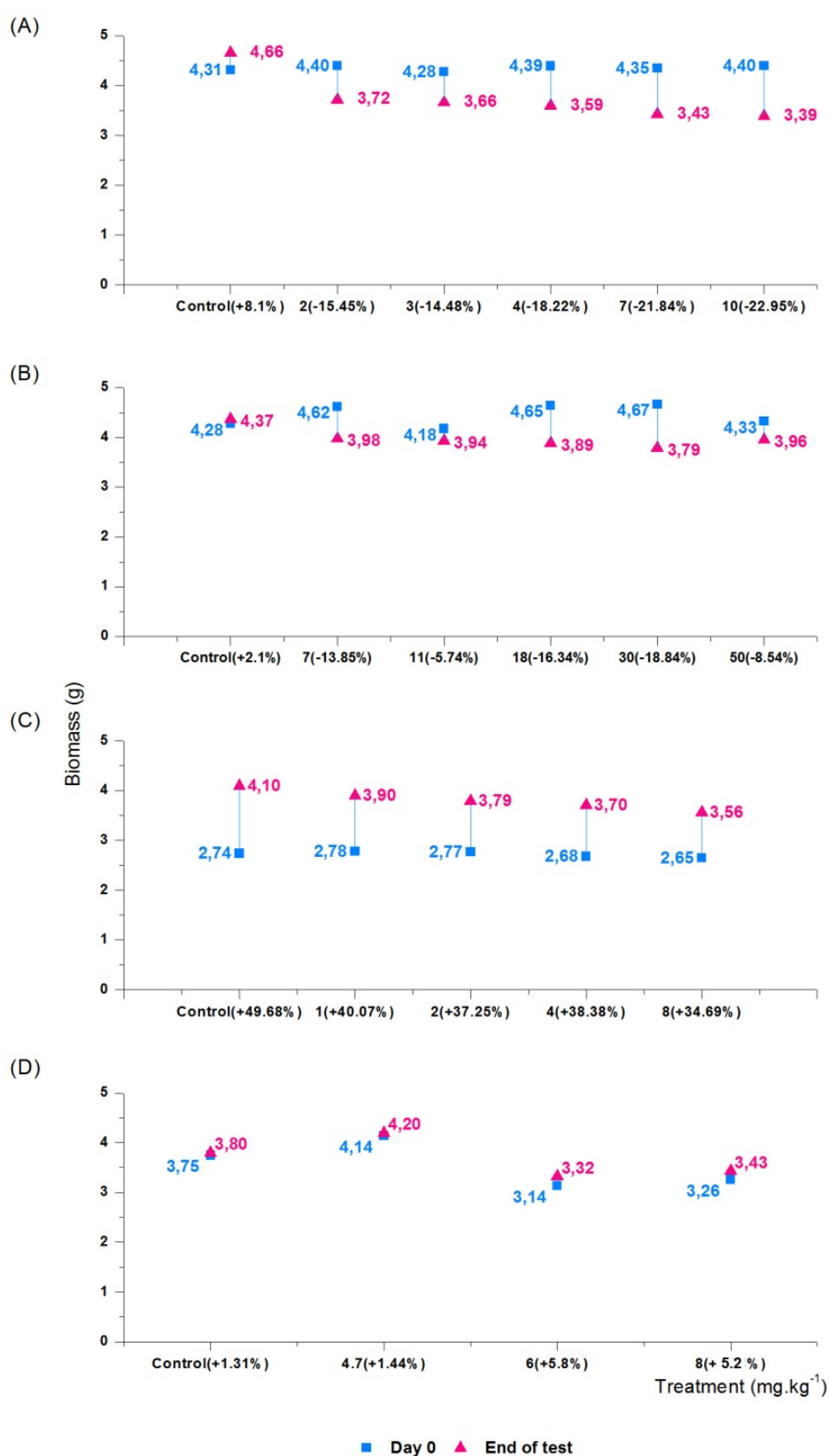
Lethal bioassay showed a  $\text{LC}_{50}$  value of 41  $\text{mg.kg}^{-1}$  at 14 days. Although these  $\text{LC}_{50}$  value is greater than those of other authors [75, 76] dead organisms showed inflamed blisters and

