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Use of the Model Organism *Caenorhabditis elegans* to Elucidate Neurotoxic and Behavioral Effects of Commercial Fungicides

Kathleen M. Raley-Susman, Eunice Chou and Hayley Lemoine

Additional information is available at the end of the chapter

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Abstract

Fungicides are widely used in agriculture and medicine and there are several different types of fungicides that are distributed globally in the soil and water through water runoff and drift from spraying as well as other avenues of distribution. Understanding the biological effects of fungicide contaminants in the environment on non-target organisms including humans is critical. This chapter considers the use of the model organism and key beneficial soil nematode, *Caenorhabditis elegans*, as an effective strategy for examining fungicide effects on growth, reproduction, nervous system and behavior. We present novel evidence of the effects of a general use fungicide, mancozeb, on behavior and neuronal structure.

Keywords: mancozeb, fungicide, dithiocarbamate, *C. elegans*, neurotoxicity, dopamine, neurodegenerative

1. Introduction

Fungal infestations of agriculturally important crops, like the infamous potato late blight (*Phytophthora infestans*) that caused widespread famine and death in the 1800s in Ireland, are estimated to be responsible for at least 13% of crop losses in the world annually. The *Magnaporthe grisea* fungus, an Ascomycota, is considered one of the most damaging in the world, infesting rice crops at a devastating annual loss [1]. In addition to agricultural blights, rusts, smuts, mildews and other types of fungal infections, fungal infestations are responsible for the Chestnut blight (*Cryphonectria parasitica*) that caused the near annihilation

of the American Chestnut tree and Dutch elm disease, caused by two different fungi carried by the elm bark beetle, that is currently decimating American elm trees. In addition to plant fungal diseases, pathogenic fungi infect many different animals, from beneficial insects to humans. For example, *Ascosphaera apis* is a fungus that devours honey bee larvae and Stonebrood is a fungal infection that affects honey bees, as well as other insects, mammals and birds [2]. Further, animals can unwittingly participate in the fungal lifecycle and so serve as reservoirs of pathogenic fungi. Many fungal infections in humans are opportunistic, like *Candida albicans* (yeast infections) and Athlete's foot (*Trichophyton mentagrophytes*) [1]. Fungal infections are often highly contagious and resist treatment and so are of major concern to public health.

Fungicides are thus used both in the home and in agriculture. Dithiocarbamates like maneb, mancozeb and thiram were introduced commercially in the mid-twentieth century and many remain popular today. Multiple classes of organic fungicides are in current use for their ease of preparation, enhanced fungal toxicity, and reduced phytotoxicity. Currently, more than 80% of the agricultural sites in the US have regular treatment with fungicides [1], the majority of them are broad spectrum with multiple modes of action. Because many fungal infestations are difficult to eradicate, use of fungicides prophylactically in agriculture is common practice. In recent years, the use of fungicides to ward off fungal infections and to increase crop yields has soared, spurred in part by the increased production of a variety of fungicides. Fungicides are routinely and frequently applied to up to 30% of the US crops of soybean, corn and wheat. A recent study revealed that 75% of surface waters tested in the US and 58% of ground water contained measurable levels of fungicides known to be toxic to aquatic animals [3].

Understanding the biological effects of fungicides and fungicide mixtures on non-target organisms, including humans, is critical. While application levels of most fungicides seem to cause little overt toxicity in mammals, much lower doses have been linked to obesity in mice [4] and Parkinson's like symptoms in mammals, including humans [5]. In addition, early exposure to a number of fungicides has been linked to epigenetic, transgenerational occurrence of obesity in mammals [5]. Because fungicide contamination is found worldwide and these chemicals are heavily used, there is an urgent need to examine the effects of fungicides on growth, development and health of organisms that are exposed, including aquatic organisms, soil organisms, birds and mammals. This chapter will review current use of several fungicides and will argue that the use of the model organism, *Caenorhabditis elegans*, is an important strategy for examining the effects of fungicides on behavior, nervous system, growth and development.

2. Uses and variety of fungicides

A large and increasing number of fungicides are used for agriculturally critical crops like soybean, rice, corn and wheat, as well as for orchard fruits and vegetables. **Table 1** lists the most commonly applied chemical categories of fungicides for commercial agricultural use and their major mode for action [6]. Many fungicides are applied topically to either the seed or the

| Category of fungicide | Specific example | Major effect |
|-----------------------|---------------------------------------|------------------------------------|
| Azole | Myclobutanil, fluconazole, flutriafol | Inhibit sterol synthesis |
| Pyrimidines | Fenarimol | Disrupt sterol synthesis |
| Strobilurins | Azoxystrobin | Inhibit mitochondrial respiration |
| Polyoxins | Polyoxin D | * |
| Benzimidazoles | Thiophanate-methyl | Inhibit cell division |
| Dicarboxamides | Vinclozolin | Inhibition of androgen signaling |
| Carbamates | Propamocarb | *Non-cholinergic mechanism |
| Dithiocarbamates | Mancozeb, thiram | Inhibit mitochondrial respiration |
| Aromatic hydrocarbons | Chloroneb | Uncouple oxidative phosphorylation |
| biofungicides | Soilguard | Microorganism defense |
| Carboxamides | Boscalid | Inhibit succinic acid oxidation |

*Unknown mechanism of toxicity.

