Mitochondrial Toxicity of Phosphorus Based Flame Retardants

by

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EXECUTIVE SUMMARY

Flame retardants (FRs) are added to various substances so that when exposed to an open flame the parent material combusts at a slower rate. However, there is concern over the safety of these chemicals. In this study the mitochondrial toxicity of Triphenyl Phosphate (TPP), Isopropylated Triphenyl Phosphate (IPP), and Tert-Butylphenyl Diphenyl Phosphate (TBDP) were assessed using the model organism *Caenorhabditis elegans*. The first experiments were growth assays utilizing mitochondrial regulation (fusion, fission, mitophagy) knock down mutants (*eat-3, fzo-1, drp-1, dct-1, pdr-1, and pink-1*). In the second set of experiments, relative ATP levels were measured using a luciferase reporter strain (PE255). The results were that *eat-3, fzo-1, drp-1, and dct-1* were more sensitive to TPP than the control (defined as growth decrease greater than 10%) at the mid to high dose range; there was decreased sensitivity to *pdr-1*. IPP and TBDP caused less toxicity in *eat-3* and *pdr-1*. There was no significant change in ATP levels after exposure to TPP, while there was a concentration-dependent decrease with IPP and a slight increase with TBDP. Overall the results of this study are consistent with the possibility that TPP is a mitochondrial toxicant; future research is needed to fully understand that relationship.
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INTRODUCTION

Flame retardants (FRs) are chemicals that are either added or chemically bound to a material so that when exposed to an open flame, the FRs form free radicals that react with oxygen. This depletes oxygen as a fuel source for the flame or creates a thermal barrier that reduces the rate at which a material burns (U.S. Environmental Protection Agency, 2013). However, many popular FRs, such as deca- and penta-DBE, are banned under the Stockholm Convention for persistent organic pollutants (United Nations Environment Programme, 2008). This has caused manufacturers to switch to different FRs such as Triphenyl Phosphate (TPP), Isopropylated Triphenyl Phosphate (IPP), and tert-Butylphenyl diphenyl phosphate (TBDP).

TPP is an additive FR that is used in application by itself or in combination with other compounds. The structure of TPP is a phosphorus atom that is double bonded to an oxygen atom and bonded to three phenol groups (Figure 1). The application for this compound is addition to different resins, textiles, polyvinylchloride and flexible and rigid polyurethane foam. The registered production volume under Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) is 1,000 to 10,000 tons per year (Danish Environmental Protection Agency, 2016). Reported mammalian toxic effects of TPP include reduced body weight when given as repeat doses, at 161.4 mg/kg-day. At 350 mg/kg-day, there was an increase in liver size in male rats (U.S. EPA, 2015). Another study found TPP causes ocular irritation when applied directly to rabbits’ eyes (U.S. Department of Health and Human Services, 2012). The mechanisms of toxicity have been studied in human cell lines, HEK293. TPP has been found to activate constitutive androstane receptor (CAR) 5.5x and pregnane X receptor (PXR) 3x baseline levels at 10 µM. This study also found that at 10 µM TPP inhibited androgen receptor activity (AR) 30-40% when in the presence of testosterone; however, glucocorticoid receptor (GR), progesterone receptor (PR) and estrogen receptor (ERα) were not affected by the addition of TPP in the presence of their native agonists (Honkakoski et al., 2004). Thus, the available in vitro data suggests that TPP may impart sex-specific endocrine activity.

IPP is an additive FR mixture comprised of TPP and different isomers of IPP. The structure of the IPP compound is derived from the TPP backbone with the three phenol groups having 0-2 isopropyl group substitutions, although the most common structure is that one to two of the phenol rings has a single isopropyl substitution (Figure 2). Commercially, IPP is added to polyurethane flexible foam, paint, and PVC/nitrile...
foam; it is used in multiple commercial FR mixtures. The REACH-reported annual production for IPP is between 10,000 to 100,000 tons per year (Danish EPA, 2016). According to the U.S. EPA, the main current toxic endpoints of concern for humans are reproductive effects and neurological effects. When female rats were exposed to an IPP mixture for 53 days with 25 mg/kg, they experienced a change in organ weights; the magnitude was not reported. In the same study, the resulting pup litter size and survival rate was reduced at 400 mg/kg/day. This was a confidential study reported to the EPA so more details on the study cannot be found. Hens experienced ataxia and axonal degeneration at oral administration of 90 mg/kg/day or inhalation for 8 hours 2.4 mg/L. This study was from the International Uniform Chemical Information Database presented to the EPA (U.S. EPA, 2015). These studies suggest that there is some risk associated with exposure to this FR. More testing is needed including: adsorption, distribution, and metabolism in humans; carcinogenicity; and inhalation exposures (U.S. EPA, 2015).

TBDP is classified as an additive FR mixture that is composed of TPP and different isomers of TBDP. The TBDP compound is based on the TPP backbone with the three phenol groups having 0-1 tert-butyl group substitutions, although the most common structure is one to two of the phenol rings substituted (Figure 3).

Firemaster 550 (Danish EPA, 2016; U.S. EPA, 2015). The main application of TBDP is on textiles or Polyvinyl chloride (PVC) (Danish EPA, 2016). TBDP has multiple endpoints for toxicity. In a study where rabbits were orally given a mixture, similar to the mixture used in this study, it was found that at 100 mg/kg/day five times a week for three weeks produced atonia and reduced plasma cholinesterase. Rabbits produced decreased levels of brain cholinesterase and erythrocyte when dermally exposed to a 10 mg/kg/day mixture that was over 80% pure monosubstituted TBPD (the rest comprising di- and tri- substituted) for five days. This study was submitted to the EPA confidentially. In a confidential study submitted to the EPA, when rats received repeated oral doses at 250 mg/kg/day for 30 days, they developed enlarged livers. Finally, there was a moderate risk of dermal irritation when the TBDP mixture was applied to rabbits; the rabbits experienced mild skin irritation which lasted 8-10 days after one dose was applied dermally. (U.S. EPA, 2015). These results suggest that there is some risk to this compound. What complicates the assessment of toxicity is that TBDP is usually tested in mixtures (US. EPA, 2015). It is difficult then to understand if the toxicity is a result of the individual chemical or the combined exposure.

