Bull Environ Contam Toxicol (2016) 96:314–319
DOI 10.1007/s00128-015-1700-8

Survival, Reproduction, Avoidance Behavior and Oxidative Stress Biomarkers in the Earthworm *Octolasion cyaneum* Exposed to Glyphosate

Carla Salvio[1] · Mirta L. Menone[2] · Sergio Rafael[1] · Fernando G. Iturburu[2] · Pablo L. Manetti[1]

Received: 13 June 2015 / Accepted: 12 November 2015 / Published online: 11 January 2016

Abstract The massive use of glyphosate (GLY) in several countries has increased the interest in investigating its potential adverse effects in non-target organisms. The aim of the present study was to assess the potential effects in survival and reproduction; avoidance behavior and oxidative stress under short-term (48 h) and subchronic exposures (28 days) to GLY in the earthworm *Octolasion cyaneum*. After 48 h no significant changes in the behavior was observed. In addition, a lower catalase activity at 498 μg GLY kg^{-1} dry soil section relative to earthworms from the control section was obtained. After 28 days of exposure inhibition of glutathione S-transferase activity was observed at 535 μg GLY kg^{-1} dry soil while no changes in the other endpoints were detected. These results indicate that environmentally relevant concentrations of GLY (up to 996 μg GLY kg^{-1} dry soil) did not exert a toxic effect to *O. cyaneum*.

Keywords Oligochaete · Avoidance behavior · Biochemical biomarkers · Herbicide

Glyphosate (GLY)-based products are the leading post-emergent herbicides to control annual and perennial weeds in the world. It is a broad-spectrum, non-selective systemic herbicide which is directly applied to foliage and it is assumed to be less toxic to the ecosystems than other herbicides (Piola et al. 2013). For example in Argentina, about 200 million kg of GLY-based herbicides were applied in 2012 (CASAFE 2013). In agricultural soils GLY was detected in concentrations up to 1502 μg kg^{-1} dry soil (Aparicio et al. 2013).

Earthworms regulate major soil processes and functions, such as structure, organic matter decomposition, nutrient cycling, microbial and invertebrate populations, and plant growth (Edwards 2004). Earthworms' presence in a wide range of soils, their high contribution to the soil biomass and other characteristics like its easy and low cost-culture makes them suitable to determine the effects of soil pollutants such as pesticides. Several earthworm species (e.g., *Eisenia fetida* and *E. andrei*) have occupied an important place in toxicity testing (OECD 1984) while earthworm biomarkers have been less investigated. Biochemical biomarkers are a valuable tool for evaluating exposure to pollutants. Recent data suggests that long-term impacts of pesticide residues on some earthworm species are likely to induce adaptation processes that may include physiological changes such as increased detoxification activity, energy allocation and behavioral changes, but yet they are poorly understood (Givaudan et al. 2014).

The aim of this study was to assess the potential effects in survival and reproduction; avoidance behavior and oxidative stress under short-term and subchronic exposures to environmentally relevant concentrations of the herbicide GLY in the earthworm *Octolasion cyaneum*. We worked under the hypothesis that biological responses (biochemical and behavior biomarkers) are indicative for toxic effects induced by GLY and can thus be valuable as early warning signals.

✉ Mirta L. Menone
mirta.menone@gmail.com

[1] Unidad Integrada Facultad de Ciencias Agrarias (UNMdP) - EEA INTA Balcarce, C.C. 276 (7620) Balcarce, Argentina

[2] Laboratorio de Ecotoxicología, Instituto de Investigaciones Marinas y Costeras, CONICET – Universidad Nacional de Mar del Plata, Funes 3350, 7600 Mar del Plata, Buenos Aires, Argentina

Materials and Methods

Specimens of *O. cyaneum* earthworms were obtained by hand-sorting from the area under no tillage located at the National Institute of Agricultural Technology (INTA), Experimental Station at Balcarce city, Argentina (37°45′S, 58°18′W). They were placed in plastic containers with 10 cm of moist soil and acclimatized at 20 ± 2°C and 14:10 h light:dark photoperiod for 2 weeks. Adults with well-developed clitellae, with individual weight between 300 and 600 mg were used (OECD 1984, 2004). The soil was a fine, mixed, thermic Typic Argiudoll (USDA classification) containing 5.1 % organic matter; 20.9 % clay, 34.9 % silt and 44.2 % sand; pH = 7.7; conductivity of 0.6 mmhos cm^{-1} and cation exchange capacity of 29.3 meq 100 g^{-1}.

