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Lethal and sub-lethal effects of five pesticides used in rice farming on the earthworm *Eisenia fetida*

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Highlights

- The toxicity of five pesticides was evaluated on the earthworm *Eisenia fetida*.
- Carbendazim was found to be highly toxic at predicted soil concentrations.
- Histopathological effects on body wall and intestinal tract were observed.
- ChE, LDH and ALP were found to be sensitive biomarkers to assess pesticide exposure.

Abstract

The toxicity of five pesticides typically used in rice farming (trichlorfon, dimethoate, carbendazim, tebuconazole and prochloraz) was evaluated on different lethal and sub-lethal endpoints of the earthworm *Eisenia fetida*. The evaluated endpoints included: avoidance behaviour after an exposure period of 2 days; and mortality, weight loss, enzymatic activities (cholinesterase, lactate dehydrogenase and alkaline phosphatase) and histopathological effects after an exposure period of 14 days. Carbendazim was found to be highly toxic to *E. fetida* (LC50 = 2 mg/kg d.w.), significantly reducing earthworm weight and showing an avoidance response at soil concentrations that are close to those predicted in rice-fields and in surrounding ecosystems. The insecticide dimethoate showed a moderate acute toxicity (LC50 = 28 mg/kg d.w.), whereas the rest of tested pesticides showed low toxicity potential (LC50 values above 100 mg/kg d.w.). For these pesticides, however, weight loss was identified as a sensitive endpoint, with NOEC values approximately 2 times or lower than the calculated LC10 values. The investigated effects on the enzymatic activities of *E. fetida* and the observed histopathological alterations (longitudinal and circular muscle lesions, edematous tissues, endothelial degeneration and necrosis) proved to be sensitive biomarkers to monitor pesticide contamination and are proposed as alternative measures to evaluate pesticide risks on agro-ecosystems.

Keywords: pesticides, histological examination, *Eisenia fetida*, biomarkers, terrestrial ecotoxicology

1. Introduction

Rice farming constitutes one of the most important agricultural production activities worldwide. Intensive rice production involves the use of synthetic pesticides for the control of fungal diseases (e.g. *Pyricularia orizae*), aphid insects and unwanted weeds. Residues of pesticides applied to rice crops may accumulate in the soil for several weeks after application and can be transported by spray-drift or water runoff into surrounding ecosystems (Gregoire et al., 2009; Guzzella et al., 2006; Schulz, 2004; Papastergiou and Papadopoulou-Mourkidou, 2001). Pesticide residues constitute a potential toxicological hazard for the non-target organisms inhabiting the rice fields and surrounding ecosystems, possibly contributing to biodiversity loss and to side-effects in higher trophic levels (Mesléard et al., 2005).

Soil invertebrates play a fundamental role for improving soil structure and fertility, and constitute an important component of the diet of a variety of animals (e.g. birds, mammals). Amongst invertebrates, earthworms are considered to be of particular interest because of their notable contribution to organic matter decomposition, nutrient cycling and soil formation (Römbke et al., 2005; Allen, 2002; Edwards, 1998). Their ecological relevance, high biomass and frequently observed sensitivity to environmental pollution make them one of the most suitable sentinel organisms for assessing the ecological risks of pesticide residues in terrestrial ecosystems (Reinecke and Reinecke, 2007; Landrum et al., 2006; Dell'Omo et al., 1999). Earthworm species such as *Eisenia fetida* or *Eisenia andrei* have been extensively used as standard test organisms for the risk assessment of pesticides, and toxicity test protocols have been derived and widely implemented to assess their sensitivity to chemical pollution (e.g. OECD 1984; ISO 1993, 1998; Edwards and Bohlen, 1992). Such standardized tests have been mainly used to assess the acute lethal effects and biomass changes for a wide range of pesticides (Wang et al., 2012 a,b); however, pesticide effects on other sub-lethal endpoints

that are potentially more sensitive and precursors of long-term individual and population-level effects have been less investigated.

The use of biomarkers constitutes a complementary approach to standard toxicity tests in the evaluation of sub-lethal effects of contaminants in earthworms, providing more information about the organism's stress response and the toxic mode of action of the evaluated substance (Gastaldi et al., 2007; Hankard et al., 2004; Kammenga et al., 2000; Scott-Fordsmand and Weeks, 2000). A variety of biomarkers have been measured in earthworms including DNA alterations, induction of metal-binding proteins, inhibition of enzymatic responses, energy reserve responses, responses in neural impulse conductivity, lysosomal membrane stability and histopathological lesions (Scott-Fordsmand and Weeks, 2000; Sanchez-Hernandez, 2006; Giovanetti et al., 2010; Kiliç, 2011). The test and use of such biomarkers, however, has mainly focused on assessing metal pollution, while the number of studies evaluating biomarker responses from organic contaminants such as agricultural pesticides is rather limited (Sanchez Hernandez, 2006).