There are a few reasons why mitochondrial toxicity was examined as a mechanism of toxicity of these FRs. These chemicals are labeled as high volume chemicals under U.S. EPA with a possibility of massive exposure. As such, they were suggested to be tested by the National Toxicology Program (NTP) for toxic effects by the Consumer Product Safety Commission (U.S. EPA, 2015).
upon testing, two of the mixtures had moderate to high neurotoxic properties (U.S. EPA, 2015). Neurodegenerative diseases, Parkinson’s and Alzheimer’s for example, are related to mitochondrial defects such as the over- and under-expression of mitochondrial-related proteins. Neurons use a large amount of energy to function properly; when the mitochondria are damaged, it often leads to a reduction of mitochondrial function, increase of reactive oxygen species, and possibly cell death. This reduction in neuron function has been attributed to the prognosis of many neural diseases (Itoh et al., 2013). Lastly, there has been little research on these chemicals and mitochondrial toxicity. In a peer-reviewed article on mitochondrial impacts resulting from exposure to FRs structurally similar to TPP, isolated human mitochondria exposed to 100 µM of tri-ortho-tolyl phosphate (mono substituted methyl groups on each phenol ring on a TPP backbone) exhibited mitochondrial swelling. (Carlson & Ehrich, 1999). Although mitochondrial swelling returned to baseline in three hours, this could be a cause for concern since the swelling could disrupt mitochondrial membranes, resulting in reduced ATP production or apoptosis. Unpublished observations by the NIEHS utilizing cells in vitro and Caenorhabditis elegans suggest that TPP, IPP, and TBDP induce mitochondrial toxicity (Meyer & Stapleton, personal communication with Mamta Behl from NIEHS, February 3, 2015). Since the initial screenings indicate these chemicals as mitochondrial toxicants, they are currently being study by the NTP for toxicity. Additionally, these compounds are neurotoxicants. Because the brain is a highly metabolic tissue and would likely be significantly impacted by mitochondrial toxicity induced by FR exposure, they were selected for further mitochondrial toxicity screening.

Mitochondria perform the essential process of oxidative phosphorylation which generates ATP, the primary fuel source for the cell (Lemire, 2005). They also participate in other cellular roles including apoptosis, production of steroids, generation of reactive oxygen species, Ca$^{2+}$ signaling (Miller, 2013; British Society for Cell Biology, n.d.). There are multiple mitochondria within each cell, and each mitochondrion contains multiple copies of their own DNA, which are physically and functionally distinct from the nuclear genome. Dysfunction of mitochondria usually does not affect the cell (in absence of other insults) until 70 to 90% of all mitochondria have that dysfunction. Since they produce so much energy, tissues and organs that depend on high energy production, such as the brain, are known to be more sensitive to mitochondrial dysfunction (Chinnery & Schon, 2006).

The model organism utilized in this study is Caenorhabditis elegans, a translucent nematode. Some of the advantages of this organism are: they are non-parasitic to humans; it has a short time period from birth to sexual maturity; its entire genome is sequenced; and is has a large brood size. In addition, it is complex enough to be organized into distinct tissues (for instance, an alimentary system) and cell populations that retain functional homology with mammals, such as dopaminergic neurons. Finally, since most C. elegans are self-fertilizing hermaphrodites, it is easy to keep a population with a mutation pure (Corsi et al., 2015). They have been used in many pharmaceutical and toxicological studies in which a whole organism offers in vivo physiological complexity that would not be necessarily present with in vitro modeling (Kaletta, & Hengartner, 2006). Human and C. elegans mitochondrial DNA are quite similar with a string degree of conservation, with most of the genes conserved (the omission being a gene that creates a protein, ATP8, for complex V of the electron transport chain) (Lemire, 2005). C. elegans have genes that are orthologous to those of humans, corresponding to 60 – 80% of human genes, along with 533 currently identified to be related to human diseases (Kaletta, & Hengartner, 2006). The
The nomenclature of the mutant strains is the gene that is knocked down within *C. elegans*. A gene being knocked down means the expression of the protein is reduced (Table 1).

Although there are many functions of mitochondria, the three mechanisms related to mitochondrial maintenance explored in this study were: fusion, fission, and mitophagy. A key way that a cell deals with damaged mitochondria is through the fusion of two mitochondria. If one happens to be damaged, the damaged material and mitochondrial DNA (mtDNA) is mixed and diluted. Then, either the mitochondrion replicates the mtDNA and undergoes fission to make two new mitochondria, or the damaged mtDNA and mitochondrial proteins are sent to be destroyed. Fission can occur in the absence of fusion to make new mitochondria (Twig et al., 2008). The last explored maintenance of mitochondria is mitophagy; this is when a single mitochondrion is tagged to be destroyed because it is malfunctioning (Ding & Yin, 2012). Overall these three processes are crucial for the normal life cycle of mitochondria and are important mechanisms to removing and creating new mitochondria that are damaged from exposure to toxicants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Human Equivalent Gene</th>
<th>Related Human Disease</th>
<th>Cellular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>N/A</td>
<td>Control/ None</td>
<td>Control</td>
</tr>
<tr>
<td>DA631 eat-3 (ad426)</td>
<td>OPA1</td>
<td>Optical nerve atrophy</td>
<td>Mitochondria Fusion</td>
</tr>
<tr>
<td>BC10210 fzo-1 (tm1133)</td>
<td>MFN1, MFN2</td>
<td>Charcot-Marie-Tooth disease</td>
<td>Mitochondria Fusion</td>
</tr>
<tr>
<td>drp-1 (tm1108)</td>
<td>DRP1</td>
<td>Parkinson’s, Huntington’s, and Alzheimer’s (overexpression).</td>
<td>Mitochondria Fission</td>
</tr>
<tr>
<td>dct-1 (tm376) X</td>
<td>NIX/BNIP3L and BNIP3</td>
<td>Loss of function in some forms of cancer; helps with proliferation</td>
<td>Mitophagy</td>
</tr>
<tr>
<td>UA86 pink-1 (tm1779)</td>
<td>pTen-induced kinase 1</td>
<td>Parkinson’s</td>
<td>Mitophagy</td>
</tr>
<tr>
<td>VC1024 pdr-1 (gk448)</td>
<td>PARK2</td>
<td>Parkinson’s</td>
<td>Mitophagy</td>
</tr>
</tbody>
</table>

(Bess et al., 2013; Bristow et al., 2011; Lemire, 2005; Lu, 2011; Palikaras, 2015; Reddy et al., 2011)

There were two assays utilized in this study. The first test was a growth screen in which a range of concentrations of the toxicants were administered to the previously mentioned mutant strains and the resulting total growth was compared to the vehicle control and control strain. This is a first pass method: if a toxicant affects a mitochondrial mutant more than it affects the wild-
type strain, this is consistent with the possibility that the chemical is a mitochondrial toxicant. For the second round of experiments, N2 PE255 strain (in which luciferase and green fluorescent protein (GFP) are expressed in the wild-type worm) were exposed to the various FRs. Once exposed, levels of adenosine triphosphate (ATP) were measured and compared to the control exposure. This strain of nematode luminescence is reflective of the amount of ATP found within the cell of the worm. This helps determine if the chemicals primarily target mitochondria or if the effects seen in the first screen are more of a secondary effect. If the reduction of ATP is seen to be significant before or at the dose that there is a significant reduction in growth, this lends evidence for the FR to be a mitochondrial toxicant. If the reduction in ATP is seen after there is significant reduction in growth, this is consistent with another mechanism causing the reduction in growth.