The commercial formulation Atanor S.C.A. was obtained from the local market. Compound characteristics are: the structure $C_6H_8NO_5P.K$, active ingredient (a. i.) CAS: 39600-42-5, formulation type: liquid soluble (LS) 48 % and 35.6 % of acid equivalent, recommended application rate: 3 L a.i. ha^{-1} (equivalent to 1440 g a.i. GLY ha^{-1}). The samples were spiked with an internal standard (40 µL of isotopically labelled standard (1,2-$^{13}C_2^{15}N$) glyphosate (98 %). The derivatization step was initiated with the addition of 0.5 mL of borate buffer (pH 9), fluorenylmethyloxycarbonyl (FMOC) (6 g/L) and acetonitrile. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses were performed using an Acquity UPLC liquid chromatograph (Waters) coupled to a triple-quadrupole mass spectrometer equipped with an ESI source (TQD, Waters Micromass, UK) (Sasal et al. 2015). Three replicates per treatment were used. The detection and quantification limit of GLY in soil samples were 0.2 and 0.6 µg kg^{-1} dry soil, respectively. Recoveries from spiked soils were greater than 105 %. Concentrations of 134, 189, 329, 440 y 535 µg GLY kg^{-1} dry soil corresponding to nominal concentrations of 166, 332, 498, 664 and 830 µg GLY kg^{-1} dry soil were measured. Taking into account the levels reported by Aparicio et al. (2013) these values are below the maximum field concentration detected.

The experiment for survival and reproduction was performed in a chamber with 14:10 h light: dark photoperiod at a temperature of 22 ± 2°C. This subchronic bioassay was performed using plastic containers (11.5 cm in diameter and 11 cm height) provided with a perforated lid. Inside, 750 g of soil mixed with the herbicide were added. Finally, 10 adult were added. Four replicates were performed. The treatments were 480, 960, 1440, 1920 and 2400 g a.i. GLY ha^{-1} which corresponded to 166, 332, 498, 664 and 830 µg GLY kg^{-1} dry soil, respectively. A solution of GLY in distilled water was prepared immediately before starting the assay in a quantity sufficient for all replicates of one concentration. The spiking of GLY was done by mixing the aqueous solutions of GLY into the pre-moistened soils, each test concentration into the whole batch of soil. After homogeneous mixing, the soil was introduced into the plastic containers. All concentration are given as a.i. per kg soil (dry weight). Controls were run in parallel and contained distilled water only. Sampling was made at 7, 14, 21 and 28 days after application (DAA) and the following variables were determined: biomass in wet weight (mg), number of individuals dead and/or alive, number of cocoons and juveniles. The bioassay was extended to 56 DAA to observe cocoons and juveniles production.