The objective of the present study was to investigate the toxicity of five pesticides typically used in rice farming on the earthworm *E. fetida* and to identify effective enzymatic and histopathological biomarkers to assess their contamination under field conditions. Pesticide effects were assessed on mortality, weight-loss and on the avoidance behavior of *E. fetida* by performing acute laboratory toxicity experiments. Furthermore, the effects of the selected pesticides were assessed on different *E. fetida* enzymatic activities, and the pesticide damage on tissues and organs were evaluated by performing histopathological examinations. The results of this study are expected to contribute to expand our knowledge on the effects of rice farming-induced pesticide pollution on earthworms as well as to identify sensitive measures

to monitor the toxicological effects of pesticides in rice production systems and in surrounding terrestrial ecosystems.

2. Material and methods

2.1 Test chemicals and solutions

Five pesticides that have been reported to be used or monitored in environmental samples taken in rice-producing areas of the Mediterranean region were selected (Andreu-Moliner et al., 1986; Ccanccapa et al., 2016). These were the insecticides trichlorfon and dimethoate, and the fungicides carbendazim, tebuconazole and prochloraz. The properties of the selected pesticides and the characteristics of the commercial products used in this study are described in Table 1. Stock solutions were prepared by diluting the commercial products in distilled water. Polysorbate 80 (Tween) was added at a concentration of 50 µg/L to the stock solution prepared with carbendazim and tebuconazole to increase their solubility. Stock solutions were stored in darkness at 4 ºC until further use in the toxicity experiments.

2.2 Test organisms

E. fetida (Savigny 1826) adults were purchased from a commercial earthworm breeding farm (Eisehumus, Alcalá de Xivert, Castellón, Spain) and maintained in a laboratory culture at 20 ± 2 ºC for at least three weeks prior to use in the toxicity experiments. Twenty-four hours prior to the start of the experiments *E. fetida* organisms of homogeneous length and weight (200-300 mg) which possessed clitellum were removed from the laboratory culture and placed on moist filter paper to allow a depuration of the gut contents. Subsequently, they were washed with distilled water, manually dried with moist paper and placed in the test units.

2.3 Toxicity tests

The toxicity tests were performed according to the OECD guideline 207 (OECD, 1984). This guideline, and the exposure duration proposed by this, was selected because it is the one recommended for regulatory pesticide risk assessment to non-target soil fauna in Europe (EC 2002). The artificial soil substrate was prepared by homogeneous mixing of 10% sphagnum peat, 20% kaolin clay, 69% fine sand, and 1% calcium carbonate. Distilled water was added and mixed with the dry soil to obtain a final moisture content of 40%. The pH of the obtained soil substrate was 6.0 ± 0.5 (mean ± SD). Two-hundred grams of artificial soil substrate were introduced into 500 mL glass vessels (15 cm diameter and 7 cm height). The artificial soil substrate was spiked with the pesticide solutions and was gently mixed to allow a homogeneous distribution of the pesticide. The pesticide exposure concentrations used in the toxicity experiments were determined based on range-finding tests performed with one replicate per treatment level. The final tests were performed in triplicate with five or six treatment levels in a geometric series (n = 3) and a control with five replicates (n = 5). A solvent-control treatment was added in the carbendazim and tebuconazole experiments (n = 5). The exposure concentrations used in the toxicity experiments performed with the five pesticides are shown in Table 1. Ten *E. fetida* individuals were randomly selected, weighed and introduced into each test vessel. The test vessels were covered with plastic lids with small holes and incubated at 20 ± 2 ºC in a continuously illuminated (400-800 Lux) climatic chamber (Sanyo Versatile Environmental Test Chamber MLR-350) for 14 days. Mortality and body weight of the *E. fetida* organisms were monitored on day 7 and 14 after the start of the experiment, and morphological changes were qualitatively evaluated. At the end of the experiments, alive worms were introduced into Eppendorf tubes, frozen with liquid nitrogen, and stored at -80 ºC for posterior biomarker and histopathological analyses.

2.4 Avoidance behaviour tests

Avoidance behaviour experiments were conducted according to the standard Guideline for the Earthworm Avoidance test (ISO, 2008) with the pesticide application dosages recommended to be used in rice production. Briefly, glass vessels were divided into two compartments by means of a removable plastic card. Next, each compartment was filled with 200 g of soil substrate. One compartment was spiked with pesticide stock solutions to reach the concentrations described in Table 1, whereas the other was only spiked with distilled water (control). The soil substrate used in these experiments was collected from an uncontaminated agricultural land located in the outskirts of the city of Valencia (Spain). Prior to its use in the experiments, the soil was sieved (< 5 mm) and carefully inspected to eliminate any organisms or particles that may interfere with the assay. The obtained soil substrate had a sandy-loam texture, a pH of approximately 8, low organic matter content (1.5–2.0%), and high calcium carbonate content (28%). After removing the plastic card, ten *E. fetida* organisms were placed on the dividing line. Then, the test units were covered with a plastic lid and incubated for 48 h at 23 ± 2 ºC under continuous light exposure. After the incubation period, the plastic card was carefully positioned within the exposed and non-exposed sections of the test unit and the number of alive worms in each compartment was counted. Each pesticide assay and additional controls (control-control) were run in triplicate (n = 3). The avoidance behavior was expressed as the percentage of worms that avoided the treated soil, expressed as the mean percentage of net responses (NR) calculated as follows:

$$NR = \left(\frac{C - T}{N}\right) x100$$

where C is the number of worms observed in the control soil; T, number of worms observed in test soil; N, total number of worms per replicate. A positive NR indicated avoidance and a negative NR indicated a non-response (or attraction) to the contaminated soil. An avoidance

response is usually judged as positive when more than 80% of the test organisms are found in the control soil compartment at the end of the test (Sánchez-Hernández, 2006).