METHODS

Chemicals and nematode strains

The composition for TBDP used in this paper was: 35.2% TPP, 49.2% TBDP, 14.4% Di-TBDP, 0.01% Tri-TBDP (MRIglobal, 2012). The composition of IPP was: 30% TPP, 32% IPP, 10% di-IPP, 2.4% tri-IPP, and 0.04% tetra-IPP (San Francisco Estuary Institute, 2009). N2 (wild-type) and VC1024 pdr-1(gk448) were obtained from the Caenorhabditis Genetics Center, University of Minnesota (Saint Paul, MN, USA). Both DA631 eat-3(ad426) and BC10210 fzo-1(tm1133) were received from Alexander van der Bliek, University of California (Los Angeles, CA, USA). Drp-1 (tm1108) was obtained from Ding Xue, University of Colorado (Boulder, CO, USA). UA86 pink-1(tm1779) was obtained from Guy Caldwell, University of Alabama (Tuscaloosa, AL, USA). Dct-1 (tm376) X was donated by Konstantinos Palikaras, University of Crete (Herakilon, Crete, Greece). For the luciferase assay Cristina Lagido from the University of Aberdeen (Aberdeen, UK) kindly donated the N2 PE255 mutants.

Growth Arrest Assay

Dosing solutions of the three different FRs were made by diluting a stock solution to desired volume with dimethyl sulfoxide (DMSO). Different strains of worms were egg prepped (Bess et al., 2013) separately in tissue culture flasks. This synchronized the worms to be at the same larval stage to reduce variability of the different strains’ growth rates. Each strain was transferred to a different 15-ml test tube where the worm concentration was calculated. This was done by shaking the tube and snipping the end of a 20 µL pipette, rinsing with 0.1% Triton-X 100 solution, and pipetting 10 µL of the worm suspension to a glass slide; afterwards, the worms were counted. This was repeated two more times with the counts averaged together to get the mean worm concentration per µL. An appropriate volume was added to a 12 well plate so that seven wells were filled with 500 worms each. Subsequently, enough K+ medium was added so that the total volume in the well was equal to 895 µL. Next, 5 µL of the drug was added to each well. Then 100 µL of ultraviolet radiation (UVR) killed bacteria was added to the wells; this was repeated for all strains. When the wells were all filled, the well plate was sealed with Parafilm, and placed in a 20°C fridge. At 24 hours 100 µL of UVR bacteria was added to each well; at 48 hours, each well was transferred to a 1 ml centrifuge tube and spun at 1000 rpm for 90 seconds. The supernatant was vacuumed off each centrifuge tube and the worm pellet was resuspended in
1 ml of K medium; this was repeated 2 more times for each dose. The tubes were then placed in the -80°C freezer to kill the worms. The growth was analyzed by thawing the worms and transferring them to a glass slide with a coverslip. The slide was then placed on a Karl Zeiss fluorescent microscope and the length of each worm was measured using Nikon Nis-Elements software (basic research version 3.22.11). Measurements were made so that each dose for each strain had at least 25 measurements, with the entire experiment repeated at least three times. The results were averaged to get the growth rate for each dose and each strain. The length for each strain was normalized to the average vehicle control length for each replicate.

**ATP Luciferase Assay**

Exposure plates were made using the same recipe for OP50 K Agar plates with the exception of no peptone added. After the agar was made and cooled down to 35°C, 25 mL of agar was added to a 50 mL conical tube; subsequently, 250 mL of the toxicant was added to the tube. A final 25 mL of agar was added in the tube and the agar was shaken up until it was fully mixed. Sixteen mL of the agar solution was added to a 15 X 100 mm plate and swirled around so that the entirety of the bottom of the plate is covered evenly. This was left to cool and dry for at least 12 hours. After the agar was cooled, 300 µL of 20x UVRA was added to each plate. This was left to dry completely with the lid off. The plates were then wrapped in Parafilm and placed in a 4°C refrigerator. For dosing the worms, around 2000 L1 N2 PE255 worms were added to an exposure plate with the volume under 400 µL. The worm solution was spread out as evenly as possible on the plate and were left to dry in the hood. Once dried, these were sealed in Parafilm and left to grow into the L4 stage in a 20°C (defined as 37.5 hours). At this time the worms were rinsed off the plate using K medium into a 15 mL tube. This was spun down for 2 minutes at 542 g. The supernatant was aspirated to around 150 µL and is transferred to a 2 ml tube for each dose which was then filled up to 2 mL with K medium. This was spun down at 2 min and supernatant was aspirated to leave ~100 µL. It was then spun down one last time and the supernatant was drawn off to around 500 µL. On a 96 well plate, samples were added to a well to a volume of 100 µL with four replicates per dose/blank. The blanks were K medium. For the exposed worms, each well had 100 worms and was filled to 100 µL with K medium. The Luciferase Buffer was made and the plate was analyzed using a BMG Labtech FLUOstar Optima plate reader. In this, an initial reading of GFP was read to normalize the subsequent GFP readings; this used a gain adjustment of 90%. The emission filter was a 520 nm emission with a 485 excitation filter and a combination optic. The plate machine injection tubes were cleaned with double deionized water and 70% ethanol. It was then primed with 1.5 mL of luciferase buffer. The combination optic, which measures fluorescence, was changed to the luminescence optic for this measurement. The plate had a gain adjustment of 40%. The reader then injected 50 µL of luciferase solution to each well; after 3 minutes, the emission was measured. The data was normalized to GFP and had blank subtractions from the luciferase measurement; three luminescence readings were normalized to the vehicle control.

**Hypothesis**

For the growth assay, the hypothesis was tested at each dosage given and for each toxicant. The hypothesis tested was that H₀: There is no difference between the percent of the control dose growth rates between the control strain and the mutant strains. Percent control
growth rates are used because some strains are slower-growing than wild-type even under control conditions. The alternative hypothesis, $H_a$, states that there is a difference between the percent control growth of the control dose of the control strain and the mutant strain. The post hoc test will conclude if $H_0$: There is no difference between the control strain and a specified mutant strain in percent control dose growth; $H_1$: There is a difference between the control strain and the specified mutant strain. The luciferase experiment had the hypothesis of $H_0$: There is no difference in the levels of (luciferase, GFP, or luciferase/GFP) between the different doses of the FRs; $H_a$: There is a difference between levels of (luciferase, GFP, or luciferase/GFP) between all treatment groups. The post hoc test has the hypothesis of $H_0$: There is no difference between the levels of (luciferase, GFP, or luciferase/GFP) between the control and a dose; $H_a$: There is a difference between the levels of (luciferase, GFP, or luciferase/GFP) between the control dose and the toxicant dose.

