ARTICLE IN PRESS

Applied Soil Ecology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Effects of different concentrations of glyphosate (Roundup 360®) on earthworms (*Octodrilus complanatus*, *Lumbricus terrestris* and *Aporrectodea caliginosa*) in vineyards in the North-East of Italy

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A R T I C L E I N F O

Keyword:
Deep-burrowing earthworms
Anecic earthworms
Glyphosate
Survival rate
Cocoon production
Resistance mechanisms
Vineyards
Octodrilus complanatus
Lumbricus terrestris
Aporrectodea caliginosa

A B S T R A C T

The study aims to analyse the effects of the herbicide glyphosate (Roundup 360®) on three key earthworm species. The taxa collected from treated and untreated sites were subsequently bred in uncontaminated soil terraria with serial concentrations of the herbicide. The chosen taxa included two anecic (*Lumbricus terrestris* and *Octodrilus complanatus*) and one endogeic (*Aporrectodea caliginosa*) earthworms. *Lumbricus terrestris* collected from uncontaminated soil (Lt1) and in a conventionally managed vineyard (Lt2). *Aporrectodea caliginosa* and *Octodrilus complanatus* collected from the same uncontaminated site where *L. terrestris* (Lt1) were collected.

Uncontaminated natural grassland soil filling the terraria was characterized for physical-chemical properties. The reproduction rate expressed in earthworms cocoon production was the measure for the effects on worms survival after 21 and 42 days of exposure to 0.59, 2.9, 5.79 g/m^2 of glyphosate, where the dose applied in vineyards in the North-East of Italy goes from 0.72 g/m^2 to 4.32 g/m^2.

Earthworms in untreated terraria were found all alive, while specimens exposed to glyphosate (Roundup 360®) showed a decreasing survival rate and a sharp decline in the number of cocoons. *L. terrestris* (Lt2) showed a survival rate between 64% and 92% after 21 days, and between 44% and 76% after 42 days. *L. terrestris* (Lt1) showed a lower resistance to herbicide (survival rate: 36%–84% after 21 days and 12%–76% after 42 days). *A. caliginosa* was also affected (survival rate: 32%–76% after 21 days and 12%–68% after 42 days). Glyphosate demonstrated severe effects on *Oc. complanatus,* collected on non-contaminated soils, with the lowest values of survival rate (33% after 21 days and 7% after 42 days of exposure). A significant reduction in the cocoons number (about 70%) was observed for *L. terrestris* (Lt1) and *A. caliginosa* after 21 days of exposure, whereas *L. terrestris* (Lt2) showed about 50% of cocoon production.

Results indicate the occurrence of some resistance mechanisms on anecic earthworms in vineyards that have been exposed to glyphosate for at least three decades.

However in spite of the long period of application of glyphosate the impact of this largely applied herbicide is still serious (up to 26% of mortality) especially on the deep-burrowing earthworms species (*Oc. complanatus* and *L. terrestris*).

1. Introduction

Intensification of soil tillage (Edwards and Bohlen, 1996), higher inputs of fertilisers and pesticides, heavy metal soil contamination (Schlegel and Manceau, 2013; Zhang et al., 2009), soil compaction exerted by heavy agricultural machinery are the most important drivers of biodiversity loss in many agroecosystems (Hole et al., 2005; Paoletti, 1999; Paoletti and Pimentel, 2000; Lindenmayer et al., 2012), especially in orchards and vineyards (Paoletti et al., 1995a,b; Altieri et al., 2005). These agricultural practices negatively affect some of different groups of soil fauna (e.g. earthworms, micro-arthropods, nematodes, microorganisms) (Ponge et al., 2013) known for their sensitivity to disturbances associated to agriculture, as soil tillage (Cortet et al., 2002a; Krogh et al., 2007; Lagomarsino et al., 2009), trampling and soil compaction (Cluzeau et al., 1992; Heisler and Kaiser, 1995), fertilizer addition (Cole et al., 2005; Van der Wal et al., 2009), and

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http://dx.doi.org/10.1016/j.apsoil.2017.07.028
Received 12 January 2017; Received in revised form 13 July 2017; Accepted 18 July 2017
0929-1393/

pesticide treatment (Frampton, 1997; Rebecchi et al., 2000; Cortet et al., 2002b). The use of pesticides (insecticides, fungicides and herbicides) in the agroecosystems represents one of the most controversial agricultural activities because of its impact on soil fauna (Paoletti and Bressan, 1996). Herbicides have pronounced negative effects on nematode soil abundance (Sánchez-Moreno et al., 2014), on interactions between earthworms and symbiotic mycorrhizal fungi (Zaller et al., 2014) on dehydrogenase activity of bacterial, fungal and actinomycetes soil communities (Sebiomo et al., 2011). Recent studies highlighted how the magnitude of micro-arthropods responses (phytophagous and predatory mites species) to weed management practices (glyphosate applications) may be linked to changing in habitat, such as loss of vegetation cover and elimination of food sources (Belden and Lydy, 2000).

In many agroecosystems and natural environments, earthworms are probably the most important group of soil biota in terms of soil formation and maintenance of soil structure and fertility (Coleman and Wall, 2015). As ecosystem engineers, especially the deep-burrowing species (anecic ones) (Lavelle et al., 1997) they have fundamental roles in soil functioning because of their burrowing activities, as well as their ingestion of soil and production of castings (Latif et al., 2009; Gavinelli et al., 2017). The importance of earthworms includes their influence on soil aeration, water infiltration and mixing of horizons that improve the soil structure. Also they represent an important source of food for birds and moles (Lavelle et al., 2006). The beneficial effects of earthworms was described for the first time by Darwin (1881) which summarized his study on earthworms after 40 years of experimental work and observations (Edwards, 2004). Since then, a vast literature has confirmed the great importance of earthworms as biological agents in soil formation (Paoletti et al., 1995a,b, 1998) and suitable bioindicators of soil pollution (Paoletti, 1999; Paoletti and Sommaggio,1996; Rodriguez-Castellanos and Sanchez-Hernandez, 2007) in terrestrial ecosystems providing an early warning of deterioration in soil quality.

Pesticides (Lüscher et al., 2014; Mosleh et al., 2003), especially fungicides (Paoletti et al., 1998) and herbicides (Pizl, 1988), have negative effects on earthworms at all organization levels (Pelosi et al., 2014) causing their death (Slimak, 1997), depressing their growth (Dalby et al., 1995; Springett and Gray, 1992; Correia and Moreira, 2010), affecting their reproduction (Yasmin and D'Souza, 2010) and their behaviour (Buch et al.,2013; Kaneda et al., 2009). Also the fatty acids content can be affected (Hossam et al., 2012). Moreover, herbicides via earthworms can achieve higher levels of the trophic chain (Jarmuł-Pietraszczyk and Jastrzebska, 2012).

