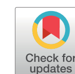
Comparative Biochemistry and Physiology, Part C 214 (2018) 1–8

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Glyphosate-based herbicides modulate oxidative stress response in the nematode *Caenorhabditis elegans*



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A R T I C L E I N F O

Keywords:
Agrochemical
Pesticide
ROS
DAF-16
Catalase
Hormesis

A B S T R A C T

Glyphosate-based formulation is used as non-selective and post-emergent herbicides in urban and rural activities. In view of its recurring applications in agricultural producing countries, the increase of glyphosate concentration in the environment stresses the need to test the adverse effects on non-target organisms and assess the risk of its use. This paper analyzes the toxicological and oxidative stress and modulatory effects of a glyphosate commercial formulation (glyphosate F) on the nematode *Caenorhabditis elegans*. We detected ROS production and enhancement of oxidative stress response in glyphosate F-treated nematodes. Particularly, we found an increased *ctl-1* catalase gene expression of a catalase specific activity. In addition, we showed that glyphosate F treatment activated the FOXO transcription factor DAF-16, a critical target of the insulin/IGF-1 signaling pathway, which modulates the transcription of a broad range of genes involved in stress resistance, reproductive development, dauer formation, and longevity. In summary, the exposure of glyphosate F induces an oxidative imbalance in *C. elegans that leads* to the DAF-16 activation and consequently to the expression of genes that boost the antioxidant defense system. In this regard, *clt-1* gene and catalase activity proved to be excellent biomarkers to develop more sensitive protocols to assess the environmental risk of glyphosate use.

1. Introduction

Glyphosate is one of the most widely used agrochemicals around the world as a result of the expansion and increased production of genetically modified crops (Coupe et al., 2012; ISAAA, 2016; US Environmental Protection Agency, 2016). It is a non-selective broad-spectrum herbicide developed for the removal of grasses, sedges, herbs and woody shrubs, especially perennials. Due to precipitation, runoff and leaching processes, glyphosate and its metabolites can be found as environmental contaminants in soil and waters, enhancing the risk of negative impacts on non-target organisms and further deteriorating ecosystem health (Peruzzo et al., 2008; Struger et al., 2015; Yang et al., 2015).

Glyphosate is a glycine analogue that harms plants by suppressing their capacity to generate aromatic amino acids (Boocock and Coggins, 1983). The potential environmental and health risk associated with their applications has been the topic of scientific and social discussion since it was first used in 1970 (Mesnage et al., 2015). Glyphosate salts alone have low to very low toxicity to mammals, but in commercial formulations, such as Roundup® or TouchDown®, they have proved to be more toxic due to the addition of surfactants (for a review see Mesnage et al., 2015). Indeed, the penetration of active ingredient through the cell membrane and subsequent intracellular action is greatly facilitated by these adjuvants (Haefs et al., 2002; Marc et al., 2002). Since the active ingredient is never used alone, further assessments are needed to determine the negative impact of the herbicide formulations on the ecosystem. Given their several advantages, toxicological experiments using the nematode *Caenorhabditis elegans* could be an excellent alternative to address this concern.

C. elegans is a free-living nematode, one of the most abundant phyla

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https://doi.org/10.1016/j.cbpc.2018.08.002
Received 13 April 2018; Received in revised form 7 August 2018; Accepted 15 August 2018
Available online 22 August 2018
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in nature, which plays an important role in ecosystem services. Due to its ubiquitous appearance and ecological relevance, this nematode is a suitable indicator to assess environmental pollution (Clavijo et al., 2017, 2016; Frézal and Félix, 2015; Leung et al., 2008; Tejeda-Benitez et al., 2016). Another interesting aspect is that many of the *C. elegans* physiological processes and stress responses are conserved in higher eukaryotes (e.g., humans) (Hunt, 2017). Therefore, the increasing amount of evidence supports *C. elegans* as a valuable toxicity model to predict outcomes in higher organisms.

Toxicological studies are greatly simplified in this nematode compared to more traditional animal models because it is a simple and small organism that has a short lifespan (4 days at 20 °C) and produces hundreds of offspring in each generation (Brenner, 1974). Additionally, powerful molecular tools have been developed for *C. elegans* that provide researchers with the means to probe the mechanisms underlying toxicity (Kaletta and Hengartner, 2006). In view of the above, it can be stated that *C. elegans* is a successful animal model for environmental mechanistic toxicology research.

To date, only a few reports have analyzed the adverse effects of glyphosate-based herbicides on *C. elegans*, but the mechanisms were not fully characterized (Ruan et al., 2009). It was demonstrated that exposure to TouchDown® during the first and second larval stages (L1 and L2) causes neuronal degeneration, particularly of dopaminergic neurons (Negga et al., 2012, 2011). McVey et al. (2016) also showed that this product affected the nervous system development of *C. elegans* embryos. Therefore, investigating the effects of glyphosate-based herbicide on nematodes may elucidate defense mechanisms and toxicological effects for this species to predict the herbicide effects on higher organisms.

Environmental pollutants, such as pesticides, induce a cellular disturbance through an increase in reactive oxygen species (ROS), commonly referred to as oxidative stress, that often precedes the onset of long term effects such as the impairment of the immune response and reproduction capacity, premature aging and a lower survival rate (Miranda-Vizuete and Veal, 2017; Monserrat et al., 2007). Several molecular mechanisms are capable of detoxifying ROS and regulating the redox balance of the cell. The enzyme superoxide dismutase degrades superoxide anion into peroxide which is then decomposed into water by the enzyme catalase and/or peroxiredoxin. These major systems act in conjunction with the thioredoxin system, composed of the thioredoxin protein and the thioredoxin reductase enzyme, and with the glutathione systems, that include glutathione, glutathione reductase, glutathione peroxidases, glutaredoxin and glutathione-S-transferases. As regards *C. elegans*, more than eighty genes are involved in the antioxidant defense systems. They include three catalase encoding genes (*ctl-1* to *ctl-3*), five superoxide dismutase encoding genes (*sod-1* to *sod-5*), 44 glutathione-S-transferase genes (*gst-1* to *gst-44*), one glutathione reductase gene (*gsr-1*), eight glutathione peroxidase genes (*gpx-1* to *gpx-8*), two thioredoxin reductase genes (*trxr-1* and *trxr-2*), three peroxiredoxins genes (*prdx-2*, *prdx-3* and *prdx-6*) (Back et al., 2012).

Hence, the aim of this paper is to analyze the toxicological and oxidative stress modulatory effects of glyphosate-based herbicides on the nematode *C. elegans*. First, we established the consequences that the glyphosate commercial formulation (glyphosate F) has on the generation of ROS. As antioxidant defense mechanisms are biomarkers widely used to assess toxic stress, we focused our attention on exploring the oxidative stress modulatory effects on acute exposure of glyphosate F. We detected ROS production and enhancement of oxidative stress response in glyphosate F-treated nematodes. Particularly, we found a pronounced increase in the expression of *ctl-1* gene and catalase specific activity. Finally, we tested if glyphosate F treatment could activate the FOXO transcription factor DAF-16, the major downstream target of the *C. elegans* insulin/IGF-1 signaling pathway, which modulates the transcription of a broad range of genes involved in stress response, reproductive development, dauer formation, and longevity (Murphy, 2006).

