doi: 10.17221/811/2016-PSE

Effects of the herbicides benfluralin, metribuzin and propyzamide on the survival and weight of earthworms (*Octodrilus complanatus*)

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ABSTRACT

Travlos I.S., Gkotsi T., Roussis I., Kontopoulou C.K., Kakabouki I., Bilalis D.J. (2017): Effects of the herbicides benfluralin, metribuzin and propyzamide on the survival and weight of earthworms (*Octodrilus complanatus*). Plant Soil Environ., 63: 117–124.

Extended pesticide use might be of high risk for several non-target organisms like earthworms. Herbicides represent a major part of the total pesticides used; however, their effects on soil organisms have been only partially studied. The effects of the herbicides benfluralin, metribuzin and propyzamide at different rates on the survival and weight of earthworms (*Octodrilus complanatus*) were determined and compared in this study. Our results revealed significant effects of benfluralin, metribuzin and propyzamide on growth and survival of earthworms. Moreover, there was a significant effect of herbicide rate and time after treatment. For several herbicides, the highest weight reduction was obtained for double the recommended rate and was up to 70% compared to the untreated soil. This study highlights the importance of not exceeding the recommended rates of herbicides, while further studies in a range of conditions are certainly required.

Keywords: weed control; toxicity; soil ecosystem; bio-indicator

Weed interference is one of the main factors of crop yield reductions, increasing production costs and reducing the quality of products (Zarea and Karimi 2012). The intensification of crop production and the need to decrease the competition between weeds and crops have increased the use of chemical herbicides worldwide. However, the use of synthetic herbicides does not affect only selected weeds. Herbicides can also affect the physicochemical properties of soils (Robidoux et al. 1999) and harm many non-target species, including beneficial soil organisms, such as earthworms, causing them combined toxicities (Reinecke and Reinecke 2007, Chen et al. 2014).

Earthworms constitute a major component of soil ecosystems and are the representative species of soil animals with the largest terrestrial faunal biomass (Edwards and Bohlen 1992). Moreover, they play important roles in nutrient cycling, organic matter decomposition, soil porosity and microbial activity (Givaudan et al. 2014). Earthworms are easily influenced by pollutants in soil such as metals, organic pollutants and pesticides and therefore they have been included in several studies (Cortet et al. 1999, Iordache and Borza 2010). In particular, their ability to incorporate toxic substances through their skin or by the consumption of large amounts of soil renders them as bio-indicators to detect soil pollutants and to study the toxic effects of several chemicals under various conditions (Stenersen et al. 1973, Lourenço et al. 2011, García-Pérez et al. 2014). *Octodrilus complanatus* is a large-sized species often found in grasslands and pastures, with a wide distribution along the Mediterranean

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basin (Pavlíček and Csuzdi 2006) and therefore it can be easily used as a bio-indicator (Bilalis et al. 2013). *O. complanatus* feeds predominantly on organic material at the soil surface (Vavoulidou et al. 2009) and body weight at maturity ranges between 6–8 g (Monroy et al. 2007). In comparison with other pesticides, it is generally considered that herbicides show relatively low toxicity toward earthworms, although there are some exceptions (Iordache and Borza 2011). In particular, their feeding behavior might be affected by herbicides and this abnormality can be reflected in the weight loss and low reproductive capacity of earthworms (Zarea and Karimi 2012). For the ecotoxicity testing of herbicides on earthworms, parameters such as density, biomass and biodiversity of earthworms, are usually studied (Paoletti 1999). Weight loss is a valuable indicator of physiological stress on earthworms, related to the degree of intoxication and time of exposure of examined species (Yasmin and D'Souza 2010). Mortality is another common parameter in studies of the chemical toxicity in earthworms (Robidoux et al. 1999, Iordache and Borza 2011).

