Acute exposure to a glyphosate-containing herbicide formulation inhibits Complex II and increases hydrogen peroxide in the model organism *Caenorhabditis elegans*

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ABSTRACT

Glyphosate-based herbicides, such as Touchdown (TD) and Roundup, are among the most heavily-used herbicides in the world. While the active ingredient is generally considered non-toxic, the toxicity resulting from exposure to commercially-sold formulations is less clear. In many cases, cell cultures or various model organisms exposed to glyphosate formulations show toxicity and, in some cases, lethality. Using *Caenorhabditis elegans*, we assessed potential toxic mechanisms through which a highly-concentrated commercial formulation of TD promotes neurodegeneration. Following a 30-min treatment, we assayed mitochondrial electron transport chain function and reactive oxygen species (ROS) production. Initial oxygen consumption studies indicated general mitochondrial inhibition compared to controls ($^*p < 0.05$). When Complex II activity was further assessed, inhibition was observed in all TD-treated groups ($^*p < 0.05$). Complex IV activity, however, was not adversely affected by TD. This electron transport chain inhibition also resulted in reduced ATP levels ($^*p < 0.05$). Furthermore, hydrogen peroxide levels, but not other ROS, were increased ($^*p < 0.05$). Taken together, these data indicate that commercially-available formulations of TD may exert neurotoxicity through Complex II (succinate dehydrogenase) inhibition, decreased ATP levels, and increased hydrogen peroxide production.

1. Introduction

The increased use of pesticide-resistant crops (*e.g.*, corn, soy, and wheat) has been accompanied by an upsurge in the total amount of pesticides applied each year (Benbrook, 2012). The majority of these pesticides, sold and applied as a combination of patent-protected formulations of active ingredients and adjuvants, are applied by workers in industrial agricultural settings (Grube et al., 2011). The most widely-used pesticides in the world are herbicides formulated with glyphosate as the active ingredient (Grube et al., 2011). While there are many proprietary mixtures of commercially-available glyphosate herbicides, the two most common formulations are sold under the names of Roundup and Touchdown (TD), which are manufactured by Monsanto

and Syngenta, respectively (Anonymous, 2013). From a human health prospective, it is important to note that numerous neurodegenerative diseases (Allen and Levy, 2013; Baltazar et al., 2014), various cancers (Alavanja et al., 2013; Engel et al., 2005), and even neurobehavioral disorders (Roberts and Karr, 2012) have been associated with pesticide exposure. Most of these diseases are marked by increased oxidative stress, reactive oxygen species (ROS) production, and/or mitochondrial inhibition (Abdul-Aziz et al., 2015; Celardo et al., 2014; Mostafalou and Abdollahi, 2013). Consequently, regulatory bodies are increasingly faced with questions regarding the potential toxicity of glyphosate formulations and their implications for human and animal health.

Similar to other studies of commercially-available pesticides, previous research on glyphosate-based herbicides focused on the toxicity of

the active ingredient, glyphosate, rather than formulations. These studies show that a large dose of glyphosate is required in rats (oral LD_{50} = 2000 mg/kg) and mice (oral LD_{50} = 10,000 mg/kg) to produce lethality (Tomlin, 2006). More recently, however, a greater appreciation for the toxicity of commercial formulations, the mixtures to which people and animals are actually exposed, has caused researchers to shift their concern from examining the toxicity of pure glyphosate to that of the applied preparations (de Liz Oliveira Cavalli et al., 2013; Mesnage et al., 2014; Peixoto, 2005). In many reports, the agricultural formulation is much more toxic than either glyphosate alone (Benachour and Seralini, 2009; Chaufan et al., 2014; Mesnage et al., 2014), or the putative surfactants that may be present in the formulation (Kim et al., 2013).

Considering the reports documenting greater potency and efficacy, our studies used commercially available TD. Our previous work showed that exposure to varying concentrations of TD led to morphological changes in multiple neuronal populations in *Caenorhabditis elegans* (*C. elegans*) (Negga et al., 2011, 2012). The mechanisms contributing to this neurodegeneration, however, have yet to be determined. As both mitochondrial inhibition and increased ROS production are common disease mechanisms (Chakraborty et al., 2013; Wang et al., 2013), our current studies assess whether TD could initiate these processes. While mitochondrial respiration studies are often conducted in isolated mitochondrial fractions, we completed these assays *in vivo* using *C. elegans* to assess the effects of TD on intact mitochondria in living organisms. As such, we first sought to determine whether exposure of *C. elegans* to TD specifically inhibited mitochondrial electron transport chain complexes. Since mitochondrial inhibition can also lead to oxidative stress, we then assessed whether TD exposure led to increases in overall ROS production or changes in levels of distinct ROS *in vivo*.