**Recipes**

**Luciferase Buffer**

(50 µL of 5% Triton 100x, 50 µL of DMSO, 50 µL of Lucifer in Salt solution, 3.483 mL 0.2 M NaPO$_4$, 1.538 mL 0.1 M Citric Acid)

K medium for 1 liter
(1 L double distilled water, 2.36 grams KCl, and 3 g NaCl);

K$^+$ medium for 50 mL
(50 mL of K medium, 25 µL cholesterol (10 g in 1L of 100% ethanol), 150 µL 1 M CaCl$_2$, 150 µL 1 M MgSO$_4$)

K agar plates for 2 liters
Before autoclave
2 L of double distilled water, 40 g of Bactor Agar, 5 g of Bacto-peptone, 6 g of NaCl, 4.72 grams of KCl).
After Autoclave
1 mL cholesterol (10 g in 1L of 100% ethanol), 1 mL 1 M CaCl$_2$, 1 mL 1 M MgSO$_4$, and 10 mL of Nystatin (12.5 mg).
17 mL are added to a 100 X 15 mm plate.
After this cools down 300 µL of OP50 bacteria is added and left to dry.

**Statistical analysis**

Statistical analysis was conducted with R v. 3.0.1 (R Development Core Team 2011). The alpha that was selected was the standard 0.05. Both percent growth and luciferase assays were first analyzed with a Kruskal-Wallis test. If found to be significant for either assay, a Dunn’s multiple comparison post hoc test with a control using the Dunn.test.control function from the PMCMR package with a Bonferroni correction (Pohlert, 2014) was performed. The test is two tailed because the mutant strain has the possibility of being more or less sensitive to the toxicant compared to the control. Line graphs and summary statistics were generated using the
RESULTS

Growth Assay

The growth assay started with 500 L1 worms placed in a 12 well plate for the number of tested doses. K⁺ was added to the wells so the total volume was 895 µL. Then 5 µL of the FR was added; subsequently 100 µL of 2x UVRA was added to each well. This was then wrapped with Parafilm (and foil to protect from light); 25 hours later another 100 µL of 2x UVRA was added to each well. At 48 hours the worms were rinsed and transferred to a freezer to be measured by a microscope at a later time. The growth assay is used as a first pass method to elucidate if the compounds could be mitochondrial toxicants. If a mutant is more or less sensitive to a compound versus the control, it suggests that the toxicant is acting directly on that mitochondrial regulation pathway. However, there is a possibility that the compound is causing damage elsewhere in the cell and the resulting change in toxicity is a secondary effect. Growth is affected by reduced mitochondrial function; the less energy there is in the cell, the less energy can be devoted into making proteins for cellular biogenesis and overall nematode growth. All experiments were run in triplicate.

All dosages of TPP were found to be statistically different in the global test (P<0.001). There was not a No Observable Effect Level (NOEL) found in the control strain. The highest difference between control strain length and a mutant was dct-1 and N2 with dct-1 having 15.8% less growth of the control versus N2. Eat-3 was found to be more than 10% sensitive at the 20 M dose, while fzo-1 was more than 10% sensitive at the 10 µM dose (P<0.001). This sensitivity was diminished at higher dosages. Pink-1 was the only mutant to not have any statistical difference between N2 at any dose for TPP. Pdr-1 was protected at the highest dose by more than 10% (P<0.001).

Table 2, Relative Change in Percent Length of Different Mitochondrial Mutant C. Elegans exposed to TPP

<table>
<thead>
<tr>
<th></th>
<th>0 uM</th>
<th>1 uM</th>
<th>5 uM</th>
<th>10 uM</th>
<th>20 uM</th>
<th>50 uM</th>
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<tbody>
<tr>
<td>dct-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>**</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>pdr-1</td>
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<td>0</td>
<td>0</td>
<td>**</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>pink-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>drp-1</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td></td>
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<td>***</td>
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</tr>
<tr>
<td>eat-1</td>
<td>0</td>
<td>+</td>
<td>**</td>
<td>***</td>
<td></td>
<td>***</td>
</tr>
</tbody>
</table>

* = p<0.025, ** = p<.005, and *** = p<0.0005

In all growth tables, there are 2 different set of symbols found in each cell on the grid. When compared to the control strain and the amount of growth reduction, there will be two set of symbols: pluses, minus, and zeroes. This will indicate that there is more or less growth inhibition for that mutant and that strain; stars indicate how statistically significant this change in growth inhibition is. One plus or minus means that the change in growth was less than 10% absolute difference, two symbols indicate more than a 10% difference. Zeroes indicate that there was not a statistically difference in growth inhibition. The p-values that are reported out of R are adjusted for Bonferroni (the values are multiplied by the number of tests-1) and as such they are reported as the multiplied values.
The summary results for IPP are as follows. IPP had different dosing concentrations than the other two FRs. The initial growth assay had IPP saturating in toxicity between 37.5 and 62.5 µM. In addition, the chemical was found to precipitate out of solution at higher concentrations, around 62.5 µM. A global test found that all doses of IPP above 0.1 µM were different (P<0.001). Eat-3 was more than 10% protected at the highest three dosages (P <0.001). The other strain that presented more than 10% protection against this IPP was pdr-1 at the highest dose tested (P<0.001). Although 0.25 was found to be the LEOL control strain, no mutants were more sensitive than the control.

Table 3, Relative Change in Percent Length of Different Mitochondrial Mutant C. Elegans exposed to IPP

<table>
<thead>
<tr>
<th></th>
<th>0 uM</th>
<th>0.1 uM</th>
<th>0.25 uM</th>
<th>9 uM</th>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
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<td>pink-1</td>
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<td>drp-1</td>
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<td>fzo-1</td>
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<td>eat-3</td>
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<td>+ ***</td>
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</tr>
</tbody>
</table>

* = p<0.025, ** = p < .005, and *** = p <0.0005

All dosages of the TBDP were found to be different in the global test (P <0.001). The NOEL for the control was found to be 1 µM, with 5 µM as the LOEL. Table 4. Interestingly pink-1 was protected more than 10% at the 20 µM dose but lost this at 50 µM (P <0.001), Table 4. As with TBDP eat-3 was more than 10% protected at the highest dose (P<0.001), Table 4.