Benfluralin is a dinitroaniline herbicide applied pre-emergence to control grasses and broad-leaved weeds in several crops and acts in the growth of weeds as a mitotic disruptor (Roberts et al. 1998a). Metribuzin, an asymmetric triazine herbicide, is used pre- and post-emergence for the control of grasses and broad-leaved weeds in several field crops (Landgraf et al. 1998) and acts as an inhibitor of photosystem II of photosynthesis (Roberts et al. 1998b). Regarding propyzamide, a benzonitrile amide herbicide, it is applied either pre-emergence or early post-emergence for the control of several weeds in many crops. It acts by means of inhibition of tubulin polymerization. The specific herbicides were selected in the present study because of their use by the farmers in a wide range of crops and their potential for pre-emergence treatment. In particular, in Greece benfluralin is registered for use in alfalfa, clover, lettuce, bean, pea and other crops; metribuzin in alfalfa, soybean, tomato, carrots and asparagus and propyzamide in trees, vines, lettuce, cabbage, lentil, sunflower and other crops. Zarea and Karimi (2012) reported that the relationship between mortality of earthworms and excessive use of several herbicides is still unclear. The aim of the present study was to determine the effects of the above-mentioned herbicides on growth and survival of earthworms *Octodrilus complanatus.* Moreover, the effects of higher than the recommended rates were also evaluated, since often in real farm practice farmers do exceed the maximum registered rates of plant protection products. Such a study could give some valuable information on the potential effects of herbicides on soil fauna and evaluate potential differences between herbicides applied at several rates.

MATERIAL AND METHODS

In November 2015 and January 2016, 12 soil samples were randomly selected from the organic (twenty-year old) experimental field of the Agricultural University of Athens (37°59'N, 23°42'E, 29 m a.s.l.) and particularly from the topmost layer (0–20 cm) and were mixed. The soil was a Leptosol and soil texture was clay loam (29.8% clay, 34.3% silt and 35.9% sand) with pH 7.29, total N 0.12%, cation exchange capacity (CEC) of 28.5 meq/100 g and electric conductance (EC) of 1.60 mS/cm. The earthworms, which were also collected from the same field, were selected by digging-out and hand-sorting within an area of 50 × 50 cm and a depth of 10–20 cm (Römbke et al. 2006) and immediately transferred to the laboratory with the moist soil they had been in at the time of sampling. Earthworms were acclimatized for 14 days under the laboratory conditions in feeding. The healthy adult earthworms with well-developed clitellum were used for the experiment in full accordance with previous studies (Xiao et al. 2006, Bagul et al. 2016). They reared in the selected soil at room temperature in containers covered with a fine nylon mesh to prevent the earthworms from escaping.

Two laboratory experiments were conducted at the Agricultural University of Athens during 2015 and 2016. The first experiment was set up in November 2015 according to a completely randomized design (CRD), with four herbicide treatments and three replications for each treatment (Table 1). The treatments were: untreated (control); benfluralin at the recommended rate of 1350 g of active ingredient (a.i.) per ha, metribuzin at the recommended rate of 750 g a.i./ha and propyzamide at the recommended rate of 1125 g a.i./ha. Each of the plastic containers was filled with 1000 g of soil and covered with a perforated fine nylon mesh. The containers' size and capac-

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Table 1. Information on herbicides used in the experiments

Active ingredient	Chemical class	Recommended rate (g a.i./ha)	Commercial product
Benfluralin	2,6-Dinitroaniline	1350	Bonalan 18 EC
Metribuzin	Triazinone	750	Sencor 70 WG
Propyzamide	Amide	1125	Kerb Flo 400 SC

ity were 20 × 14 × 10 cm and 2.8 L, respectively. Three earthworms with similar weight were added to each container as suggested in previous studies (Bilalis et al. 2013). Moreover, 20 g of crushed dried leaves (2.5 g administered once per week) of mulberry (*Morus alba* L.) were placed on the soil surface of each container from the acclimatization period until the end of experiment. Herbicide applications were performed with an air-pressurized hand-field plot sprayer, with a boom fitted with two flat fan nozzles, calibrated to deliver 400 L/ha of water at 250 kPa pressure. As recommended, herbicides were applied in a way that soil surface was homogeneously covered. The experiment was repeated twice. The experimental room temperature range was 18–22°C, and the moisture content of the soil was monitored throughout the study and if necessary adjusted at 45–65% of the water holding capacity. The second experiment, set up on January 2016, was arranged under a completely randomized design, with ten treatments and three replications. For each of the three herbicides, three different rates were studied: the recommended, half and double the recommended rate. In particular, the treatments were as follows: untreated (control); benfluralin at 675, 1350 and 2700 g a.i./ha; metribuzin at 375, 750 and 1500 g a.i./ha; propyzamide at 562.5, 1125 and 2250 g a.i./ha. The above-mentioned procedure for the first experiment was also followed here.