2. Materials and methods

2.1. Chemicals

Commercial formulations of glyphosate (GlifosatoAtanor II®, distributed by Atanor, Munro, Buenos Aires province, Argentina), containing monopotassium salt of *N*-phosphonomethyl glycine at 43.8 g L^{-1} as the active ingredient, and of paraquat (Gramoxone Super®, distributed by Syngenta Chemicals B.V., Belgium), containing paraquat dichloride at 276 g L^{-1}, were used. The Sigma-Aldrich Chemical Company supplied 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) and Roche and Trizol Reagent from Invitrogen supplied the complete mini protease mix. All other chemicals were of the highest purity available.

A stock solution of commercial herbicide with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 mL of 1 M $MgSO_4$, and H_2O to 1 L, pH 7.2) was freshly made. Different volumes of the stock solution were mixed with M9 buffer to achieve working concentrations expressed in terms of the final glyphosate concentration presented in each assay. The initial glyphosate concentration in stock solution was analytically determined at LABFAUBA (Facultad de Agronomía, Universidad de Buenos Aires) by high performance liquid chromatography technique (HPLC) on an Agilent 1100 with a C18 reverse column and a fluorescence detector (excitation 266 nm, emission 305 nm). Elution programs were developed using solvent A (acetonitrile and a 0.002 M KH_2PO_4 with 7% acetonitrile, pH 7) and solvent B (acetonitrile) with a flow rate of 0.5 mL min^{-1}. Quantization of glyphosate in samples was calculated by comparing the peak areas for each compound with those obtained from the injection of standard solutions after derivatization. The actual values were 97 ± 2% of their theoretical values.

2.2. Organism and culture conditions

The *C. elegans* strains used in the current study were N2 var. Bristol, CF1038 [*daf-16*(*mu86*)*I*] and TJ356 [*zIs356 IV* (*pdaf-16-daf-16*::*gfp*; *rol-6*)] obtained from the Caenorhabditis Genetics Centre at the University of Minnesota (Minneapolis, MN, USA). *C. elegans* was routinely propagated on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50 at 20 °C, using the standard method previously described by Brenner, 1974. Synchronization of worm culture was achieved by treating gravid hermaphrodites with alkaline bleach solution as described by Stiernagle (2006).

2.3. Nematode pesticide acute exposure

Age-synchronized L4-staged nematodes were treated with sublethal concentrations of glyphosate F or paraquat in M9 buffer (500 worms mL^{-1}) for 16 h at 20 °C supplemented with *E. coli* OP50 as food source (DO_{600nm} = 1). Worms exposed to M9 buffer without any herbicide were assayed as a control group and the ones exposed to paraquat as a positive control group for oxidative stress induction (Park et al., 2009). Three replicates were performed per treatment, and independent experiments were made three times. After exposure periods, the survival was always checked under a dissecting microscope by scoring the percentage of dead worms under a dissecting microscope as alive if moving or dead if unresponsive to gentle probing. None of the treatments presented a survival lower that 99%.

2.4. Reactive oxygen species detection

H2DCF-DA was used to quantify intracellular ROS in nematodes using a microplate reader (Schulz et al., 2007). Age-synchronized L4-staged N2 nematodes were treated with glyphosate F (4.8 mM) or

paraquat (2.0 mM) as described above. Additionally, we repeated the herbicides treatments and the control in the presence of 50 mM ascorbic acid, a prototypic antioxidant treatment (Sem and Rhen, 2012). The nematodes were harvested, washed five times with M9 buffer, and then 30 animals were transferred to wells of a 96-well plate containing 50 µL of PBS buffer. Subsequently, 50 µL of fresh 100 µM H2DCF-DA solution in PBS buffer was added to each well. In all plates, control wells containing nematodes from each treatment without H2DCF-DA and wells containing H2DCF-DA without animals were prepared in parallel. The fluorescence from each well was measured at 20 °C immediately after incorporation of the reagent and 60 min later, using 485 and 535 nm as excitation and emission wavelengths, respectively. The initial fluorescence and the fluorescence signals of the control wells were subtracted from the corresponding signals of each well after the second measurement. For each experiment ROS measurements were made in quadruplicate. The measurements were performed in a Spark20M Multimode Microplate Reader (Tecan, NC, USA).

2.5. Quantitative real-time RT-PCR analysis

L4-staged nematodes were treated with glyphosate F (1.0, 4.8 and 9.6 mM, according to each experiment) or paraquat (2.0 mM) as described above. Then, 4000 nematodes were harvested, washed five times with M9 buffer, transferred to an eppendorf, resuspended into 500 µL of Trizol Reagent and kept at −80 °C until further use. Total RNA was isolated with Trizol Reagent protocol and was quantified with a qubit RNA HS assay kit using the qubit fluorometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) synthesis was performed using the Superscript II Reverse Transcriptase (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. cDNA was diluted 1:4 and1 µl of the resulting cDNA preparation was used for quantitative real-time PCR using the Fast-Plus EvaGreen® Master Mix (Biotium, USA) and an Applied Biosystems 7500 Fast Real-Time PCR system. The oligonucleotide sequences of all the primers were designed according to the sequences retrieved from www.wormbase.org. Supplementary Table S1 shows the list of primers with their corresponding PCR-efficiencies. Gene expression of oxidative stress-related genes was assessed relative to the non-herbicide treatment control and a stable transcript, *cdc-42*, was used as reference gene (Hoogewijs et al., 2008). The relative quantification was calculated using the ΔΔCt method with correction for efficiency as described by Pfaffl (2001). For each replicate every gene was amplified in triplicate.

2.6. Catalase specific activity

L4-staged nematodes were treated with glyphosate F (1.0, 4.8 and 9.6 mM) or paraquat (2.0 mM) as described above. Then 4000 worms were harvested, and the pellet was washed five times with M9 buffer. The remaining pellet was transferred to an eppendorf resuspended in 500 µL of M9 buffer with protease inhibitors and kept at −80 °C until further use. Afterward, samples were stirred and sonicated six times for 10 s in a Sonifier cell disruptor (Branson 450) to obtain a homogenate. The homogenate was centrifuged (13,000 ×*g*, 5 min, 4 °C) and the supernatant was collected for catalase activity determination. Catalase activity was determined by the rate of disappearance of H_2O_2 measured at 240 nm during 3 min at 15 s intervals; each sample was measured in triplicate (Aebi, 1984). Protein content was quantified according to Bradford's method using bovine serum albumin as standard. The enzyme activity was calculated with the molar extinction coefficient at 43.6 $M^{-1} cm^{-1}$ and the results were expressed as µmol of H_2O_2 consumed min^{-1} mg $protein^{-1}$.

2.7. Intracellular localization of daf-16::GFP

Transgenic strain TJ356 [zIs356 IV (pdaf-16-daf-16::gfp; rol-6)] was used to detect the intracellular localization of GFP tagged DAF-16 protein. L4-staged nematodes of this strain were treated with glyphosate F or paraquat as described above. A heat-shock treatment (30 min exposure at 37 °C) was used as positive control of completed nuclear translocation of DAF-16 (Pant et al., 2014). The nematodes were washed thrice with M9 buffer and mounted on 2% agar pads in PBS with 25 mM NaN_3. The cellular localization of DAF-16::GFP was detected with a Nikon Eclipse 50i fluorescence microscopy (excitation wavelength 460–495 nm; emission wavelength 510–550 nm). Worms were classified into three groups (nuclear, intermediate and cytoplasmic) according to the localization of the DAF-16::GFP protein. The nuclear category included the worms exhibiting nuclear fluorescence and the cytoplasmic category included worms with delocalized fluorescence. Worms that did not fit any category were classified as intermediate (Pant et al., 2014). Images were taken with a Nikon CoolPix S10 digital camera. One hundred worms were analyzed for each sample and the percentage of GFP relocalization was calculated.