2.5 Biomarker analysis

The pesticide effects on the *E. fetida* organisms that survived the toxicity experiments were evaluated on three different enzymatic biomarkers: cholinesterase activity (ChE), lactate dehydrogenase activity (LDH) and alkaline phosphatase activity (ALP). The earthworm samples were homogenised in a phosphate buffer, pH 7.2 (1:10 w/v). Then, the samples were centrifuged at 3500 rpm during 10 min (temperature: 4 ºC). The supernatant was poured off and used for the analyses described below.

Prior to the biomarker analysis, the protein content (PC) was analysed according to the method described by Herbert et al. (1995). Dilutions of the homogenates were prepared with phosphate buffer (1:10, 1:100, 1:1000, 1:10000) in quadruplicate. Microplates of 400 µL well-volume were filled with 10 µL of the diluted homogenates and 250 µL of Bradford reagent dissolved in deionized water (1:4 v/v). After 15 min, the absorbance of the samples was read in a spectrophotometer (TECAN Infinite M200) at a wave-length of 595 nm, and the protein concentration was calculated based on a previously made calibration curve using Bovine Serum Albumin (BSA) as standard.

The ChE activity in the earthworm samples was measured according to the method described by Ellman et al. (1961). Similarly to the procedure followed for the PC analysis, the samples were diluted with phosphate buffer, and 50 µL of the diluted samples were added to the microplate wells. Next, 250 µL of a reagent composed by 1000 µL of dithiobis-2-nitrobenzoic acid (DTNB) 200 µL of iodide acetylcholine and 30 mL of phosphate buffer (pH

7.2) were added. Finally, the enzymatic activity was read once per minute for 10 min in the spectrophotometer at a wave-length of 414 nm, and the final results were expressed as nmols of hydrolysed acetylcholine/min/mg of protein.

The LDH activity was measured according to Vassault (1983). Briefly, 50 µL of the homogenate, 2.5 mL of a TRIS/NaCl/NADH solution and 0.5 of a TRIS/NaCl/pyruvate solution were added to a quartz cuvette. Subsequently, the absorbance was read at a wave-length of 340 nm every 30 seconds for 3 minutes. The results of the spectrophotometer were recalculated to nmols of reduced pyruvate/min/mg of protein.

The analyses of the ALP were performed with a commercial kinetic optimized test (SPINREACT S.A.). Briefly, 20 µL of the homogenate were introduced in a cuvette and mixed with 1.2 mL of a reagent formed by a solution of diethanolamine buffer (1 mmol/L; pH 10.4) with magnesium chloride (0.5 mmol/L) and p-nitrophenil phosphate (10mmol/L) in a proportion of 9:1 (v/v). Finally, the absorbance was measured once per minute for 3 minutes at 405 nm, and the ALP activity was expressed in µmols of hydrolysed p-nitrophenyl/min/mg of protein.

2.6 Histopathological examination

After exposure to pesticides, the survived earthworms were rinsed with distilled water and fixated with 10% formaldehyde. One worm belonging to the control group, one belonging to the lowest exposure concentration, and one belonging to the highest exposure concentration were selected from each toxicity test, and were embedded into paraffin. Subsequently, each worm was sliced vertically 4 or 5 times. Each slice had a thickness of approximately 5-7 µm. Sections were mounted on glass microscope slides with one drop of albumin and stained with

haematoxylin-eosin. Finally, the samples were examined by an optical microscope (x4 and x10) connected to a digital camera (NIKON ECLIPSE E400), which allowed to take pictures of the earthworm sections. The differences between the pesticide exposed earthworm samples and the control earthworm samples were qualitatively described.

2.7 Statistical analyses

The calculation of the concentrations causing 10% and 50% of mortality (LC10 and LC50, respectively) in the toxicity experiments and their corresponding 95% confidence intervals (CI) were calculated by Probit analysis using SPSS (version 16.0). The weight loss data and the biomarker response data were analysed by using a one-way ANOVA followed by a post-hoc analysis using the Fisher's least significant difference (LSD) test with STATGRAPHICS PLUS (version 5.1). Prior to this analysis, the data were checked for normality by using the Shapiro-Wilk test and for homogeneity of the variance by the Cochran test. The No Observed Effect Concentration (NOEC) was derived as the highest tested pesticide concentration that did not show significant effects as compared to the control. The data obtained from the avoidance behaviour test was analysed using a Chi-squared test to compare the observed and expected number of individuals in the two soils and to determine whether an avoidance response was present. All statistical tests were performed using a significance level of 0.05.