2. Materials and methods

2.1. Worm and Escherichia coli strains

Wild-type (N2) nematodes, as well as NA22 *Escherichia coli (E. coli)* and OP50-1 *E. coli* were obtained from the *Caenorhabditis* Genetics Center (CGC).

2.2. Synchronization

C. elegans were maintained at 20 °C and synchronized according to standard protocols (Brenner, 1974), and as previously published (Negga et al., 2011). Briefly, *C. elegans* were grown on 8 P plates (51.3 mM NaCl, 25.0 g bactoagar/L, 20.0 g bactopeptone/L, 1 mM $CaCl_2$, 0.5 mM KH_2PO_4 (pH 6), 13 μM cholesterol (95% ethanol), 1 mM $MgSO_4$) with NA22 *E. coli* (cultured in 16 g tryptone/L, 10 g yeast extract/L, 85.5 mM NaCl) until gravid. Eggs were isolated from gravid worms using a hypochlorite solution (0.55% NaOCl, 500 μM NaOH). The egg/bleach mixture was diluted with M9 solution (20 mM KH_2PO_4, 40 mM Na_2HPO_4, 68 mM NaCl) followed by separation of eggs using a 30% sucrose solution. Eggs were then resuspended with M9 and nutated for 18 h at 20 °C.

2.3. Treatments

Worms were exposed to TD as previously published (Negga et al., 2011; Settivari et al., 2013). Briefly, 5000 worms/treatment group were exposed for 30 min to 3.0% glyphosate as TD, 7.0% glyphosate as TD, or 10.0% glyphosate as TD (Touchdown® Hitech, formulation of 52.3% glyphosate, Syngenta AG, Wilmington, DE). To facilitate comparison of our results with other glyphosate-containing pesticides, concentrations are reported as percent active ingredient (glyphosate) rather than percent overall pesticide formulation. At the conclusion of the incubation, worms were washed with sterile dH_2O and returned to nematode growth media plates (NGM plates; 51.3 mM NaCl, 17.0 g bactoagar/L,

2.5 g bactopeptone/L, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 500 μM KH_2PO_4 (pH 6.0), 13 μM cholesterol (95% ethanol), 1.25 mL nystatin/L, 200 mg streptomycin/L) with a lawn of OP50-1 *E. coli* for 24 h at 20 °C to recover from the treatment. Since concentrated TD used in industrial agriculture is diluted to the appropriate application concentration for spraying with water (Monsanto, 2014; Syngenta et al. 2010), control worms were treated with sterile dH_2O.

2.4. Mitochondrial assays

2.4.1. Oxygen consumption studies

Worms post-treatment and recovery were counted and normalized to yield standardized groups of 10,000 live worms/mL/treatment concentration. Worms and M9 were added to heated (22 °C) water bath chambers (YSI 5301B Standard Bath), and an oxygen probe (YSI 5304) was inserted and re-equilibrated in the sample chamber. Oxygen consumption measurements were recorded every ten seconds for a total of five minutes to assess oxygen consumption and general inhibition of mitochondrial respiration.

2.4.2. Complex II activity

Following the 24-h recovery period, worms were washed from plates with sterile M9, and samples were normalized to yield 1000 live worms/mL/treatment concentration. Following fixation and permeabilization (Grad et al., 2007), Complex II activity was assayed using 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM KCN, 200 μM phenazine methosulfate, 50 mM sodium succinate, 250 μM nitroblue tetrazolium (NBT) in 10 mL phosphate buffered saline (PBS) per published protocols (Grad et al., 2007). To verify that the assay measured succinate dehydrogenase activity, a negative control was used containing sodium malonate without the inclusion of sodium succinate. After incubation in the assay solution, stained worms were washed and counted to yield 250 worms/treatment group. Worms were pipetted into a 96-well plate, and absorbance read at 560 nm using a Promega® GloMax-Multi$^+$ Detection System (Promega Corporation, Madison, WI).