sores all over their bodies, surviving earthworms showed either broad zones or small segments with inflammations. At sublethal exposure, survival of earthworms was not affected but the behaviour was affected in all tested concentrations. Control organisms remained vivacious, mobile and clitellated, T<sub>1</sub> organisms (2 mg.kg<sup>-1</sup>dw) exuded abundant mucus when contacted the soil and remained mobile during the entire test. After the first week, the rest of the treatments (3; 4; 7 and 10 mg.kg<sup>-1</sup>dw) showed a rigid aspect with abundant coelomic fluid excretion by dorsal pores and mucus through the body wall, symptom that accentuated during the exposure period. Some organisms presented ulcerated inflammations and yellow creamy exudates, they were rigid or moved by rolling either the whole body or the caudal portion. Furthermore Liu *et al.* [77] determined that endosulfan induce DNA damage in earthworms. During the test only control organism increased their weight, while exposed organisms accused significant weight loss (F 38.28  $p < 0.05$ ) with a notable change after day 14 (F 5.58  $p = 0.003$ ). Soil surface at the treatments showed traces of food. Stereomicroscope observation of the anterior region of the gut of the exposed earthworms showed no traces of food. The main weight loss (23.01%) corresponded to 10 mg.kg<sup>-1</sup>dw treatment. In the remaining treatments weight loss oscillated between 14 and 21% (Fig. 2-A). Biomass changes can be a good indicator of chemical stress, which may link chemical effects to energy dynamics and ultimately inhibit growth [78]. Earthworms usually show a recovery a few weeks after being removed of treated soil, however in real life, earthworms cannot be removed from soil exposed to pesticides. Instead, they would continuously be exposed to chemicals until the chemicals degraded [79]. At the start of the experiment it is common that there was no significant difference between the mean biomass of the control group and test group. But at the end of the experience the mean biomass in exposed group is significantly lower because of earthworms are able to resist the toxicant in terms of diminishing energy necessary to support other processes [78-81].

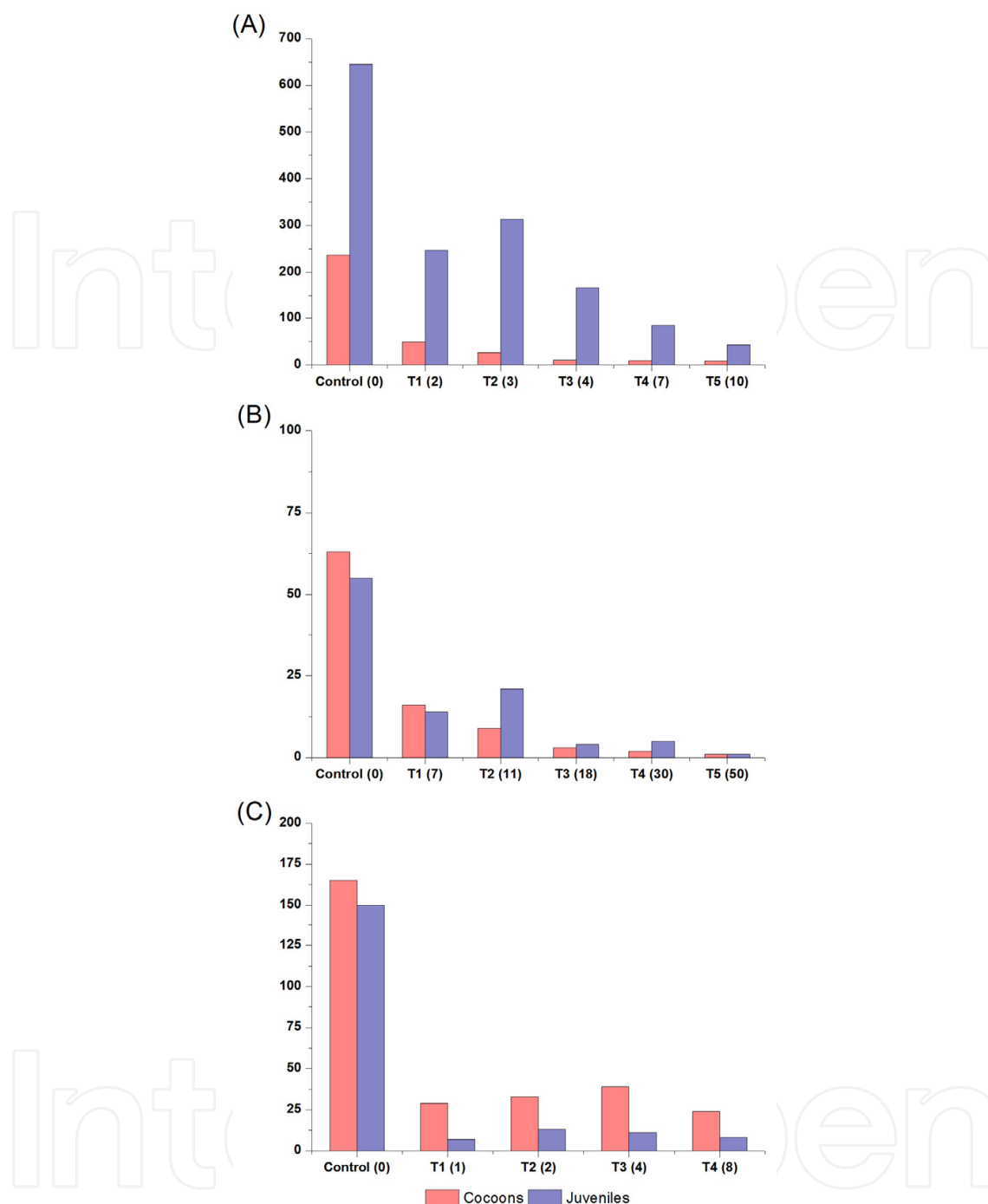
After two week exposed organisms began to lose their clitellum, condition that increased with exposure time and concentration. At the end of the test only the control organisms remained clitellated while in treatments showed a loss of clitellum that ranged between 30 and 100%.

