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Exposure to Mn/Zn Ethylene-*bis*-Dithiocarbamate and Glyphosate Pesticides Leads to Neurodegeneration in *Caenorhabditis elegans*

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Abstract

Epidemiological evidence suggests positive correlations between pesticide usage and the incidence of Parkinson's disease (PD). To further explore this relationship, we used wild type (N2) *Caenorhabditis elegans* (*C. elegans*) to test the following hypothesis: Exposure to a glyphosate-containing herbicide (TD) and/or a manganese/zinc ethylene-*bis*-dithiocarbamate-containing fungicide (MZ) may lead to neurotoxicity. We exposed N2 worms to varying concentrations of TD or MZ for 30 min (acute) or 24 hours (chronic). To replicate agricultural usage, a third population was exposed to TD (acute) followed by MZ (acute). For acute TD exposure, the LC₅₀ = 8.0% ($r^2 = 0.6890$), while the chronic LC₅₀ = 5.7% ($r^2 = 0.9433$). Acute MZ exposure led to an LC₅₀ = 0.22% ($r^2 = 0.5093$), and chronic LC₅₀ = 0.50% ($r^2 = 0.9733$). The combined treatment for TD + MZ yielded an LC₅₀ = 12.5% ($r^2 = 0.6367$). Further studies in NW1229 worms, a pan-neuronally green fluorescent protein (GFP) tagged strain, indicated a statistically significant ($p < 0.05$) and dose-dependent reduction in green pixel number in neurons of treated worms following each paradigm. This reduction of pixel number was accompanied by visual neurodegeneration in photomicrographs. For the dual treatment, Bliss analysis suggested synergistic interactions. Taken together, these data suggest neuronal degeneration occurs in *C. elegans* following treatment with environmentally-relevant concentrations of TD or MZ.

Keywords

Glyphosate; mancozeb; *C. elegans*; pesticides; NW1229; neurodegeneration

1.0. Introduction

1.1. Pesticide Usage and Parkinson's Disease (PD)

Epidemiological studies support a relationship between pesticide usage and Parkinson's disease (PD) (Ascherio et al., 2006; Hancock et al., 2008; Tanner et al., 1999); however, it is unclear as to which pesticides and what mechanisms of action may contribute to the

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disease's etiology. Other studies suggest that occupational exposure to various metals, including iron (Fe) or manganese (Mn), is risk a factor for PD and/or parkinsonism (Gorell et al., 2004; Hernandez et al., 2002; Racette et al., 2005). Additionally, it is well-documented that various mitochondrial complex I inhibitors induce parkinsonian symptoms in humans and animals (Keeney et al., 2006; Piccoli et al., 2008; Testa et al., 2005). Taken together, data from these studies suggest that pesticides containing Mn (Ferraz et al., 1988; Israeli et al., 1983a, 1983b) or Fe, or those capable of inhibiting mitochondrial respiration (Astiz et al., 2009; Liou et al., 1997; Richardson et al., 2005), may be putative contributors to the onset of neurodegeneration associated with PD.

1.2. Model Organism: *C. elegans*

One difficulty of studying mechanisms of pesticide toxicity *in vivo* is the complexity of most organisms. Although rodents and non-human primates have been used with much success in toxicology studies, downsides include the significant cost of maintaining them throughout their life span, from 14 months to many years, respectively. As parkinsonism is associated with increasing age, this makes chronic, low-level exposure studies difficult and expensive. Recently the nematode *C. elegans* has gained popularity in neurotoxicology testing (Leung et al., 2008; Peterson et al., 2008). While a handful of labs (Easton et al., 2001; Ruan et al., 2009; Saffih-Hdadi et al., 2005; Saha et al., 2009; Svendsen et al., 2010) have examined the gross toxicity of pesticides in *C. elegans*, to our knowledge no labs have looked in this model system at specific neurodegeneration related to PD following exposure to various herbicides or fungicides.

Such studies are greatly simplified in *C. elegans*, compared to more traditional animal models, because it is a simple organism whose genome, which has significant homology to the human genome, and cell lineage are completely mapped. Additionally, its nervous system is quite simple compared to that of humans and mammals as it contains only 302 (hermaphrodite) or 381 (male) neurons (Chalfie and White, 1988). Other useful features of *C. elegans* include the fact that they are transparent, have a short lifespan, and produce hundreds of offspring in each generation (Wood, 1988). With this in mind, we initially investigated whether *C. elegans* would be an appropriate model for studying a glyphosate-containing herbicide, TouchDown (TD), and a manganese (Mn)-containing fungicide, mancozeb (MZ). Thus, we used N2 (wild-type) worms to determine dose response curves, and later used NW1229 (pan-neuronal green fluorescent protein (GFP) tagged) worms to determine whether exposure to our pesticides of interest could induce regionally specific neurodegeneration.

1.3. Glyphosate-containing herbicides

Glyphosate-containing herbicides are some of the most prevalently used agrochemicals in the world (Woodburn, 2000). In fact, many important food crops (wheat, corn, soybeans) have been genetically modified so they are resistant to these herbicides, providing weed control without crop damage (Dewar, 2009; Gardner and Nelson, 2008). Glyphosate (Figure 1A), a glycine analogue and active ingredient in RoundUp and TouchDown (TD), is relatively non-toxic (oral LD₅₀ = 4320 to 5600 mg/kg) to rats (Committee, 1979; Worthington, 1983). For pesticide formulations, however, glyphosate is combined with additional chemicals, which are typically listed only as “inert ingredients” and whose toxicity may be unknown in combination with the active ingredient.