A short-term bioassay was performed evaluating avoidance behavior of *O. cyaneum*. Rectangular plastic containers (18 cm long × 12 cm wide × 12 cm height) were used, provided with a perforated lid. Each container was divided in two equal sections by a removable plastic split (Loureiro et al. 2005). One half of the container received 750 g of soil with concentration of herbicide and the other half 750 g of soil control (without herbicide). The following treatments were tested: 720, 1440 and 2880 g a.i. GLY ha^{-1} which corresponded to 249, 498 and 996 µg GLY kg^{-1} dry soil, respectively. Analytical measurements of GLY were not carried out in the short-term bioassay samples. The application of herbicide was made following the same procedure used in the sub-chronic bioassay. After the separator was removed, ten earthworms of 300–600 mg were placed in the center of the container. The design was completely randomized with five replicates per treatment following the methodology by Amorim et al. (2005). The experiment was performed in a chamber in a 14:10 h light:dark photoperiod at a temperature of 22 ± 2°C. After 48 h the removable split was reintroduced and the individuals of each section were counted and weighed. The results of the counting were expressed as net response (NR) in percentage according to ISO (2005): NR = ((C − T)/N) × 100 where C is the number of observed worms in the control soil, T is the number of worms in GLY treated soil and N is the total number of worms (Amorim et al. 2005). A positive NR indicates avoidance of the treated soil whereas 0 % or a negative value indicates a non-response or attraction to the pesticide tested. Only average values >80 % could be considered as indication of an impact on behavior (Amorim et al. 2005; ISO 2005).

The activities of catalase (CAT) and glutathione S-transferase (GST), the total protein content and the lipid peroxidation (LPO) were examined 28 DAA in the subchronic bioassay and after 48 h in the avoidance behavior experiment. Whole body tissues were flash–frozen in liquid N_2 and stored at −80°C. Cytosolic enzymes were extracted following Wiegand et al. (2000) without the purification step. The activity of GST was determined in duplicate at

340 nm, using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). CAT activity was determined in duplicate, following the decomposition of H_2O_2 at 240 nm (Claiborne 1985). The total protein content was assessed spectrophotometrically following the method of Bradford (1976), using bovine serum albumin solution as standard. Enzyme activities were reported in nano katals per mg of protein (nkat. mg^{-1} prot), where 1 katal correspond to the conversion of 1 mol of substrate per second. Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARs) following Oakes and Van Der Kraak (2003). TBARs was measured in the spectrophotometer ($\lambda_{abs} = 532$ nm). TBARs content was expressed as nanomoles per mg of fresh tissue using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

To test significant differences between mean biomass values, a repeated measures – ANOVA test was performed ($\alpha = 0.05$). For cocoon and juvenile numbers ANOVA test was used ($\alpha = 0.05$). When differences between the means were detected the least significance difference (LSD) test was used ($\alpha = 0.05$). Data normality and homogeneity of variances were always confirmed. For biochemical biomarkers in the short-term experiment, pair-wise Student's *t* test was used ($\alpha = 0.05$). For biochemical biomarkers at the 28 DAA the Dunnett's test was performed ($\alpha = 0.05$). All analyses were done with R version 3.1.1(R Development Core Team 2014).

Results

No mortality of *O. cyaneum* was observed during the subchronic assay-irrespective of the GLY concentration. The average earthworm biomass (measured in mg of wet weight) ranged from 350 to 490 mg, being not significantly different at all time points ($p > 0.05$) (Table 1). In addition, repeated measure analysis was performed to check for differences in the biomass development over time but no changes were observed ($p > 0.05$). At 28 and 56 DAA, the cocoons and juveniles numbers did not show differences among treatments ($p > 0.05$) (Table 1).

No mortality of *O. cyaneum* was observed at the termination of the avoidance test. A slight deviation from zero (NR = 17.5 %) was observed at 249 µg GLY kg^{-1} dry soil (Fig. 1). A negative net response at 498 and 996 µg GLY kg^{-1} dry soil was observed (Fig. 1).

Biochemical biomarkers were studied in *O. cyaneum* in both the subchronic and the short-term bioassays. Inhibition of GST was observed in earthworms exposed to 535 µg GLY kg^{-1} dry soil at 28 DAA ($p = 0.035$) (Table 2). No differences in total protein content, CAT activity or TBARs content were detected 28 DAA for any

Table 1 Biomass, cocoon and juveniles numbers of *Octolasion cyaneum* exposed to glyphosate (GLY)