2.4.3. Complex IV activity

Similar to Complex II preparation, worms were washed and counted to yield 1000 worms/mL/treatment group. Following fixation and permeabilization, Complex IV activity was assayed using 10 mg 0.1% (w/v) 3,3′-diaminobenzidine (DAB), 10 mg 0.1% (w/v) cytochrome-C, 2 mg 0.02% (w/v) catalase in 10 mL PBS (Grad et al., 2007). Potassium cyanide (KCN), without the addition of cytochrome c, was used as a negative control. Stained worms were read at 450 nm as described in Section 2.4.2.

2.4.4. Proton gradient integrity

Post-recovery worms normalized to yield 1000 worms/mL/treatment group. To determine proton gradient integrity, worms were assayed using 9.71 mM tetramethylrhodamine ethyl ester (TMRE; Biotium, Hayward, CA) in dimethyl sulfoxide (DMSO; yielding a final concentration of 50 μM TMRE in 0.5% DMSO). *C. elegans* were incubated for 1 h, and then photographed using a digital camera attached to a fluorescence microscope (see Section 2.6).

2.4.5. Relative ATP amount

Worms were washed and counted to yield 500 live worms/treatment group. Relative ATP concentration was measured using the Promega Mitochondrial ToxGlo™ Assay kit (Promega Corporation, Madison, WI). To verify the assay, a negative control was used containing either KCN (30 min incubation) or sodium azide (24 h incubation). Per the kit protocol provided, 20 μL of the fluorogenic peptide substrate was added and incubated at 20 °C for 60 min to determine percent live worms compared to control. Fluorescence was measured with an excitation wavelength of 495 nm and an emission wavelength of 520–530 nm using a Promega® GloMax-Multi$^+$ Detection System.

Next, 100 μL of a luciferin-containing ATP detection reagent was added to each well and incubated at room temperature for 15 min. Luminescence was also measured using the Promega® GloMax-Multi$^+$ Detection System.

2.5. Reactive oxygen species detection

2.5.1. Superoxide detection

Following recovery, worms normalized to 5000 live worms/mL/ treatment group. One mL of this worm solution was centrifuged, and 10 μL of supernatant was removed and replaced with 10 μL of 500 μM dihydroethidium (DHE; Merck KGaA, Darmstadt, Germany) for a final concentration of 5 μM DHE. Tubes were then vortexed and placed on a nutator at 20 °C for 3 h. DHE fluorescence was assessed by photomicrograph analysis at the conclusion of the incubation period.

2.5.2. Hydrogen peroxide detection

Following recovery, worms were removed from NGM plates, washed, and normalized to yield an average of 4 live worms/μL/treatment group. A total of 200 worms per group was added to a 96-well plate. To the negative control, 50 μL of 1X reaction buffer supplied with the Amplex® Red kit (Life Technologies, Grand Island, NY) was added, while the positive control had 50 μL of 10 μM H_2O_2. Next, 50 μL of the Amplex® Red mixture was added to all groups. The plate was covered with aluminum foil to protect the reaction mixture from light, placed on a nutator, and incubated at room temperature for 1 h. Afterwards, fluorescence was read with an excitation wavelength of 525 nm and an emission wavelength of 580–640 nm using a Promega® GloMax-Multi$^+$ Detection System.

2.5.3. Hydroxyl radical detection

Immediately following the 30-min incubation with TD (no recovery period), worms were washed with sterile dH_2O, and live worms were counted to yield 1000 worms/mL/treatment group. Worms were placed in microcentrifuge tubes with 500 μL of hydroxyphenyl fluorescein (HPF) probe (Life Sciences, Grand Island, NY) and 500 μL of worms. Each group was incubated for 1.5 h at 20 °C. Following incubation, worms were washed and counted again to yield 250 live worms in each well. Fluorescence was read at an excitation wavelength of 490 nm and an emission wavelength of 510–570 nm using a Promega® GloMax-Multi$^+$ Detection System.