For example, a recent study examined the effects of RoundUp or glyphosate alone on mitochondrial oxidative phosphorylation (Peixoto, 2005). This *in vitro* data demonstrated that the commercial formulation, but not glyphosate alone, significantly decreased the ADP/oxygen ratio at concentrations as low as 0.5 mM glyphosate (the lowest concentration

tested), suggesting that exposure to the pesticide, but not the active ingredient alone, lead to mitochondrial dysfunction. These data confirm previous reports that commercial formulations of glyphosate inhibit mitochondria (Bababunmi et al., 1979; Olorunsogo and Bababunmi, 1980; Olorunsogo et al., 1979). Since those who use these pesticides are exposed to the commercial formulations, and not pure glyphosate, this gap in the literature indicates that further studies are necessary to examine whether treatment with the pesticide formulation may lead to neurodegeneration similar to that observed in PD.

1.4. Mn-Containing Fungicides

Mancozeb (MZ), whose active ingredient is Mn/zinc (Zn)-ethylene-*bis*-dithiocarbamate (Mn/Zn-EBDC; Figure 1B) is a contact fungicide that inhibits enzyme activity by forming a complex with enzymes involved in ATP production (Cornell, 1987). It is a widely-used fungicide, with a total application over the past ten years hovering around 3.6 million kg annually (Gianessi and Reigner, 2006; Giannesse and Marcelli, 2000). Studies indicate that MZ may have pro-apoptotic effects (Calviello et al., 2006), induce neurodegeneration in the nigrostriatal dopamine system, and lead to subsequent vulnerability to additional environmental toxicants (Domico et al., 2007; Domico et al., 2006; Soleo et al., 1996). Data also suggest that, like glyphosate-containing herbicides, MZ inhibits mitochondrial respiration (Domico et al., 2006; Zhang et al., 2003).

1.5. Overview of Treatment Paradigms

In order to investigate the hypothesis that the commercial formulation of these pesticides (TD and MZ), and not simply their active ingredient(s), may contribute to neurodegeneration, we initially established LC₅₀s for acute and chronic treatments of N2 *C. elegans* with TD or MZ. As most agricultural applications include exposure to multiple pesticides, we also examined *C. elegans* acutely treated with TD followed by an acute exposure to MZ. This latter paradigm is based on the order in which farmers or other agricultural workers are exposed to these chemicals during growing seasons. Lastly, we repeated the exposures in NW1229 worms to determine whether TD and/or MZ contribute to general neurodegeneration in this model organism.

As practically no data relating pesticide usage to neurodegeneration have been generated in *C. elegans*, the focus of the current work was to determine whether TD and/or MZ could produce toxicity in *C. elegans* at environmentally relevant concentrations, and to determine whether neurodegeneration was apparent at the same concentrations. Thus, this work provides systematically established concentration parameters for the commercial formulations of TD and MZ, as well as sets the stage for delineation of potential mechanisms of general toxicity or neurotoxicity in the model organism *C. elegans*.

2.0. Materials and Methods

2.1. Worm and *E. coli* Strains

Wild-type *C. elegans* (N2) and NW1229 worms, as well as NA22 *E. coli* and OP50 *E. coli* (an uracil auxotroph) were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by the National Institutes of Health (NIH) National Center for Research Resources (NCRR). NW1229 expresses a pan-neuronal green fluorescent (GFP) pattern due to the integration of Ex[F25B3.3::GFP; dpy-20(+)] in dpy-20(-) background.

2.2. Worm Maintenance and Treatment

Worms were maintained at 20°C and synchronized according to standard protocols (Brenner, 1974). Briefly, gravid worms grown on 8P plates (51.3 mM NaCl, 25.0 g bactoagar/L, 20.0 g bactopectone/L, 1 mM CaCl₂, 0.5 mM potassium phosphate buffer (pH

6), 0.013 mM cholesterol (in 95% ethanol), 1 mM MgSO_4) with a lawn of NA22 *E. coli* (grown in 16 g tryptone/L, 10 g yeast extract/L, 85.5 mM NaCl), were synchronized by exposure to a solution of sodium hypochlorite (4.0 mM) and sodium hydroxide (0.5 mM), and monitored under the microscope until worms released eggs. Approximately 18 h following isolation and purification of eggs, synchronous L2 *C. elegans* were exposed to varying concentrations of TD (Touchdown Hitech, formulation with [52.3% glyphosate] from Syngenta AG, Wilmington, DE), MZ (Mancozeb with Zinc Flowable [37% Mn/Zn-EBDC] from Bonide Products, Inc., Oriskany, NY), or a sequential treatment of the two (TD/MZ). In the combination (dual) treatment, all worms were initially exposed to 2% glyphosate (recommended application concentration of glyphosate in TD) for 30 min (acute), followed by varying concentrations of Mn/Zn-EBDC (as mancozeb) for 30 min (acute). To facilitate comparison of our results with other glyphosate or Mn/Zn-EBDC pesticides, concentrations are reported as percent active ingredient rather than percent pesticide.

For acute pesticide treatments, worms were exposed to the respective pesticide for 30 min, washed at least 3× with sterile dH_2O , then placed on fresh nematode growth media (NGM; 51.3 mM NaCl, 17.0 g bactoagar/L, 2.5g bactopectone/L, 1 mM CaCl_2 , 1 mM MgSO_4 , 0.5 mM potassium phosphate buffer (pH 6.0), 12.9 mM cholesterol in 95% ethanol, 1.25 mL nystatin/L, 0.2 g streptomycin/L) plates, with a lawn of OP50 *E. coli* (grown in 25 g Luria broth/L, 200 mg streptomycin/L). Following a 24-h incubation at 20°C, either the number of live worms was counted using a binocular dissection microscope, or pictures were taken using a digital camera attached to a fluorescence microscope (see below).

For chronic treatments, synchronous L2 worms were exposed to the respective pesticide for 30 min, then, without additional washes, placed on NGM plates seeded with a lawn of OP50 *E. coli* and counted or photographed after a 24-h incubation period at 20°C.