Glyphosate (GLY) (N-phosphonomethyl-glycine) base product is the leading non-selective aminophosphonate-type herbicide for the annual and perennial weeds control both in agriculture and in non-agricultural landscapes (Piola et al., 2013). It has been registered as a broad spectrum herbicide in the U.S. since 1974 and in the early twenty-first century, glyphosate became the largest-selling single crop protection chemical product on the market (Woodburn, 2000). Glyphosate is generally regarded as an environmentally-friendly herbicide due to its rapid biodegradation, strong adsorption to soil minerals, especially Fe and Al oxides (Norgaard et al., 2014) and fast microbial degradation (Barja and Santos, 2005; Giesy et al., 2000; Vereecken, 2005). Some studies demonstrated that the presence of glyphosate could significantly reduce the acute toxicity of Cu to earthworms (Zhou et al., 2012, 2013).

However, many common glyphosate-containing products (Roundup360®) are made with a polyethoxylated tallow amine surfactant that is more toxic than glyphosate itself and the combination of the two is yet more toxic (Monsanto, 1996). These chemicals are acutely adverse to animals (IBR, 1991a,b; Martin, 1982; Morowati, 2000); Monsanto (1988a,b) demonstrated that Roundup increases the frequency of recessive lethal mutations in fruit flies. Acute toxicity tests on laboratory rats showed that the absorbed glyphosate dose had distributed throughout the body seven days after administration (Monsanto, 1988a,b). In humans, the main effects involve cardiac depression, accumulation of excess fluid in the lungs, eye and skin irritation, gastrointestinal pain and vomiting (Cox, 1995a). Moreover, residues of the commonly-used herbicide glyphosate can be detected long after glyphosate treatments have been made (Cox, 1995b; Landry et al., 2005). However, some earthworms species (*Aporrectodea caliginosa* and *Allolobophora chlorotica*) showed the ability to acclimate to residual glyphosate contamination in agricultural soils through accelerated activation of detoxification and antioxidant enzymes (Bon et al., 2006; Givaudan et al., 2014; Omar et al., 2012).

The current study aimed at testing the effects of a commercial formulation of glyphosate (Roundup 360 Power, Monsanto Europe N.V. Belgium), on the growth and survival rate of three key earthworms species (two being large burrowing species: *Lumbricus terrestris* and *Octodrilus complanatus* and one, *A. caliginosa* an endogeic) collected in either treated or untreated sites and subsequently reared within terraria supplemented with increasing dosages of glyphosate.

Concentration-dependent weight loss has been reported for glyphosate intoxication in *Eisenia andrei* (Piola et al., 2013) and *Eisenia foetida* (Yasmin and D'Souza, 2007). On *E. foetida* a reduced locomotor activity was also observed (Verrell and Van Buskirk, 2004). Negative impact of glyphosate on reproductive ability and on growth inhibition and in nervous system has been reported in *Dendrobaena veneta* and the production of deformed cocoons under laboratory conditions was observed (Jarmuł-Pietraszczyk and Jastrzebska, 2012). *Lumbricus terrestris* featured a significant decrease in sperm numbers (Sherwan, 2013).

2. Material and methods

2.1. Species analyzed

Lumbricus terrestris is a deep-burrowing, anecic species native in Europe; an earthworm that recently has invaded some areas of Canada, parts of US and South America, New Zeland, Australia. It is pinkish to reddish-brown in colour and typically reaches 10–25 cm in length. It is found in deciduous natural forests, planted forests, range/natural grasslands, riparian zones, ruderal/disturbed, scrub/shrub lands, urban areas, wetlands and it is often present also in agricultural fields, although it is negatively affected by fungicides, herbicides, tillage operations and lack of leaf litter. It can inhabit all soil types except coarse sands, bare rock and acidic peat (*Sphagnum*), but it is not frost-tolerant and hibernates into deep soil layers during the winter (Addison, 2009; Tiunov et al., 2006; Wironen and Moore, 2006). By rapidly consuming leaf litter, *L. terrestris* affects biochemicals cycles and plant communities through its interaction with seeds. It may also displace native earthworm species.

Oc. complanatus is a large deep-burrowing species and it is present on hills, mountains, woodlot remnants and in rural landscapes. Although large specimens also disappear upon shifting to agricultural intensification, they generally recolonize fields abandoned to fallow (Paoletti, 1999). It lives into deep soil galleries but occasionally feeds on surface litter when soil humidity is high enough, e.g., in the North-Eastern Italy (Paoletti, 1985).

A. caliginosa is the most common endogeic species in European, North American and New Zealand fields (Paoletti and Pimentel, 2000).

2.2. Experimental design

The experiment consisted of two trials. Every trial had the aim to analyze two groups of taxa. Adults earthworms thoroughly identified by morpho-anatomical characters using the LOMBRI software (Paoletti and Gradenigo, 1996) were collected in farms under different agricultural management and geographical areas:

Lumbricus terrestris (Lt1 group), and *Aporrectodea caliginosa* were collected from non-treated natural grassland close to the experimental farm at Legnaro (45°20′42.87″N; 11°57′20.26″E);

Table 1
Mean of three replicates of physical and chemical properties of the soil added in the terraria.

	Depth (cm)	pH (H_2O)	EC ($dS\ m^{-1}$)	Texture (%)			TOC (% dw)	N (% dw)	C/N ratio	Organic matter ($g\ kg^{-1}$)	Total limestone ($g\ kg^{-1}$)
				Clay	Silt	Sand					
Mean	0 – 15	7.63	0.97	34.33	18.33	47.33	2.71	0.32	8.64	46.63	338

L. terrestris (Lt2 group) was isolated from a conventionally-managed glyphosate-treated vineyard on the Euganean hills (45°18′37.59″N; 11°38′35.14″E);

Octodrilus complanatus was collected in natural non-treated grassland (45°20′43.69"N; 11°40′49.73″E) on the Euganean hills, both located in areas in which no herbicides had been applied.

The trial was carried out at the experimental farm of University of Padova, at Legnaro (45°20′42.87″N, 11°57′20.26″E).