2.6. Fluorescence microscopy

For studies involving TMRE fluorescence, photomicrographs were taken with worms on a 4% agarose pad per the protocol already published (Negga et al., 2012). Fluorescence photomicrographs were obtained using a Leitz & Wetzlar (Halco Instruments, Inc) microscope equipped with a 50-W AC mercury source lamp (E. Leitz, Rockleigh, NJ), filter for red fluorescence, and 40X objective (Leitz & Wetzlar, Halco Instruments, Inc.) coupled to a digital camera (Micrometrics, MilesCo Scientific, Princeton, MN) operated by Micrometrics software (SE Premium, v2.7). Images were obtained under identical levels of exposure time, gain, gamma, and saturation. Photomicrographs were then imported into Adobe Photoshop 6.0.1, to determine red (TMRE) pixel intensity $\pm$ standard error of the mean (SEM).

2.7. Statistical analysis

All data were analyzed using GraphPad Prism (version 6.07 for Windows, GraphPad Software, San Diego California USA, www. graphpad.com). For oxygen consumption studies, data are presented as mean consumption, and represent N ≥ 4 separate synchronizations, with n $\geq$ 3 intra-experimental replicates. Differences in slope were determined using linear regression followed by one-way analysis of variance (ANOVA) of the regression lines. If the ANOVA resulted in an
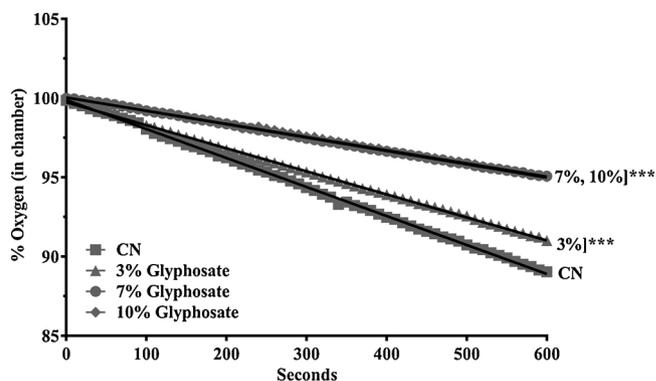

Fig. 1. TD-Induced Mitochondrial Inhibition. Following TD exposure, N2 (wildtype) worms consumed statistically significantly more oxygen consumed compared to any of the TD treated groups. Data are presented as mean percent oxygen consumed with the respective regression lines, and represent N ≥ 4 separate synchronizations. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to controls.

overall significant p value ($p < 0.05$), a *post-hoc* Dunnett's multiple comparison test was performed.

Data are presented as mean $\pm$ SEM, and represent N ≥ 3 separate synchronizations with n $\geq$ 3 intra-experimental replication. One-way ANOVA, followed by a *post-hoc* Dunnett's multiple comparison test, was used for plate reader-based studies. For TMRE studies, pixel intensity of individual worms (n > 5 for each independent replication) was measured. Data were considered to be statistically significantly different from control when $^*p < 0.05$.

3. Results

3.1. Decreased oxygen consumption

To assess overall oxygen consumption and mitochondrial respiration following exposure to TD, oxygen consumption in whole worms was measured (Fig. 1). Linear regression analysis indicated that acute treatment with all TD concentrations resulted in a statistically significant decrease in percent oxygen consumed during the equilibration phase of the assay ($^{***}p < 0.001$).

3.2. Decreased Complex II activity

While oxygen consumption data provided us with a general indication of mitochondrial function, we wanted to interrogate Complex II individually because previous reports using glyphosate herbicide formulations showed Complex II inhibition in isolated mitochondria (Peixoto, 2005). Following treatment and recovery, *C. elegans* were incubated with the electron acceptor NBT. In the presence of functioning Complex II, NBT is reduced, resulting in the formation of a blue precipitate (Grad et al., 2007). Absorbance data were analyzed for each TD concentration (Fig. 2), and indicated that all concentrations resulted in statistically significant decreases in NBT conversion compared to controls ($^*p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$), but not to each other.