3. Results and discussion

3.1 Individual-level responses

The results of the toxicity experiments performed with the five tested pesticides are shown in Table 2. Mortality in the control test units was not recorded during the 14-day experimental period. Recorded mortality on day 7 was in most cases not sufficient to fit a dose-response curve and, therefore, the LC10 and LC50 values for this time point were, for the majority of

the studied pesticides, not calculated. The exception was the fungicide prochloraz, which induced the fastest toxic response with a very steep dose-response curve, resulting in an LC10-7d value of 280 mg/kg d.w. and an LC50-7d value of 285 mg/kg d.w. Carbendazim was found to be highly toxic to *E. fetida*, with an LC50-14d of 2.0 mg/kg d.w and an LC10-14d of 1.1 mg/kg d.w. The insecticide dimethoate showed a moderate toxicity to *E. fetida*, with and LC50-14d of 28 mg/kg d.w. The rest of the studied pesticides were found to exert relatively low toxicity to *E. fetida* on day 14 after the start of the exposure period, with LC50 values higher than 100 mg/kg d.w. The results of this study are in agreement with previous studies, which already identified a high toxicity of carbendazim to *E. fetida* (Garcia et al., 2008; Ellis et al., 2007; Van Gestel, 1992; Van Gestel et al., 1992; Vonk et al., 1986).

Morphological changes were assessed at day 7 and 14. No morphological changes were clearly observed at day 7 for the majority of the pesticides, except at the highest tested concentration for carbendazim (6 mg/kg) and tebuconazole (142 mg/kg), at which worms exhibited body constrictions, slimming, coiling and curling. On day 14, an excessive mucus secretion was observed at the 1.2 and 1.8 mg/kg treatment levels for carbendazim, and at the 5.0 and 11 mg/kg treatment levels for dimethoate.

All pesticides resulted in a significant weight loss in the exposed worms as compared to the controls (Table 2). Weight loss in the control worms ranged between 3% and 9% on day 7, and increased up to 20% on day 14 of exposure. Weight loss in the exposed worms showed a clear dose-response relationship in all experiments. Average weight-loss percentages for the exposed organisms reached 36% and 61% on day 7 and 14 after the start of the experiment, respectively. At the end of the experiment, significant effects on weight loss were found to be below the lowest exposure concentration for trichlorfon, tebuconazole and prochloraz. A

NOEC of 1.2 and 5 mg/kg d.w. was calculated for carbendazim and dimethoate, respectively (Table 2). Our results indicate that the weight loss endpoint was for some pesticides (e.g. trichlorfon, dimethoate, tebuconazole) two times more sensitive than mortality, confirming this endpoint as a valuable indicator for field monitoring, as also indicated by Frampton et al. (2006) and Xiao et al. (2006).

The results of the avoidance behaviour test performed with the control soil (control-control) showed that *E. fetida* were randomly distributed among both soil compartments. A significant avoidance response was measured for the fungicide carbendazim (Fig. 1). On average, 87% of the tested worms avoided the soil compartment contaminated with carbendazim at a concentration of 2.3 mg/kg d.w. These results are in close agreement with the calculated avoidance NOECs reported by Garcia et al. (2008) for artificial tropical soils and European natural soils (<1 mg/kg d.w.). As for the rest of studied pesticides, a significant avoidance behaviour could not be identified. For tebuconazole a slight attraction effect was observed, however, this effect was not significant when compared to the controls (Fig. 1).

Our results indicate a clear correspondence between the observed mortality effects and the avoidance behaviour. Carbendazim showed an elevated avoidance response (87%) at a concentration near its LC50, whereas the other pesticides were tested at concentrations between 10 and 200 times below their respective LC50, thus showing no avoidance response. The avoidance test has a number of advantages such as its short duration and lower laboriousness in comparison to the standard mortality or reproduction tests. Moreover this test is based on the fact that organisms possess chemoreceptors highly sensitive to chemicals in their environment. This test is proposed as a short-term screening tool in ecological risk assessment schemes for contaminated land, for triggering other tests in case of pollution

298 concerns, and for the identification of concentration ranges to be investigated in longer-term
299 experiments (Da Luz et al., 2004; Amorim et al., 2005).
300
301 **3.2 Biomarker and histopathological responses**
302 All tested pesticides significantly inhibited the ChE activity of *E. fetida* at the lowest exposure
303 concentration (Table 3). As expected, trichlorfon and dimethoate (acethylcholinesterase
304 inhibitors) resulted in the highest toxic effects on the acethylcholine metabolism, with a
305 percentage of ChE activity inhibition of approximately 50% at the lowest tested concentration
306 (Fig. 2a,b). Such levels of ChE inhibition have also been observed for other organophosphate
307 insecticides, such as chlorpyrifos or malathion, in *E. fetida* and other earthworm species e.g.
308 *Drawida willsi* (Rao et al., 2003; Panda and Sahu, 2004). LDH activity was significantly
309 inhibited by the exposure to trichlorfon, dimethoate and prochloraz (e.g. Fig. 2c), with
310 NOECs below the lowest tested pesticide concentration (Table 3) and percentages of
311 inhibition at the lowest exposure concentration of about 70% for trichlorfon, and 20-25% for
312 dimethoate and prochloraz. Carbendazim also resulted in a decrease of the LDH activity,
313 however, significant effects only occurred at soil concentrations higher than 0.8 mg/kg d.w.
314 Exposure to tebuconazole significantly increased LDH activity in soil concentrations up to
315 142 mg/kg d.w., but a significant decrease was observed in the highest exposure concentration
316 (Fig. 2d), indicating a possible hormesis effect. Pesticide exposure to trichlorfon, dimethoate,
317 carbendazim and prochloraz resulted in a significant decrease of the ALP activity (e.g. Fig
318 2e), with NOECs below the lowest tested concentration (Table 3). Tebuconazole, however,
319 did not alter the ALP activity at the tested soil concentration range (63-213 mg/kg d.w.; Fig.
320 2f). The majority of the biomarker investigations on earthworm organisms have focused on
321 assessing ChE effects (e.g. Ribera et al., 2001; Rao et al., 2003; Panda and Sahu, 2004),
322 whereas the inhibition of other enzymatic activities has hardly been evaluated (Sanchez-