Concentration of GLY (µg a.i. kg^{-1}soil)	Biomass in wet weight (mg)				Cocoon number		Juveniles number	
	7 DAA	14 DAA	21 DAA	28 DAA	28 DAA	56 DAA	28 DAA	56 DAA
Control	464.1 ± 22.2 a	431.4 ± 25.0 a	394.6 ± 30.5 a	340.9 ± 17.0 a	2.0 ± 1.0 a	6.0 ± 1.0 a	1.0 ± 1.3 a	5.0 ± 1.0 a
134	480.5 ± 53.2 a	449.8 ± 53.0 a	392.4 ± 41.7 a	353.5 ± 24.8 a	2.0 ± 1.3 a	9.0 ± 3.0 a	2.0 ± 1.3 a	5.0 ± 2.0 a
189	493.1 ± 9.8 a	454.8 ± 14.4 a	398.8 ± 28.5 a	371.1 ± 11.8 a	2.0 ± 1.7 a	9.0 ± 1.0 a	1.0 ± 0.8 a	6.0 ± 2.0 a
329	471.4 ± 30.2 a	420.7 ± 28.0 a	371.3 ± 32.7 a	338.2 ± 16.2 a	1.0 ± 1.3 a	8.0 ± 3.0 a	1.0 ± 0.5 a	6.0 ± 2.0 a
440	460.4 ± 21.5 a	428.3 ± 20.9 a	381.7 ± 25.3 a	341.7 ± 14.4 a	1.0 ± 1.0 a	5.0 ± 1.0 a	1.0 ± 0.6 a	3.0 ± 2.0 a
535	493.1 ± 12.9 a	456.6 ± 20.0 a	402.8 ± 23.6 a	356.5 ± 26.1 a	1.0 ± 0.6 a	7.0 ± 5.0 a	1.0 ± 0.5 a	6.0 ± 4.0 a

Values are expressed as mean ± standard deviation. The same letters indicate no significant differences among treatments ($p > 0.05$, LSD test)

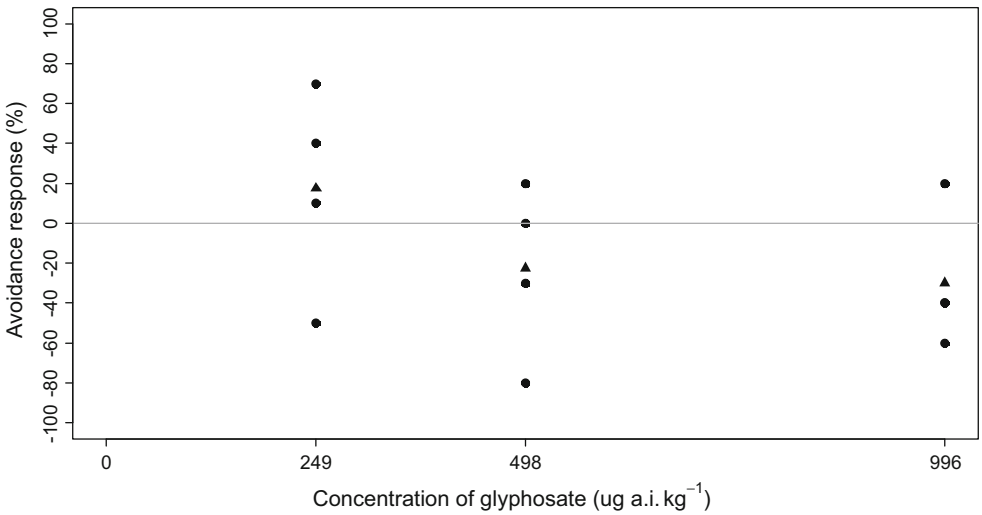

Fig. 1 Avoidance behavior of *Octolasion cyaneum* when exposed in a control versus contaminated with glyphosate (μg a.i. kg^{-1} soil) after 48 h. *Triangles* mean. *Circles* replicates