2.8. Statistical analysis

Statistical analysis was performed using the GraphPad Prism® software, version 6.01 (GraphPad Software, San Diego, CA). Differences among treatments were tested using analysis of variance (ANOVA) followed by Tukey's multiple comparisons test except for Fig. 5A, where a chi-square test was performed. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Glyphosate commercial formulation induces reactive oxygen species formation in C. elegans

C. elegans growth and reproduction have been affected upon glyphosate F treatment (supplementary Fig. S1). The dose-response model that included hormesis was the best fit to both responses (the lack-of-fit *F* test for growth was $F = 17.44$; $p < 0.001$, and for reproduction was $F = 25.81$; $p < 0.0001$) with EC50s of 6.8 ± 0.1 mM and 4.0 ± 0.2 mM for growth and reproduction respectively. Considering that the hormesis mechanism could be a stress-related over-compensation response, we investigated the redox balance in the nematodes treated with glyphosate and the induction of its possible toxicity mechanism (Mattson, 2008). To this end, we treated L4 nematodes for 16 h with a sublethal dose of glyphosate F (4.8 mM) and evaluated intracellular ROS levels. During the experiment, paraquat was used as a positive control of ROS generator (Park et al., 2009). As shown in Fig. 1, both herbicides significantly increased ROS production when compared to the control ($p < 0.0001$). Results showed that intracellular ROS production were 3.4 and 4.4 times higher in worms treated with glyphosate F and paraquat than in the control. Furthermore, we also tested whether a prototypic antioxidant treatment could delay ROS production in glyphosate F-treated nematodes (Sem and Rhen, 2012). In order to do that, we repeated the experiments in the presence of ascorbic acid and we realized that this antioxidant protected the nematodes from glyphosate-induced ROS production (Fig. 1).

3.2. C. elegans stress-response gene expression upon exposure to glyphosate commercial formulation

C. elegans has different molecular mechanisms that can be induced in response to increasing ROS concentration to restore the redox balance of the organism. To analyze which pathways are predominantly up-regulated upon exposure to glyphosate F, we performed a stress-response gene expression analysis on selective genes, representing the main stress-response pathways, such as glutathione (*gsr-1*, *gpx-7*, *gst-28* and *gst-36*) and thioredoxin (*trxr-1* and *trxr-2*) antioxidant systems, and ROS detoxifying enzymes (*prdx-2*, *sod-3*, *ctl-1*, *ctl-2* and *ctl-3*). Once again, paraquat was used as an oxidative stress inductor.

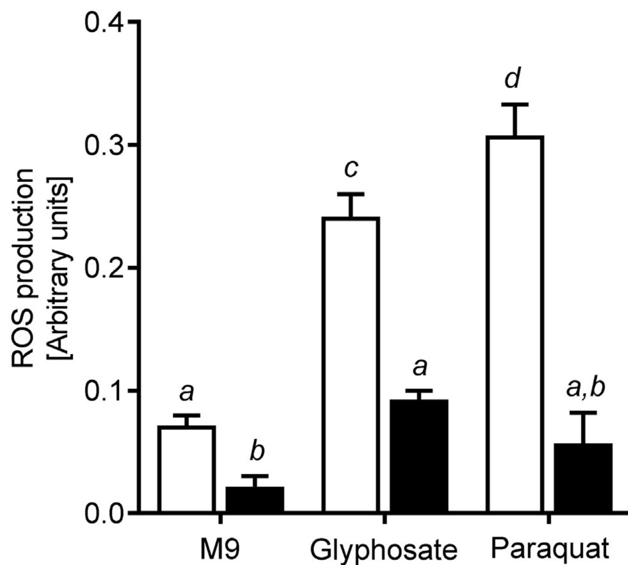

Fig. 1. ROS production in glyphosate F and paraquat exposed nematodes in the absence (unfilled bars) or presence of 50 mM ascorbic acid (filled bars). L4-staged nematodes were treated with glyphosate F (4.8 mM), paraquat (2.0 mM) or without treatment (M9 buffer) for 16 h at 20 °C, and then were harvested, stained with H2DCF-DA and the fluorescence were measured with a Microplate Reader. ROS production is expressed as described in Material and methods. The values represent means ± SD of 3 independent assays. Bars with same letter are not different at $p < 0.05$ based on ANOVA followed by Tukey's multiple comparisons test.

Gene expression analyses revealed that *gpx-7*, *gst-36*, *ctl-1* and *ctl-3* were significantly up-regulated in nematodes exposed to glyphosate F (Fig. 2). The most overexpressed gene was *ctl-1* as the mRNA level was 3.2 higher than the control. Notably, these four genes were also positively regulated in paraquat stressed animals, indicating that both herbicides could trigger a common signaling pathway. However, three of the eleven genes analyzed were overexpressed in animals treated with paraquat but not with glyphosate F, being *sod-3* gene one of them, which is a well-known oxidative stress marker. Remarkably, *sod-3* gene also presented the highest induction level in the analysis, as its mRNA expression was about 4 times higher compared to the control. On the other hand, neither glyphosate treatment nor paraquat treatment affected significantly the transcriptional expression of *gst-28*, *trxr-2*, *prdx-2* and *ctl-2* genes.

Since *ctl-1* and *ctl-3* significantly responded to glyphosate F exposure, we further investigated catalases genes expression to three sublethal concentrations of the pesticide. The expression of *ctl-1* and *clt-3* genes were highly induced with the three doses of glyphosate F (white bars in Fig. 3A and C). Meanwhile, *ctl-2* gene expression did not significantly change with any of the tested glyphosate F doses (white bars in Fig. 3B). In order to confirm whether the change in the genetic expression of *ctl-1* and *ctl-3* is reflected in catalase activity, the enzymatic specific activity was evaluated after the nematode exposure to the three concentrations of glyphosate F mentioned above. A significant increased in a dose-dependent manner was observed with 4.8 and 9.6 mM glyphosate F treatment (white bars Fig. 4).

3.3. Glyphosate formulation induced stress-response gene expression is regulated by DAF-16

In *C. elegans* the FOXO transcription factor DAF-16 is the major target of insulin-like signaling pathway, which modulates stress response and promotes reproductive development (Panowski and Dillin, 2009). In order to determine if the glyphosate F induction of the catalase gene expression was dependent on DAF-16, experiments were performed using a *daf-16* deficient mutant CF1038 strain [*daf-16*(*mu86*) *I*]. Unlike what happened in the wild-type strain, the glyphosate F treatment was not able to induce a strong catalase gene expression (compare white and grey bars in Fig. 3). In fact, only with the 4.8 mM glyphosate F treatment the *ctl-1* gene was slightly induced in the *daf-16*(*mu86*) *I* mutant strain. Moreover, catalase specific activity upon 9.6 mM glyphosate F treatment was induced 1.7 times in the *daf-16*(*mu86*) *I* mutant strain while in the wild-type strain it was induced 2.8 times (Fig. 4). A similar tendency was observed with the 4.8 mM glyphosate F treatment.