All trials were performed in terraria. Each terrarium consisted in a plastic box of 5 L in volume with 0.0288 m^2 (18 × 16 cm) of surface. Terraria were filled with a layer of about 2 cm of gravel with 8–15 mm of diameter and 3 kg of natural non contaminated soil sieved. In order to permit the restrain of the minimal size of the coocons, then the size of the mesh was 4 mm. To prevent earthworms escape terraria where closed at the top with a 0.5 mm mesh net and wrapped with aluminium foil to avoid the lateral effects of light. The terraria were placed outside, under a roof covering to take shelter from atmospheric agents and ensuring temperatures compatible with the thermal requirements of earthworms (from 10 °C to 18 °C). The relative humidity the soil moisture was kept constant (80%), near to optimal humidity for most of the earthworms species (Reinecke and Venter, 1987). After the calculation, Earthworms were reintroduced into the same terraria.

The number of earthworms in each terrarium is set analysing the natural earthworms density.

In natural conditions the density goes from 50 to 500 specimens per m^2.

After 7 days of earthworms acclimation, a glyphosate commercial formulation (Roundup 360® Power, Monsanto, 360 $g\ l^{-1}$) was sprinkled on the soil surface of the terraria.

The set up included the following treatments of glyphosate concentration in the terrarium soil: (a) untreated control: distilled water; (b) dosage 1: 0.59 g/m^2; (c) dosage 2: 2.9 g/m^2; (d) dosage 3: 5.79 g/m^2; (e) dosage 4: 11.59 g/m^2 of herbicide. The dosage could include the maximum and minimum dose suggested of Roundup 360 applied by the farmers, and this dose suggested is from 0.72 g/m^2 to 4.32 g/m^2 is per treatment (Monsanto, 2013).

The dimension of the terraria intended to simulate the soil herbicide exposition present in the real vineyards.

2.2.1. First incubation trial

25 terraria were arranged for five treatments. In each terrarium, five adult specimens of a single species were placed for *A. caliginosa* or *L. terrestris*, while three specimens per terrarium were used for the larger *Oc. complanatus*.

For each treatment, five replicates were made. At days 21 and 42 after treatment, terrarium were manually sorted and accurately examined, and the number of adult earthworms was recorded. With these data the survival rate was calculated.

2.2.2. Second incubation trial

12 terraria, filled with uncontaminated soil, were arranged for each group Lt1, Lt2 and *A. caliginosa*. Ten adult earthworms were transferred to each terrarium prepared by applying three different concentrations of glyphosate. Three replicates were set up for each concentration and three control boxes were prepared under identical conditions but without the addition of glyphosate. After 7 days of acclimation and 21 days of exposure to herbicide the percentage of survival adults and number of cocoons were recorded by hand-sorting method by expert eye check.

2.3. Terraria soil characterization

The soil used for terraria was collected in a natural non-treated grassland in the experimental farm of the University of Padova at Legnaro (45°20′42.87″N, 11°57′20.26″E).

Mean of seventy-two soil cores were collected to 15 cm of depth, dried, sieved to 2 mm and analyzed for the main physical-chemical properties. The soil was characterized by a sub-alkaline pH (7.60–7.68) and high electrical conductivity values (EC: 0.91–0.95). It showed a medium-high content of sand (46–48%) and clay (34–35%) but it presented low silt values (18–19%), being therefore classified as a sandy-loam soil. All subsamples were rich in organic carbon (TOC: 2.62–2.85%), organic matter (45.0–49.0 $g\ kg^{-1}$) and had C/N ratio values from 8.45 to 8.88. The amount of limestone was between 334 and 342 $g\ kg^{-1}$, therefore the soil can be defined as very calcareous. These soil features are largely shared in vineyards farms around in the hilly Euganean area from which the earthworms where captured (Table 1).

2.4. Data analysis

The data were analyzed using the non-parametric Mann-Whitney test. All results were expressed as means ± SE (standard error). P-values less than 0.05 were taken as statistically significant. Data on the mortality rate (%) were used to obtain a concentration-curve response and analyzed using Statistica v. 13.0 software (Dell Inc., Tulsa, OK, USA). Median lethal (LC_{10} and LC_{50}) glyphosate concentrations were determined using the statistical package XLSTAT 2016v. 18.6 software (Addinsoft Sarl, Paris, France).

3. Results

The trials provided strong evidence that glyphosate induced pronounced negative effects on the survival rate and cocoons production of earthworms (Tables 2 and 3).

3.1. Effects on first incubation trial

The percentage of adult survival showed a concentration-dependent decrease for all groups studied compared to the untreated control. No mortality was observed in control terraria. There was a sharp decline in the number of survived adults at the highest concentrations of herbicide (0.59 g/m^2; 2.9 g/m^2; 5.79 g/m^2; 11.59 g/m^2 but measurable even at lower concentrations (0.59 g/m^2) in *A. caliginosa* and *Oc. complanatus* (Fig. 1 and Table 2). After 21 days *L. terrestris* (Lt2) showed survivors numbers decreasing by −7% in the 0.59 g/m^2 and −36% in the 11.59 g/m^2 dosage, while the most susceptible species proved to be *A. caliginosa* [Δ = −24% to 0.59 g/m^2 and Δ = −68% to 11.59 g/m^2] and *Oc. complanatus* [Δ = −40% to 0.59 g/m^2 and Δ = −67% to 11.59 g/m^2].

The herbicide induced a further decrease in the percentage of surviving individuals, especially at higher concentrations after 42 days of exposure for *A. caliginosa* [Δ = −40% to 0.59 g/m^2 and Δ = −88% to 11.59 g/m^2] and *Oc. complanatus* [Δ = −53% to 0.59 g/m^2 and

Table 2
Effects of glyphosate on the survival rate (%) of earthworms. Data are expressed as means ± SE (standard error). The means followed by the same letter are not significantly different according to Mann-Whitney test ($p < 0.05$).

Species	survival rate (after 21 days of exposure) – First Sampling				
	Untreated control	dosage1 (treatment 1)	dosage 2 (treatment 2)	dosage 3 (treatment 3)	dosage 4 (treatment 4)
L. terrestris (Lt1)	100.0 a	84.0 ± 4.0 a	72.0 ± 4.9 a	60.0 ± 4.9 b	36.0 ± 4.0 b
L. terrestris (Lt2)	100.0 a	92.0 ± 4.9 a	76.0 ± 4.0 a	68.0 ± 4.9 a	64.0 ± 4.0 a
A. caliginosa	100.0 a	76.0 ± 7.5 b	64.0 ± 4.0 a	52.0 ± 4.9 b	32.0 ± 4.9 b
O. complanatus	100.0 a	60.0 ± 6.7 b	47.0 ± 8.2 b	47.0 ± 8.2 b	33.0 ± 0.0 b
	survival rate (after 42 days of exposure) − Second Sampling				
L. terrestris (Lt1)	100.0 a	76.0 ± 4.0 a	44.0 ± 4.9 b	32.0 ± 4.0 b	12.0 ± 4.0 b
L. terrestris (Lt2)	100.0 a	76.0 ± 4.0 a	68.0 ± 4.0 a	56.0 ± 4.9 a	44.0 ± 4.9 a
A. caliginosa	100.0 a	68.0 ± 8.2 a	44.0 ± 6.7 b	28.0 ± 0.0 b	12.0 ± 6.7 b
O. complanatus	100.0 a	47.0 ± 4.9 b	40.0 ± 4.0 b	27.0 ± 4.9 b	7.0 ± 4.9 b

Table 3
Effects of glyphosate on the LC10 and LC50 of earthworms (Lt1 and Lt2) based on 21 and 42 day of exposure. Values in g/m^2 with 95% confidence interval.