Regarding the measurements, earthworms were removed from the containers, washed with distilled water and then blotted with filter paper. Their growth was assessed weekly by measuring weight change in earthworms according to the method proposed by Mosleh et al. (2003). The survival was also determined every week, by counting the surviving earthworms in each container. Consequently, there were growth and survival measurements at 1, 2, 3, 4 and 5 weeks after treatment (WAT).

For the statistical analysis, JMP 8 software (SAS Institute Inc., Cary, USA) was used. The experimental data were analysed according to the completely randomized design to examine the effect of different herbicides on weight and survival of earthworms *O. complanatus*. Values were compared by the analysis of variance (ANOVA) and differences between means were separated using the Student's *t*-test. All comparisons were made at the 5% level of significance ($P < 0.05$).

RESULTS AND DISCUSSION

The effects of several herbicides on the weight of earthworms are given in Figure 1. ANOVA revealed

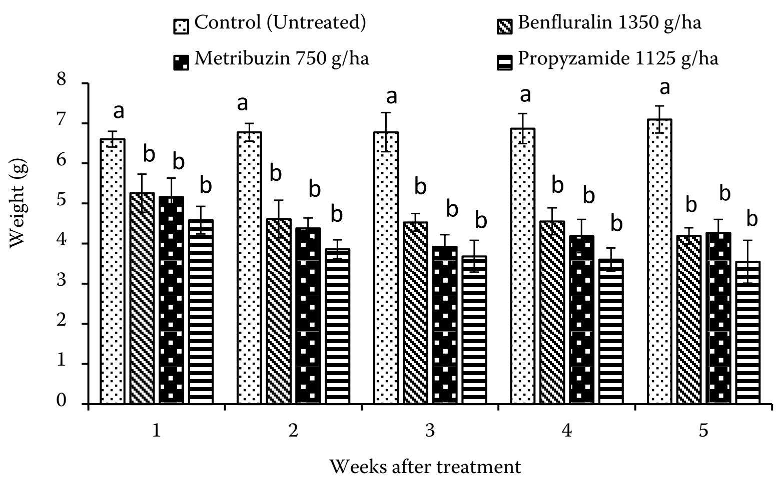

Figure 1. Weight of earthworms as influenced by different herbicides in the first experiment. Vertical bars and different lower case letters denote standard errors of the means and statistically significant differences ($P < 0.05$), respectively

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Table 2. Analysis of variance for herbicide treatment and time effects on survival and weight of earthworms according to the results obtained in the first experiment

Source	*df*	Weight		Survival	
		F	*P*	*F*	*P*
Treatment	3	24.3687	< 0.0001***	15.0767	< 0.0001***
Time	4	0.8532	ns	2.7292	0.0424*
Treatment × time	12	0.2615	ns	0.7014	ns

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns – not significant

significant effects of the treatments on earthworm weight and survival (Table 2). Comparing the results of weight measurements in soils treated with the three different herbicides, no statistically significant differences were observed among them. However, a gradual reduction in weight of treated worms was obtained during this experiment. The highest weight reduction was observed in worms treated with propyzamide at 1125 g a.i./ha, where the percentage of weight loss at the end of experiment was 50% of the untreated control. With the exception of the last week, the smallest reduction was obtained in worms treated with benfluralin at a rate of 1350 g a.i./ha followed by worms treated with metribuzin at a rate of 750 g a.i./ha. A negative impact of herbicides on earthworm growth has been reported by various researchers. Xiao et al. (2006) suggested that growth can be regarded as one of the most sensitive parameters in order to evaluate the toxicity of acetochlor on earthworms.

Regarding survival, with the exception of the first week, statistically significant differences between the different herbicide treatments were also observed in the first experiment. Consequently, and based on the results of this experiment, the time of exposure had a significant effect on earthworm survival. After five weeks of exposure the highest survival percentage was found in earthworms treated with benfluralin (86.6%), followed by earthworms treated with metribuzin and propyzamide (Figure 2).