The DAF-16 protein normally traces in the cytoplasm, but under adverse environmental stress conditions, it translocates to the nucleus where it activates transcription of a large number of genes that increase stress resistance and longevity (Panowski and Dillin, 2009). To evaluate whether glyphosate F exposure would induce the translocation of DAF-16, we analyzed protein localization in a *C. elegans* transgenic strain carrying a DAF-16::GFP reporter gene. As indicated in Fig. 5, glyphosate F induced DAF-16 translocation from cytoplasm to nucleus in 48.2% of the treated nematodes compared to the 4.8% of the M9 buffer control worms. Regarding the positive controls, the group of nematodes exposed to a short heat shock at 37 °C presented approximately 100% of DAF-16 nuclear localization while in paraquat-treated worms it reached 71%.

4. Discussion

In order to meet the food demand for the growing world population, it is necessary to increase crop production. This is one of the main reasons why herbicides-resistant transgenic crop technologies have been developed in the last 20 years. Among all these technologies, the ones that include glyphosate are the most widely used. Moreover, glyphosate-based herbicides are applied in urban spaces and in household. As a consequence, large amounts of glyphosate-based herbicides are released into the environment annually. In fact, the ecotoxicological and human health effects of glyphosate are at the focus of social

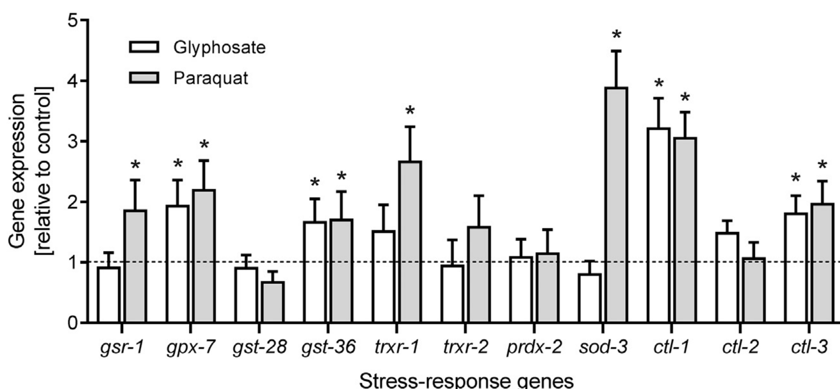

Fig. 2. Quantitative real-time RT-PCR analysis of oxidative stress-related genes in glyphosate F and paraquat exposed nematodes. L4-staged nematodes were treated with glyphosate F (4.8 mM), paraquat (2.0 mM) or without treatment (M9 buffer) for 16 h at 20 °C, and then were harvested and the levels of each of the mRNA were assessed by quantitative real-time RT-PCR as described in Material and methods. The expression level of each target PCR product was normalized to that of the housekeeping gene, *cdc-42* and related to the control. The dotted line denotes de normalized control value. The values represent means ± SD of 3 independent assays. *$p < 0.05$ based on ANOVA followed by Tukey's multiple comparisons test.

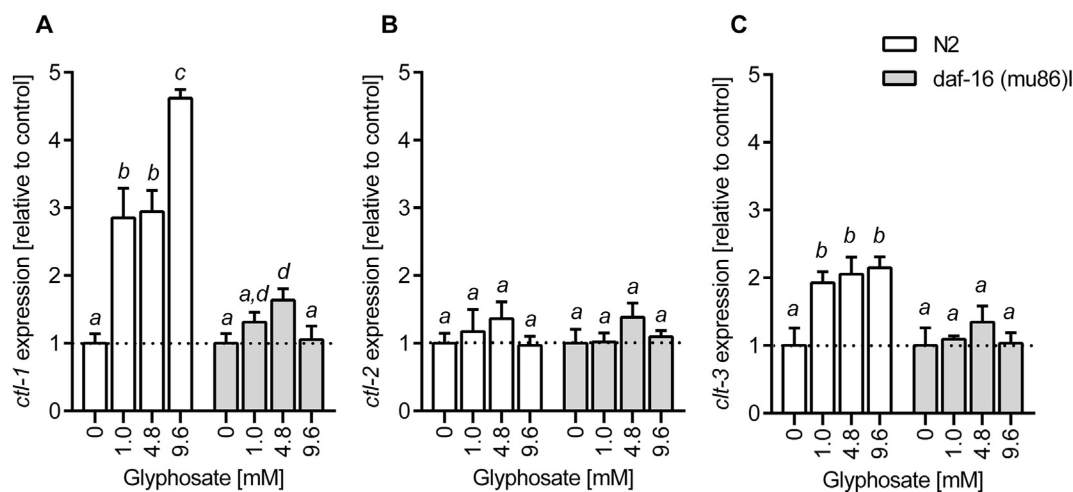

Fig. 3. Quantitative real-time RT-PCR analysis of *ctl-1* (A), *ctl-2* (B) and *ctl-3* (C) genes expression in L4-staged N2 wild-type (white bars) and *daf-16* (*mu86*)*I* mutant (grey bars) nematodesexposed to increasing concentrations of glyphosate F in M9 buffer for 16 h at 20 °C as described in Material and methods. The expression level of each target PCR product was normalized to that of the housekeeping gene, *cdc-42*. The dotted line denotes the normalized control value. The values represent means ± SD of 3 independent assays. Bars with same letter are not different at $p < 0.05$ based on ANOVA followed by Tukey's multiple comparisons test.

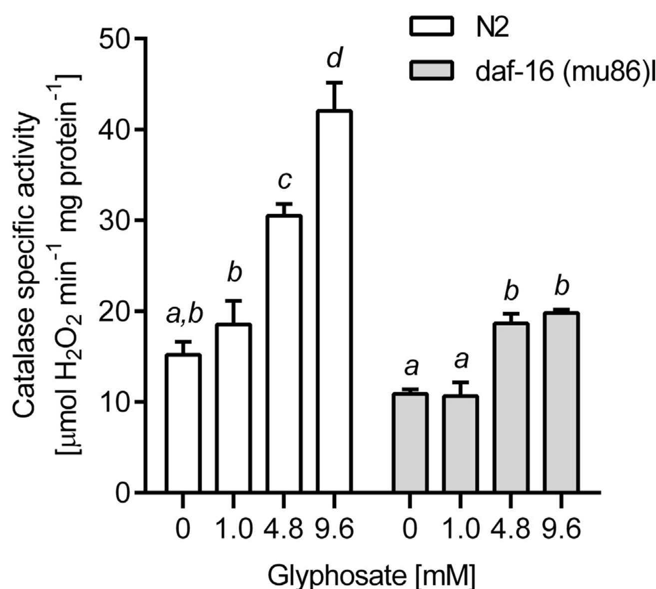

Fig. 4. Catalase specific activity in L4-staged N2 wild-type (white bars) and *daf-16* (*mu86*)*I* mutant (grey bars) nematodes exposed to increasing concentrations of glyphosate F in M9 buffer for 16 h at 20 °C as described in Material and methods. The values represent means ± SD of 3 independent assays. Bars with same letter are not different at $p < 0.05$ based on ANOVA followed by Tukey's multiple comparisons test.

concern (Battaglin et al., 2016).