Table 1. Classes of commonly applied fungicides and major mode of action (adapted from [6]).

plants and the ground surrounding and act to repel fungal spores and prevent fungal growth and infection. These are considered less toxic to humans, except for workers involved in their application. Contact fungicides include the dithiocarbamates and aromatic hydrocarbons [6]. These compounds wash off and can enter the water supplies and intercalate into the soil. Acute neurotoxicity has been reported for the Mn²⁺-containing ethylene-bis-dithiocarbamate fungicides mancozeb [7] and thiram [8] via selective dopaminergic and gabanergic neuron signaling associated with perturbations in mitochondrial respiration. Other fungicides can be absorbed into plant tissues, with either limited penetrance (strobilurins, dicarboxyamides) or systemic distribution (phosphonates). Vinclozolin is a dicarboximide fungicide that has antiandrogen activity in developing male brain [9] and is used primarily to treat diseases caused by *Botrytis cinerea* and *Monilinia* spp. [10]. In mammalian brain, the preoptic/anterior hypothalamic area (POA/AH) is one of the most sexually dimorphic areas as reported previously for male and female rabbit offspring with sexual dimorphism and endocrine disruption induced by vinclozolin. A previous report noted that secondary products of vinclozolin activity may result in lipid peroxidation as well as oxygen free radical turnover in *B. cinerea*. Further investigations are warranted to reveal whether metabolites of vinclozolin induced signaling mechanisms that lead lipid peroxidation and/or oxygen free radicals associated with neurotoxicity. Another carbamate fungicide, propamocarb, induced decreased motor activity and vacuolization of the choroid plexus in rats and interference with glycolysis and formation of fibrillary proteins like neurofilaments through interaction with SH-groups [11]. Fungicides are also applied post-harvest to reduce rot during food storage and transport, and residues persist after food processing. A few specific examples are discussed below. A comprehensive review of all the major types of fungicides is beyond the scope of this review and the reader is directed to several recent reviews [6, 12].

2.1. Azole fungicides

In general, azole fungicides inhibit the formation of the fungal cell wall by interfering with a key enzyme involved in ergosterol biosynthesis [13]. There are a large number of different azole fungicides, used both agriculturally and in medicine. Despite their widespread use, relatively little work has been done to examine the toxicity of these compounds to wildlife and humans. One commonly used azole, flutriafol, has been associated with liver toxicity, reduced body weight of offspring and possible endocrine disruption of female fertility in rats [13, 14]. Another azole, triadimefon, disrupts dopamine neurotransmission function in rats, with subsequent effects on the nigrostriatal system that governs behaviors like general locomotor activity, as well as stereotyped, repetitive behaviors [15]. One recent study demonstrated edema and necrosis of circular and longitudinal muscle layers in earthworms exposed to tebuconazole for 14 days [16]. Further, epoxiconazole, a commonly applied triazole, acts as a male reproductive toxicant in rats [17] and damaged spermatogenesis in *C. elegans* [18]. Pursuing studies using *C. elegans* is a very useful and important avenue of research.

2.2. Strobilurins

This class of fungicide, developed in the 1990s has rapidly become the second most prevalent type of fungicide, accounting for a \$600 million industry that includes treatment of soybean, wheat and other cereal crops. Compounds like kresoxim-methyl and pyraclostrobin are broad-spectrum fungicides that have long-term stability [19] and were recently shown to be neurotoxic to cultured mammalian neurons [20]. Pyraclostrobin fed to rodents was also associated with stomach ulcerations, reduced weight gain and decreased food consumption [21]. In contrast, azoxystrobin did not cause significant toxicological health risks to humans from levels measured in the environment [22]. These compounds, while heavily used, have not been studied extensively by researchers, so much more work is needed.

2.3. Dithiocarbamates

Dithiocarbamate fungicides have been in use globally since the 1940s and are often considered to be only mildly toxic, or a moderate irritant, to mammals and human agricultural workers [23]. However, numerous reports have linked the exposure to these compounds to neurodegenerative damage to dopaminergic systems, including Parkinson's disease ([5, 24]; reviewed in Ref. [25]). Because many of these compounds chelate metal ions, particularly divalent cations like manganese, magnesium and zinc, the commercial formulations often contain these metal ions [26], which can exert toxic effects independently and in conjunction with the organic moieties in the formulations [27]. These compounds, including maneb, mancozeb and manate, have been shown to alter ion channel function [28] and inhibit mitochondrial function [29], as well as generate ROS (reactive oxygen species) [30]. The subsequent sections of this chapter focus on this fungicide as an example of the utility of *C. elegans* for examining fungicide effects on nervous system and behavior.