Table 4, Percent Change in Percent Length of Different Mitochondrial Mutant C. Elegans exposed to TBDP

<table>
<thead>
<tr>
<th></th>
<th>0 uM</th>
<th>1 uM</th>
<th>5 uM</th>
<th>10 uM</th>
<th>20 uM</th>
<th>50 uM</th>
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<tr>
<td>dct-1</td>
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<td>0</td>
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<tr>
<td>pdr-1</td>
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<td>- *</td>
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<tr>
<td>pink-1</td>
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<td>- *</td>
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<tr>
<td>drp-1</td>
<td>0</td>
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<td>+ *</td>
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<tr>
<td>fzo-1</td>
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<td>+ ***</td>
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<td>eat-3</td>
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<td>+ ***</td>
</tr>
</tbody>
</table>

* = p<0.025, ** = p < .005, and *** = p <0.0005

To see if one class of mutants were more sensitive to FRs overall, the comparative toxicity was grouped by function.

Fusion Mutants

The two fusion mutants, eat-3 and fzo-1, elicited similar results when exposed to the same compounds (Figure 4). The difference is the extent of the change. These mutants were both protected and sensitive to TPP, depending on the dose. Both were protected at 1 µM, but to a small extent: 6.13% growth protection for fzo-1 and 3.96% for eat-3. At higher doses, the strains
were more sensitive with \textit{fzo-I} being more sensitive at 10 \(\mu\)M (15.46% growth inhibition compared to control) and \textit{eat-3} more sensitive at 20 \(\mu\)M with 10.2% more growth inhibition than control (Figure 4). Both fusion mutants were protected when exposed to IPP and TBDP. However, only \textit{eat-3} was protected from exposure by more than 10% each time; 11.01% TBDP at 50 \(\mu\)M and 16.24% at 37.5 \(\mu\)M of IPP. IPP (Figure 4) did not reduce growth very much in \textit{eat-3} compared to \textit{fzo-I}. Additionally, both \textit{fzo-I} and \textit{eat-3} have larger inter-quartile range (IQR) than the control strain except at the highest dose.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Fusion Mutants Growth Exposed to TPP, IPP, TBDP}
\end{figure}

\textit{Fission Mutants}

The only fission mutant used in this assay was \textit{drp-1}. With IPP, the sensitivity was only observed at 9 \(\mu\)M; at higher dosages the sensitivity was not observed (Figure 5). When exposed to TBDP, \textit{drp-1} was protected at 5, 20, and 50 \(\mu\)M with none of the protection reaching above 10% (Figure 5). \textit{Drp-1} was more sensitive to TPP than the control at the two highest dosages; at the highest dose of TPP (50 \(\mu\)M), there was a 12.28% more sensitivity. As seen in (Figure 5), \textit{drp-1} had a smaller IQR than the control at the two highest dosages of TPP.
Three different mitophagy mutants (pdr-1, pink-1, dct-1) were tested. For TBDP, at 1 µM, pdr-1 and pink-1 were more sensitive while at higher dosages pink-1 and pdr-1 were protected. Dct-1 was only slightly protected at 5 µM; this is similar to what was seen in IPP where pdr-1 and pink-1 were more sensitive at lower dosages. This time only pdr-1 and dct-1 were protected at the highest dose with 12.12% less growth inhibition for pdr-1. For TPP, all three mutants reacted differently. The pink-1 mutant not significantly different than the control at any dose. The pdr-1 mutant protected from 10 to 50 µM, with the most protection at 50 µM (13.07% less growth inhibition). Dct-1 was found significantly different than N2 at all doses above 10 µM with the most sensitivity at 50 µM (increased sensitivity of 15.8%). As seen in (Figure 6), the IQR of dct-1 at 50 µM is the highest of any the mitophagy strains and dose of TPP. Pdr-1 changed very little in both the amount of growth and spread of data; pink-1 seems to match the control of N2 at dosages above 5 µM of TPP, (Figure 6).
In the luminescence assay, all the worms have luciferase enzyme attached to a green fluorescent protein (GFP). The GFP proteins are located in the cytosol and are activated by being exposed to luciferase. At this point, the protein starts to luminesce and the wavelength is measured by a probe; however, for the enzyme to have the ability to metabolize, it needs a supply of ATP. Therefore, by exposing same amount of worms to the same amount of luciferin, ATP should be the limiting factor. Lower emissions mean that there is less ATP being produced (Lagido et al., 2015). This is roughly used as a measure of mitochondrial function; however, this does not account for other processes using ATP such as damaged proteins being repaired. This requires ATP and would lower the amount of free ATP in the cytosol. With less ATP able to interact with GFP, there would be a lower emission signal; the lower signal could falsely be attributed to mitochondrial toxicity when the mitochondria are functioning normally. The measurement would be measuring the reduced ATP from being used in other biological processes and not direct toxicity to the mitochondria. This would be considered a secondary side effect of exposure to the FRs.

For the luciferase assay, L1 N2 PE255 worms were plated onto plates that contained various concentrations of the FRs. Thirty-seven hours later or about L4, the worms were rinsed off the plate and cleaned 2 times. The worms were plated onto a 96 well plate and placed into a plate reader. The first reading was of GFP, the second reading had a luciferase mixture injected into each well and then 3 minutes later the luciferase reading was taken. The following assays were analyzed in a similar manner. The GFP and luminescence could not be transformed to have a normal distribution; accordingly, a non-parametric test was conducted. GFP readings were taken as a way to normalize the amount of luciferase proteins in the wells. Since GFP will fluoresce when exposed to a set wavelength, one can measure the amount of luciferase enzyme.
There has been a correlation shown that for there is a linear relationship between the amount of luciferase enzyme and the size of the worm (Lagido et al., 2015).

There was a significant difference in the levels of GFP, luminescence, and luminescence/GFP on a global scale. However, there was not a statistical difference between the control and any of the doses (p = 6.26 * 10^{-3}; p = 2.15 * 10^{-6}; p = 6.80 * 10^{-5}). At the 20 µM dose there was found to be 45.25% less than the control and was trended towards significance. This is seen in (Figure 7), in which the highest dose is the lowest median value. Even though the test did not yield a statistically significant result, it does appear that ATP does decreases with increasing dosages of TPP.

**Figure 7, Luminescence TPP**

Luminescence/GFP were found to be different on the global scale for IPP (, p = 1.24 * 10^{-7}; p = 9.81 * 10^{-6}). The 40 µM dose was the only dose to be found different in the post hoc analysis (P<.001). This compared significant result in the levels of GFP and Luciferase (P<0.001). All doses were significantly different for GFP (P<0.05). The results of this indicate that IPP does decrease the amount of ATP with increasing dosages, yet this is only prevalent at the highest dose (Figure 8).
The main significant difference in all the tests was that there was an increase in luciferase/GFP at 5 µM (P<0.5). This was discovered after a significant global test (p = 2.73 * 10^{-4}). GFP levels did not significantly change with the increasing dose of TBDP (p = 0.11). The difference in luminescence levels was found to be statistically different at (p = 1.67 * 10^{-2}) but none of the doses were significant. These results indicate that TBDP does not decrease the amount of ATP with increasing dosages (Figure 9).