Days of exposure	LC_{10}	LC_{50}
21	0.496 (0.389–0.527)	26.804 (23.437–27.821)
42	0.317 (0.226–0.364)	7.001 (6.551–7.237)

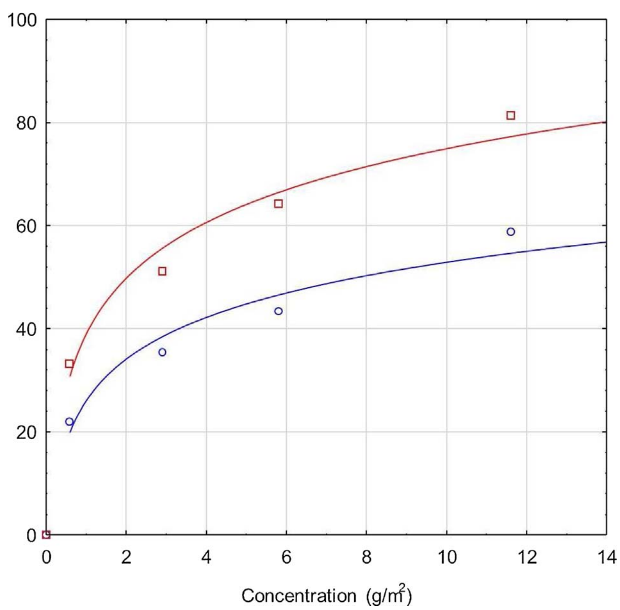

Fig. 1. Effects of glyphosate on the total mean of mortality rate (%) the earthworms based on 21 and 42 days of exposure. Blue line with circled dots: 21 days exposure mortality rate (25.9738 + 26.8692*log10(x)); Red line with squared dots: 42 days exposure mortality rate (38.9362 + 35.9893*log10(x)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$\Delta = -93\%$ to 11.59 g/m^2] compared to untreated controls (Figs. 2–5 and Table 2).

A further analysis on the earthworms mean for each concentration, showed a global decreasing survival rate especially at the concentration of 5.79 and 11.59 g/m^2.

Effects of glyphosate on the LC_{10} and LC_{50} of earthworms (Lt1 and Lt2) based on 21 and 42 day of exposure are shown in Table 3.

3.2. Effects on second incubation trial

The count of the cocoons provided an index to the reproductive output of the earthworms. A sharp decline in their number in

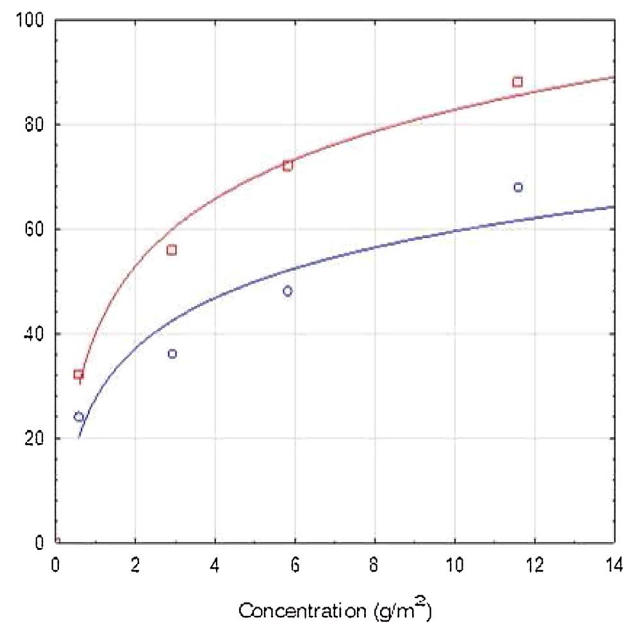

Fig. 2. Effects of glyphosate on the mortality rate (%) of *A. caliginosa* based on 21 and 42 days of exposure. Blue line with circled dots: 21 days exposure mortality rate (y = 27.4761 + 32.0345*log10(x)); Red line with squared dots: 42 days exposure mortality rate (y = 39.9071 + 42.8309*log10(x)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

glyphosate-treated soils compared to the untreated control was observed (Table 4). The reproductive output was significantly impacted due to exposure to 2.9 g/m^2; 5.79 g/m^2 ($p < 0.05$) in all groups of worms tested.

In soil treated with glyphosate, the reduction in the cocoons number was higher than 70% for *L. terrestris* (Lt1) and *A. caliginosa* when exposed to highest concentrations of herbicide, after 21 days of exposure. About 50% of cocoons production was observed for *L. terrestris* (Lt2) when compared with control. A significant reduction in cocoons number was recorded also at lower concentrations (0.59 g/m^2).

The percentage of survival measured for all groups of earthworms exposed to glyphosate is shown in Fig. 1. When the concentration of glyphosate was set at the same level for all groups, *L. terrestris* (Lt1) showed not significantly higher ($p < 0.05$) tolerance to herbicide than the other two species.

This experimental evidence was confirmed after 42 days of exposure (Fig. 4).

As shown in Figs. 2, 3, 4, 5, the survival rate of three species studied (*L. terrestris, A. caliginosa, Oc. complanatus*) exposed to herbicide showed a concentration-dependent decrease not significantly different