3. Use of *C. elegans* as a model to assess fungicide effects on development, lifespan and the nervous system

A number of pesticide and fungicide toxicology studies utilize the earthworm (e.g., *Eisenia fetida*) because of the importance of earthworms in soil health [16]. However, this organism lacks the ease of cultivation and powerful genetics of the model organism, *C. elegans*. The soil nematode, *C. elegans*, is an excellent model organism for neurotoxicology study of fungicides [31]. *C. elegans* is an important mesofaunal soil nematode that consumes bacteria and is predated by fungi, other nematodes and a host of other soil organisms [32]. *C. elegans* is a free-living, non-parasitic nematode that grows from egg to adult in about 3.5 days at 20°C, with a lifespan of about 18 days at that temperature. The nematode is transparent, 1 mm in length as an adult and is easily grown on agar plates with small patches of *Escherichia coli* as food. Hermaphroditic with the possibility of sexual mating with a low frequency male phenotype, the nematodes each produce 300 progeny through self-fertilization and up to 1000 progeny if mated with a male. The genome has been fully sequenced and a number of laboratories have generated a rich variety of mutants, including strains that express green fluorescent protein under different promoters. The GFP strains allow researchers to examine particular cell types or tissues using fluorescence microscopy. While a simple organism with only 302 neurons and a total of 959 cells, *C. elegans* nonetheless exhibits numerous behaviors and sensory functions, including associative and non-associative learning. It uses all the major neurotransmitters found in other invertebrates and shares at least 60% genes with mammals. *C. elegans* can self-fertilize or reproduce sexually, making genetic-level studies straightforward. Its short life span, transparent body and ease of cultivation have made *C. elegans* a key model for neurotoxicological study [33].

3.1. *C. elegans* dopamine neurons

Fungicides can have long-term effects on soil organisms, including nematodes [34]. Early reports of parkinsonism and other dopaminergic neuron-mediated neurological conditions in agricultural workers exposed to these compounds [35–37] generated interest in examining the unintended mechanisms by which these fungicides exert neurotoxicological effects. *C. elegans* is an ideal organism for experimentally examining the neurotoxicological effects of these fungicides on dopamine neurons and the behaviors they govern. *C. elegans* have four pairs of dopaminergic neurons, two pairs of CEP (cephalic sensilla) neurons, one pair of ADE (anterior deirids) neurons and one pair of PDE (posterior deirids) neurons [38]. These neurons modulate a variety of behavioral responses including foraging behavior, locomotion rate upon entering food, transition from swimming to crawling, egg-laying and defecation [39, 40]. The neurons are also large, readily identified in the light microscope and can be evaluated for neurodegeneration using established morphological criteria [33].

The CEP neurons reside anterior to the circumpharyngeal nerve ring in the head region of the animal and have ciliated processes that extend to the mouth/nose tip region (**Figure 3A**). The neurons

synthesize and release dopamine [41] as well as express acetylcholinesterase, a target of many pesticides. These neurons modulate a variety of behavioral responses including foraging behavior and locomotion rate upon entering food [39, 40]. They regulate a characteristic feeding behavior called basal slowing, wherein the animal detects the presence of bacteria via mechanosensation transduced by these neurons and slows its foraging locomotion. CEP neurons modulate feeding/search behavior by altering the overall activity of a 48 neuron circuit [42]. The ADE neurons, posterior to CEP neurons but still slightly anterior to the circumpharyngeal nerve ring, serve as mechanoreceptors that regulate the animal's response to nose touch and the presence of bacteria. Like the CEP neurons, they regulate the basal slowing response to food, and in addition, respond to harsher nose touch with a recoil response. These neurons also seem to participate in responses to nematode pheromones that regulate foraging and the recovery from dauer formation [43].

The PDE neurons, found in the posterior half of the nematode, also regulate the basal slowing response upon nematode entry to a lawn of bacterial food [39]. These dopaminergic neurons have also been found to regulate more complex behaviors and functions like locomotion, sensory perception, and learning [39, 44–48]. Because there are straightforward behavioral assays of dopaminergic neuron function, and because there are readily available strains of nematodes that express GFP in dopaminergic neurons, *C. elegans* serve as a powerful model organism for examining the effects of fungicides on dopaminergic systems.