Our research seeks to contribute knowledge of this complex issue by defining molecular pathways modulated by glyphosate-based formulation exposure. To this end, the nematode *C. elegans* was selected because of the important role it plays in developing a hazard assessment of the pesticide-induced effects on the environment. Actually, many authors have demonstrated that this free-living soil nematode is a valuable bioindicator organism in ecological risk assessment in both aquatic and soil environments (Clavijo et al., 2017, 2016; Leung et al., 2008; Tejeda-Benitez et al., 2016; Xu et al., 2017; Yu et al., 2016). Besides, *C. elegans* studies are useful to predict a pollutant mode of action as many basic physiological processes are conserved in higher organisms, including humans (Kaletta and Hengartner, 2006).

In our previous report we described the toxicological effects of a commercial formulation of glyphosate in *C. elegans* post-embryonic development. The report shows that this herbicide interferes with normal growth, fecundity and reproduction in a dose-response manner (Kronberg et al., 2014). According to US EPA's scale of ecotoxicity categories for terrestrial and aquatic organisms for pesticide classification, glyphosate F falls into the practically non-toxic category (US Environmental Protection Agency, 2008). In order to explain differences in growth, fecundity and reproduction, we investigated which molecular mechanism could be modified by glyphosate-based herbicides. We focused our attention on the redox balance and cellular stress response. To this end, we measured ROS generation after glyphosate F treatments. Our results show the high intracellular ROS production in glyphosate F-treated nematodes is similar to the nematodes treated with paraquat, a well-known generator of reactive oxidants (Park et al., 2009). Moreover, a prototypic antioxidant treatment with ascorbic acid protected the nematodes from glyphosate-induced ROS production. The induction of ROS generation after exposure to different pesticides has been examined in *C. elegans* (McVey et al., 2012; Meyer and Williams, 2014). Several authors reported ROS production after treatment with chlorpyrifos insecticides (Jadiya and Nazir, 2012) and with monocrotophos insecticides (Leelaja and Rajini, 2013). Recently, Bailey et al. (2018) stated that exposure of *C. elegans* to TouchDown® inhibits mitochondrial respiration and leads to the accumulation of hydrogen peroxide. In this sense, our data confirms that ROS production is a key molecular mechanism incurred by glyphosate that affects intracellular homeostasis. Furthermore, this mechanism of action in which glyphosate induces redox imbalance is conserved in other non-target organisms. For example, exposure to glyphosate can affect antioxidant defense systems and induce ROS and oxidative damage in oysters (Mottier et al., 2015), blackworms (Contardo-Jara et al., 2009) and several fish species (Glusczak et al., 2011; Modesto and Martinez, 2010; Sinhorin et al., 2014). In fact, damage to lipids and DNA was reported as a consequence of the glyphosate-induced oxidative stress in *Anguilla anguilla* and *Drosophila melanogaster* (Guilherme et al., 2012; Mattos de Aguiar et al., 2016). Moreover, Heu et al. (2012) showed that glyphosate affects the mitochondrial membrane integrity in human cells, increases ROS production and promotes apoptosis. In this regard, it would be interesting to establish the relationship between glyphosate ROS production and apoptosis induction in *C. elegans*. Additionally, it has been demonstrated that ROS may induce a hormetic compensatory endogenous response leading to a lifespan extension on *C. elegans*(Gems and Partridge, 2008). In fact, our next research will focus on experiments to better understand the relationship between glyphosate F, oxidative stress and lifespan.

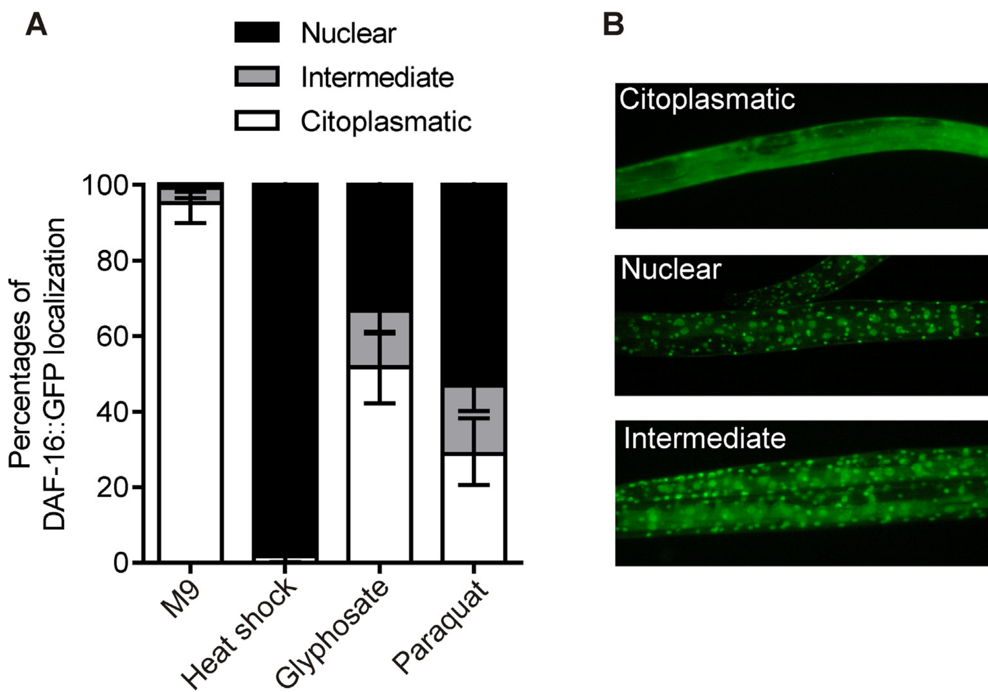

Fig. 5. DAF-16::GFP translocation induced by glyphosate F (A) and representative images of transgenic strain TJ356 with cytoplasmic (upper) nuclear (center) and intermediate (down) DAF-16::GFP localization in an L4 worm (B). L4-staged nematodes were treated with glyphosate F (4.8 mM), paraquat (2.0 mM), exposure 30 min at 37 °C or without treatment (M9 buffer) for 16 h at 20 °C. The values represent means ± SD.

Next, we analyzed which cellular detox pathways were activated upon exposure to glyphosate F to restore the redox balance in the nematode. To this end, we performed a stress-response gene expression analyses on selective genes that represent the main stress response pathways. We found that *gpx-7*, *gst-36*, *ctl-1* and *ctl-3* were significantly up-regulated in animals exposed to glyphosate F (Figs. 2 and 3). These findings corroborate recent published data that showed an increase in hydrogen peroxide, but not in superoxide, in *C. elegans* exposed to TouchDown® (Bailey et al., 2018). In this sense, we confirmed that the change in the genetic expression of *ctl-1* and *ctl-3* was correlated with catalase specific activity after exposure to the three concentrations of glyphosate F in dose-response manner (Fig. 3). These results suggest an earlier increase of the antioxidant defense system to face up to the oxidative stress caused by glyphosate F. Furthermore, supporting our finding García-espiñeira et al. (2018) demonstrated using transgenic GFP reporter strains of *C. elegans* an induction in oxidative response genes *sod-1*, *sod-4* and *gpx-4*with Roundup® treatment.