323 Fernandez, 2006). Our results indicate that LDH and ALP, are also sensitive biomarkers of
324 pesticide exposure and can be used to complement ChE evaluations for several pesticides with
325 different toxic mode of action.
326
327 The results of the histopathological examination showed that the tested organophosphate
328 insecticides affected the epidermis and resulted in serious damage of the circular and
329 longitudinal muscular layers (e.g. Fig. 3c and d). Exposure to high trichlorfon and dimethoate
330 concentrations also resulted in internal damage, with a degradation of the tiflosol, a
331 deformation of the dorsal blood vessel (Fig. 3c), and a degradation of the muscular layer
332 protecting the digestive system (Fig. 3d). These damages potentially resulted in a disorder of
333 the nervous and digestive systems. Exposure to the fungicides carbendazim and tebuconazole
334 resulted in similar effects, with hemolimphatic edemas and occasional necrosis in the circular
335 and longitudinal muscular layers. In the case of carbendazim, a clear flattening of the dorsal
336 blood vessel and the ventral nerve cord was also observed (Fig. 3e). Exposure to prochloraz
337 also resulted in effects on the muscular layers, but effects on internal tissues and organs were
338 less noticeable at the tested exposure concentration (286 mg/kg d.w.; Fig. 3f).
339 Histopathological examination of transverse sections of the control earthworms showed
340 normal architecture of body wall, showing continuous cuticular membrane, intact circular and
341 longitudinal muscles, and intact blood vessels (Fig. 3a,b).
342
343 A number of studies with different earthworm species have shown comparable
344 histopathological responses when exposed to organic pollutants (Scott-Fordsmand and
345 Weeks, 2000; Kiliç, 2011; Saxena et al., 2014). The most common responses were
346 disintegration of the cuticular membrane and the ectoderm layers, damages in the circular and
347 longitudinal muscles due to necrosis, deformation in chloragogenous cells and tissue erosion,

the latter usually leading to body fragmentation (Morowati, 2000; Amaral and Rodrigues, 2005; Muthukaruppan et al., 2005; Reddy and Rao, 2008; Gao et al., 2013; Saxena et al., 2014). In our study, earthworms exposed to high pesticide concentrations, particularly carbendazim and tebuconazole, showed comparable histopathological damages. A study conducted with the earthworm *Metaphire posthuma* exposed to 0.5 mg/kg of carbofuran revealed loss of normal architecture and disintegration of cuticular membrane, epidermal cells, circular and longitudinal muscles at 14-day of exposure in soil medium, which can result in bleeding and fragmentation of the body (Saxena et al., 2014). Similar symptoms were also observed by the same authors when using the *E. fetida* contact test with 1.20 μg/cm^2 of carbofuran, and by earlier studies using carbaryl and metal treated earthworms (Gupta and Sundararaman, 1988, 1990; Lourenço et al., 2011). Dittbrenner et al. (2011) observed significant impairment of the midgut tissue, cuticula, mucocytes and epidermal cells at imidacloprid soil concentrations ranging between 0.2 and 4.0 mg/kg in *Aporrectodea caliginosa*, *E. fetida* and *L. terrestris* in laboratory toxicity tests. Previous studies also revealed damages in the intestines of *E. fetida* exposed to organophosphate pesticides (Rao et al., 2003; Reddy and Rao, 2008).

Earthworms are continuously exposed to soil chemicals through their digestive mucoses and skin, and are dependent on efficient detoxification systems for their survival (Kiliç, 2011). Any cell death or necrosis that is not rapidly repaired usually produces failures in the osmotic regulation (Morowati, 2000). As a mechanism to prevent osmotic failures, earthworms present a large regeneration capacity. In case of tissue damage, the chloragogen cells are able to migrate to the wound or lost tissue and regenerate it (Vogel and Seifert, 1992; Cancio et al., 1995; Morgan et al., 2002; Reddy and Rao, 2008). Alterations in the chloragogen cell activity produced by exposure to high pesticide concentrations are likely to be responsible of the

observed impairment in enzymatic activities (i.e., ChE, LDH and ALP) and can be considered precursors of lethal and sub-lethal effects.