A number of dithiocarbamate fungicides, including maneb, ziram and Mancozeb, have been evaluated using the *C. elegans* model system. Mancozeb is a commercial formulation of the main active ingredients ethylene-bis-dithiocarbamate, zinc, manganese, as well as other “inactive” compounds that are not published by the company (Bonide Products, Inc., NY). It is used in agricultural and residential garden settings to control a variety of fungal infections that infest ornamental plants and vegetables, like blossom end rot and downy mildew [49]. Mancozeb is currently approved for use in over 120 countries including the United States. It is used on over 70 different crops and protects against more than 400 different fungal infections [50]. Typically, even though it has limited solubility in water, Mancozeb is applied as a mixture with water to plants and the soil around them, where it forms a film on the plants and intercalates into the upper layers of soil. According to the application instructions, Mancozeb is most typically applied at a final concentration in the range of 0.5–1.5% (v/v). Mancozeb has an active half-life of 1–7 days depending on weather conditions. The targeted mechanism of action of Mancozeb is the disruption of key metabolic enzymes by altering sulfhydryl groups. There is also evidence that the metabolite, ethylenethiourea (ETU) might also have toxic effects. The organic moiety of Mancozeb is a potential estrogen disrupting chemical and suspected carcinogen [51]. Further, the manganese and zinc present in the formulation also cause toxicity via multiple mechanisms. For example, zinc modulates glutamate neurotransmission and interacts with numerous enzyme systems [52] and manganese can exacerbate dopamine neuron degeneration [53] possibly via enhanced oxidative stress [54]. Mancozeb was shown to be lethal to nematodes at concentrations experienced by agricultural workers [55] and sub-lethal doses led to degeneration of neurons, including dopamine neurons [55], induction of heat shock responses [55] and inhibition of larval growth [56, 57]. In many instances, the fungicide is applied repeatedly, so organisms are exposed multiple times and cumulatively over long periods of time. It is likely that many people are exposed to low levels of Mancozeb

remaining on unwashed fruits and vegetables. Farmers, pesticide sprayers and home gardeners are exposed to higher levels when they work with the formulations. Thus, while soil and aquatic organisms may be most vulnerable to the toxicity of sublethal concentrations of mancozeb, human populations are also at risk from exposure to concentrated formulations and from the accumulated effects of small doses from ingesting fruits and vegetables that have been in contact with the fungicide [25, 58].

We explored locomotory, mechanosensory, and egg-laying behaviors in response to a 24 h exposure to Mancozeb to better understand the impacts of sublethal amounts of this widely used fungicide on this important soil organism [33] on particular neuronal populations.

4. Materials and methods

4.1. *C. elegans* strains and maintenance

The following strains were obtained from the *C. elegans* Genetics Center: wild-type (N2); OH7547 ((otIs199)[*cat2*::GFP + *rgef-1*(R25B3.3)::dsRed + *rol-6*(su1006)]). *C. elegans* was maintained on NGM petri plates containing spots of *E. coli* (OP50) bacteria for food at 20°C [59]. We prepared small developmentally-synchronized cultures by transferring 10 gravid worms to NGM plates with *E. coli* and allowing them to lay 30–50 eggs for approximately 2 h. Resulting synchronized cultures were exposed to mancozeb as described in the following section.

4.2. Mancozeb (MZ) exposure

Mancozeb treatments were made as volume:volume percent solutions with water, as recommended by the manufacturer. 0.5–1.5% mimic the application dose to garden soils [33] and are within the LC50 of nematode sensitivity of 1% [33, 55]. Lower concentrations correspond to putative residual concentrations present beyond the garden or application site. The solutions were spread evenly using a sterile glass rod to coat the entire surface of the NGM agar plate. In some experiments, the Mancozeb was also combined with the *E. coli* mixtures, while in others the *E. coli* were spread onto the plate already coated with Mancozeb. We saw no differences in experimental outcome among these different exposure conditions. In the experiments reported in this study, the Mancozeb-treated plates were allowed to air-dry overnight at 20°C prior to the placement of nematodes. Young adult nematodes, just past the L4 transition, were exposed to different concentrations of Mancozeb for 1, 3, 6 or 24 h. Some nematodes were tested directly after exposure and some were given a comparable recovery time on plates lacking Mancozeb. For example, a 24 h exposure to Mancozeb was followed by a 24 h recovery period on untreated plates prior to behavioral or microscopic assessment. For all experiments, at least three separate replicates were performed.

4.3. Behavioral measurements

For each assay, unexposed, age-matched nematodes were measured during the same time frame as the Mancozeb-exposed nematodes. More than one investigator conducted the

behavioral measurements to prevent investigator bias. All measurements were obtained at room temperature (22°C). Nematodes were visualized with an Olympus SZ-1 dissecting microscope.