DISCUSSION

Utilizing C. elegans to test for mitochondrial toxicity of FRs lead to a range of discoveries. The first was that no mutant was more than 10% more or less sensitive to either TBDP or IPP than was the wild-type strain (Table 3 and 4). However, eat-3 was less sensitive to
both FRs, and pdr-1 was less sensitive to IPP. TPP caused more than a 10% increased sensitivity in multiple mutants that spanned the three tested functions of mitochondrial regulation (Table 2). Results from the ATP assays were less clear; exposure to TBDP at 5 µM resulted in more ATP than the controls (Figure 9); exposure to TPP had no detectable effect on ATP (Figure 7); and IPP at the highest dose reduced steady-state ATP (Figure 8).

**TPP**

Overall, TPP had the most evidence for being a mitochondrial toxicant. First, there were multiple mitochondrial mutants that were more sensitive to exposure (Table 2). Second, despite the very wide error bars for GFP normalization (Figure 7), there is a very strong decrease in luminescence levels and standardized luminescence levels. The reduction in standardized luminescence levels is higher than the average of percent control reduction in growth for N2 (65.88% vs 76.91%). There is over a 10% difference, which we defined as a biologically significant decrease in ATP levels. One hypothesis for why fzo-1 and drp-1 were sensitive to TPP at higher dosages than the wild-type strain is that both mutants have low spare respiratory capacity (Luz et al., 2015). As such, these strains have little capacity to increase respiration to meet energy demands associated with normal physiological function, or resulting from damage to other processes. Additional evidence for mitochondrial toxicity of TPP comes from a recent study performed on zebrafish, in which exposure to TPP resulted in increased blood concentration of glucose and pyruvate in addition to fat droplets in the liver. These three occurrences taken together were thought to be related to impaired breakdown of energy sources from the mitochondria (Du et al., 2016). Thus, our data and an independent study examining different endpoints in a different species lend evidence to mitochondrial toxicity for TPP. Adding to urgency for further testing, there are possible sensitive sub-populations of people that have mutations in OPA1 and MFN1/MFN2. These genes are homologs of the two mutants eat-3 and fzo-1, which were found to be significantly sensitive to TPP exposure.

As mentioned in the results section, the three mitophagy mutants responded differently to the three studied FRs. The currently proposed pathway for mitophagy activation in *C. elegans* is seen in the (figure 10). In this dct-1 is activated by three different pathways. One of these includes both pink-1 and pdr-1. Thus it is presumed that even if pdr-1 or pink-1 is reduced, dct-1 can still be activated (Palikaras et al., 2015a). In addition, the alternative pathway which results from oxidative stress from damaged mitochondria can activate skn-1. This can cause the creation of new mitochondria to help balance out the under functioning ones (Palikaras et al., 2015a). Damaged mitochondria can leak Ca²⁺ ions into the cytosol. In cells that lack mitophagy this level is elevated (Palikaras et al., 2015b). *Unc-43* is activated by increased Ca²⁺ levels in the cytosol (Palikaras et al., 2015b). *Unc-43* increases the activity and transcription of SKN-1(Palikaras et al., 2015b). *Skn-1* bins with *daf-16* which then initiate the transcription of proteins that promote mitochondria genesis (Palikaras et al., 2015b). Toxicants like TPP, affecting the master switch of dct-1 at the highest dose may prevent a high enough turnover rate of mitochondria to meet up with a growing worms needs. This may have been normal with pink-1 because with enough time enough ROS could be caused by the mitochondria to induce the second pathway. This pathway starts with oxidative stress, activates skn-1, which then activates dct-1. Once dct-1 is activated, mitophagy can occur Also they are known to have increased proton leak which may contribute to a reduced amount of energy in the long term (Lutz et al., 2015). The protection caused by pdr-1
might be related to having more mitochondria than wild-type (Palikaras et al., 2015a). Out of the three mitophagy mutants, pdr-1 has the most nematodes with normal mitochondrial morphology (Palikaras et al., 2015a). This could mean that within the mutant, other mechanisms are activated that keep a high level of functional mitochondria as discussed above. It could also mean that a reduction in pdr-1 caused another compensatory mechanism that can also adept with dealing with TPP toxicity.

Figure 10, Dct-1 and Mitophagy activation (Palikaras et al., 2015)

TBDP

These experiments did not result in support for the hypothesis that TBDP is a mitochondrial toxicant. In the ATP assay, there was an 18% increase after exposure to TBDP. This would indicate that the amount of ATP present is higher than the control. This could result from a compensatory mechanism in which the worm is trying to deal with the toxicant insult. In an experiment in which L4 stage N2 PE255 nematodes were exposed to processed sewage sludge, a similar trend was observed when measured at 22 hours. The amount of luminescence increased with concentration until it hit a maximum concentration luminescence level. After this point, with increasing concentrations, the ATP levels returned to baseline. When measured 1 hour later the differences between the concentration dose and luciferase levels were not significantly different from the control (McLaggin et al., 2012). This validates that what was observed in this work is not necessarily an anomaly. This also means that this increase could be temporary as the organism may not be able to continue high production of ATP to deal with the toxicant stress. At the same concentration, there was a significant reduction in growth levels for N2. Based on these results it would appear that mitochondria are not a primary target. Rather, this is consistent with growth effects being secondary. However, this does not mean that these effects should be ignored as there were differences in the way the different mutants were affected.

The fact that eat-3 was protected against TBDP toxicity seems surprising. Although this mutant is not noted for having widespread resistance to chemical insults, and in our hands has been sensitive to other stressors (e.g., Bess et al., 2012), there is one published example: in the case of hemiasterlin, eat-3 had a 2 µM higher LD_{50}. This chemical inhibits microtubule assembly (Zubovych et al 2010). The increased protection from the various mutants for TBDP has at least
two potential explanations. The first is that for TBDP, the majority of the protection was less than 10%. This, as indicated in the results, is likely due to statistical spread rather than real biological meaning. The other possibility for this protection, that it is an artifact of differential growth rates between strains, is discussed in the strengths and weaknesses section.

*IPP*

Although IPP was the only FR tested that caused a significant decrease in ATP levels, we do not argue that it is a mitochondrial toxicant, for two reasons. First, the mitochondrial mutants were not more sensitive than wild-type, although our results do not rule out the possibility that strains with mutations in other mitochondrial processes might be more sensitive. Second, the reduction in ATP levels was dramatic but occurred at the highest dose tested for both growth and the luciferase assay; this could indicate that there is wide systemic toxicity occurring and not specifically mitochondrial toxicity. The explanation for why *pdr-1* is protected for IPP is similar to the explanation proposed for TPP; mitophagy can still occur through the alternative pathway and the additional mitochondria produced might help produce enough ATP to help with coping mechanisms against the toxicant exposure.