3.3 Relevance for risk assessment

Acute Toxicity Exposure Ratios (TERs) for the tested pesticides in the rice fields were calculated by dividing the calculated LC50-14d by the recommended pesticide application dosages shown in Table 1. For the pesticides that have a logKow larger than 2 (i.e., tebuconazole and prochloraz; Table 1), the LC50 values were divided by 2 as proposed in EC (2002). The calculated TERs were equal or larger than 10 for all pesticides, indicating no short-term risks for the rice-field earthworm populations, except for carbendazim which had a TER of 0.9 (Table 2). Mortalities of about 50% of the in-field population are expected at the recommended dosages of carbendazim. Burrows and Edwards (2004) calculated a Predicted Environmental Concentration (PEC) for carbendazim of 0.76 mg a.i./kg d.w. in terrestrial ecosystems surrounding agricultural fields and found an EC50-28d for earthworm biomass of 1.9 mg/kg d.w. using terrestrial microcosms. Based on the chemical fate calculations of their study and the acute weight loss NOEC calculated here, it is expected that carbendazim results in sub-lethal effects (e.g. growth impairment) in earthworm populations after application. Therefore, its ecotoxicological impacts should be further evaluated under field conditions. Daam et al. (2011) demonstrated that the sensitivity of other earthworm species can be up to two orders of magnitude higher than that of *E. fetida*, and De Silva et al. (2009) indicated that lethal and sub-lethal responses of earthworms are largely dependent on temperature and soil properties. These findings suggest that the preliminary risk calculations performed here could be somewhat underprotective. Therefore, further research should be dedicated to identify sensitive earthworm species that can be used for the risk assessment of pesticides in rice

paddies, preferably using soils with the same characteristics as those found under natural conditions.

Biomarkers are an important element in the ecological risk assessment of organic pesticide pollution. This study has demonstrated that ChE, LDH and ALP can effectively be used as biomarkers of carbendazim exposure at environmentally relevant concentrations (i.e., PEC calculated by Burrows and Edwards, 2004), and shows that, with few exceptions (e.g. LDH and ALP for tebuconazole), the evaluated enzymatic responses have a sensitivity that is at least two times higher than the measured acute lethal endpoints. Furthermore, this study shows that morphological changes in the body wall and gastrointestinal tract could be used as early warning signals of pesticide contamination and could be added to earthworm's standardized tests for the evaluation of contaminated ecosystems, and used in a multi-biomarker approach to assess individual-level effects of pesticide pollution. The next challenge, however, remains on establishing a mechanistic link between the biochemical and morphological responses observed here and behavioural responses (e.g. feeding, mating), to quantify effects on earthworm populations and their mediated ecological functions (e.g. organic matter decomposition, soil formation).

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Conflicts of interest

The authors declare no conflicts of interest.

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Tables

Table 1. Characteristics of the pesticide active ingredients and formulations used in this study, and exposure concentrations used in the laboratory experiments.

	Trichlorfon	Dimethoate	Carbendazim	Tebuconazole	Prochloraz
Pesticide type	Organophosphate insecticide	Organophosphate insecticide	Benzimidazole fungicide	Triazole fungicide	Imidazole fungicide
Mode of action	Acetylcholinesterase inhibitor	Acetylcholinesterase inhibitor	Inhibition of mitosis and cell division	Disrupts membrane function	Disrupts membrane function
Pesticide properties [a]					
Molecular mass (g/mol)	257.4	229.3	191.2	307.8	376.7
Solubility in water (mg/L)	120000	39800	8	36	26.5
K_{ow} (-)	2.69	5.06	30.2	5010	3160
K_{oc} (L/kg)	10	28.3	225	769	500
Laboratory soil DT50 (d)	18	2.6	40	73	120
Pesticide formulations					
Commercial name	Dipterex 80 PS	Citan 40	KAR-50	Folicur 25 EW	Octagon
Active ingredient (%)	80	40	50	25	45
Formulation form	Powder	Liquid	Powder	Liquid	Liquid
Purchased from	Bayer	Inagra	Kenogard	Bayer	Aventis
Exposure concentrations					
Toxicity tests (mg/kg d.w.)	33, 50, 75, 113, 169, 253	5.0, 11, 25, 57, 128	0.8, 1.2, 1.8, 2.6, 4.0, 6.0	63, 95, 142, 213, 320	188, 216, 249, 286, 329
Avoidance tests (mg/kg d.w.) [b]	4.6	2.7	2.3	1.4	2.3

[a] Pesticide properties obtained from the PPDB database: http://sitem.herts.ac.uk/aeru/ppdb/en/. Last accessed on 15th June 2014.
[b] Recommended pesticide application dosages.

Table 2. Results of the toxicity experiments performed with *E. fetida* and calculated acute Toxicity Exposure Ratios (TERs) based on the recommended application dosages. Concentrations are provided in mg/kg d.w.

	Mortality			Weight loss		Acute TERs
	14 days			7 days	14 days	
Pesticide	Dose-response slope±s.e.	LC10 (95% CI)	LC50 (95% CI)	NOEC	NOEC	
Trichlorfon	6.5 ± 0.9	77 (64-88)	122 (110-136)	33	<33	27
Dimethoate	3.1 ± 0.4	11 (7.1-14)	28 (23-35)	<5.0	5.0	10
Carbendazim	5.1 ± 0.6	1.1 (0.9-1.2)	2.0 (1.7-2.2)	0.8	1.2	0.9
Tebuconazole	5.4 ± 0.7	104 (83-121)	180 (161 -204)	63	<63	64
Prochloraz	23 ± 3.4	229 (216-239)	261 (252-270)	<188	<188	57