4.3.1. Basal slowing behavior

When nematodes enter a patch of *E. coli* food, they dramatically slow their locomotion, a behavior called basal slowing, whose mechanism involves the activation of the dopaminergic mechanosensory neurons, CEP, ADE and PDE [39]. After exposure to Mancozeb or control conditions, nematodes were transferred individually to plates lacking food for several seconds to allow the adhering Mancozeb to be cleared. Control nematodes also were transferred to food-free plates for this brief “clearing” period. Then, nematodes were transferred individually to petri plates containing a circular patch of *E. coli*. They were placed outside of the food circle. Basal slowing was measured by counting body bends for 20 s outside of the food and then again when the nematode entered the patch of food. Body bends were defined as one complete sinusoidal movement of the nematode’s tail. We operationally defined basal slowing as the difference in body bends/20 s on the food as compared with off the food, as determined by earlier studies [60]. Basal slowing responses were compared with untreated animals using a one-way ANOVA followed by Bonferroni tests for multiple comparisons using Prism 5.0 statistical software (GraphPad, Inc.) for experiments testing more than one concentration of Mancozeb and the Student’s *t*-test for the reversibility assay, as described in specific figure legends.

4.3.2. Tail drag (or paralysis) after swimming

Our earlier work determined that Mancozeb exposure interfered with overall locomotion frequency [33]. We further examined locomotion by developing a behavioral measure of tail motion. We noticed that, following exposure to liquid-induced swimming behavior, the return to crawling behavior was abnormal. In particular, the tail appeared stiffer or paralyzed. We measured this change in tail movement as a novel assay of locomotory deficit. Individually, worms were removed from the treatment plate and placed on a 2 cm petri dish lacking *E. coli*. The dish was placed on an Olympus SZ-1 dissecting microscope, and locomotion was digitized as short video clips using a digital camera (Insight, Inc.). 2 µl of M9 buffer solution were placed over the worm to induce swimming behavior, and left to dry (about 30 s). Directly after the M9 buffer solution had dried, we collected 100 frames (at 60 frames/s) of worm movement using Spot (2.0) software (Insight, Inc.). The worm was then removed from the dish, and a new worm was put in its place. This continued until all the worms from the Mancozeb-coated petri dish or from control conditions were filmed.

The computer program LoggerPro (Vernier, Inc.) tracked the movement of the worms from the short video clips. Two different data points marking the head and the tail were placed on each worm image for each frame of the video and the *X* and *Y* coordinates of the points were determined. We calculated the distance each worm traveled between frames ($\text{distance} = \sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2}$). This allowed the speed of the head and the tail to be

found for each worm at each time point ($\text{speed} = d/(t_2 - t_1)$). These values were then averaged for each worm. We focused our statistical analyses on the tail movements. The tail speeds were analyzed with ANOVA followed by Bonferroni's post-hoc tests for multiple comparisons to determine significance between different treatments. All statistical analyses were performed using Prism 5.0 software (GraphPad, Inc.). A p -value of less than 0.05 was considered statistically significant.

4.4. Confocal microscopy

Dopamine neurons were evaluated immediately after a 24 h exposure to Mancozeb or control conditions by immobilizing young adult hermaphrodite nematodes of the OH 7547 strain on a 2% agarose pad within a drop of buffer containing 2.5 mM NaN_3 to paralyze the worms. Images were acquired with a Nikon PCM2000 confocal laser-scanning microscope equipped with an argon laser. For each neuron analyzed, we collected 18-image z-stacks using a 60 \times immersion oil lens and analyzed the merged images. Confocal images were processed to adjust contrast using simple PCI software. In all cases, whole images were processed, rather than particular portions of images, to ensure faithful representation of the results.

5. Results and discussion

We discovered that sublethal concentrations of Mancozeb impaired locomotion, both exploratory locomotion and the transition from swimming to crawling behavior, a transition that is activated by dopamine neurons [33]. With further analysis using our tail drag assay, we documented a significant tail rigidity or paralysis upon return to crawling behavior (**Figure 1A**). The tail paralysis persisted even after 24 h recovery from the exposure to Mancozeb (**Figure 1B**). In addition, the basal slowing response of fungicide-exposed nematodes was significantly impaired (**Figure 2**). If nematodes were exposed to the fungicide for 24 h and allowed to recover for 24 h, the behavior did not recover, suggesting a long-lasting and potentially permanent impairment.

The locomotory transition from swimming to crawling, a process governed by dopamine neurons, was also permanently impaired [33]. In keeping with these long-term functional impairments, the dopaminergic neurons exhibited characteristic indications of neurodegeneration, including blebbing along the neural processes and changes in soma morphology (**Figure 3**) [33]. Other recent studies have also documented neuronal impairments in response to maneb and manganese exposure, measured by a loss of GFP fluorescence intensity [27, 55].

While dopaminergic neurons were most sensitive to fungicide exposure [33, 56], other neuron groups, including serotonergic [33], glutamatergic and GABA neurons [50, 61] exhibited morphological damage. Vulnerability to neurodegenerative effects varies depending on the dose of fungicide, duration of exposure and time of life of exposure [25, 33].