Cocoon production started at day 7 in all treatments decreasing with exposure time and concentration (F 21.49  $p \leq 0.05$ ). The number of juveniles hatching from treated cocoons was also lower (F 40.59  $p \leq 0.05$ ); immature organisms were less mobile, exuded coelomic fluid and mucus, being their size smaller than control groups. Survival of treated juveniles ranged from 90 to 75%, their growth experimented a significant delay (F 27.24  $p \leq 0.05$ ) and never reached sexual maturity (Fig. 3-A, Table 3).

Fecundity in earthworms is sensitive to pesticides even though the earthworms may be not immediately impacted, changes in the reduction of population in the longer term might occur [79]. The effects on the reproductive output can be interpreted either as a direct effect to an interaction with key mechanisms for reproduction, or as an indirect effect, via assimilation of nutrients, growth, and maintenance of the energetic balance [82].



**Figure 2.** Biomass changes in the chronic toxicity test (percents in brackets). (A-C) experiences with *Eisenia fetida*: (A) Endosulfan (B) Glyphosate, (C) Lambda-cyhalothrin, (D) experiences with *Aporrectodea trapezoides* and lambda-cyhalothrin.



**Figure 3.** Results of reproduction bioassay: number of cocoons and juveniles. (A) Endosulfan, (B) Glyphosate, (C) Lambda-cyhalothrin. Test concentration  $\text{mg.kg}^{-1}$  are in brackets.

Degradation in soil assay with  $2 \text{ mg.kg}^{-1}$  dw endosulfan indicated half-life times of 40 and 60 days for isomers  $\alpha$  and  $\beta$ , 47 and 87 days for  $4 \text{ mg.kg}^{-1}$  and 45 and 86 days for  $10 \text{ mg.kg}^{-1}$ . In all the treatments the toxic metabolite endosulfan sulphate was detected at days 57, 46 and 66 respectively. According to the bibliography half-life range from 1-2 months for endosulfan and 4-6 months for endosulfan sulphate, being a high threat for soil organisms because of their effect over reproduction.

Treatment (mg.kg <sup>-1</sup> dw)	Increase of biomass (%)	Development of clitellum (days)	Cocoon production (days)
Control	97.35	112	155
2	92.54	-	-
3	90	-	-
4	81.90	-	-
7	66.79	-	-
10	52.27	-	-

**Table 3.** Biomass, sexual development of juveniles, cocoon production.

Volatilization is important for endosulfan  $\alpha$  at soils with high humidity and their mobility is higher than isomer  $\beta$ , being the mobility of isomer  $\beta$  higher than endosulfan sulphate [83]. Tests performed in Australia and Brazil indicates that soil microorganisms degrade endosulfan. Fungi oxidized it to sulphate (high toxicity) and bacteria to diol (30 days), sulphate (60 days) and unknown metabolites (90 days). Microbial activity, humidity and temperature lead to a delay on the recovery of earthworm populations which impacts over the quality of the soil and the trophic relations [1, 83, 84].