3.3. Increased Complex IV activity

Since others had reported that Complex IV activity was unaffected in isolated mitochondria treated with Roundup (Peixoto, 2005), we wanted to assess Complex IV activity in worms to verify that our results were similar to those from isolated mitochondria. Here, we used the electron donor DAB, which when oxidized by active Complex IV forms a brown precipitate (Grad et al., 2007). Analysis of DAB conversion absorbance data (Fig. 3) indicated that treatment with 7.0% TD resulted in a statistically significant increase in absorbance compared to
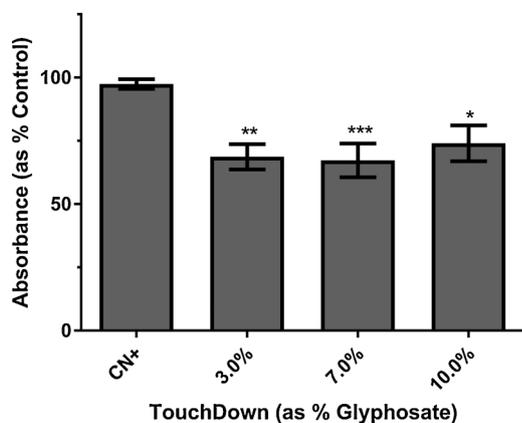
Fig. 2. Direct Complex II activity assessment. Treatment with TD indicated a statistically significant decrease in absorbance compared to control worms. Data are presented as mean absorbance $\pm$ SEM and represent N ≥ 4 separate synchronizations. [**]$p < 0.01$, [***]$p < 0.001$ compared to controls.
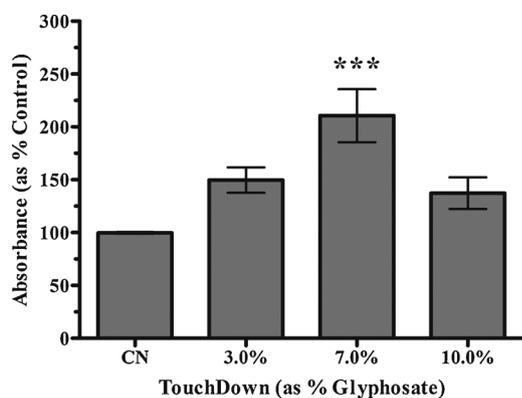

Fig. 3. Complex IV activity following TD treatment. Following treatment with 7.0% TD, N2 worms showed a statistically significant increase in absorbance. Data are presented as mean absorbance $\pm$ SEM and represent N ≥ 4 separate synchronizations. [***]$p < 0.001$ compared to controls.

control ([***]$p < 0.001$). Although a trend was present for the other two concentrations, increased DAB oxidation did not reach the level of statistical significance.

3.4. Decreased proton gradient integrity

To further ascertain whether mitochondrial function was impaired by TD treatment, TMRE was used to assay the integrity of the proton gradient. TMRE accumulates within active mitochondria due to the presence of an intact proton gradient ($\Delta\psi$). Following exposure to TD and TMRE, analysis of red pixel number indicated that only treatment with 10.0% TD resulted in a statistically significant decrease ([*]$p < 0.05$) compared to control (Fig. 4).

3.5. Decreased relative ATP levels

Relative ATP concentrations were measured using a luminescent probe specific for ATP to further assess mitochondrial function (Fig. 5A). Results showed a statistically significant decrease in ATP production compared to control for all TD concentrations. To verify that decreased ATP levels were not due to increased mortality, a fluorogenic peptide substrate was used to detect necrotic protease activity (Fig. 5B). Results indicated no statistically significant difference among any groups, suggesting that decreased ATP was not due to increased lethality.
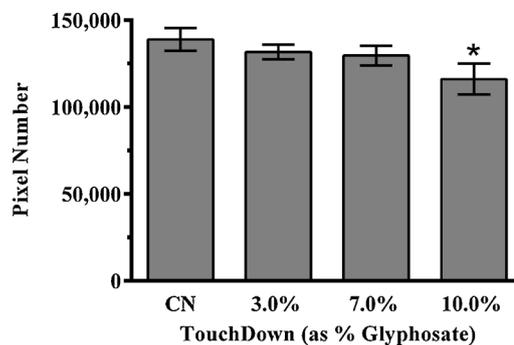

Fig. 4. Proton gradient integrity following TD treatment. Only worms treated with the highest concentration of TD showed a statistically significant decrease in number of red pixels. Data are presented as mean pixel intensity $\pm$ SEM, and represent N ≥ 3 separate synchronizations. [*]$p < 0.05$ compared to control.