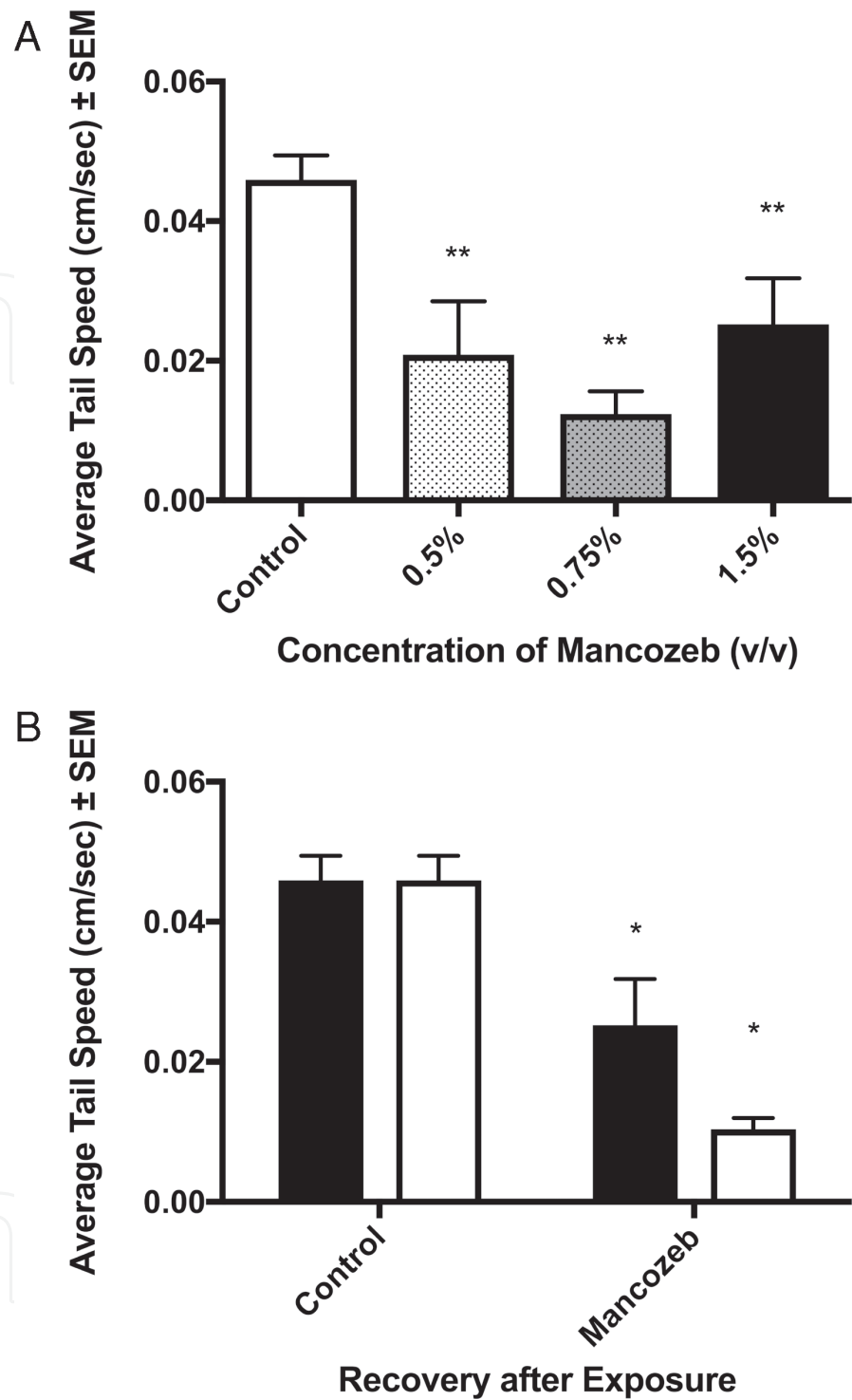


Figure 1. Long-lasting tail paralysis after exposure to Mancozeb. Tail speed after swimming in droplet of M9 buffer (as described more fully in Section 4; $n=12-21$...replicates.) individual nematodes per treatment group across at least three separate replicates. (A) Immediately following Mancozeb exposure; (B) 24 h after Mancozeb exposure. Black bars indicate nematodes exposed to 1.5% Mancozeb for 24 h and assayed immediately thereafter. White bars are nematodes moved to untreated plates with food for 24 h after the Mancozeb exposure. Data are presented as the mean \pm SEM. ** $p < 0.01$ when compared with untreated controls using one-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons.