Our ability to carry out cross comparison to published studies for the ATP luciferase assay was limited. Other studies either used different strains of nematodes, dosed different life stages, or dosed for a much shorter duration (McLaggin et al., 2012; Lagido et al., 2008; Lagido et al., 2015). The study that was the most similar to the one conducted here exposed L1 N2 PE255 to Cd and measured normalized ATP levels 19 hours later. In that study, GFP decreased to 56-58% of the control levels while the luminescence decreased as well (Lagido et al., 2009). This reduction of GFP levels is not always seen in acute exposure until very high levels (Lagido et al., 2008; Lagido et al., 2015). Although N2 PE255 and N2 PE254 have similar expression levels of GFP in some experiments, McLaggin et al., 2012, they can have quite drastic different response slopes (Lagido et al., 2009). A steeper response slope indicates it takes less of a chemical to elicit the same response as another chemical. If the two GFP luciferase nematodes have different dose response slopes for some chemicals, then this reduces the validity of comparing relative toxicity values between the two strains. This again reduces the ability to cross compare results to other studies.

*Strengths and Weaknesses of Experiments*

The strengths of the growth assay for the experiments conducted throughout the study are as follows. Analysis of the effect of the exposures in the context of the mutations allow for a first-pass mechanistic understanding of toxicity of a chemical: if a mutant in a specific pathway is more sensitive than wild-type, it is likely that pathway is targeted by the chemicals being tested, and approach that has been described as “functional toxicology” (Gayton & Vilpe, 2014). Of course, this does not preclude more precise experiments in the future to elucidate the exact mechanism. In addition, the experiment is relatively quick as the assay was done in 48 hours. However, the growth assay has some limitations as well. Standardizing to percent length of control helps to normalize across mutant strains that have different maximal lengths and slight variation in growth rates. It would be expected that their respective controls would be smaller so the length that a dosed worm grows would be proportional to that strains control. This facilitated
comparison of wild-type to these various mutants. However, this becomes an issue when the growth rate is drastically slower, as with eat-3 and fzo-1 (Avery, 1993). The rate of growth is so much slower that it takes about an extra day to reach adulthood versus control. This means that these strains would not have grown as much as the control even without exposure. This reduced total length means that the difference between the hatching length and the measured length of the nematode after the 48-hour mark will be much lower percent difference. So when an exposure retards growth, this magnitude of growth retardation will be smaller for the mutants, in particular if the growth deficit in wild-type is quite large already. If the effect in wild-type is not large, then this is not an issue; for example, we could easily detect exacerbated growth delay after exposure to low-dose ultraviolet C radiation in fzo-1 and eat-3 nematodes (Bess et al., 2012). One way to account for this, as long as growth inhibition is not too drastic in wild-type, is to analyze percent growth. This involves taking the length of the control, subtracting the L1 length and dividing that by the amount of time to get a growth rate. That rate could then be set to 100% and all subsequent dosed worms growth rates could be compared to that. There was also a possible issue with equal exposure in the eat-3 strain. Eat-3 mutants have sluggish and irregular pharyngeal pumping (Avery, 1993), and were in fact named based on the phenotype of eating slowly. This is an issue if one of the exposure pathways is through ingestion and the FRs bind to the food. In this case eat-3 will have lower exposure levels despite equal exposure medium concentrations. Finally, this assay does not include mutants that are involved in ATP production or other mitochondrial functions. As such there could be mitochondrial toxicity that was affected that would not have been elucidated by these mutants. The ATP assay thus was a complement to the growth assay in assessing if mitochondria was a target for these mutants these other results as a possible.

Strengths of the ATP assay for these experiments are as follows. The assay is more sensitive than traditional lysing of C. elegans to measure relative ATP levels (Lagido et al., 2008). In addition, the real time measurement allows for short acute exposure or chronic (Lagido et al., 2008; Lagido et al., 2009). This allows one to track possible recovery at a rapid pace. However, a few sources of error could have occurred with the ATP measurements. The first one was that the length of the nematodes were not measured, as such there could have been significant differences in length of the nematode between doses of the FRs. Reduced length would have mean less mitochondria and thus less ATP. Since ATP drives the luminescence of luciferase, there could have been a lower value that was more related to growth arrest and not mitochondrial toxicity. This should have been accounted for by GFP but ideally should also be accounted for with taking length measurements of the exposed worms, because GFP expression varies between different life stages and thus serves to normalize much better within than between life stages. In particular, the L4 stage is noted for a large increase in mitochondria (i.e., mitochondrial biogenesis). If the worms were borderline L3/L4 or even late L3 there would be a disproportionately less mitochondria that even length would not have accounted for. Imaging of the worms could help clear up the proportion of worms that fell into the category. If there were a disproportionately large percentage of worms that fell into this category, the ATP measurements can be made at a different time point or the dose could be thrown out. This could have made the higher doses have a larger difference from the control. In addition, the number of worms could have been different in each well that was measured. Following the previously mentioned protocol should have reduced this error, but it is still possible that there was a difference in the number of worms in each well. This again could be accounted for with GFP normalization but
this compounded with differences of size could skew ATP measurements more. Finally, there is the possibility that any of these FRs actually target the luciferase tagged enzyme and modulate the functionality. There is a possibility that the FRs either acted as a competitive inhibitor and prevented ATP binding or acted as a cofactor and changed the shape of the binding pocket so that ATP no longer fit. Both of these would reduce the efficacy of the protein and thus reduce the fluorescence lower than the actual level of ATP would dictate. This could be elucidated by either performing an x-ray crystallography, and looking for structural changes, or by performing in vitro ATP assays as outlined in Lagido et al, 2009.