The *C. elegans* genome contains three genes encoding different catalases. *Ctl-2*, the peroxisomal catalase, realized approximately 80% of total catalase activity in the nematode whereas the remaining activity is contributed by cytosolic catalase *ctl-1* and *ctl-3* (Petriv and Rachubinski, 2004). These authors have also demonstrated that a lack of *ctl-2* accelerates aging and reduced lifespan contrary to *ctl-1* mutant, and that *ctl-3* is mainly expressed in pharyngeal muscles and neuronal cells, indicating that these three catalases have different functions in the nematode biology. Our results indicated that only the two cytosolic catalase genes are induced and proteins activated with glyphosate F (and paraquat) treatments. Similar results were reported by Han et al. (2017)who detected a significant increase in *clt-1* and *ctl-3* genes but not in *ctl-2* gene after treating *C. elegans* with phoxim, an organophosphorus insecticide. All together these data suggested that *ctl-1* play an important role in stress adaptation when faced with chemical insults and underline the predictive potential of this gene as a biomarker to evaluate the environmental risk of glyphosate among other pesticides.

Since the FOXO transcription factor DAF-16 modulates several stress response genes, including the ones that were up-regulated with glyphosate F treatment, we assessed the activation of this master key regulator. To this end, we traced DAF-16 protein localization using a transgenic strain carrying a DAF-16::GFP reporter gene. Our results show a clear protein relocalization from cytoplasm to nucleus after treatment and proved that glyphosate F, like paraquat, could trigger activation of DAF-16 (Fig. 5). Furthermore, the activation of *ctl-1*and*ctl-3* genes and the catalase activity induced by glyphosate F exposure was significantly lower in *daf-16* deficient mutant strain compared to the wild-type strain (Figs. 3 and 4). This is consistent with the fact that DAF-16 is the major target of the insulin-like signaling pathway which modulates stress response and promotes reproductive development in *C. elegans* (Murphy, 2006).

It is interesting to note that the pattern of induced DAF-16 targets might change depending on the stimulus. While known DAF-16 target genes such as *ctl-1* and *ctl-3* were activated in glyphosate F-treated nematodes, the classic target *sod-3* gene remained unchanged. On the other hand, paraquat induced the expression of all three genes. Actually, the phenomenon where a nuclear localization of DAF-16 induced by certain conditions did not increase *sod-3* gene expression had already been described (Henderson et al., 2006; Kampkötter et al., 2008). This observation suggests that nuclear localization alone may not be enough to activate DAF-16 target genes. It has been proposed that other factors, like combinatorial interaction with other effectors, phosphorylation state or different isoforms, can modulate the capacity of DAF-16 to induce transcription and orchestrate a fine-tune detox defense (Berdichevsky and Guarente, 2006; Kwon et al., 2010; Lewis et al., 2013; Riedel et al., 2013; Van Der Heide et al., 2004).

In conclusion, we demonstrated that glyphosate F exposure can induce an oxidative imbalance in *C. elegans*, leading to the activation of DAF-16 transcription factor and consequently to the expression of genes that boosts the antioxidant defense system. *C. elegans* stress-response pathways mediated by DAF-16/FOXO are conserved in vertebrates including mammals, so our approach could provide a framework to develop biomarkers for early detection of oxidative damage. This could provide a more sensitive tool for the assessment of agrochemicals risks and hazards and help improve environmental management policies.

Conflict of interest statement

We do not have any conflicts of interest to declare.

Acknowledgements

This study has been financially supported by Ministry of Science, Technology and Productive Innovation, Argentina through the National Agency for the Promotion of Science and Technology. These institutions awarded two grants to E. Munarriz [PICT-PRH 2014/0002 and PICT 2014/3293] and one grant to E. Pagano [ANPCyT-PID 0032/2011]. We are especially grateful to Priva Braunfeld from Water National Institute for critically reviewing the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2018.08.002.

References

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol. 105, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3.

Back, P., Braeckman, B.P., Matthijssens, F., 2012. ROS in aging *Caenorhabditis elegans*: damage or signaling? Oxidative Med. Cell. Longev. 2012, 608478. https://doi.org/10.1155/2012/608478.

Bailey, D.C., Todt, C.E., Burchfield, S.L., Pressley, A.S., Denney, R.D., Snapp, I.B., Negga, R., Traynor, W.L., Fitsanakis, V.A., 2018. Chronic exposure to a glyphosate-containing pesticide leads to mitochondrial dysfunction and increased reactive oxygen species production in *Caenorhabditis elegans*. Environ. Toxicol. Pharmacol. 57, 46–52. https://doi.org/10.1016/j.etap.2017.11.005.

Battaglin, W., Bradley, P., Meyer, M., 2016. Glyphosate and other contaminants of concern in water, sediment, and soil in the United States. In: Plant and Animal Genome XXIV Conference. San Diego, CA, USA, pp. W646.

Berdichevsky, A., Guarente, L., 2006. A stress response pathway involving sirtuins, forkheads and 14-3-3 proteins. Cell Cycle 5, 2588–2591. https://doi.org/10.4161/cc.5.22.3513.

Boocock, M.R., Coggins, J.R., 1983. Kinetics of senolpyruvylshikimate-3-phosphate inhibition by glyphosate. FEBS Lett. 154, 127–133. https://doi.org/10.1016/0014-5793(83)80888-6.

Brenner, S., 1974. The genetics of *Caenorhabditis elegans*. Genetics 77, 71–94. https://doi.org/10.1002/cbic.200300625.

Clavijo, A., Kronberg, M.F., Rossen, A., Moya, A., Calvo, D., Salatino, S.E., Pagano, E.A., Morabito, J.A., Munarriz, E.R., 2016. The nematode *Caenorhabditis elegans* as an integrated toxicological tool to assess water quality and pollution. Sci. Total Environ. 569–570, 252–261. https://doi.org/10.1016/j.scitotenv.2016.06.057.

Clavijo, A., Rossen, A., Calvo, D., Kronberg, M.F., Moya, A., Pagano, E.A., Munarriz, E.R., 2017. Water quality and toxicological impact assessment using the nematode *Caenorhabditis elegans* bioassay in a long-term intensive agricultural area. Water Air Soil Pollut. 228. https://doi.org/10.1007/s11270-017-3512-4.

Contardo-Jara, V., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its formulation roundup ultra in *Lumbriculus variegatus* and its effects on biotransformation and antioxidant enzymes. Environ. Pollut. 157, 57–63. https://doi.org/10.1016/j.envpol.2008.07.027.

Coupe, R.H., Kalkhoff, S.J., Capel, P.D., Gregoire, C., 2012. Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basins. Pest Manag. Sci. 68, 16–30. https://doi.org/10.1002/ps.2212.

Frézal, L., Félix, M.A., 2015. *C. elegans* outside the petri dish. elife 4, 1–14. https://doi.org/10.7554/eLife.05849.

García-espiñeira, M., Tejeda-benitez, L., Olivero-verbel, J., 2018. Ecotoxicology and environmental safety toxicity of atrazine- and glyphosate-based formulations on *Caenorhabditis elegans*. Ecotoxicol. Environ. Saf. 156, 216–222. https://doi.org/10.1016/j.ecoenv.2018.02.075.