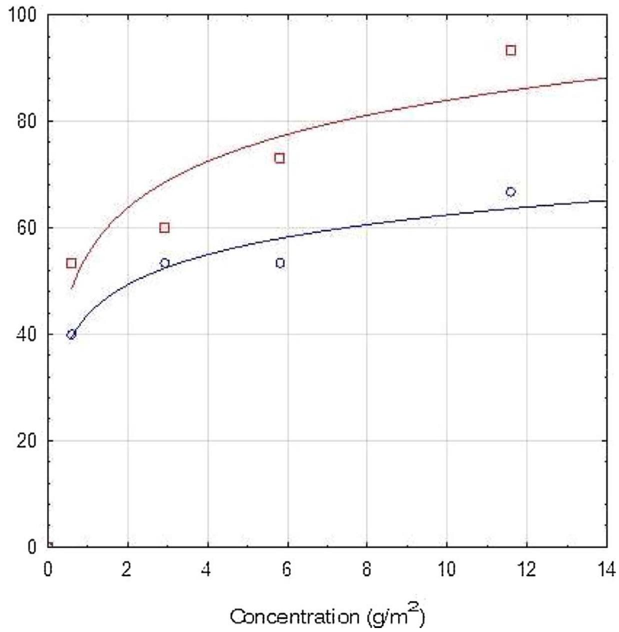

Fig. 3. Effects of glyphosate on the mortality rate (%) of *O. complanatus* based on 21 and 42 days of exposure. Blue line with circled dots: 21 days exposure mortality rate (y = 43.6605 + 18.7524*log10(x)); Red line with squared dots: 42 days exposure mortality rate (y = 55.1092 + 28.8685*log10(x)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

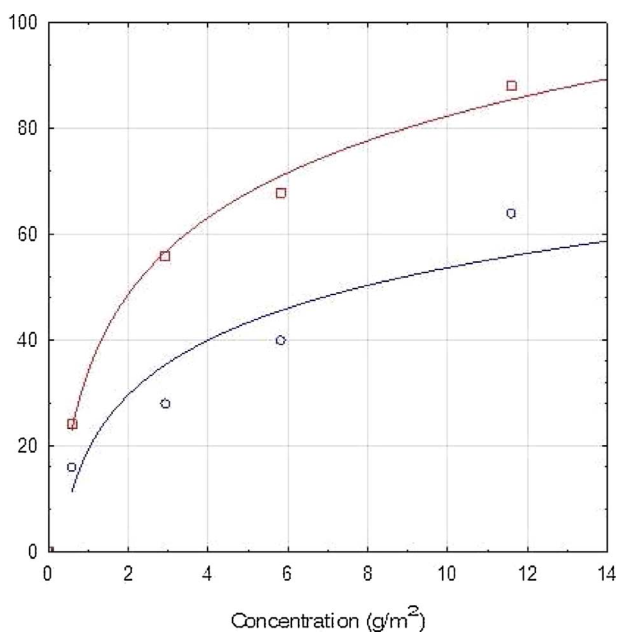

Fig. 4. Effects of glyphosate on the mortality rate (%) of Lt1 based on 21 and 42 days of exposure. Blue line with circled dots: 21 days exposure mortality rate (y = 19.2455 + 34.4202*log10(x)) Red line with squared dots: 42 days of exposure mortality rate (y = 34.1198 + 48.2347*log10(x)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

($p < 0.05$) for all the assayed concentrations (0.59 g/m^2; 2.9 g/m^2; 5.79 g/m^2; 11.59 g/m^2) comparing to control.

4. Discussion

The three taxa showed a different response to the same concentration and exposure time. Especially Lt2 showed an higher survival rate than Lt1, *A. caliginosa* and even more than *Oc. complanatus* to long-term

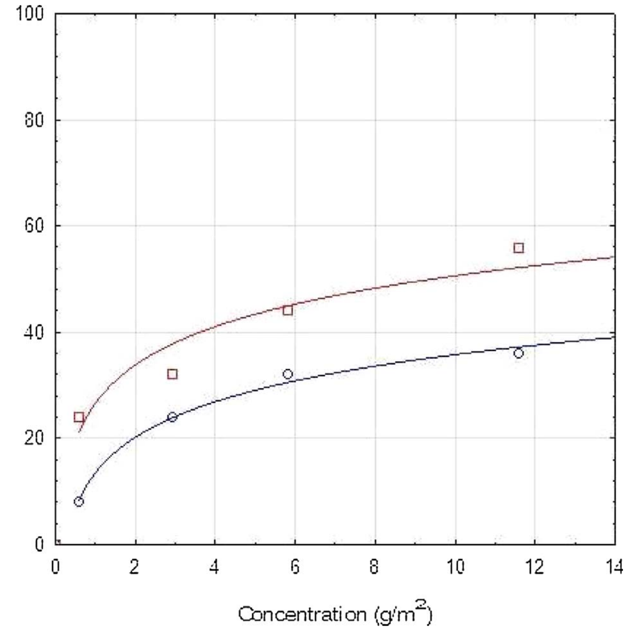

Fig. 5. Effects of glyphosate on the mortality rate (%) of Lt2 based on 21 and 42 days of exposure. Blue line with circled dots: 21 days exposure mortality rate (y = 13.5129 + 22.2698*log10(x)) Red line with squared dots: 42 days of exposure mortality rate (y = 26.6085 + 24.02317*log10(x)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

exposure. These differences appear determined by the collection area and not by the species. In fact Lt1 and Lt2 belong to the same species but they showed different levels of susceptibility to glyphosate, whereas Lt1 and *A. caliginosa* had similar behaviour. The glyphosate induced similar effects on different species collected in areas sharing the same soil management (herbicide treatment or its absence). A lower survival rate was measured in *Oc. complanatus*, *A. caliginosa* and Lt1 collected in natural grassland and therefore not covered by common agricultural practices, including glyphosate weed control. Lt2 was collected in a vineyard where the Roundup 360® was distributed 2–3 times per year. The herbicide demonstrated severe effects on reproduction of *L. terrestris* and *A. caliginosa* during terraria trials. Mohamed et al. (1995) explored impacts on the survival and body mass of the earthworm *A. caliginosa* and observed toxic effects at doses close to LC50 (100–1000 μg a.e./cm^2) due to glyphosate. Compared with soil controls, the count of cocoons number in treated soils showed a sharp decline of reproductive activity in all worms collected from natural grassland, although a lower reproductive ability was observed also for specimens collected from cultivated soil. The reduction in cocoons could result from interferences by this substance and the earthworms reproductive mechanisms although the present study cannot reveal the mechanism behind the decrease in cocoon production. These results suggest that the specimens collected from areas interested by the same agronomic practices show similar reproductive behaviour. It may therefore be hypothesized that a long-term exposure to glyphosate has led the evolution of resistance mechanisms in the studied earthworm species (*L. terrestris*) and improved their ability to live and reproduce in the presence of the compound.

However they still decrease consistently in the numbers due to the key herbicidal application.

The small terraria we used are not representative of real vineyard fields but can provide a simplified model, with however environmental factors under control than representing what happens in nature.

We could not fully confirm some past studies although made on other species, as the historical summarization in Table S1 recapitulates but we confirm that glyphosate (Roundup 360®) application can severely affect earthworm fauna.

Table 4
Effects of glyphosate on the survival rate and reproductive output (%) of earthworms. Data are expressed as means ± SE (standard error). The means followed by the same letter are not significantly different according to the Mann-Whitney test ($p < 0.05$).