In agricultural settings, concentrated TD and MZ are diluted with water to the appropriate application concentrations. As such, for all treatments paradigms (acute, chronic or dual), control worms were treated with sterile dH_2O .

2.3. Counting Worms

Twenty-four hours post-treatment, the number of living worms was counted using a binocular dissection microscope with transparent graph paper placed beneath the plates to facilitate counting. During counting, live worms (those that were rounded and moved in sigmoidal patterns) were differentiated from dead worms (those that were motionless and had a straightened body). From each plate, 8 - 10 different representative blocks, picked at random from the entire plate, were counted. The average number of worms from these blocks was calculated, and multiplied by the total number of blocks to approximate the number of live worms on each plate. Treatment counts were normalized to controls ([mean treated number of worms/mean control of worms] × 100) from each synchronization in order to account for variation among unique synchronizations.

2.4. Fluorescence Microscopy

For studies involving NW1229 worms, photomicrographs were taken of 4 - 10 worms on 4% agarose pads from at least four separate synchronizations. Fluorescence observations were performed using an epifluorescence microscope (Leitz & Wetzlar, Halco Instruments, Inc) equipped with a 50-W AC mercury source lamp (E. Leitz, Rockleigh, NJ) and 40× objective (Leitz & Wetzlar, Halco Instruments, Inc). The microscope was coupled to a digital camera (Micrometrics, MilesCo Scientific, Princeton, MN) operated by Micrometrics software (Micrometrics SE Premium, v2.7) for image acquisitions. In order to standardize

image acquisition, the Micrometrics software was programmed so that pictures were obtained under identical levels of exposure time, gain, gamma, saturation and color gain. Images were processed with Adobe Photoshop 6.0.1, and green pixel number $\pm$ the standard deviation was obtained following selection of green pixels.

2.5. Statistical Analysis

A dose-response curve, including non-linear curve fitting, LC_{50} s, 95% confidence intervals (CIs) and regression coefficients, was generated using GraphPad Prism (v5.03 for Windows, GraphPad Software, San Diego, CA). Each point on the dose-response curve is presented as mean $\pm$ SEM, where $N \geq 4$ separate synchronizations completed in at least triplicate ($n \geq 3$) for the respective pesticide concentrations. Data are presented as percent active ingredient (% glyphosate for TD, and % Mn/Zn-EBDC for MZ) in order to facilitate future comparisons with other pesticides with similar active ingredients.

For studies involving number of green pixels, data are presented as mean $\pm$ SEM and represents $N \geq 4$ separate synchronizations, of at least three independent replicates ($n \geq 3$) of 8 - 10 worms per intra-experimental replication, for each treatment paradigm. Differences in pixel number were determined by one-way analysis of variance (ANOVA), followed by a *post-hoc* Bonferroni test. Data was considered to be statistically significant when $p < 0.05$.

The Bliss additivism model was used to classify the effect of combining TD and MZ as additive, synergistic or antagonistic (Buck et al., 2006). Using the equation $E_{\text{Bliss}} = E_{\text{TD}} + E_{\text{MZ}} - (E_{\text{TD}} \times E_{\text{MZ}})$, where E_{TD} and E_{MZ} are the fractional inhibitions obtained by TD alone and MZ alone at specific concentrations of each of the pesticides used in the dual treatment protocol. E_{bliss} is defined as the fractional inhibition expected if the combination of the two pesticides was exactly additive. If the experimentally measured inhibition was less than E_{bliss} , the combination was considered as synergistic. If the experimentally measured inhibition was greater than E_{bliss} , the combination determined to be antagonistic.

Additionally, the potential contribution of acute treatments of either TD or MZ to the dual treatment was determined by calculating the percent change in pixel numbers compared to control ($[\text{total pixel number/average pixel number of control}] \times 100$), for the LC_{25} , LC_{50} and LC_{75} for either MZ or the TD/MZ concentrations. The results for TD were only calculated at 3%, the approximate concentration of TD used for all dual treatments (Table 2). For each concentration (LC_{25} , LC_{50} or LC_{75}) data were compared to each other using one-way ANOVA, followed by Bonferroni post-test, with the independent variable of interest being the treatment (TD vs. MZ vs. dual).

Results

3.1. Acute Pesticide Treatments

To determine the LC_{50} for acute exposure to TouchDown (TD) or Manzate (MZ), worms were treated as described. For worms acutely treated with TD (Figure 2A), the $LC_{50} = 8.0\%$ glyphosate (95% confidence interval (CI): 6.4 - 9.7%; $r^2 = 0.6890$); for MZ, (Figure 2B), the $LC_{50} = 0.22\%$ Mn/Zn-EBDC (95% CI: 0.072 - 0.65%; $r^2 = 0.5093$), which was lower than the recommended application concentration of 0.69% Mn/Zn-EBDC for MZ. It should be noted that concentrations greater than 1% Mn/Zn-EBDC did not result in any additional worm death, even at the highest concentration 30% of MZ tested (Figure 2B).

3.2. Chronic Pesticide Treatments

In order to model the multiple pesticide applications to which workers are exposed throughout a significant fraction of their life time, we also used a chronic paradigm to

generate LC₅₀s for both pesticides. For worms treated with TD (Figure 3A), the LC₅₀ = 5.7% glyphosate (95% CI: 5.3 - 6.2%; $r^2 = 0.9433$); for MZ, (Figure 3B), the LC₅₀ = 0.50% Mn/Zn EBDC (95% CI: 0.14 - 0.87%; $r^2 = 0.9733$ for curve).