Gems, D., Partridge, L., 2008. Stress-response hormesis and aging: "that which does not kill us makes us stronger". Cell Metab. 7, 200–203. https://doi.org/10.1016/j.cmet.2008.01.001.

Glusczak, L., Loro, V.L., Pretto, A., Moraes, B.S., Raabe, A., Duarte, M.F., da Fonseca, M.B., de Menezes, C.C., Valladao, D.M. de S., 2011. Acute exposure to glyphosate herbicide affects oxidative parameters in piava (*Leporinusobtusidens*). Arch. Environ. Contam. Toxicol. 61, 624–630. https://doi.org/10.1007/s00244-011-9652-4.

Guilherme, S., Gaivão, I., Santos, M. a, Pacheco, M., 2012. DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide-elucidation of organ-specificity and the role of oxidative stress. Mutat. Res. 743, 1–9. https://doi.org/10.1016/j.mrgentox.2011.10.017.

Haefs, R., Schmitz-Eiberger, M., Mainx, H.G., Mittelstaedt, W., Noga, G., 2002. Studies on a new group of biodegradable surfactants for glyphosate. Pest Manag. Sci. 58, 825–833. https://doi.org/10.1002/ps.539.

Han, Y., Song, S., Wu, H., Zhang, J., Ma, E., 2017. Antioxidant enzymes and their role in phoxim and carbaryl stress in *Caenorhabditis elegans*. Pestic. Biochem. Physiol. 138, 43–50. https://doi.org/10.1016/j.pestbp.2017.02.005.

Henderson, S.T., Bonafe, M., Johnson, T.E., 2006. Daf-16 protects the nematode *Caenorhabditis elegans* during food deprivation. J. Gerontol. A Biol. Sci. Med. Sci. 61, 444–460. https://doi.org/10.1093/gerona/61.5.444.

Heu, C., Berquand, A., Elie-Caille, C., Nicod, L., 2012. Glyphosate-induced stiffening of HaCaT keratinocytes, a peak force tapping study on living cells. J. Struct. Biol. 178, 1–7. https://doi.org/10.1016/j.jsb.2012.02.007.

Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J., Vanfleteren, J.R., 2008. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in *C. elegans*. BMC Mol. Biol. 9, 9. https://doi.org/10.1186/1471-2199-9-9.

Hunt, P.R., 2017. The *C. elegans* model in toxicity testing. J. Appl. Toxicol. 37, 50–59. https://doi.org/10.1002/jat.3357.

ISAAA, 2016. Global Status of Commercialized Biotech/GM Crops: 2016, ISAAA Briefs. ISAAA Briefs, Ithaca, NY. https://doi.org/10.1017/S0014479706343797.

Jadiya, P., Nazir, A., 2012. Environmental toxicants as extrinsic epigenetic factors for parkinsonism: studies employing transgenic *C. elegans* model. CNS Neurol. Disord. Drug Targets 11, 976–983.

Kaletta, T., Hengartner, M.O., 2006. Finding function in novel targets: *C. elegans* as a model organism. Nat. Rev. Drug Discov. 5, 387–398. https://doi.org/10.1038/nrd2031.

Kampkötter, A., Timpel, C., Zurawski, R.F., Ruhl, S., Chovolou, Y., Proksch, P., Wätjen, W., 2008. Increase of stress resistance and lifespan of *Caenorhabditis elegans* by quercetin. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 149, 314–323. https://doi.org/10.1016/j.cbpb.2007.10.004.

Kronberg, M.F., Clavijo, A.M., Moya, A., Heredia, O., Eduardo, A., 2014. Utilización del nematodo Caenorhabditis elegans en ensayos de toxicidad de muestras de agua. In: 2do. Encuentro De Investigadores En Formación En Recursos Hídricos, . https://www.ina.gob.ar/legacy/ifrh-2014/Eje2/2.19.pdf.

Kwon, E.-S., Narasimhan, S.D., Yen, K., Tissenbaum, H.A., 2010. A new DAF-16 isoform regulates longevity. Nature 466, 498–502. https://doi.org/10.1038/nature09184.

Leelaja, B.C., Rajini, P.S., 2013. Biochemical and physiological responses in *Caenorhabditis elegans* exposed to sublethal concentrations of the organophosphorus insecticide, monocrotophos. Ecotoxicol. Environ. Saf. 94, 8–13. https://doi.org/10.1016/j.ecoenv.2013.04.015.

Leung, M.C.K., Williams, P.L., Benedetto, A., Au, C., Helmcke, K.J., Aschner, M., Meyer, J.N., 2008. *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. Toxicol. Sci. 106, 5–28. https://doi.org/10.1093/toxsci/kfn121.

Lewis, J., Gehman, E., Baer, C., Jackson, D., 2013. Alterations in gene expression in *Caenorhabditis elegans* associated with organophosphate pesticide intoxication and recovery. BMC Genomics 14, 291. https://doi.org/10.1186/1471-2164-14-291.

Marc, J., Mulner-Lorillon, O., Boulben, S., Hureau, D., Durand, G., Bellé, R., 2002. Pesticide roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chem. Res. Toxicol. 15, 326–331. https://doi.org/10.1021/tx015543g.

Mattos de Aguiar, L., Figueira, F.H., Gottschalk, M.S., Da Rosa, C.E., 2016. Glyphosate-based herbicide exposure causes antioxidant defence responses in the fruit fly *Drosophila melanogaster*. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 185–186, 94–101. https://doi.org/10.1016/j.cbpc.2016.03.006.

Mattson, M.P., 2008. Hormesis defined. Ageing Res. Rev. 7, 1–7. https://doi.org/10.1016/j.arr.2007.08.007.

McVey, K.A., Mink, J.A., Snapp, I.B., Timberlake, W.S., Todt, C.E., Negga, R., Fitsanakis, V., 2012. *Caenorhabditis elegans*: an emerging model system for pesticide neurotoxicity. J. Environ. Anal. Toxicol. S4. https://doi.org/10.4172/2161-0525.

McVey, K.A., Snapp, I.B., Johnson, M.B., Negga, R., Pressley, A.S., Fitsanakis, V.A., 2016. Exposure of *C. elegans* eggs to a glyphosate-containing herbicide leads to abnormal neuronal morphology. Neurotoxicol. Teratol. 55, 23–31. https://doi.org/10.1016/j.ntt.2016.03.002.

Mesnage, R., Defarge, N., Spiroux De Vendômois, J., Séralini, G.E., 2015. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food Chem. Toxicol. 84. https://doi.org/10.1016/j.fct.2015.08.012.

Meyer, D., Williams, P.L., 2014. Toxicity testing of neurotoxic pesticides in *Caenorhabditis elegans*. J. Toxicol. Environ. Health B Crit. Rev. 17, 284–306. https://doi.org/10.1080/10937404.2014.933722.

Miranda-Vizuete, A., Veal, E.A., 2017. *Caenorhabditis elegans* as a model for understanding ROS function in physiology and disease. Redox Biol. 11, 708–714. https://doi.org/10.1016/j.redox.2016.12.020.

Modesto, K.A., Martinez, C.B.R., 2010. Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochiloduslineatus*. Chemosphere 78, 294–299. https://doi.org/10.1016/j.chemosphere.2009.10.047.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 221–234. https://doi.org/10.1016/j.cbpc.2006.08.012.