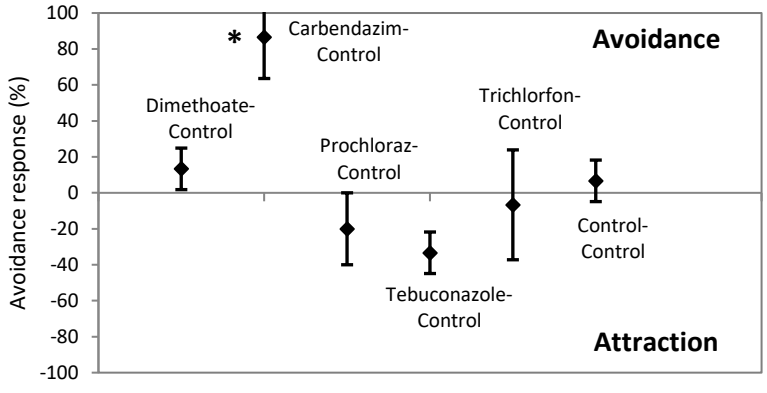
Table 3. Results of the biomarker analysis with *E. fetida* [a].

		Trichlorfon	Dimethoate	Carbendazim	Tebuconazole	Prochloraz
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001
ChE	Effect	↓	↓	↓	↓	↓
	NOEC (mg/kg d.w.)	< 33	< 5	<0.8	<63	<188
	p-value	<0.001	<0.001	0.08	<0.001	<0.001
LDH	Effect	↓	↓	↓	↑/↓	↓
	NOEC (mg/kg d.w.)	< 33	< 5	0.8	<63	< 188
	p-value	0.001	0.007	<0.001	0.23	<0.001
ALP	Effect	↓	↓	↓	NS	↓
	NOEC (mg/kg d.w.)	<33	< 5	<0.8	> 213	<188

[a] A *p*-value lower than 0.05 indicates that the pesticide had a significant effect on the evaluated biomarker. The arrows ↑ and ↓ indicate an increase or decrease of the measured enzymatic activity, respectively, and NS indicates a non-significant increase or decrease in the tested concentration range. The NOEC values correspond to the highest pesticide concentration that did not result in significant effect as compared to the controls.