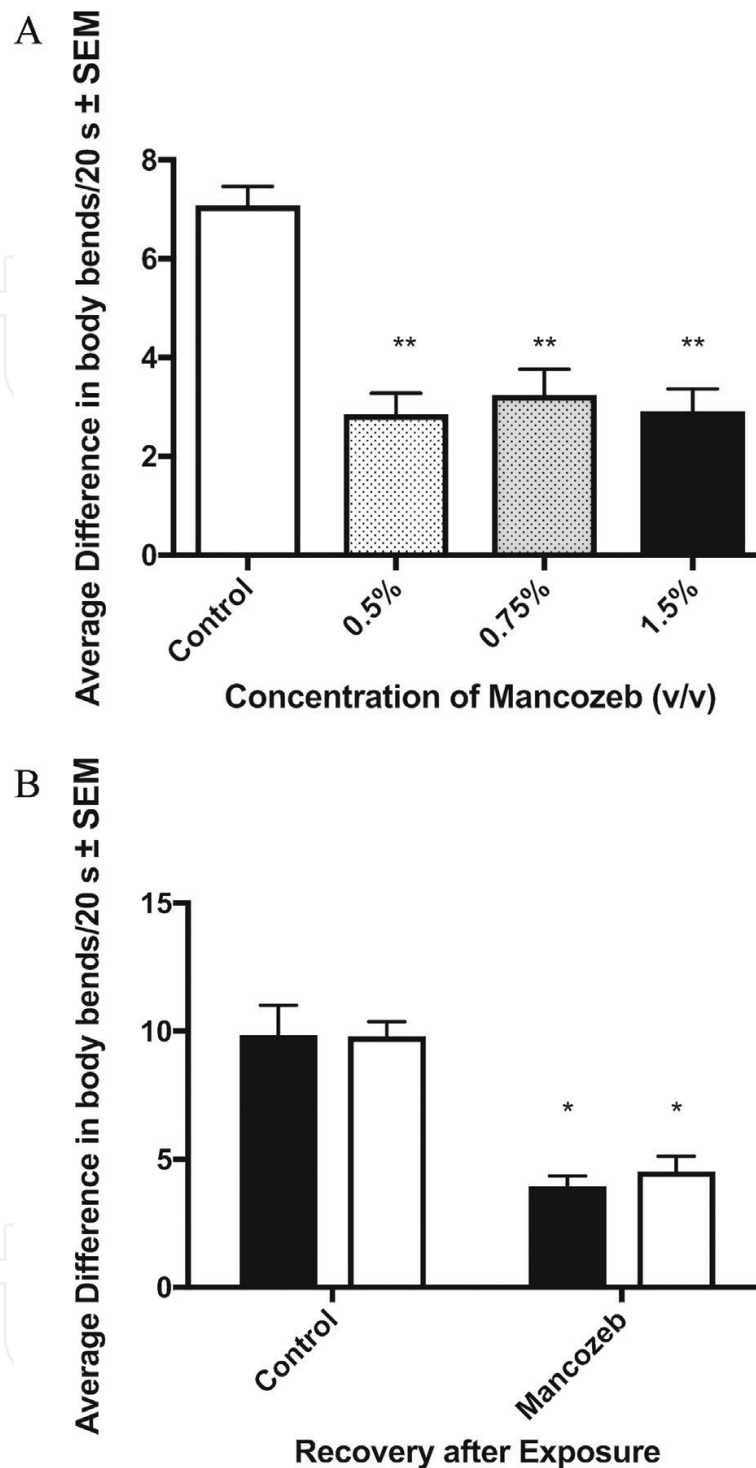


Figure 2. Basal slowing behavior impairments after 24 h exposure to 1.5% Mancozeb are not reversible after 24 h recovery. Day 4 nematodes at the L4/adult transition were exposed to 1.5% Mancozeb for 24 h and then basal slowing behavior was measured as the change in body bends upon entry to a bacterial lawn. Black bars indicate worms analyzed immediately following exposure to 1.5% Mancozeb. White bars show nematodes treated and then measured immediately following exposure (A) or moved to untreated plates with food for a 24 h recovery period prior to measurement (B). Data are averages of $n = 20$ independent worms per treatment across three separate replications. * $p < 0.01$ when compared with time-matched controls using a 2-way ANOVA followed by Bonferroni multiple comparisons tests.