Mottier, A., Seguin, A., Devos, A., Le Pabic, C., Voiseux, C., Lebel, J.M., Serpentini, A., Fievet, B., Costil, K., 2015. Effects of subchronic exposure to glyphosate in juvenile oysters (*Crassostreagigas*): from molecular to individual levels. Mar. Pollut. Bull. 95, 665–677. https://doi.org/10.1016/j.marpolbul.2014.10.026.

Murphy, C.T., 2006. The search for DAF-16/FOXO transcriptional targets: approaches and discoveries. Exp. Gerontol. 41, 910–921. https://doi.org/10.1016/j.exger.2006.06.040.

Negga, R., Rudd, D.A., Davis, N.S., Justice, A.N., Hatfield, H.E., Valente, A.L., Fields, A.S., Fitsanakis, V.A., 2011. Exposure to Mn/Zn ethylene-bis-dithiocarbamate and glyphosate pesticides leads to neurodegeneration in *Caenorhabditis elegans*. Neurotoxicology 32, 331–341. https://doi.org/10.1016/j.neuro.2011.02.002.

Negga, R., Stuart, J.A., Machen, M.L., Salva, J., Lizek, A.J., Jayne, S., Osborne, A.S., Mirallas, O., McVey, K.A., Fitsanakis, V.A., 2012. Exposure to glyphosate- and/or mn/zn-ethylene-bis- dithiocarbamate-containing pesticides leads to degeneration of γ-aminobutyric acid and dopamine neurons in *Caenorhabditis elegans*. Neurotox. Res. 21, 281–290. https://doi.org/10.1007/s12640-011-9274-7.

Panowski, S.H., Dillin, A., 2009. Signals of youth: endocrine regulation of aging in

Caenorhabditis elegans. Trends Endocrinol. Metab. 20, 259–264. https://doi.org/10.1016/j.tem.2009.03.006.
Pant, A., Saikia, S.K., Shukla, V., Asthana, J., Akhoon, B.A., Pandey, R., 2014. Beta-caryophyllene modulates expression of stress response genes and mediates longevity in *Caenorhabditis elegans*. Exp. Gerontol. 57, 81–95. https://doi.org/10.1016/j.exger.2014.05.007.
Park, S.K., Tedesco, P.M., Johnson, T.E., 2009. Oxidative stress and longevity in *Caenorhabditis elegans* as mediated by SKN-1. Aging Cell 8, 258–269. https://doi.org/10.1111/j.1474-9726.2009.00473.
Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ. Pollut. 156, 61–66. https://doi.org/10.1016/j.envpol.2008.01.015.
Petriv, O.I., Rachubinski, R.A., 2004. Lack of peroxisomal catalase causes a progeric phenotype in *Caenorhabditis elegans*. J. Biol. Chem. 279, 19996–20001. https://doi.org/10.1074/jbc.M400207200.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45. https://doi.org/10.1093/nar/29.9.e45.
Riedel, C.G., Dowen, R.H., Lourenco, G.F., Natalia, V., Heimbucher, T., West, J.A., Bowman, S.K., Robert, E., Dillin, A., Asara, J.M., Ruvkun, G., 2013. DAF-16/FOXO employs the chromatin remodeller SWI/SNF to promote stress resistance and longevity. Nat. Cell Biol. 15, 491–501. https://doi.org/10.1038/ncb2720.DAF-16/FOXO.
Ruan, Q.-L.L., Ju, J.-J.J., Li, Y.-H.H., Liu, R., Pu, Y.-P.P., Yin, L.-H.H., Wang, D.-Y.Y., 2009. Evaluation of pesticide toxicities with differing mechanisms using *Caenorhabditis elegans*. J. Toxicol. Environ. Heal. Part A Curr. Issues 72, 746–751. https://doi.org/10.1080/15287390902841532.
Schulz, T.J., Zarse, K., Voigt, A., Urban, N., Birringer, M., Ristow, M., 2007. Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab. 6, 280–293. https://doi.org/10.1016/j.cmet.2007.08.011.
Sem, X., Rhen, M., 2012. Pathogenicity of *Salmonella enterica* in *Caenorhabditis elegans* relies on disseminated oxidative stress in the infected host. PLoS One 7, e45417. https://doi.org/10.1371/journal.pone.0045417.
Sinhorin, V.D.G., Sinhorin, A.P., Teixeira, J.M. dos S., Miléski, K.M.L., Hansen, P.C., Moreira, P.S.A., Kawashita, N.H., Baviera, A.M., Loro, V.L., 2014. Effects of the acute exposition to glyphosate-based herbicide on oxidative stress parameters and antioxidant responses in a hybrid Amazon fish surubim (*Pseudoplatystoma*sp). Ecotoxicol. Environ. Saf. 106, 181–187. https://doi.org/10.1016/j.ecoenv.2014.04.040.
Stiernagle, T., 2006. Maintenance of *C. elegans*. WormBook 1–11. https://doi.org/10.1895/wormbook.1.101.1.
Struger, J., Van Stempvoort, D.R., Brown, S.J., 2015. Sources of aminomethylphosphonic acid (AMPA) in urban and rural catchments in Ontario, Canada: glyphosate or phosphonates in wastewater? Environ. Pollut. 204, 289–297. https://doi.org/10.1016/j.envpol.2015.03.038.
Tejeda-Benitez, L., Flegal, R., Odigie, K., Olivero-Verbel, J., 2016. Pollution by metals and toxicity assessment using *Caenorhabditis elegans* in sediments from the Magdalena River, Colombia. Environ. Pollut. 212, 238–250. https://doi.org/10.1016/j.envpol.2016.01.057.
US Environmental Protection Agency, 2008. Technical Overview of Ecological Risk Assessment Analysis Phase: Ecological Effects Characterization [WWW Document]. URL. http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm.
US Environmental Protection Agency, 2016. Glyphosate issue paper: evaluation of carcinogenic potential EPA's Office of Pesticide Programs. In: EPA's Off. Pestic. Programs. 227.
Van Der Heide, L.P., Hoekman, M.F.M., Smidt, M.P., 2004. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem. J. 380, 297–309. https://doi.org/10.1042/bj20040167.
Xu, T., Zhang, M., Hu, J., Li, Z., Wu, T., Bao, J., Wu, S., Lei, L., He, D., 2017. Behavioral deficits and neural damage of *Caenorhabditis elegans* induced by three rare earth elements. Chemosphere 181, 55–62. https://doi.org/10.1016/j.chemosphere.2017.04.068.
Yang, X., Wang, F., Bento, C.P.M., Meng, L., van Dam, R., Mol, H., Liu, G., Ritsema, C.J., Geissen, V., 2015. Decay characteristics and erosion-related transport of glyphosate in Chinese loess soil under field conditions. Sci. Total Environ. 530–531, 87–95. https://doi.org/10.1016/j.scitotenv.2015.05.082.
Yu, C.W., How, C.M., Liao, V.H.C., 2016. Arsenite exposure accelerates aging process regulated by the transcription factor DAF-16/FOXO in *Caenorhabditis elegans*. Chemosphere 150, 632–638. https://doi.org/10.1016/j.chemosphere.2016.01.004.