3.6. Unchanged superoxide levels

In light of mitochondrial inhibition, we also wanted to assess whether oxidative stress was increased in *C. elegans* following exposure to TD. Initially we used DHE to determine superoxide ($\cdot^-O_2$) production. DHE is oxidized in the presence of $\cdot^-O_2$ resulting in the formation of a red fluorescent product (Michalski et al., 2014; Peshavariya et al., 2007). Analysis of red pixel number indicated that there was no statistically significant difference between treatment groups compared to controls (data not shown).

3.7. Increased hydrogen peroxide levels

Since no significant $\cdot^-O_2$ production was detected, we assessed whether hydrogen peroxide (H_2O_2) was formed. We chose to look at this ROS next because a one-electron transfer to $\cdot^-O_2$ could yield H_2O_2, and thus explain the lack of $\cdot^-O_2$ in the previous studies. Following exposure of worms to TD, Amplex®Red was added to assay tubes. Analysis indicated that TD treatment at all three concentrations resulted in a statistically significant increase in H_2O_2 production (Fig. 6) compared to control worms ([***]$p < 0.001$ for 3.0% TD; [*]$p < 0.05$ for 7.0% TD; and [**]$p < 0.01$ for 10.0% TD).

3.8. Unchanged hydroxyl radical levels

Finally, since hydroxyl radicals (·OH) can be produced from a one-electron transfer to H_2O_2, we measured this ROS following exposure to TD. To this end, *C. elegans* exposed to varying concentrations of TD were further exposed to HPF. In the presence of ·OH, this probe is oxidized and fluoresces. Analysis indicated that there was no statistically significant difference among any of the groups (data not shown).

4. Discussion

Previous research comparing the toxicity of glyphosate alone or the commercially-available formulation of RoundUp (RU; Monsanto) showed that RU was significantly more toxic than glyphosate alone in numerous cell culture systems (Benachour and Seralini, 2009; Kim et al., 2013; Mesnage et al., 2014). This differential toxicity was also observed *in vitro* using isolated mitochondria (Olorunsogo et al., 1979; Peixoto, 2005). Based on these reports and our previous research showing various levels of neurodegeneration in *C. elegans* (Negga et al., 2011, 2012), we sought to test the hypothesis that mitochondrial inhibition and/or oxidative stress could be possible mechanisms through which the glyphosate-containing herbicide Touchdown (TD) exerts its toxicity. The concentrations used in the current studies are consistent with application concentrations recommended for agricultural use (Monsanto, 2014; Syngenta et al., 2010). For example, glyphosate
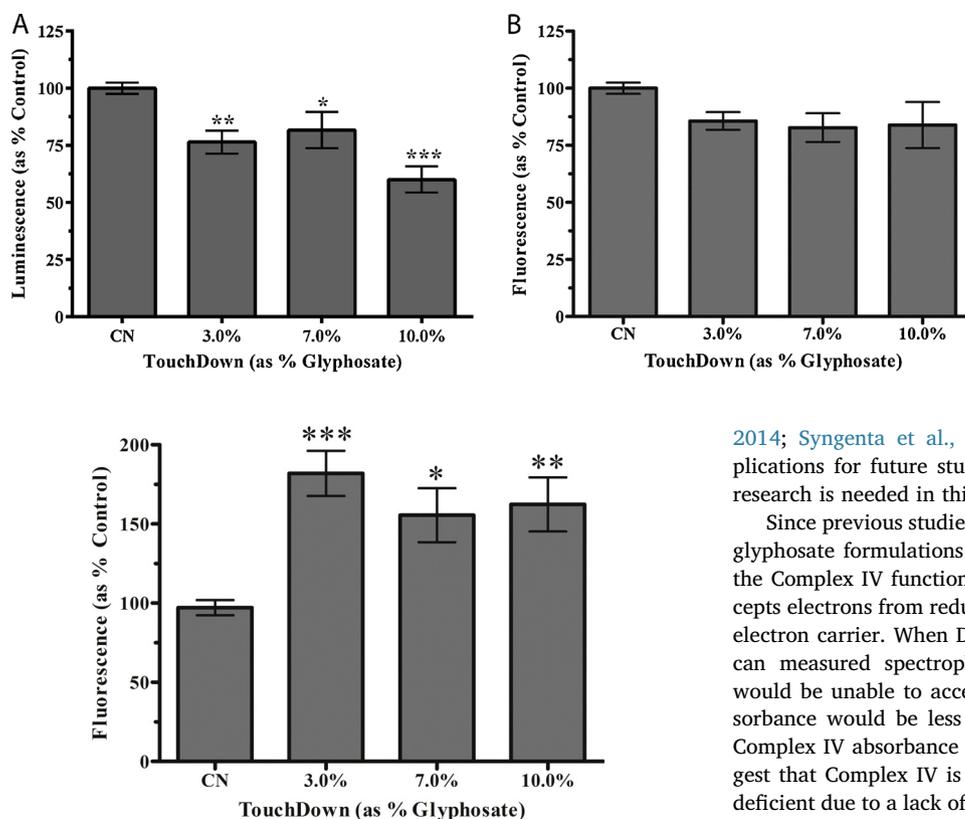
Fig. 5. Relative ATP amount following TD treatment of N2 worms. Following treatment with 3.0% or 10.0% TD, analysis indicated a statistically significant decrease in luminescence, a measure of ATP levels (A), but no statistically significant change in fluorescence, a measure of viability/lethality (B). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to controls.