### 3.2.2. Glyphosate

No mortality was registered. Organisms at control group and 7 mg.kg<sup>-1</sup> remained active (burrows all over the substrate) while at higher concentrations organisms exudated a high amount of mucus and their burrowing activities decreased with time and concentration corresponding with the observed by Correia & Moreira [85] over different concentrations of glyphosate (10-1000 mg.kg<sup>-1</sup>). Morowati [86] found that glyphosate induce histochemical changes in the intestine of *Pheretima elongate* which affect survival, feeding and mobility. In the other hand, Pereira *et al.* [87] found that glyphosate (ai) within a range of 6-46 mg.kg<sup>-1</sup> and Spasor (commercial glyphosate) ranging from 4-162 mg.kg<sup>-1</sup> did not have a negative impact over *Eisenia andrei* behaviour. Pesticides enter to the organisms through ingestion and absorption [85] leading to alterations in the metabolism of earthworms which decrease appetite, biomass and growth [79, 80, 88]. Control organisms gained weight during the test and were fed weekly. Treated organisms showed a no significant decreased of their weight with a mean value of 390mg (F 48.47  $p=0.78$ ) and represent about a -12.6% from the initial weight (Fig. 2-B). It should be stated that undigested food traces were found in treatment boxes. Glyphosate interfere with the normal development and reproduction rates of *E. fetida* with direct impact over their poblational dynamics and indirectly over soil fertility [82, 85]. Treated earthworms began to lose their clitellum after 7 days of exposure, tendency that increased with time and concentration. At the end of chronic bioassay, 100% of the organisms from the highest concentration (50 mg.kg<sup>-1</sup>) lost their clitellum. In the other treatments the percent of non clitellated organisms ranged from 75 to 97%. Control organisms showed a loss of clitellum of 7.5% due the normal reproductive cycle of the specie. Fecundity parameters also showed significant differences between contaminated and control soils. The highest cocoon production was found at control group representing a

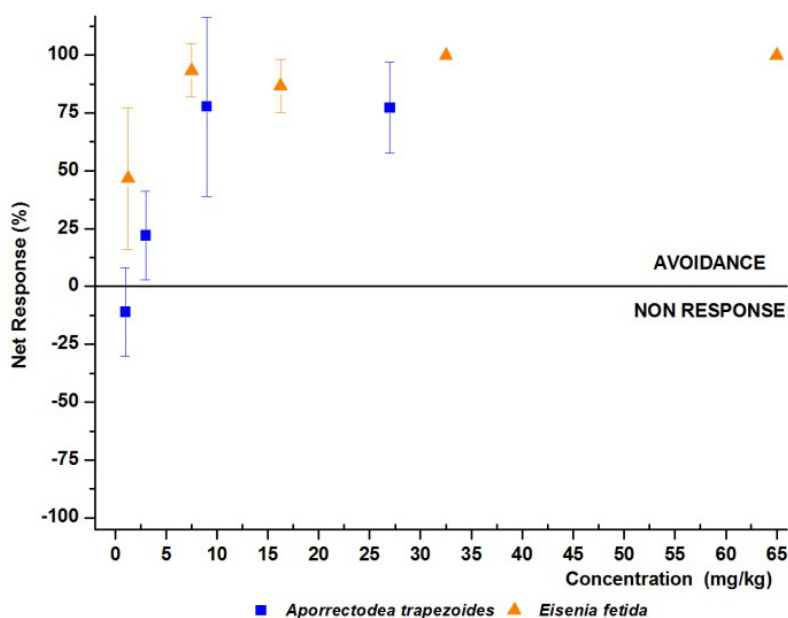
67.02% of total cocoons (Fig.3-B). In treatments the number of cocoons decreased with exposure time and concentration but the inhibitory effect is already shown at the 7 mg.kg<sup>-1</sup>dw, with a significant difference respect to the control (F 25.12  $p<0.05$ ). Cocoon size in the treatments was lower (F 45.72  $p<0.05$ ) with a mean of 3.87 mm x 1.95 mm, representing a decrease of the 25% respect to control (4.56 mm x 2.18 mm). Juveniles production was lower and showed a smaller size at all treatments (F 18  $p<0.05$ ).