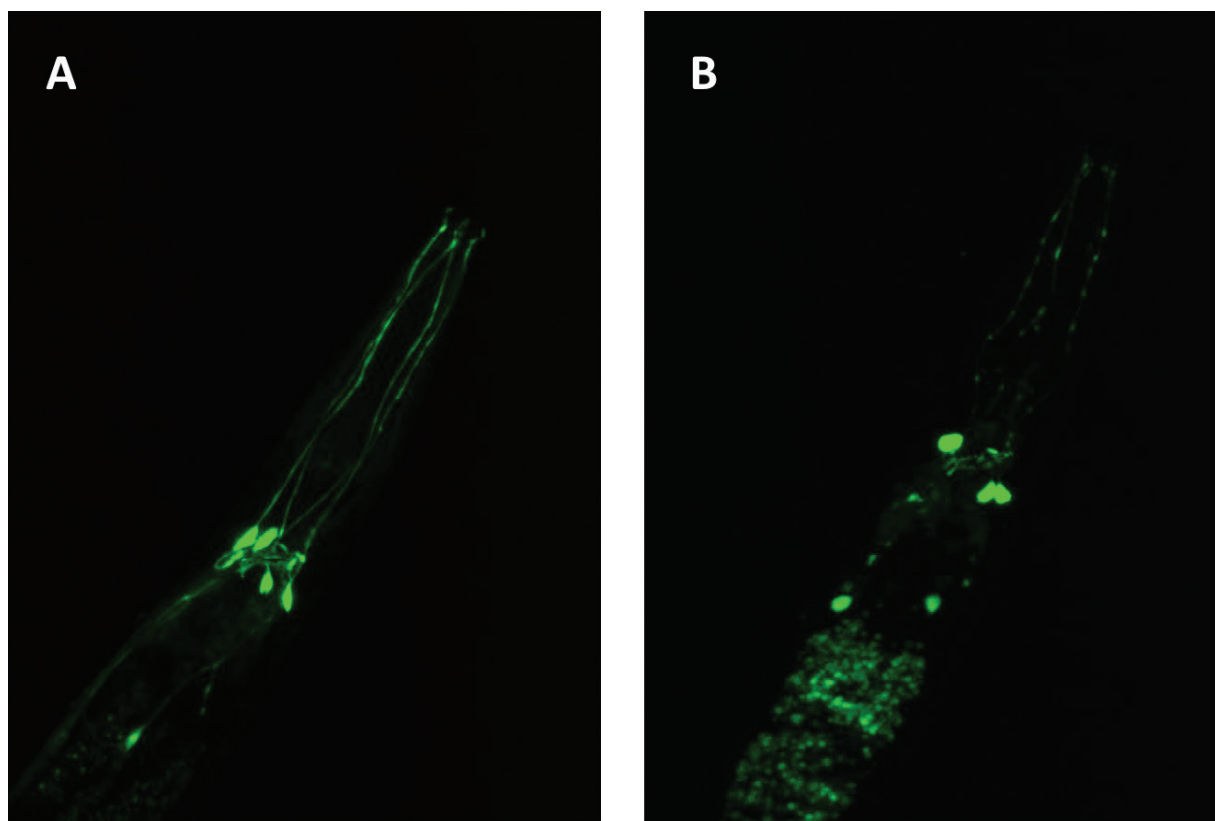


Figure 3. Mancozeb-induced degeneration of dopamine neurons. Late L4/young adult nematodes of the OH7547 strain, which express GFP in dopamine neurons were exposed to 1.5% Mancozeb as described in Section 4. Individual nematodes were picked to 2% agarose pads in a droplet of M9 buffer containing 2.5% NaN_3 to paralyze them and viewed using confocal microscopy. (A) Untreated nematode. The two pairs of CEP neurons and one of the ADE neurons are visible, along with their long processes. (B) Nematode exposed to 1.5% Mancozeb. 60 \times oil immersion lens.

6. Conclusions

Dithiocarbamate fungicides are widely used globally in large part because they are thought to be safe for wildlife and humans [7]. However, there is growing evidence that these compounds, and their breakdown products, harm wildlife and may even lead to Parkinson's like symptoms in humans [62, 63]. Further, a number of reports indicate that these compounds in combination with other widely used pesticides like paraquat or several organophosphate pesticides [64] are associated with cellular and organismal toxicity.

Mancozeb and maneb, the two most commonly used dithiocarbamate fungicides, have adverse effects on a number of non-target organisms, including protozoans [65], soil arthropods [66], birds [67], rodents [68] and fish [69]. The toxic effects include neurotoxicity [7], immunotoxicity, reproductive defects and endocrine disruption [67] for both high doses and low doses. Even though these compounds have a relatively short half-life, the metabolites and the metal ions persist in the environment. In addition, in part because of the short half-life, farmers and gardeners tend to apply the pesticides multiple times during a growing season. Further, the behavioral damage we documented in *C. elegans* is permanent and occurs well

within the active life of the pesticides. As a result, exposure to the compounds and their toxic byproducts is far greater and far more long-lasting, particularly during growing seasons, which correspond to breeding, growing and active periods of many organisms.

These studies have underscored the utility of examining the effects of commonly used fungicides like the dithiocarbamates and metal-containing compounds (particularly zinc and manganese) on *C. elegans* behavior, development and neural function. More recently, researchers evaluated the effects of triazole fungicides, particularly epoxiconazole, on nematode growth and reproduction [18] and determined that spermatogenesis is inhibited by epoxiconazole. These findings agree with reports of endocrine-disrupting effects of this family of fungicides in rats and birds [17, 70, 71]. The advantages of short life span, comprehensive genetics and ease of study make these nematodes an ideal system for continued study of the effects of fungicides, necessary agents for agriculture and medicine, on organismal development, health and behavior.

Author details

Kathleen M. Raley-Susman*, Eunice Chou and Hayley Lemoine

*Address all correspondence to: kasusman@vassar.edu

Vassar College, Department of Biology, Poughkeepsie, NY, USA

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