3.3. Dual Pesticide Treatments

As most agricultural workers are exposed to multiple pesticides during a growing season, we also treated worms with TD (used agriculturally to prepare a field for planting and halt initial weed growth) followed by MZ (used to protect crops mid- to late season from fungi). This environmentally relevant treatment paradigm was used to better model field applications. Worms were treated with 2% glyphosate (recommended application concentration) followed by varying concentrations of MZ (Figure 4). For this study, the LC₅₀ of the dual treatment was 12.5% (95% CI = 10.3 - 22.6%; $r^2 = 0.6367$).

3.4. Decreased GFP Pixel Number Following TD Treatment

We next determined whether exposure to TD could lead to general neurotoxicity in *C. elegans* NW1229 worms following treatment with varying concentrations (approximately equal to the LC₂₅, LC₅₀ or LC₇₅ for each respective paradigm) of glyphosate either acutely or chronically (Figure 5). The number of green pixels in the nerve ring, which contains mainly dopaminergic (DAergic) neurons, was analyzed separately from those of the ventral nerve cord, which contains GABAergic, cholinergic and serotonergic neurons (Rand and Nonet, 1997a). Following acute treatment with TD, a statistically significant reduction in green pixel number was first observed in the nerve ring (Figure 5A) at 3% glyphosate (**p < 0.01).

Compared to control worms (Figure 5B), photomicrographs taken at 3% glyphosate revealed the formation of minor gap between both the ventral and dorsal nerve ring and the cuticle (Figure 5C). When the glyphosate concentration was increased to 7% or 10%, the number of green pixels in the ventral nerve cord (Figure 5A) also decreased in a statistically significant and dose-responsive manner (**p < 0.001). Photomicrographs of worms treated 10% glyphosate (Figure 5D) also exhibited an increased gap between the ventral and dorsal nerve ring and the cuticle. Furthermore a decrease in length of the retrovesicular ganglia was evident (Figure 5D). Finally, ventral nerve cord projections were much thinner than those of control worms, and the corresponding soma were less defined (Figure 5D).

Following chronic treatments, a reduction of pixel number in the nerve ring was observed (Figure 5E). This reduction reached statistical significance (*p < 0.05) at the concentration corresponding with the LC₇₅. Similar to the degeneration observed in acutely-treated worms, photomicrographs (Figure 5G) of worms treated with 9.8% glyphosate indicated the formation of a gap between the dorsal and ventral nerve ring and the cuticle, and thinning of the ventral nerve cord.

3.5. Decreased GFP Pixel Number Following MZ Treatment

Similar studies were completed using NW1229 worms treated either acutely or chronically with MZ (Figure 6). As before, data were analyzed in groups separated into nerve ring or ventral nerve cord. Compared to control worms, those treated acutely with MZ showed a statistically significant and dose-dependent reduction in pixel numbers only in the DAergic neurons of the nerve ring (Figures 6A) from 1.6% to 17% Mn/Zn-EBDC (*p < 0.05). The initial value corresponded to the first concentration of MZ at the beginning of the 50% live/dead asymptote (Figure 2B). Compared to control worm (Figure 6B), photomicrographs of worms treated with 7.5% (Figure 6C) or 17% (Figure 6D) Mn/Zn-EBDC indicated the formation of a gap between the ventral and dorsal nerve ring and the cuticle, as well as a decreased extension of the retrovesicular ganglia. Interestingly, worms treated with 30%

Mn/Zn-EBDC more closely resembled control worms than *C. elegans* treated with lower concentrations (data not shown).

In contrast to acute treatments, worms chronically exposed to varying concentrations of MZ showed a dose-dependent statistically significant decrease in pixel number, beginning at 0.1% Mn/Zn-EBDC (a concentration below the LC_{25}), in neuron in both the nerve ring ($***p < 0.001$) and ventral nerve cord ($^{\wedge}p < 0.01$; Figure 6E). Compared to control worms (Figure 6F), photomicrographs of worms treated with 0.1% (Figure 6G) or 1.7% (Figure 6H) Mn/Zn-EBDC indicate the appearance of a gap between the ventral and dorsal nerve ring and cuticle. This is accompanied by an apparent thinning of projection, and a decrease in the size of the soma along the ventral nerve cord. In addition, photomicrographs of worms treated with 1.7% Mn/Zn-EBDC show a decrease in the length of the retrovesicular ganglia (Figure 6H).

3.6. Decreased GFP Pixel Number Following Dual TD/MZ Treatment

For the dual treatment, a statistically significant decrease ($*p < 0.05$) in green pixel number was only observed for the highest concentration of Mn/Zn-EBDC (20%), and only in the nerve ring (Figure 7A). Compared to control worms from this treatment paradigm (Figure 7B) photomicrographs of worms treated with 20% Mn/Zn EBDC (Figure 7C) indicated the formation of a small gap between the dorsal nerve ring and the cuticle, with a shortening of the dorsal extension of the nerve ring.

3.7 Bliss Analysis and Potential Contribution of Individual Pesticides

Further analysis of the dose-dependent relationship in the acute dose response curves, and the pixel analysis for the nerve ring and nerve cord was completed. For the dose-response curve data, only the acute curves were used since the dual treatment involved only acute treatments with either TD or MZ. E_{Bliss} for the combination (Table 1) confirmed that the treatment of TD followed by MZ resulted in a synergistic effect in the dual treatment. This synergism was also observed in the nerve ring analysis of the NW1229 worms, where greater reduction in pixel number was detected following the dual treatment compared to exposure to either pesticide alone. Interestingly, this was not the case in the nerve cord, where the pesticide interaction depended on the MZ concentration (Table 1). Rather treatment with the first two concentrations of MZ (10% or 12% Mn/Zn-EBDC) resulted in an apparent synergistic interaction, while the 20% Mn/Zn-EBDC with the 2% glyphosate was interpreted as antagonistic.