As in the first experiment, the herbicide treatment had a significant effect on the weight of earthworms, while there was a clear dose-response (Table 3). This finding is in full accordance with the results of previous studies with other herbicides like metolachlor (Xu et al. 2010). Moreover, the increase of time exposure to herbicides was another factor that reduced the weight. It has to be noted that there was a significant interaction between the rate of herbicide and time both for survival and growth (Table 3). Based on the results presented in Figure 3, it was generally observed that in benfluralin treatments there was the lowest reduction in mean weight of worms compared to the control. On the contrary, the highest reduction was found after propyzamide treatments. Between the treatments with each of the herbicides,

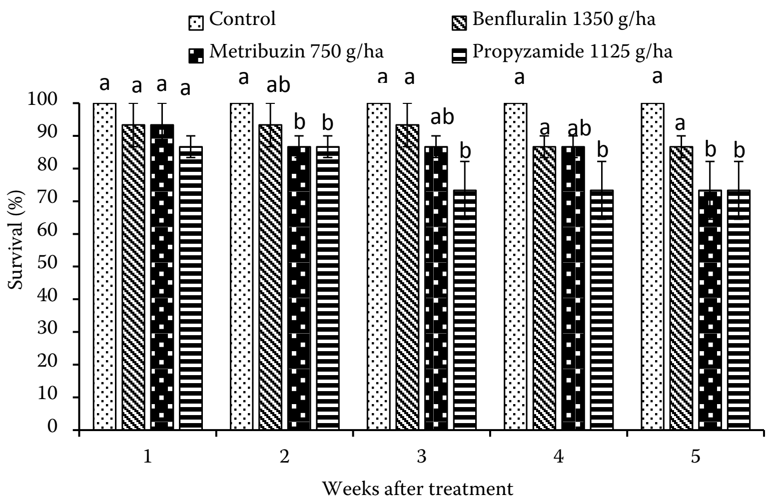

Figure 2. Survival of earthworms as influenced by different herbicide treatments in the first experiment. Vertical bars and different lower case letters denote standard errors of the means and statistically significant differences ($P < 0.05$), respectively

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Table 3. Analysis of variance for herbicide, rate and time effects on survival and weight of earthworms according to the results obtained in the second experiment

Source	*df*	Weight		Survival	
		F	*P*	*F*	*P*
Herbicide	2	3.3519	0.0383*	5.9295	0.0035*
Rate	3	186.1123	< 0.0001***	59.4081	< 0.0001***
Time	4	4.3820	0.0024**	17.9808	< 0.0001***
Herbicide × rate	6	0.5880	ns	0.8184	ns
Herbicide × time	8	0.1311	ns	0.5288	ns
Rate × time	12	3.5282	0.0002***	3.3825	0.0003***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns – not significant

the highest and lowest reductions were observed in the double and half the recommended rate, respectively. This significant reduction in the earthworm growth in a dose-dependent manner is in full accordance with previous studies with the earthworm *Eisenia fetida* and pesticides like carbendazim, glyphosate and dimethoate (Yasmin and D'Souza 2007).

Concerning benfluralin, it was observed that there was a gradual reduction in all treatments during the experiment. Particularly, the effects of benfluralin treatments were significantly different at 3 WAT. The highest weight loss was observed in the worms treated with double the recommended rate of the specific active ingredient at 5 WAT with weight approximately 45% of untreated earthworms' weight. At the same time, the lowest weight loss was revealed in soil treated with half the recommended rate. During the experiment, it was observed that after treatment with half the recommended and recommended rate of metribuzin there was a gradual reduction at 3 WAT; while after that there was a progressive increase until the end of the experiment (Figure 3). For double of the recommended rate there was a progressive reduction throughout the experimental period.