Table 2 Effect of concentration of glyphosate (GLY) (μg a.i. kg^{-1} soil) on the Glutathione S-transferase (GST) and Catalase (CAT) activities, thiobarbituric acid reactive substances (TBARs) and total protein (TP) contents in *Octolasion cyaneum* at 28 days after application during the subchronic bioassay

Concentration of GLY (μg a.i. kg^{-1} soil)	GST (nkat mg^{-1} protein)	CAT (nkat mg^{-1} protein)	TBARS (mmol mg^{-1} FW)	TP (mg g^{-1} FW)
Control	0.031 ± 0.005	7.43 ± 2.4	0.120 ± 0.038	1106.13 ± 295.5
134	0.034 ± 0.003	8.42 ± 2.3	0.083 ± 0.013	1384.06 ± 116.9
189	0.034 ± 0.011	8.29 ± 1.9	0.076 ± 0.006	1878.50 ± 875.6
329	0.035 ± 0.004	10.06 ± 0.3	0.107 ± 0.026	1357.72 ± 159.7
440	0.025 ± 0.005	6.84 ± 0.1	0.075 ± 0.004	2015.54 ± 171.9
535	0.015 ± 0.004*	9.51 ± 0.5	0.112 ± 0.004	1134.90 ± 1177.3

Values are expressed as mean ± standard deviation

* Significant differences from the control ($p < 0.05$, Dunnett's test)

GLY treatment relative to the controls ($p > 0.05$) (Table 2). In the short-term experiment, *O. cyaneum* showed lower CAT activity in the 498 μg GLY kg^{-1} dry soil section relative to the respective control section ($p = 0.005$) (Table 3). No differences in GST activity or TBARS and total protein contents between GLY sections and their respective control sections were found ($p > 0.05$) (Table 3).

Discussion

Zhou et al. (2013) found no significant differences in weight or the cocoon production in *E. fetida* exposed to 25×10^3–200×10^3 μg GLY kg^{-1} dry soil after 28 DAA. Even though, 100 % mortality at 7 DAA have been reported by García-Torres et al. (2014) in *O. tyrtaeum* exposed to 50×10^6 μg GLY kg^{-1} dry soil, certainly concentrations in both cases much higher than those found in the field and used in the present work. Therefore, at very high concentrations GLY can exert differential responses in earthworms. In contrast, recommend field applications rates of GLY have been reported to reduce the weight (Correia and Moreira 2010), and to decrease the feeding activity (Casabé et al. 2007) in the epigeic specie *E. fetida.* Therefore, species sensitivity constitutes a critical factor in the response to pesticides in soil. While *E. fetida* is internationally recognized for its use in ecotoxicological tests, the genera *Octolasion* is found in many Argentine agricultural ecosystems from Buenos Aires and Córdoba provinces as well as in other countries such as Uruguay, Australia, United States of America, India and in Europe (Mischis 2000). Also *E. fetida* is not a truely soil-dwelling species (De Silva et al. 2010), *O. cyaneum* is an endogeic earthworm. Therefore, its wide geographical distribution and its prefered habitat encourage the consideration of *O. cyaneum* as an alternative test species.

The low NR observed at 249 μg GLY kg^{-1} dry soil indicates an avoidance behavior, but according to Amorim et al. (2005) it should be interpreted as no evidence of an

Table 3 Effect of concentration of glyphosate (GLY) (μg a.i. kg^{-1} soil) on the glutathione S-transferase (GST) and catalase (CAT) activities, thiobarbituric acid reactive substances (TBARs) and total protein (TP) contents in *Octolasion cyaneum* after 48 h of exposure in the avoidance test