Species	survival rate (after 21 days of exposure)			
	Untreated control	dosage1 (treatment 1)	dosage 2 (treatment 2)	dosage 3 (treatment 3)
L. terrestris (Lt1)	100.0 a	66.7 ± 3.3 b	53.3 ± 3.3 b	36.7 ± 3.3 b
L. terrestris (Lt2)	100.0 a	93.3 ± 3.3 a	76.7 ± 3.3 a	66.7 ± 3.3 a
A. caliginosa	100.0 a	60.0 b	50.0 b	33.3 ± 3.3 b
	Cocoons (after 21 days of exposure)			
L. terrestris (Lt1)	100.0 a	54.9 ± 2.5 b	36.6 ± 1.3 b	26.9 ± 0.7 b
L. terrestris (Lt2)	100.0 a	91.5 ± 1.6 a	77.8 ± 0.6 a	52.1 ± 0.4 a
A. caliginosa	100.0 a	57.3 ± 0.7 b	41.4 ± 2.2 b	29.4 ± 2.0 b

Acknowledgment

We would like to thank dr. Mariano Mutton for the technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.07.028.

References

Addison, J.A., 2009. Distribution and impacts of invasive earthworms in Canadian forest ecosystems. Biol. Invasions 11, 59–79.
Altieri, M.A., Clara, I., Nicholls, C.I., Ponti, L., York, A., 2005. Designing biodiverse, pest-resilient vineyards trough habitat management. Pract. Winery Vineyard J. 27, 16–30.
Barja, B.C., Santos, M.A., 2005. Aminomethylphosphonic acid and glyphosate adsorption onto goethite: a comparative study. Environ. Sci. Technol. 39, 585–592.
Belden, J.B., Lydy, M.J., 2000. Impact of atrazine on organophosphate insecticide toxicity. Environ. Toxicol. Chem. 19, 2266–2274.
Bon, D., Gilard, V., Massou, S., Pérès, G., Malet-Martino, M., Martino, R., Desmoulin, F., 2006. In vivo ^{31}P and ^{1}H HR-MAS NMR spectroscopy analysis of the unstarved *Aporrectodea caliginosa* (Lumbricidae). Biol. Fertil. Soils 43, 191–198.
Buch, A.C., Brown, G.G., Niva, C.C., Sautter, K.D., Sousa, J.P., 2013. Toxicity of three pesticides commonly used in Brazil to *Pontoscolex corethrurus* (Müller, 1857) and *Eisenia andrei* (Bouché, 1972). Appl. Soil Ecol. 69, 32–38.
Cluzeau, D., Binet, F., Vertes, F., Simon, J.C., Riviere, J.M., Trehen, P., 1992. Effects of intensive cattle trampling on soil-plant-earthworms system in two grassland types. Soil Biol. Biochem. 24, 1661–1665.
Cole, L., Buckland, S.M., Bardgett, R.D., 2005. Relating microarthropod community structure and diversity to soil fertility manipulations in temperate grassland. Soil Biol. Biochem. 37, 1707–1717.
Coleman, D.C., Wall, D.H., 2015. Soil fauna: occurrence, biodiversity, and roles in ecosystem function. In: Paul, E.A. (Ed.), Soil Microbiology, Ecology and Biochemistry, fourth ed. Academic Press, London, pp. 111–150.
Correia, F.V., Moreira, J.C., 2010. Effects of glyphosate and 2,4-D on earthworms (Eisenia foetida) in laboratory tests. Bull. Environ. Contam. Toxicol. 85, 264–268.
Cortet, J., Ronce, D., Poinsot-Balaguer, N., Beaufreton, C., Chabert, A., Viaux, P., Cancela De Fonseca, J.P., 2002a. Impacts of different agricultural practices on the biodiversity of microarthropod communities in arable crop systems. Eur. J. Soil Biol. 38, 239–244.
Cortet, J., Dominique Gillon, D., Joffre, R., Ourcival, J.-M., Poinsot-Balaguer, N., 2002b. Effects of pesticides on organic matter recycling and microarthropods in a maize field: use and discussion of the litterbag methodology. Eur. J. Soil Biol. 38, 261–265.
Cox, C., 1995a. Glyphosate, part 1: toxicology. J. Pestic. Reform 15 (3), 14–20.
Cox, C., 1995b. Glyphosate, part2: human exposure and ecological effects. J. Pestic. Reform 15 (4), 14–20.
Dalby, P.R., Baker, G.H., Smith, S.E., 1995. Glyphosate, 2,4-DB and dimethoate: effects on earthworm survival and growth. Soil Biol. Biochem. 27, 1661–1662.
Darwin, C.R., 1881. The Formation of Vegetable Mould, Through the Action of Worms, with Observations on Their Habits. John Murray, London.
Edwards, C.A., Bohlen, P.J., 1996. Biology and Ecology of Earthworms, third ed. Chapman and Hall, London.
Edwards, C.A., 2004. Earthworm Ecology. CRC Press, Boca Raton.
Frampton, G.K., 1997. The potential of Collembola as indicators of pesticide usage: evidence and methods from the UK arable ecosystem. Pedobiologia 41, 179–184.
Gavinelli, F., Barcaro, T., Dorigo, L., Dreon, A.L., Toniello, V., Pamio, A., Csuzdi Cs Blakemore, R., Squartini, A., Moretto, E., Fernandez Marchan, D., De Sosa, I., Nicolussi, G., Paoletti, M.G., 2017. Importance of the large deep-burrowing (anecic) earthworms in forested and cultivated areas (especially vineyards) of the North-Eastern Italy. Appl. Soil Ecol (submitted).
Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup® herbicide. In: In: Ware, G.W. (Ed.), Reviews of Environmental Contamination and Toxicology. Reviews of Environmental Contamination and Toxicology, vol. 167 Springer, New York.