### 3.2.3. Lambda-cyhalothrin

Exposure time at behavioural test should be between 24 and 48 hours. At longer exposure time the natural behaviour of the organisms to mix the soil, could cause a 'soft mixing' of both soils along time, inducing a decrease in the difference between soil from both sections of the test containers [89, 90]. Since the only difference between test soil and control is the presence of the investigated chemical, a statistical difference between the soils indicates an effect caused by the test chemical. Since avoidance test and reproduction test had a comparable sensitivity, avoidance tests can be used as suitable screening test [44]. Temperature, moist content and organic matter content of the soil modify the bioavailability of pesticides which impacts over poblational parameters of earthworms [82, 91, 92]. Survival was not affected at lambda-cyhalothrin exposure as observed in previous researchs [20, 41, 93, 94].

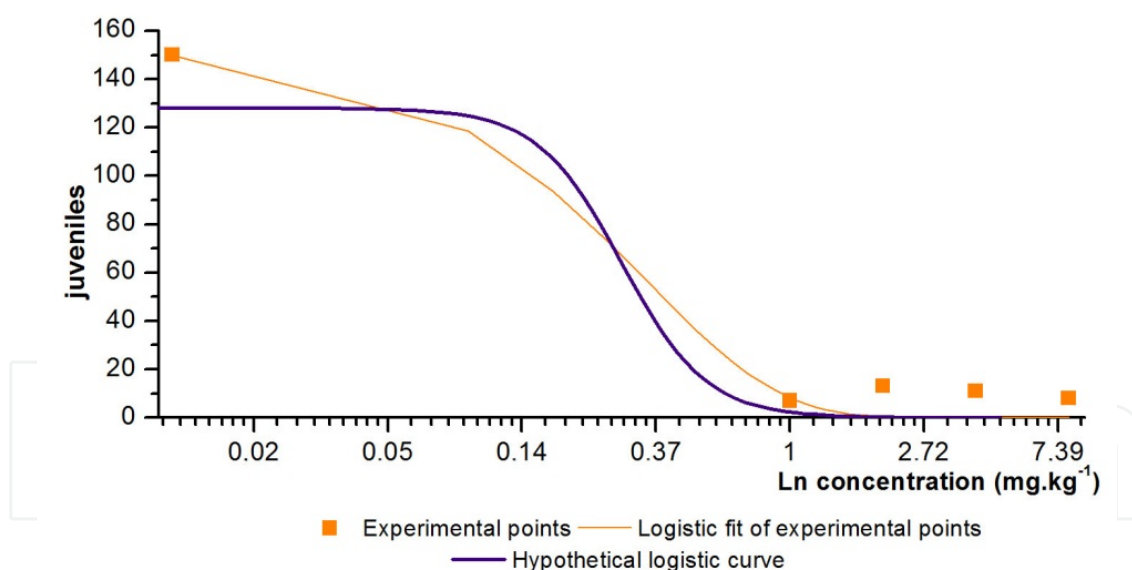
#### 3.2.3.1. *Eisenia fetida*

Significant avoidance responses (F 21.35  $p<0.05$ ) were observed even at the lowest concentration (Fig. 4). Probit estimation EC<sub>50</sub> was 1.36 mg.kg<sup>-1</sup> (C.L.0.24-2.80 mg.kg<sup>-1</sup>) for tested concentrations which indicates that lambda-cyhalothrin is easily detected by *E. fetida* sensorial organs even at concentrations that are close to agricultural application rates [34; 38].



**Figure 4.** Avoidance or attraction response of *E. fetida* and *A. trapezoides* exposed to lambda-cyhalothrin concentrations in OECD and reference soil (mean net response and standard deviation bars).

Chronic test showed no significant differences in biomass ( $F 2.37 p>0.05$ ), even so the weight on the control group was higher (49.68%) than the treatments (34 -40%) (Fig.2-C). Organisms from the control group showed well developed clitellums at the end of the tests, some of the treated organisms lose their clitellum. Exposed organisms started to recover their clitellum from the 7<sup>th</sup> week of the test, tendency that could be related to a reduction of the concentration of pesticide on contaminated soils. Cocoons production was higher in control groups (Fig. 3-C), decreasing significantly in all the tested lambda-cyhalothrin concentrations ( $F 11.94 p<0.05$ ). Cocoons hatched after 21 days both in control group as in 1mg.kg. In the rest of treatments, a delay in the normal hatching period [92] was observed with a range of 28-35 days, which increased with concentration. The observed results indicate that lambda-cyhalothrin has a direct effect over fecundity on *E. fetida* affecting cocoon production and their viability. These effects are usually related to testicular malformations but due to the limitations of this study cannot be determined. Similar results were found by others authors [80, 82, 91, 92, 95]. The lowest observed effect concentration value (LOEC) for reproduction was estimated at 1 mg.kg<sup>-1</sup> corresponding with the lower tested concentration. Furthermore soils are considerate toxic when <50% of the number of juveniles determined for the control were counted [44]. Number of juveniles was significantly below the 50% threshold (4.67–8.67% of the control) in all treatments. Experimental points could not be fitted with the regression model from equation (3) making the EC<sub>50</sub> unable to be calculated (Fig. 5).