Fig. 6. Hydrogen peroxide following TD treatment. Treatment at all concentrations of TD showed a statistically significant increase in fluorescence compared to control. Data are presented as mean intensity $\pm$ SEM and represent $N \geq 3$ separate synchronizations. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control.

herbicide formulations are typically sold as "ready-for-use" concentrations of approximately 3% glyphosate, or as concentrated formulations of 37.0–53.2% glyphosate, which are diluted with water to final concentrations ranging from 0.4 to 2.2% for field application and 20–30% for spot application (Syngenta et al., 2010). While some human data are available for glyphosate blood levels in women and children (Knudsen et al., 2017; Kongtip et al., 2017), these populations are typically not the same one that are applying the pesticide in agricultural or industrial settings. Doses for rodent studies are often based on good laboratory practice (GLP) no-observed adverse effect levels (NOAELs), which can be less than a single exposure level to which workers can be exposed (Romano et al., 2010; Tang et al., 2017). It is for these reasons that we used application concentrations for these studies.

Our initial oxygen consumption studies allowed us to assess general mitochondrial inhibition. The results (Fig. 1) showed an overall decrease in oxygen consumption (Fig. 1), suggesting that basal mitochondrial respiration is reduced because of TD treatment. Since oxygen consumption measures the downstream effect of electron transfer to oxygen, we moved to histochemical assays to determine the function of individual complexes. Based on previous studies using isolated mitochondria exposed to RU (Peixoto, 2005), our primary focus next shifted to Complex II and Complex IV.

To assess Complex II activity, we used NBT, which changes color following Complex II-dependent reduction. Consistent with data from Peixoto (2005), we also measured a decrease in Complex II activity related to its ability to reduce NBT. Furthermore, the inhibition was not concentration-dependent at the concentrations we used, suggesting these were above the no observable adverse effect level (NOAEL) for this particular endpoint. Given the fact that our concentrations were within those typically recommended by manufacturers (Monsanto,

2014; Syngenta et al., 2010) these data could have important implications for future studies in rodents, and for human health. More research is needed in this area, however.

Since previous studies indicated that Complex IV was not a target of glyphosate formulations (Peixoto, 2005), we next used DAB to assess the Complex IV function in our *in vivo* model system. Complex IV accepts electrons from reduced cytochrome c, but in our assay DAB is the electron carrier. When DAB is oxidized it leads to a color change that can measured spectrophotometrically. Thus, inhibited Complex IV would be unable to accept electrons from DAB, and the resulting absorbance would be less than that of controls. On the other hand, if Complex IV absorbance is increased relative to controls, the data suggest that Complex IV is functional, but electron starved, *i.e.*, electron deficient due to a lack of electrons from up-stream complexes. Not only did we show that Complex IV is not a target of TD (Fig. 3), we showed that it had a greater capacity to accept electrons relative to controls, as evidenced by the increased absorption. Data from this assay therefore implies that electron transport chain (ETC) enzymes upstream of Complex IV (*e.g.*, Complex III) may be more sensitive targets.