Givaudan, N., Binet, F., Le Bot, B., Wiegand, C., 2014. Earthworm tolerance to residual agricultural pesticide contamination: field and experimental assessment of detoxification capabilities. Environ. Pollut. 192, 9–18.
Heisler, C., Kaiser, E.A., 1995. Influence of agricultural traffic and crop management on collembola and microbial biomass in arable soil. Biol. Fertil. Soils 19, 159–165.
Hole, D.G., Perkins, A.J., Wilson, J.D., Alexander, I.H., Grice, P.V., Evans, A.D., 2005. Does organic farming benefit biodiversity? Biol. Conserv. 122, 113–130.
IBR, 1991a. Final Report. Acute Toxicity in Earthworms According to OECD 207-Test Article: Technical Isopropylamine Salt of Glyphosate. Monsanto Unpublished Study IB-91-053. International Bioresearch, Hannover, Germany.
IBR, 1991b. Final Report. Acute Toxicity in Earthworms According to OECD 207-Test Article. Roundup Monsanto Unpublished Study IB-90-57 I. International Bioresearch, Hannover, Germany.
Jarmuł-Pietraszczyk, J., Jastrzebska, A., 2012. Herbicide toxicity to the California earthworms *Eisenia foetida* Sav. nd *Dendrobaena veneta* Rosa. Ecol. Chem. Eng. A 19, 1133–1137.
Kaneda, S., Okano, S., Urashima, Y., Murakami, T., Nakajima, M., 2009. Effects of herbicides, glyphosate, on density and casting activity of earthworm, *Pheretima (Amynthas) carnosus*. Jpn. J. Soil Sci. Plant Nutr. 80, 469–476.
Krogh, P.H., Griffiths, B., Demsar, D., Bohanec, M., Debeljak, M., Andersen, M.N., Sausse, C., Birch, A.N.E., Caul, S., Holmstrup, M., Heckmann Lars-Henrik Cortet, J., 2007. Responses by earthworms to reduced tillage in herbicide tolerant maize and Bt maize cropping systems. Pedobiologia 51, 219–227.
Lüscher, G., Jeanneret, P., Schneider, M.K., Turnbull, L.A., Arndorfer, M., Balázs, K., Báldi, A., Bailey, D., Bernhardt, K.G., Choisis, J.P., Elek, Z., Frank, T., Friedel, J.K., Kainz, M., Kovács-Hostyánszki, A., Oschatz, M.L., Paoletti, M.G., Papaja-Hülsbergen, S., Sarthou, J.P., Siebrecht, N., Wolfrum, S., Herzog, F., 2014. Responses of plants, earthworms, spiders and bees to geographic location, agricultural management and surrounding landscape in European arable fields. Agric. Ecosyst. Environ. 186, 124–134.
Lagomarsino, A., Grego, S., Marhan, S., Moscatelli, M.C., Kandeler, E., 2009. Soil management modifies micro-scale abundance and function of soil microorganisms in a Mediterranean ecosystem. Eur. J. Soil Sci. 60, 2–12.
Landry, D., Dousset, S., Fournier, J.C., Andreux, F., 2005. Leaching of glyphosate and AMPA under two soil management practices in Burgundy vineyards (Vosne-Romanée, 21-France). Environ. Pollut. 138 (November (2)), 191–200.
Latif, R., Ezzatpanah, S., Malek, M., Parsa, H., 2009. Earthworms of the Central Elburz Mountains, Iran. Iran. J. Anim. Biosyst. 5, 1–5.
Lavelle, P., Bignell, D., Lepage, M., Wolters, V., Roger, P., Ineson, P., Heal, O.W., Dhillion, S., 1997. Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur. J. Soil Biol. 33, 159–193.
Lavelle, P., DecaËns, T., Aubert, M., Barot, S., Blouin, M., Bureau, F., Margerie, P., Mora, P., Rossi, J.P., 2006. Soil invertebrates and ecosystem services. Eur. J. Soil Biol. 42, 3–15.
Lindenmayer, D., Cunningham, S., Young, A., 2012. Land Use Intensification: Effects on Agriculture, Biodiversity and Ecological Processes. CSIRO Publishing, Australia.
Martin, N.A., 1982. The effects of herbicides used on asparagus on the growth rate of the earthworm *Allolobophora caliginosa*. Proceedings of the 35th New Zealand Weed and Pest Control Conference 328–331.
Mohamed, A.I., Nair, G.A., Kassem, H.H., Nuruzzaman, M., 1995. Impacts of pesticides on the survival and body mass of the earthworm *Aporrectodea caliginosa* (Annelida: Oligochaeta). Acta Zool. Fenn. 196, 344–347.
Monsanto Co, 1988a. Material Safety Data Sheet: Pondmaster Aquatic Herbicide.
Monsanto Co, 1988b. The Metabolism of Glyphosate in Sprague Dawley Rats—Part I. Excretion and Tissue Distribution of Glyphosate and Its Metabolites Following Intravenous and Oral Administration. WHO by Monsanto Ltd., prepared by Monsanto Environmental Health Laboratory/Monsanto Life Sciences Research Center, St. Louis, MO (Unpublished report no. MSL-7215 submitted to).
Monsanto Co, 1996. Roundup Ultra Ingredients Summary Sheet. Monsanto Company, Agricultural Products, St. Louis, MI.
Monsanto Co, 2013. ROUNDUP POWER 2.0. Erbicida Sistemico Per Applicazioni in Post-emergenza Delle Infestanti. Liquido Solubile Concentrato. http://www.roundup.it/