Further analysis of the dose-dependent decrease in pixel number from each concentration of MZ or TD in the dual treatment paradigm indicated a differential contribution of each pesticide, depending on whether the nerve ring or the ventral nerve cord was analyzed. Although none of the one-way ANOVAs reached statistical significance for the nerve ring, the data suggest that changes in the nerve ring are more closely related to the influence of MZ rather than that of TD (Table 2). Conversely, analysis for the ventral nerve cord showed a statistically significant decrease in pixel number at both the LC_{50} ($p = 0.0153$) and LC_{75} ($p = 0.003$) that was similar between the dual and the TD (Table 2).

Discussion

4.1. Mitochondrial Inhibition as a Link to Glyphosate and Parkinson's Disease (PD)

Pesticide usage and PD have been positively correlated in numerous studies (Elbaz et al., 2009; Gatto et al., 2009); however, due to the tremendous numbers of pesticides available, it has been difficult to link a specific pesticide to PD. Although studies have examined the potential role of organophosphates (Manthripragada et al., 2010) and Mn-containing

fungicides (Ferraz et al., 1988; Meco et al., 1994) in the etiology of PD or parkinsonism, few have investigated the widely-used glyphosate-containing herbicides, e.g. RoundUp and TouchDown (TD).

Interestingly, in 1987, 2,700 - 3,600 metric tons of glyphosate-containing herbicides were applied in the United States (US), making it the 17th most-commonly used conventional pesticide (Donaldson et al., 2004; Kiely et al., 2004). By 2001, 39,000 - 41,000 metric tons of glyphosate were applied, making it the most-commonly applied pesticide (Donaldson et al., 2004; Kiely et al., 2004). Recent *in vitro* data demonstrate that RoundUp, but not the active ingredient glyphosate, may be a potent mitochondrial inhibitor (Peixoto, 2005). Furthermore, previous studies with *N*-(phosphonomethyl)-glycine also suggest that at least one glycine analogue may lead to mitochondrial inhibition (Olorunsogo, 1982; Olorunsogo, 1990; Olorunsogo et al., 1979; Olorunsogo et al., 1980).

Considering the relationship between mitochondrial inhibition and PD (Moraes and De Strooper, 2010), we chose to examine the potential of a representative glyphosate-containing herbicide (TD) to induce neurotoxicity in the model organism, *C. elegans*. It is well-documented that neurodegeneration in PD patients occurs in the neurons of the *substantia nigra*. In *C. elegans* most of the dopaminergic (DAergic) neurons are located in the nerve ring. There are additional DAergic neurons in the tail of the male, but we chose not to examine these, and rather focused our analysis on the head region. Our goal was to determine whether exposure to TD could induce DAergic neurodegeneration in *C. elegans*.

4.2. The relationship of Mn-EBDC-Containing Fungicides and PD

While the fungicide maneb (Mn-EBDC) is currently being phased out of usage in the US (Billingslea, 2009), application of manzate (MZ), a closely-related analogue containing Mn/Zn-EBDC is actually increasing. For example, MZ usage in 1987 was estimated at 1,800 - 2,700 metric tons, making it the 21st most widely applied pesticide; by 2001, an estimated 2,700 - 3,600 metric tons were applied, raising it to 20th (Donaldson et al., 2004; Kiely et al., 2004). Additionally, MZ exposure has been linked to PD and parkinsonism *in vivo* (Israeli et al., 1983a;1983b), and to nigrostriatal degeneration *in vitro* (Domico et al., 2006; Soleo et al., 1996). Thus, if MZ induces DAergic neurodegeneration in *C. elegans*, then neurons in the anterior region of the worm should be affected by treatment with this fungicide, as outlined in section 4.1. Mn, however, is known to induce GABAergic neurodegeneration in the neurons of the *globus pallidus*. In *C. elegans*, GABAergic neurons are found in the ventral nerve cord, along with some cholinergic neurons. Thus, our lab analyzed the ventral nerve cord of the NW1229 worms to determine if MZ affected GABAergic, DAergic or both neuronal populations.

As a result of the growing usage of both of these pesticides (TD and MZ), we sought to determine whether exposure to these individual pesticides, or to a sequential application of TD followed by MZ, would lead to changes in green fluorescent protein (GFP) expression in the neuronal populations described above. As most previous research using these pesticides has been completed either in cell culture or *in vivo* (mainly using rats or mice), our work also reinforces the importance of *C. elegans* as a model system for pesticide-linked neurotoxicity studies.

4.3. LC₅₀s for Each Treatment Paradigm

Our initial studies (Figures 2-4) were designed to establish LC₅₀s in wild-type worms (N2) for each treatment paradigm. This was important in order to verify that *C. elegans* were susceptible to concentrations to those pesticide formulations within environmentally-relevant ranges. This was indeed the case, particularly for the chronic exposure paradigms.

The recommended application concentration for TD for fields with weeds over 15 cm tall is 0.6-0.9% glyphosate, although the concentration can be as high as 4-10% glyphosate for spot spraying (Syngenta, 2010). Typically, however, glyphosate products for use by the general public are sold with 2% glyphosate, an apparent general use concentration. The acute and chronic LC₅₀s for TD were 8.0% and 5.7%, respectively (Figures 2A and 3A), which, while higher than the general use concentration, were well within the recommendation for agricultural spot spraying. As such, the LC₅₀ data suggest that *C. elegans* are vulnerable to TD at levels well within environmentally relevant concentrations.

The recommended application concentration for MZ is 0.29-0.49% Mn/Zn-EBDC (Bonide, 2010). Data from the LC₅₀ studies indicate that the acute LC₅₀ = 0.22%, while the chronic LC₅₀ = 0.50% (Figures 2B and 3B). Both of these are also well within the recommended concentration applications, again suggesting that *C. elegans* are a relevant and useful model organism for these studies. Typically, it would be anticipated that the chronic LC₅₀ would be lower than that of the acute LC₅₀, due to the increased exposure time. Interestingly, and somewhat unexpectedly, the LC₅₀ for acute MZ treatment was lower than that of the chronic treatment. Further investigation into the mechanism of action of MZ will be necessary to explain this observation, but this was beyond the scope of the current studies.