Concentration of GLY (μg a.i. kg^{-1}soil)	Side of the experimental unit	GST (nkat mg^{-1} protein)	CAT (nkat mg^{-1} protein)	TBARS (mmol mg^{-1} FW)	TP (mg g^{-1} FW)
249	Control soil	0.62 ± 0.2	2086.6 ± 436.7	0.22 ± 0.2	4.07 ± 0.5
249	GLY treated soil	0.63 ± 0.1	2083.9 ± 777.9	0.17 ± 0.1	4.25 ± 0.5
498	Control soil	0.44 ± 0.1	2765.4 ± 605.9*	0.17 ± 0.1	3.96 ± 0.6
498	GLY treated soil	0.50 ± 0.1	2040.6 ± 830.2*	0.20 ± 0.1	3.98 ± 0.6
996	Control soil	0.54 ± 0.2	1726.3 ± 373.0	0.25 ± 0.1	5.61 ± 1.1
996	GLY treated soil	0.58 ± 0.2	1507.7 ± 345.1	0.18 ± 0.1	5.07 ± 0.7

Values are expressed as mean ± standard deviation

* Indicates statistical differences between control and treated with glyphosate in the experimental unit (paired *t* test, $p < 0.05$)

effect in the tested concentration because the value was not significant. Although GLY was found to be non-toxic for *Pontoscolex corethrurus* and *E. andrei*, a significant avoidance behavior at a concentration of 47×10^3 μg GLY kg^{-1} dry soil was reported (Buch et al. 2013), certainly a higher concentration than the currently applied.

Although GST enzymes are part of the phase II metabolism, these enzymes can also function as antioxidant enzymes catalyzing the reduction of organic hydroperoxides (Koenig and Solé 2012). The effect of oxidative stress has also been tested in the endogeic earthworm *Aporrectodea caliginosa* exposed to a low concentration of 2.5×10^3 μg GLY kg^{-1} dry soil (Givaudan et al. 2014). Their results at 28 DAA showed only inhibition of GST, similarly to our data in *O. cyaneum,* indicating that the biomarkers used did not give a clear indication of oxidative stress under subchronic exposure.

However, *O. cyaneum* exposed during 48 h to 498 μg GLY kg^{-1} dry soil (~0.5 μg GLY g^{-1} dry soil) showed a significant decrease of CAT activity, noteworthy at a test concentration corresponding to the recommended application rate of the commercial formulation. A similar response was reported in *A. caliginosa* exposed to 2.5×10^3μg GLY kg^{-1} dry soil during 72 h (Givaudan et al. 2014). In both cases the same response in the antioxidant enzyme was observed, although the specimens of *A. caliginosa* used in their bioassay proceed from a GLY pre-exposed population. Therefore, the response observed for CAT and the lack of positive response in TBARS content and GST activity suggest the effect of oxidative stress without oxidative damage. The differential responses observed between the short-term and subchronic assays are expected because GST enzymes can show an adaptive response while antioxidants like CAT mostly react after short-time exposures.

Under stress invertebrates can mobilize proteins as an energy source, although lipids are generally utilized first (Mayer et al. 1992). Both biomolecules have been observed to be low at highest soil pesticide contents, indicating higher energetic demands under pesticide exposure (Givaudan et al. 2014). However, these parameters are known to react only when the organisms are near death (Mayer et al. 1992), and this was not the case of *O. cyaneum* in the present work. Therefore, the use of total protein as a biomarker is suggested not to be included in future studies.

Finally, our results indicate that environmentally relevant concentrations of GLY (up to 996 μg GLY kg^{-1} dry soil) did not exert a toxic effect to *O. cyaneum*. Therefore, the battery of biochemical and behavior biomarkers selected was not useful to detect potential sublethal effects induced by GLY.

Acknowledgments Thank you to N. Clemente for the statistical analysis. This work was supported by CONICET (PIP 112-201-101-01084) and UNMdP (EXA 608/12 and AGR 390/12).