files/gamma/RoundupPower2.0_etichetta_DPP.pdf.
Morowati, M., 2000. Histochemical and histopathological study of the intestine of the earthworm (*Pheretima elongata*) exposed to a field dose of the herbicide glyphosate. Environmentalist 20, 105–111.
Mosleh, Y.Y., Ismail, S.M.M., Ahmed, M.T., Ahmed, Y.M., 2003. Comparative toxicity and biochemical responses of certain pesticides to the mature earthworm *Aporrectodea caliginosa* under laboratory conditions. Environ. Toxicol. 18, 338–346.
Norgaard, T., Moldrup, P., Ferré, T.P.A., Olsen, P., Rosenbom, A.E., de Jongea, L.W., 2014. Leaching of glyphosate and aminomethylphosphonic acid from an agricultural field over a twelve-year period. Vadose Zone J. 13. http://dx.doi.org/10.2136/vzj2014.05.0054.
Omar, Hossam El-Din M., Ibraheim, Zedan Z., El-Shimy, Nasser A., Ali, Rouwaida S., 2012. Analysis of FAME for two species of earthworms *Allolobophora caliginosa* savigny and *Pheretima hawayana* rosa (Annelida-Oligochaeta). J. Biol. Earth Sci. 2 (1), B37–B44.
Paoletti, M.G., Bressan, M., 1996. Soil invertebrates as bioindicators of human disturbance. Crit. Rev. Plant Sci. 15, 21–62.
Paoletti, M.G., Pimentel, D., 2000. Environmental risks of pesticides versus genetic engineering for agricultural pest control. J. Agric. Environ. Ethics 12, 279–303.
Paoletti, M.G., Sommaggio, D., 1996. Biodiversity indicators for sustainability. Assessment of rural landscapes. In: Van Straalen, N.M., Krivolutsky, D.A. (Eds.), Bioindicator Systems for Soil Pollution. Kluwer Academic Publishers, Dordrecht pp. 123–140.
Paoletti, M.G., Schweigl, U., Favretto, M.R., 1995a. Soil macroinvertebrates, heavy metals and organochlorines in low and high input apple orchards and a coppiced woodland. Pedobiologia 39, 20–33.
Paoletti, M.G., Sommaggio, D., Petruzzelli, G., Pezzarossa, B., Barbafieri, M., 1995b. Soil invertebrates as monitoring tools for agricultural sustainability. Pol. Pismo Entomol. 64, 113–122.
Paoletti, M.G., Sommaggio, D., Favretto, M.R., Petruzzelli, G., Pezzarossa, B., Barbafieri, M., 1998. Earthworms as useful bioindicators of agroecosystem sustainability in orchards and vineyards with different inputs. Appl. Soil Ecol. 10, 137–150.
Paoletti, M.G., 1985. Soil invertebrates in cultivated and uncultivated soils in northeast Italy. Redia 71, 501–563.
Paoletti, M.G., 1999. The role of earthworms for assessment of sustainability and as bioindicators. Agric. Ecosyst. Environ. 74, 137–155.
Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., Vandenbulckeet, F., 2014. Pesticides and earthworms. A review. Agron. Sustain Dev. 34, 199–228.
Piola, L., Fuchs, J., Oneto, M.L., Basack, S., Kesten, E., Casabé, N., 2013. Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. Chemosphere 91, 545–551. http://dx.doi.org/10.1016/j.chemosphere.2012.12.036.
Pizl, V., 1988. Interactions between earthworms and herbicides 1. Toxicity of some herbicides to earthworms in laboratory tests. Pedobiologia 32, 227–232.
Ponge, J.F., Pérès, G., Guernion, M., Ruiz-Camacho, N., Cortet, J., Pernin, C., et al., 2013. The impact of agricultural practices on soil biota: a regional study. Soil Biol. Biochem. 67, 271–284.
Rebecchi, L., Sabatini, M.A., Cappi, C., Grazioso, P., Vicari, A., Dinelli, G., Bertolani, R., 2000. Effects of a sulfonylurea herbicide on soil microarthropods. Biol. Fertil. Soils 30, 312–317.
Reinecke, A.J., Venter, J.M., 1987. Moisture preferences, growth and reproduction of the compost worm *Eisenia foetida* (Oligochaeta). Biol. Fertil. Soils 3, 135–141.
Rodriguez-Castellanos, L., Sanchez-Hernandez, J.C., 2007. Earthworm biomarkers of pesticide contamination: current status and perspectives. J. Pestic. Sci. 32, 360–371.
Sánchez-Moreno, S., Castro, J., Alonso-Prados, E., Alonso-Prados, J.L., García-Baudín, J.M., Talavera, M., Durán-Zuazo, V.H., 2014. Tillage and herbicide decrease soil biodiversity in olive orchards. Agron. Sustain. Dev. 35 (2), 691–700.
Schlegel, L.M., Manceau, A., 2013. Binding mechanism of Cu(II) at the clay-water interface by powder and polarized EXAFS spectroscopy. Geochim. Cosmochim. Acta 113, 113–214.
Sebiomo, A., Ogundero, V.W., Bankole, S.A., 2011. Effect of four herbicides on microbial population, soil organic matter and dehydrogenase activity. Afr. J. Biotechnol. 10, 770–778.
Sherwan, T.A., 2013. The impact of four pesticides on the earthworm *Lumbricus terrestris* (Annelida: Oligochaeta). Int. J. Curr. Res. Rev. 5 (21), 1–5.
Slimak, K.M., 1997. Avoidance response as sublethal effect of pesticides on *Lumbricus terrestris* (Oligochaeta). Soil Biol. Biochem. 29, 7131–7715.
Springett, J.A., Gray, R.A.J., 1992. Effect of repeated low doses of biocides on the earthworm *Aporrectodea caliginosa* in laboratory culture. Soil Biol. Biochem. 24, 1739–1744.
Tiunov, A.V., Hale, C.M., Holdsworth, A.R., Vsevolodova-Perel, T.S., 2006. Invasion patterns of Lumbricidae into the previously earthworm-free areas of northeastern Europe and the western Great Lakes region of North America. Biol. Invasions 8, 1223–1234.
Van der Wal, A., Geerts, R.H.E.M., Korevaar, H., Schouten, A.J., Jagers op Akkerhuis, G.A.J.M., Rutgers, M., Mulder, C., 2009. Dissimilar response of plant and soil biota communities to long term nutrient addition in grasslands. Biol. Fertil. Soils 45, 663–667.
Vereecken, H., 2005. Mobility and leaching of glyphosate: a review. Pest Manag. Sci. 61, 1139–1151.
Verrell, P., Van Buskirk, E., 2004. As the worm turns: *Eisenia foetida* avoids soil contaminated by a glyphosate-based herbicide. Bull. Environ. Contam. Toxicol. 72, 219–224.
Wironen, M., Moore, T.R., 2006. Exotic earthworm invasion increases soil carbon and nitrogen in an old-growth forest in southern Quebec. Can. J. For. Res. 36, 845–854.
Woodburn, A.T., 2000. Glyphosate: production, pricing, and use worldwide. Pest Manag. Sci. 56, 309–312.
Yasmin, S., D'Souza, D., 2007. Effect of pesticides on the reproductive output of Eisenia foetida. Bull. Environ. Contam. Toxicol. 79, 529–532.
Yasmin, S., D'Souza, D., 2010. Effects of pesticides on the growth and reproduction of earthworm: a review. Appl. Environ. Soil Sci. 678360.
Zaller, J.G., Heigl, F., Ruess, L., Grabmaier, A., 2014. Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. Sci. Rep. 4, 5634. http://dx.doi.org/10.1038/srep05634.
Zhang, Z.S., Zheng, D.M., Wang, Q.C., Lv, X.G., 2009. Bioaccumulation of total and methyl mercury in three earthworms species (*Drawida* sp., *Allolobophora* sp. and *Limnodrilus* sp.). Bull. Environ. Contam. Toxicol. 83, 937–942.
Zhou, C.-F., Wang, Y.-J., Yu, Y.-C., Sun, R.-J., Zhu, X.-D., Zhang, H.-L., Zhou, D.-M., 2012. Does glyphosate impact on Cu uptake by, and toxicity to, the earthworm *Eisenia fetida*? Ecotoxicology 21, 2297–2305.
Zhou, C.F., Wang, Y.J., Li, C.C., Sun, R.J., Yu, Y.C., Zhou, D.M., 2013. Subacute toxicity of copper and glyphosate and their interaction to earthworm (*Eisenia fetida*). Environ. Pollut. 180, 71–77.