The dual treatment paradigm (Figure 4) was designed to mimic the exposure common to agricultural workers. To prevent potential cross-reaction between TD and MZ, worms were thoroughly washed following exposure to 2% glyphosate, a mid-range concentration that took into consideration the various application procedures. Interestingly, the LC₅₀ for the dual treatment (12.5%) was shifted to the right compared to that of either acute treatment alone. This may suggest that, following treatment with TD, compensatory mechanisms were induced that ameliorated the toxicity previously observed with MZ treatment alone. Such induction is not uncommon following exposure to a toxicant, but remains to be seen for this model system.

4.5. Analysis of Photomicrographs of Treatment Paradigms

In addition to dose-response curves using wild-type (N2) worms, pan-neuronal GFP-tagged *C. elegans* were also treated with various concentrations (approximately equal to the LC₂₅, LC₅₀ or LC₇₅) of the respective pesticides or dual treatment concentrations. In each paradigm, we observed statistically significant decreases in the number of green pixels in the nerve ring, ventral nerve cord, or both. As mentioned previously, the anteriorly-located nerve ring is predominantly dopaminergic (DAergic) neurons, while the ventral nerve cord contains GABAergic, cholinergic and serotonergic neurons (Rand and Nonet, 1997a; Rand and Nonet, 1997b). As such, we analyzed the two regions separately to determine if there was potential differential neuronal sensitivity to the two pesticides. In order to approximate neuronal degeneration, we analyzed photomicrographs to determine the number of green pixels in each region following treatment (Figures 5-7). While not definitive evidence of neurodegeneration, this method, along with visual analysis of photomicrographs, suggests that treatment with pesticides used in these studies may lead to neurotoxicity.

In both the acute and chronic treatments with TD, loss of green pixel number was observed at concentrations corresponding to either the LC₂₅ (3% glyphosate in acute studies) or the LC₇₅ (9.8% glyphosate in chronic studies). Photomicrographs of the acute LC₂₅ and chronic LC₇₅ glyphosate treatments indicate the formation of a gap between the ventral and dorsal nerve ring and the cuticle (Figures 2 and 5). Overall, however, these data suggest that exposure to TD may lead to a general neurotoxicity, with the DAergic neurons perhaps slightly more vulnerable to TD exposure than other populations.

There was a marked difference between the acute and chronic MZ treatment, however. Following acute MZ exposure, decreases in green pixel number were observed only in the nerve ring (Figure 6A), which was visually confirmed in photomicrographs by the presence of a gap between the ventral and dorsal nerve ring and the cuticle. A decrease in length of the retrovesicular ganglia (Figure 6 C-D) was also observed. Chronic treatment resulted in loss of green pixels in both the nerve ring and ventral nerve cord (Figure 6E). Even at the lowest concentrations of MZ tested (0.1% Mn/Zn-EBDC), photomicrographs indicate the formation of a gap between the dorsal nerve ring and the cuticle along, coincident with thinning of the ventral nerve cord and the decrease size of the soma (Figure 6G). This suggests that in *C. elegans*, nerve ring neurons (DAergic) may be more sensitive to treatment with this fungicide than other neuronal populations, supporting observations previously reported in mammalian models (Morato et al., 1989; Zhang et al., 2003) and humans (Meco et al., 1994).

Interestingly, the dual treatment protocol had a neuronal pixel profile completely different from that of either pesticide alone (Figure 7). As worms were initially treated with TD followed by MZ, it was expected that the trends observed in the dual treatment would be similar to those from acute treatments with the individual pesticides. Thus, we hypothesized that green pixels associated with the nerve ring would decrease with the lowest concentration of Mn/Zn-EBDC (1.6%; Figure 6A), particularly since the worms were pre-treated with TD. Rather, in the dual treatment paradigm, the nerve ring was only affected at the highest concentration of MZ (20% Mn/Zn-EBDC; Figure 7), and there was no measurable loss of pixels in the ventral nerve cord for any fungicide amount studied. Compared to controls (Figure 7B), photomicrographs of 20% Mn/Zn-EBDC (Figure 7C) indicate the formation of a small gap between the dorsal nerve ring and the cuticle with the commonly observed shortening of the dorsal extension of the nerve ring.

Further analysis of the data from the dual treatment suggested that the sequential application of the two pesticides generally resulted in a synergistic effect. The only exception was that of 20% Mn/Zn-EBDC in the dual treatment paradigm in the nerve cord (Table 1). Additionally, decreased pixel number in the nerve ring may be due to the influence of MZ rather than TD, while the decrease in pixel number observed in the ventral nerve cord is perhaps more reflective of the influence of TD (Table 2). Similar to the shift in the dose-response curve of the dual treatment compared to that of MZ, the synergistic interactions observed in the NW1229 strain (Table 1) may suggest that is likely that initial treatment with TD primes the worms for further environmental injury. Since both TD and MZ are suspected mitochondrial inhibitors, and mitochondrial inhibition leads to increases in oxidative stress, perhaps TD treatment initiates mitochondrial injury or inhibition that is exacerbated by the subsequent application of MZ. Further work will be necessary to fully examine this hypothesis, however.