Based on the oxygen consumption and ETC data, we were somewhat surprised by the fact that the proton gradient was only statistically significantly decreased at the highest TD concentration (Fig. 4). All of the ETC enzymes except Complex II are responsible for maintaining the proton gradient (Fig. 7). As such, our TMRE data are entirely compatible with Complex II inhibition, and further suggests that Complex III may also be involved. Although the proton gradient was minimally adversely affected by TD, we still measured lower ATP levels in all treatment groups (Fig. 5). This was most prominent at the highest TD concentration, and is consistent with the general decrease in mitochondrial inhibition we observed in the oxygen consumption data (Fig. 1). Consistent with mitochondrial inhibition, we also observed increased ROS production (Fig. 6).

It is important to note that many pesticides used to model Parkinson's disease inhibit mitochondria (Keeney et al., 2006; Testa et al., 2005) and/or increase oxidative stress (McCormack et al., 2005). Classic toxicants used in Parkinson's disease research (*e.g.*, 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) also exert their toxicity through similar mechanisms of action (Schober, 2004). Clinically, both mitochondrial inhibition and increased ROS are observed in patients with Parkinson's disease (Celardo et al., 2014; Hauser and Hastings, 2013), as well as other neurodegenerative diseases (Barnham et al., 2004; Lin and Beal, 2006). As occupational exposure to glyphosate-containing herbicides (and other pesticides) often occurs multiple times during a growing season, it is imperative to determine if these agrochemicals contribute to mitochondrial inhibition or increased oxidative stress in order to better understand what types of personal protective equipment may be advisable for these workers.

While *C. elegans* is a simple model organism that is quite evolutionarily distant from humans, these worms still provides valuable information for future studies in higher organisms (Teschendorf and Link, 2009). Furthermore, numerous human disease-relevant gene
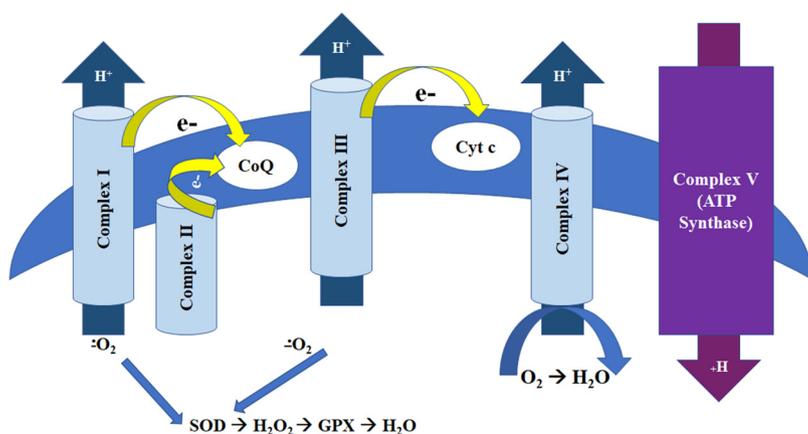
Fig. 7. Schematic of the electron transport chain. Complex II does not contribute to the formation of the proton gradient, which may explain our TMRE data. Reactive oxygen species are normally formed during mitochondrial respiration, particularly at Complexes I and III. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) are responsible for the detoxication of these ROS. Typically an increase in superoxide ($^{\bullet}O_2$) is associated with Complex I inhibition, and hydrogen peroxide (H_2O_2) formation is more common with Complex III inhibition.

homologues and orthologues related to neurotransmitter synthesis, transport, catabolism and clearance, and receptors (Bargmann and Kaplan, 1998; Chase and Koelle, 2007; Jin, 2005) are highly conserved in *C. elegans*. As such, *C. elegans* are widely-used in various toxicology and neurodegenerative studies (Wolozin et al., 2011) to refine and reduce the number of studies required in mammals. Here, we demonstrate that *C. elegans* acutely exposed to occupationally-relevant concentrations of TD exhibit mitochondrial inhibition and oxidative stress *in vivo*. Given these findings, we suggest that additional work is required in higher organisms using the pesticide formulations to better characterize potential risk of neurodegeneration in humans.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.etap.2018.12.019.

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