**Figure 5.** Graphic estimation of the EC<sub>50</sub> for juveniles production using a logistic regression model.

Bioaccumulation factors of lambda-cyhalothrin at the end of the test were 0.0076; 0.0056 and 0.0845 for the 2; 4 and 8 mg.kg<sup>-1</sup> treatments respectively. Soil lambda-cyhalothrin degradation was estimated at 86 days (99%).

Persistence of lambda-cyhalothrin in soil ranged from 4 to 12 weeks with a half-life value of 30 days in most soils [18] which matches our results. Some studies indicate that endogeic earthworms do not accumulate pyrethroids [96] but their lyphophilic characteristics made

them available to be absorbed by epigeic earthworms with preference by some isomers [1, 97, 98] which could be related to the traces of lambda-cyhalothrin found in *E. fetida* at the end of the test.

3.2.3.2. *Aporrectodea trapezoides*

Estimated EC<sub>50</sub> at avoidance test was 7 mg.kg<sup>-1</sup> being significantly above than the found for *E. fetida* [99]. Control and treated groups gained weight (Fig. 2-D) with significant differences in 6 and 8 mg.kg<sup>-1</sup> (F=11.67 *p*<0.05). Presence of organic clusters was recorded at control and 4.7 mg.kg<sup>-1</sup> which indicate high mobility and intensive burrowing activity of the organisms, no traces of food were found. The burrowing behaviour from earthworms induce physicochemical and biochemical changes in the soil. Their depositions and mucus production increase the content of nutrients which are necessary to the growth of microbial biomass [100, 101].



**Figure 6.** (A) Production and distribution of organic clusters in control and treatments (B) General aspect and size of organism in control and treatments.

The mobility was reduced at 6 and 8 mg.kg<sup>-1</sup> where the burrows were restricted to the middle and lower fractions of the substrate and the soil surface showed food traces and few organic clusters. Pesticides affect the detoxification processes of earthworms leading to a decrease of feeding, biomass and mobility (diapauses or migrations to lower layers of soil) [102, 103].

Organisms exposed to 8 mg.kg<sup>-1</sup> curled their bodies to minimize the contact between pesticide and body wall. Stress generated from lambda-cyhalothrin exposure had a lower impact over *A. trapezoides* (endogeic) than in *E. fetida* (epigeic) that could be explained by the fast reaction associated to ingestion/egestion rate of epigeic earthworms as a consequence of their direct contact with the pesticide [7, 102, 104-111]. Researches performed by Cerón Rincón & Melgarejo Muñoz [112] and Renella *et al.* [113] registered stress symptoms at the microfloral, microbial and enzymatic activities which could explain the results of the higher exposure concentrations (6 and 8 mg.kg<sup>-1</sup>).

As stated at field results, *A. trapezoides* is the dominant earthworm specie of agricultural soils from Santa Fe playing a crucial role in their fertility (68-72). According to Tripathi *et al.* [97] earthworm sensitivity is related to the characteristics of the organisms and their ecological category. Results of the bioassay indicate that *A. trapezoides* ecophysiology is severely affected by lambda-cyhalothrin with negative impact over soil fertility [75, 114].

## 4. Conclusions

Effects of soil exploitations on different environments influence the composition and taxocenosis structure of earthworms affecting their density and diversity.

Conventional production practices for agriculture (minimum tillage, non tillage, organic fertilizers) and non intensive livestock benefit the conservation and increase of edaphic fauna.

Endosulfan, glyphosate and lambda-cyhalothrin induced negative changes over feeding, reproduction and behaviour of *Aporrectodea trapezoides* and *Eisenia fetida* at tested concentrations and exposure times.

These changes lead to detrimental effects on the population dynamics of earthworms that affect trophic relationship

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