5.1. Conclusion

Our studies demonstrate that *C. elegans* are vulnerable to glyphosate-containing herbicides and Mn/Zn-EBDC-containing fungicides at environmentally relevant concentrations, suggesting that these worms are a valuable and viable model system for future testing involving these pesticides. In relationship to those concentrations tested in our studies, it is important to note, especially for TD, that both agrochemicals can be readily purchased in concentrations much higher than those used in common applications. Thus, humans (and local fauna) could potentially be exposed to concentrations much higher than those reported here. While concentrations at the upper end of the dose-response curve were higher than recommended application concentrations, they are likely still relevant in human exposure paradigms.

For studies involving the NW1229 worms, however, we focused only on those concentrations that were at the LC₂₅, LC₅₀ and LC₇₅, rather than repeating all concentrations used in the dose-response curve. Based on the dose-dependent loss of green pixels in these worms, our data suggest that, similar to result in mammalian models, treatment with TD and/or MZ leads to neurotoxicity in *C. elegans* as well. The most notable visible signs of degeneration resulted in the formation of gaps between the nervous system and the inner cuticle, and a thinning of the nerve cord. While this general pattern was observed in all treatment paradigms, it was most pronounced for worms treated with MZ.

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(A)

(B)

Figure 1.

Chemical structures of pesticide active ingredients. **(A)** Structure of glyphosate, the active ingredient in the herbicides Touchdown (TD) and RoundUp. **(B)** Structure of manganese/zinc-ethylene-*bis*-dithiocarbamate (Mn/Zn-EBDC), the active ingredient in mancozeb and mancozate.

(A)

(B)

Figure 2.

Acute treatment with TouchDown (TD) or Manzate (MZ). (A) Dose-response curve of acute TD indicated that the LC_{50} = 8.0% glyphosate (95% confidence interval (CI): 6.4 - 9.7%; r^2 = 0.6890). (B) Dose-response curve of acute MZ indicated that the LC_{50} = 0.22% Mn/Zn-EBDC (95% CI: 0.072 - 0.65%; r^2 = 0.5093).

(A)

(B)

Figure 3.

Chronic treatment with TouchDown (TD) or Manzate (MZ). (A) Dose-response curve of chronic TD indicated that the LC₅₀ = 5.7% glyphosate (95% CI: 5.3 - 6.2%; $r^2 = 0.9433$). (B) Dose-response curve of chronic MZ indicated that the LC₅₀ = 0.50% Mn/Zn EBDC (95% CI: 0.14 - 0.87%; $r^2 = 0.9733$).

Figure 4. Dual treatment with TouchDown (TD) and Manzate (MZ). Dose-response curve of 2% glyphosate followed by varying concentrations of Mn/Zn EBDC indicated an $LC_{50} = 12.5\%$ (95% CI: 10.3 - 22.6% and $r^2 = 0.6367$).

(A)

(B) *C. elegans* (Control Acute Exposure)

(C) *C. elegans* (3% TD Acute Exposure)

(D) *C. elegans* (10% TD Acute Exposure)

(E)

(F) *C. elegans* (Control Chronic Exposure) (G) *C. elegans* (9.8% TD Chronic Exposure) **Figure 5.**

TD treatment of NW1229 nematodes. Following acute (A-D) or chronic (E-G) treatment with TD, number of green pixels was determined as described. Data in (A) and (E) are presented as mean pixel number $\pm$ SEM for nerve ring (dark gray bars) or ventral nerve cord (black bars). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to nerve ring of controls, and ^ $p < 0.0001$ compared to ventral nerve cord of controls. (B) Photomicrographs of control worms for acute treatments show the nerve ring in close proximity to the cuticle (long blue arrows). The red arrows point to ventral nerve cord soma and their projections. (C-D) Photomicrographs of worms treated with 3% (C) or 10% (D) glyphosate indicate the formation of a minor gap between the ventral and dorsal nerve ring and the cuticle (long

blue arrows). Short blue arrow emphasizes the decrease in length of the retrovesicular ganglia, while red arrows highlight the thinning of the projections and the less defined soma along the ventral nerve cord. **(F)** Photomicrographs of control worms for chronic treatments show the nerve ring in close proximity to the cuticle (long blue arrows). Short blue arrow emphasizes the extension from the nerve ring, red arrows point to ventral nerve cord soma and their projections. **(G)** Photomicrographs of worms treated with 9.8% glyphosate indicate the formation of an increased gap between the dorsal nerve ring and the cuticle (long blue arrows). The red arrow points to the minor thinning of the ventral nerve cord, and loss of somal definition.

(A)

(B) *C. elegans* (Control Acute Exposure)

(C) *C. elegans* (7.5% Mn/Zn-EBDC Acute Exposure)

(D) *C. elegans* (17% Mn/Zn-EBDC Acute Exposure)

(E)

(F) *C. elegans* (Control Chronic Exposure)

(G) *C. elegans* (0.1% Mn/Zn-EBDC Chronic Exposure) (H) *C. elegans* (1.7% Mn/Zn-EBDC Chronic Exposure) **Figure 6.**

MZ treatment of NW1229 nematodes. Following acute (A-D) or chronic (E-H) treatment with MZ, number of green pixels was determined as described. Data in (A) and (E) are presented as mean pixel number $\pm$ SEM for nerve ring (dark gray bars) or ventral nerve cord (black bars). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to nerve ring of controls, and ^ $p < 0.01$, ^^ $p < 0.001$ compared to ventral nerve cord of controls. (B) Photomicrographs of control worms for acute treatments show the nerve ring in close proximity to the cuticle (long blue arrows). The red arrows point to ventral nerve cord soma and their projections. Photomicrographs of worms treated with 7.5% (C) or 17% (D) Mn/Zn-EBDC indicate the formation of an increased evident gap between the ventral and dorsal nerve ring and the cuticle (long blue arrows). The short blue arrow emphasizes the decreased length of the retrovesicular ganglia. (F) Photomicrographs of control worms for chronic treatments show

the nerve ring in close proximity to the cuticle (long blue arrows). The short blue arrow emphasizes the extension from the nerve ring, red arrows point to ventral nerve cord soma and their projections. Photomicrographs of worms treated with 0.1% (**G**) or 1.7% (**H**) Mn/Zn-EBDC indicate the formation of an increased gap between the dorsal nerve ring and the cuticle (long blue arrows), while red arrows highlight the thinning of ventral nerve cord projections and the decreased size of the soma.