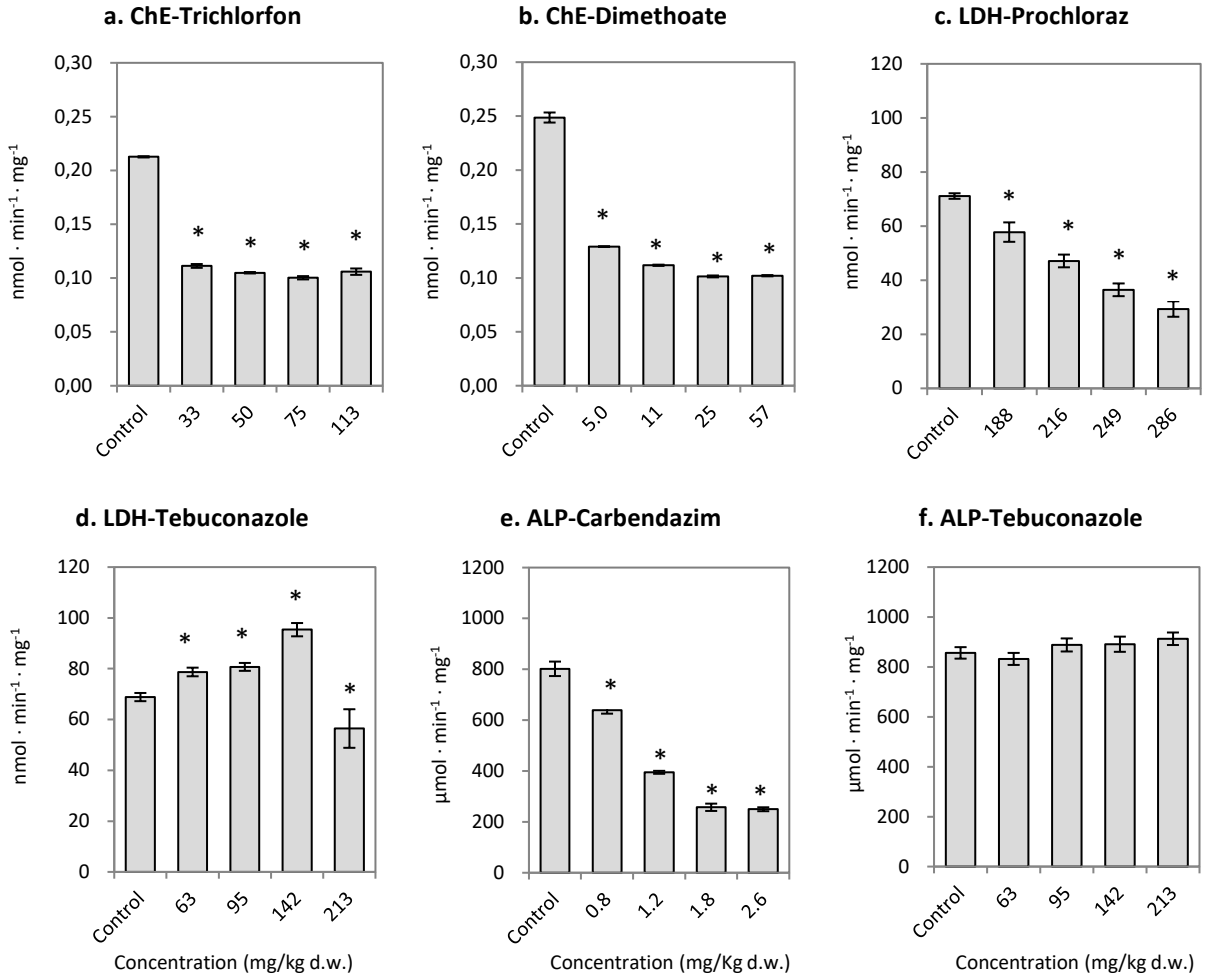
Figures

Figure 1. Results of the avoidance behaviour test (average ± relative standard deviation).

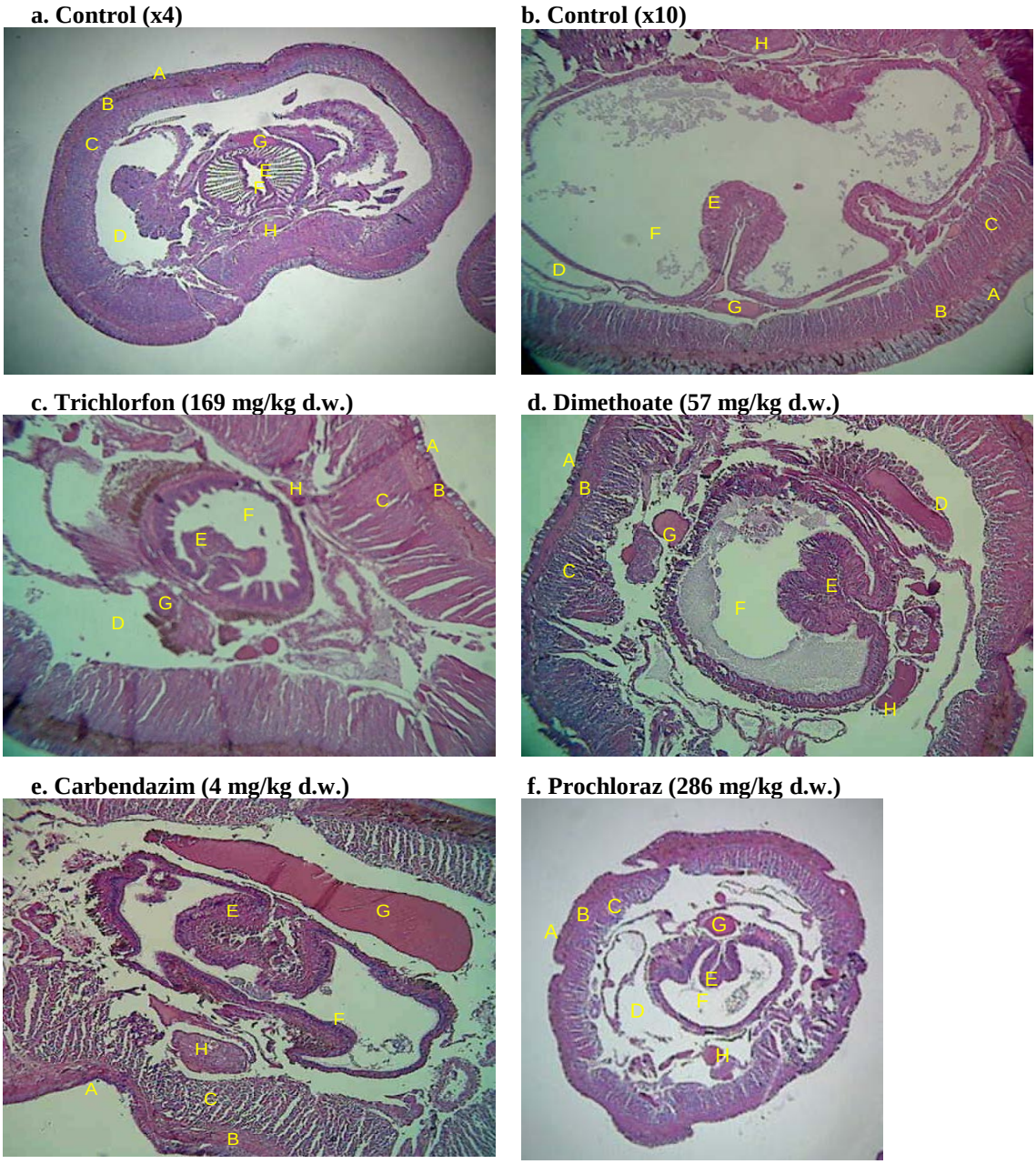


* Significant avoidance response caused by the tested pesticide concentration ($p<0.05$).

Figure 2. Biomarker activity of *E. fetida* organisms after a 14 d pesticide exposure period (mean ± SD). The asterisk indicates significant differences with the control ($p<0.05$).



686 **Figure 3.** Histological sections of earthworms. A: Epidermis; B: Circular muscular layer; C:
687 Longitudinal muscular layer; D: Celoma; E: Tiflosol; F: Intestinal space; G: Dorsal blood
688 vessel; H: Ventral nerve cord.



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