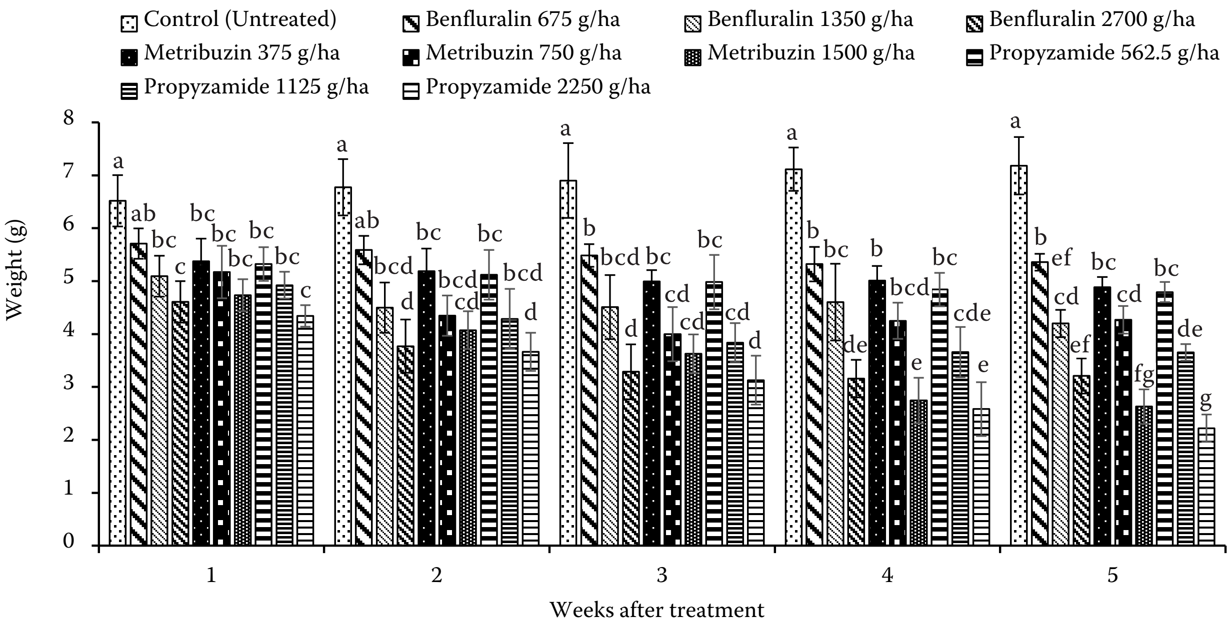

Figure 3. Weight of earthworms as influenced by different herbicides in the second experiment. Vertical bars and different lower case letters denote standard errors of the means and statistically significant differences ($P < 0.05$), respectively

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Metribuzin treatments were significantly different at 4 WAT. In particular, the highest weight was observed in earthworms treated with half the recommended rate of metribuzin after five weeks of exposure. At that time, the weight of earthworms was approximately 22% lower than the corresponding value for the untreated ones. Moreover, the weight of these earthworms was significantly different from the weight of earthworms treated with recommended and double the recommended rate. As in the case of benfluralin, it was observed that there was a gradual reduction in all propyzamide treatments (except the half of the recommended rate at 4 WAT) during the experiment. There were significant differences between the three propyzamide rates at 3 WAT (Figure 3). The highest weight reduction was obtained for double the recommended rate at 5 WAT, where the weight was approximately 70% lower than the untreated control. The earthworms in soils treated with recommended rate had the half weight of untreated earthworms, while the earthworms treated with half of recommended rate were approximately 35% lighter than control.

Regarding the effects of the several herbicides on mortality, the survival of worms was also reduced with the increase of rate of each herbicide (Figure 4). In addition, the increase of time exposure of worms to herbicides was another factor that reduced the survival and had a significant effect in the first experiment (Table 2). It has also to be noted that in the second experiment a significant interaction was found between the rate of herbicide and time as shown in Table 3. The highest and lowest survival reduction of worms was observed after treatment with propyzamide and benfluralin, respectively. Also, it has to be noted that at 3 WAT, the lowest survival was found after the treatment with double the recommended rate of metribuzin.

Regarding the behavior of several herbicides in the soil, it seems to be rather variable. In particular, metribuzin degradation in surface soils is considered to be influenced by temperature and organic substrate in the soil; while its half-life (DT_{50}) value is from 16 to 50 days (Hyzak and Zimdahl 1974, Savage 1976). The half-life of propyzamide was found to range from 10 to 40 days at 25°C, while temperature and cultivation significantly affect the persistence of propyzamide residues (Hance et al. 1978). Vischetti et al. (2002) found half-life values for benfluralin varying from 11.4 to 37.9 days, depending on soil type, incubation conditions and initial herbicide concentration. The above-mentioned data could partially explain our findings. However, in many cases the behavior of several pesticides in soil is rather controversial. For instance, Hyzak and Zimdahl (1974) showed that most of the herbicide residues were found in the