(A)

(B) *C. elegans* (Control Dual Exposure)

(C) *C. elegans* (20% Mn/Zn-EBDC Chronic Exposure) **Figure 7.**

Dual treatment of NW1229 nematodes. (A) Following treatment with acute TD (2% glyphosate), worms were treated with varying concentrations of MZ. Number of green pixels was determined as described. Data are presented as mean pixel number $\pm$ SEM in the nerve ring (dark gray bars) or ventral nerve cord (black bars). * $p < 0.05$ compared to nerve ring of controls. (B) Photomicrographs of control worms show the nerve ring in close proximity to the cuticle (long blue arrows). Short blue arrow emphasizes the dorsal extension of the nerve ring, while red arrows point to ventral nerve cord soma and their projections. (C) Photomicrographs of worms treated with 20% Mn/Zn EBDC indicate the formation of a small gap between the dorsal nerve ring and the cuticle (long blue arrows). The short blue arrow highlights a shortening of the dorsal extension of the nerve ring.

Table 1

Results from Bliss analysis demonstrating putative interaction between TD and MZ in the dual treatment paradigm. Data is presented as lethal concentrations (LC) at various levels rather than specific concentrations due to the fact that the concentrations among the paradigms (acute TD, acute MZ, dual TD/MZ) were so different. The LC₂₅ for TD $\approx$ 3% glyphosate; LC₂₅ for MZ $\approx$ 2.7% Mn/Zn-EBDC, LC₅₀ for MZ $\approx$ 1.6% Mn/Zn-EBDC, LC₇₅ for MZ $\approx$ 7.5% Mn/Zn-EBDC; LC₂₅ for dual $\approx$ 10% Mn/Zn-EBDC, LC₅₀ for dual $\approx$ 12% Mn/Zn-EBDC, LC₇₅ for dual $\approx$ 20% Mn/Zn-EBDC. Data only for LC₂₅ of TD is presented due to the fact that only that concentration was used in the dual treatment protocol.

Dose Response Curves					
	TD Inhibition	MZ Inhibition	Dual Inhibition	Calculated E _{Bliss}	Interpretation
LC ₂₅	0.804	0.465	0.695	0.895	E _{Bliss} > Observed Synergistic
LC ₅₀	N/A	0.465	0.635	0.895	
LC ₇₅	N/A	0.465	0.275	0.895	
Nerve Ring (Predominantly DAergic Neurons)					
	TD Inhibition	MZ Inhibition	Dual Inhibition	Calculated E _{Bliss}	Interpretation
LC ₂₅	0.825	0.793	0.649	0.964	E _{Bliss} > Observed Synergistic
LC ₅₀	N/A	0.553	0.728	0.922	
LC ₇₅	N/A	0.561	0.632	0.923	
Nerve Cord (Predominantly GABAergic Neurons)					
	TD Inhibition	MZ Inhibition	Dual Inhibition	Calculated E _{Bliss}	Interpretation
LC ₂₅	1.04	1.15	0.971	0.993	E _{Bliss} > Observed Synergistic
LC ₅₀	N/A	1.33	0.793	0.985	
LC ₇₅	N/A	0.505	1.15	1.02	E _{Bliss} < Observed Antagonistic

Table 2

Results from the one-way ANOVAs comparing the TD concentration with the respective MZ concentrations used in either the MZ alone, or dual treatment paradigms. Data is presented as lethal concentrations (LC) at various levels rather than specific concentrations due to the fact that the concentrations among the paradigms (acute TD, acute MZ, dual TD/MZ) were so different. The LC₂₅ for TD $\approx$ 3% glyphosate; LC₂₅ for MZ $\approx$ 2.7% Mn/Zn-EBDC, LC₅₀ for MZ $\approx$ 1.6% Mn/Zn-EBDC, LC₇₅ for MZ $\approx$ 7.5% Mn/Zn-EBDC; LC₂₅ for dual $\approx$ 10% Mn/Zn-EBDC, LC₅₀ for dual $\approx$ 12% Mn/Zn-EBDC, LC₇₅ for dual $\approx$ 20% Mn/Zn-EBDC. Data only for LC₂₅ of TD is presented due to the fact that only that concentration was used in the dual treatment protocol.

	Nerve Ring	Ventral Nerve Cord
LC₂₅	ANOVA: $p = 0.0542^{\wedge}$ $t = 2.459$ (TD vs Dual) Results: Dual similar to MZ	ANOVA: $p = 0.6501$ Results: Dual similar to TD and MZ
LC₅₀	ANOVA: $p = 0.0874$ $t = 2.239$ (TD vs MZ) Results: Dual similar to TD and MZ	ANOVA: $p = 0.0153^*$ $t = 3.007$ (MZ, Dual) Results: Dual similar to TD
LC₇₅	ANOVA: $p = 0.1692$ $t = 1.580$ (TD vs MZ) $t = 1.501$ (Dual vs TD) Results: Dual similar to MZ	ANOVA: $p = 0.003^{**}$ $t = 3.607$ (TD vs MZ) $t = 3.607$ (MZ vs Dual) Results: Dual similar to TD
Summary	For the nerve ring, it appears that changes are more closely related to the contribution of MZ than TD.	For the ventral nerve cord, it appears that changes are more closely related to the contribution of TD.

Notes for statistical significance:

*
 $p < 0.05$,

**
 $p < 0.01$,

$\wedge$
 p is marginally significant