References

Amorim MJB, Römke J, Soares AMVM (2005) Avoidance behaviour of *Enchytraeus albidus*: effects of benomyl, carbendazim, phenmedipham and different soil types. Chemosphere 59: 501–510

Aparicio VC, De Gerónimo E, Marino D, Primost J, Carriquiriborde P, Costa JL (2013) Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. Chemosphere 93:1866–1873

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Buch AC, Brown GG, Niva CC, Sautter KD, Sousa JP (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

Casabé N, Piola L, Fuchs J, Oneto ML, Pamparato L, Basack S, Jiménez R, Massaro R, Papa JC, Kesten E (2007) Ecotoxicological assessment of the effects of glyphosate and chlorpyrifos in an Argentine soya field. J Soils Sediments 7(4):232–239

CASAFE (2013) http://www.casafe.org/pdf/estadisticas/Informe%20Mercado%20Fitosanitario%202012.pdf. Retrieved 25 Aug 2014

Claiborne A (1985) Catalase activity. In: Greenwald RA (ed) CRC handbook of methods in oxygen radical research. CRC Press, Boca Raton, pp 283–284

Correia FV, Moreira JC (2010) Effects of glyphosate and 2,4-D on earthworms (*Eisenia foetida*) in laboratory tests. Bull Environ Contam Toxicol 85:264–268

De Silva PMCS, Pathiratne A, Van Gestel CAM (2010) Toxicity of chlorpyrifos, carbofuran, mancozeb and their formulations to the tropical earthworm *Perionyx excavates*. Appl Soil Ecol 44:56–60

Edwards CA (2004) Earthworm ecology, 2nd edn. St. Lucie Press, New York

García-Torres T, Giuffré L, Romaniuk R, Ríos RP, Pagano EA (2014) Exposure assessment to glyphosate of two species of annelids. Bull Environ Contam Toxicol 93(2):209–214

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

Habig W, Pabst MJ, Jakoby WB (1974) Glutathione S-transferase, the first step in mercapturic acid formation. J Biol Chem 249: 1730–1739

ISO (2005) Soil quality-avoidance test for testing the quality of soils and toxicity of chemicals-test with earthworms (*Eisenia andrei*). ISO, Geneve, Switzerland. N° 17512, ISO, Geneva, Switzerland. s.p

Koenig S, Solé M (2012) Natural variability of hepatic biomarkers in Mediterranean deep-sea organisms. Mar Environ Res 79:122–131

Loureiro S, Soares AMVM, Nogueira AJA (2005) Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ Pollut 138:121–131

Mayer FL, Versteeg DJ, McKee MJ, Folman LC, Graney RL, McCume DC, Rattner BA (1992) In: Huggett RJ, Mehrle RA, Bergman HL (eds) Biomarkers, biochemical, physiological and histological markers of anthropogenic stress. Lewis Publishers, Boca Raton, pp 5–85

Mischis CC (2000) Las lombrices de tierra (Annelida, Oligochaeta) de la República Argentina. Nat Neotrop 31:17–27

Oakes KD, Van Der Kraak GJ (2003) Utility of TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquat Toxicol 63:447–463

OECD (1984) Earthworm, acute toxicity tests, test guidelines N° 207. OECD Test in Soil Guidelines for Testing Chemicals, Paris

OECD (2004) Earthworm reproduction test (*Eisenia fetida*/*Eisenia andrei*), test guidelines N° 222. OECD Test in Soil Guidelines for Testing Chemicals, Paris

Piola L, Fuchs J, Oneto ML, Basack S, Kesten E, Casabé N (2013) Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. Chemosphere 91:545–551

R Development Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Version 3.1.1

Sasal MC, Demonte L, Cislaghi A, Gabioud EA, Oszust JD, Wilson MG, Michlig N, Beldoménico HR, Repetti MR (2015) Glyphosate loss by runoff and its relationship with phosphorus fertilization. J Agric Food Chem. doi:10.1021/jf505533r

Wiegand C, Pflugmacher S, Oberemm A, Steinberg CEW (2000) Activity development of selected detoxication enzymes during the ontogenesis of the zebrafish (*Danio rerio*). Int Rev Hydrobiol 85:413–422

Zhou CF, Wang YJ, Li CC, Sun RJ, Yu YC, Zhou DM (2013) Subacute toxicity of copper and glyphosate and their interaction to earthworm (*Eisenia fetida*). Environ Pollut 180:71–77