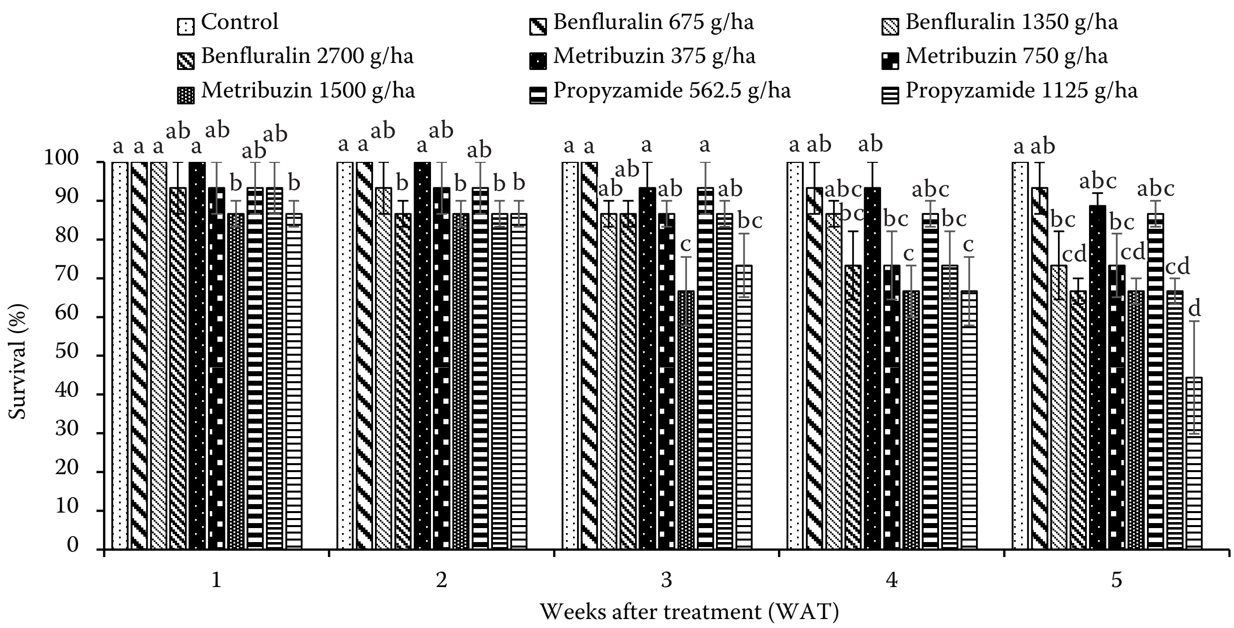

Figure 4. Survival of earthworms as influenced by different herbicide treatments in the second experiment. Vertical bars and different lower case letters denote standard errors of the means and statistically significant differences ($P < 0.05$), respectively

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upper 5 cm of the profile, while in another study; Hernandez et al. (1998) proved that metribuzin can be very mobile in soil.

According to Mosleh et al. (2003), even if the importance of earthworms in ecotoxicological studies is well recognized, there is still a need for further data on the toxicity of specific pesticides to such non-target organisms. Most published ecotoxicological studies on earthworms have focused on metals, while the effects of pesticides have been less studied (Pelosi et al. 2014). Results from previous studies indicate that pesticides uptake by earthworms cannot be generalized between species and that the influence of species traits can vary depending on the nature of the studied chemicals (Carter et al. 2016) and exposure temperatures (Velki and Ecimovic 2015). Furthermore, as reviewed by Hickman and Reid (2008), even case-specific, there are a number of potentially positive physical effects of earthworms upon agrochemical fate, especially sorption and release. Our results revealed significant effects of benfluralin, metribuzin and propyzamide on growth and survival of earthworms. Moreover, there was a significant interaction of herbicide rate and time after treatment, with the highest reductions in treatments with double the recommended rates. However, extrapolation of effects of pesticides observed in laboratory studies to effects in the field studies may be impeded by environmental variables such as soil parameters and weather conditions as stated by Kula (1995) and Yasmin and D'Souza (2010) and consequently further studies in a range of environments are required. Nevertheless, the use of plant protection products like herbicides should be always in accordance to the registrations in order to ensure their sustainability and minimize any adverse effects, being along with other cultural or no-chemical methods in an integrated crop and weed management context.

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Received on December 28, 2016
Accepted on March 17, 2017
Published online on April 3, 2017