**PBPK Model for C. elegans**

Physiologically-based pharmacokinetic (PBPK) are models that help to estimate the absorption, distribution, metabolism, storage, and excretion of a compound given to a model organism. This allows for the estimation of how much of a compound will be found in various tissues based on an administered dose and how long it will take for the compound to be removed from the system. There is currently not a PBPK model developed for *C. elegans*, which limits our ability to understand actual internal dose levels. There are two main routes for exposure in *C. elegans*: ingestion and dermal uptake. A literature search yielded few values for the volume of these compartments, which hinders the usefulness of any PBPK. The most information is available on ingestion as an exposure route. When the chemical was ingested, the first area that the toxicant would reach would be the pharynx. This area has two sections with pharynx 1 having a pH of 5.71 while pharynx 2 had a pH of 4.95 (Chauhan et al., 2013). After this point the food moves into the lumen. The pH is found to be between 3.92 and 4.05 (Pfeiffer et al., 2008; Chauhan et al., 2013). The pH drops in this compartment to 2.07 when defecating (Pfeiffer et al., 2008). The retention time of food is three to ten minutes (Avery & Shtonda, 2003), with the amount of time increasing significantly in older nematodes. The chemical can be taken up by intestinal cells which have an internal pH of 7.4 (Johnson & Nehrke, 2010; Pfeiffer et al., 2008). The pH of this compartment falls down to 7 during defecation (Johnson & Nehrke, 2010). Metabolism and detoxification can occur in these cell types thus reducing the chance of exposure to other components (McGhee, 2007). The drug can then be transferred to the pseudocoelom which has a volume of 40 to 80 picoliters (Banse & Hunter, 2012). The pH of this compartment is 7.29 at rest and decreased to 6.32 while defecating (Pfeiffer et al., 2008). This compartment acts as a crude circulatory system and can deliver the toxicant to other components of the nematode; this can include the muscle (pH of 7.49) or the neuron (pH of 7.52) (Johnson & Nehrke, 2010). As seen from the different studies the biggest difference in pH levels is between the lumen of the intestine and the intestinal cells as would be expected. The pseudocoelom can have significant differences in pH from the intestinal, muscle, or neuron cells. This difference in pH could shift equilibrium of a toxicant to have a higher concentration in one of previously mentioned cell types. Fat is stored mainly in fat droplets inside intestinal cells (Mak, 2012). Many toxicants are lipophilic and can be transported into these cells but without knowing the volume, the storage of these molecules is unknown. Chemicals such as FRs used in this study have a high Kow and are lipophilic. These chemicals would more likely be found stored in fat than in the cell. This could reduce their rate of transfer and cause massive release of the chemical if there was a starvation event or transfer to another growth stage.
In addition, some of the mutants have different internal environments than the wild-type. *Fzo-1(tm1133)* and *eat-3 (ad426)* were 0.31 pH units more acidic in the intestine cells with a value of 7.09. *Fzo-1* neurons were 0.18 pH units more acidic, with a value of 7.34. The muscles were the most different, with *fzo-1* having slightly more acidic with a 0.17 more acidic with a pH value of 7.32; *eat-3* was 0.39 more acidic with a pH of 7.10. *Drp-1(tm1108)* was found to have similar values to wild-type (Johnson & Nehrke, 2010). This complicates toxicokinetic understanding because different acidity levels mean different levels of partitioning. Although the absolute magnitude difference is not large between wild-type and some of the mutants, if the pKa is between 6 and 8, there could be noticeable differences in the amount of toxicant absorbed from the lumen and deposited into intestinal and muscle cells. Unfortunately, without knowledge if there is a difference in pH pseudocoelom or intestinal lumen, the extent of this difference is unknown.

There are additional parameters that would be important to the development of a PBPK model. More needs to be known about approximate volume of various compartments, the pH of the compartments for different mutants, and the approximate volume of fat in the organism. Furthermore, dermal absorption remains unexplored. This adds another layer of complexity, as the cuticle changes not only in composition and thickness as the worms mature (Page & Jhonstone, 2007). This means at different life stages the rate of a molecule with a very high apparent partition coefficient would get into the worm faster. The shedding of the cuticle with each molting stage would allow for a chance for molecules with lower apparent partition coefficient that would be degraded by one of the cell layers of the cuticle to possibly enter the worm; it also offers the opportunity for elimination. As such there is still much to be discovered in how these changes in the cuticle and normal penetration could help understand the extent that this exposure route plays to internal dosing at molecular targets for toxicants. With this known there could be a much more accurate model made which could help predict target organs and approximate internal dosing. Although this is not a complete PBPK, it does lay the groundwork for the construction of one.

**Comparison of Methods**

Although the drug delivery method was different in the growth and luciferase assay, Zheng et al. found that dosing through toxic plates like prepared in this paper and liquid medium were similar. Worms had about the same internal exposure to compounds that they tested (Zheng et al., 2013). This is not taken that all chemicals will behave like this; these chemicals are semi (TPP) to not volatile (IPP, TBDP) (UK EA 2009a; UKEA, 2009b; USA EPA 2008). This means that there should not have been evaporation of the FRs when mixed into the agar. Due to this, the comparison between the luciferase and growth assays are more substantiated when comparing N2 to N2 results.

**Future Directions**

Given our data supporting a mitochondrial mechanism of TPP toxicity, future work should further investigate the mitochondrial effects of TPP. One direction would be to measure more mitochondrial parameters using the methods outlined by Luz et al., 2015 to analyze the fundamental respiratory-related function of mitochondria in *C. elegans*. This assay could give
insight into potential effects of TPP such as spare respiratory capacity and basal oxygen (Luz et al., 2015). This would help define which mitochondrial functions are affected, and also provide hints about how TPP exposure could affect how much stress an organism can tolerate energetically. Proton leak reduces the amount of ATP that a mitochondrion can make per unit of sugar/fat/carbohydrate. This means that more energy sources have to be consumed to maintain an adequate energy level. The second direction would be to investigate reactive oxygen species (ROS) generation in the mitochondria. Both fzo-1 and eat-3 are sensitive to ROS (Kanazawa et al. 2008). Further support for this comes from a study of TPP dosing in TM3 Leydig cells. A dose of 60 µg/ml caused over a two-fold increase in transcription of superoxide dismutase (SOD) 2 (mitochondrial) and a 21% increase of SOD activity in the cell (Chen et al., 2015). However, this study did not separate the activity of the different SODs which means that the activity of the cytosolic SOD could have diluted the increased activity of SOD 2; increased activity of this enzyme would lend support to mitochondria ROS production. An experiment could be conducted in which either a probe measured ROS production in the mitochondria, or the functional importance of ROS is tested by attempting a pharmacological rescue. The rescue experiment would involve dosing C. elegans with TPP and then a mitochondrial-targeted antioxidant. Length measurements would involve three treatments: a control, a TPP dosed group, and a TPP dosed group with the rescue. If the length of the group with the antioxidant was less retarded in growth than the TPP only group, then one could conclude that the TPP results in ROS production in the mitochondria.

CONCLUSION

TPP, IPP, and TBDP are high volume FRs that are in current use. Based on preliminary data from the NTP, the toxicity of these chemicals on mitochondria was explored. C. elegans were utilized because of the rapidness of experiments with this model, the availability of mitochondrial mutants, and the fact that it is an in vivo model. The results of the growth assay using mitochondrial regulation mutants support the possibility that TPP is a mitochondrial toxicant. This was not fully supported by the ATP luciferase data, but this may be due to high variability in the luciferase data. Further mitochondrial toxicity should be explored using either an assay to explore specific mitochondrial functions or by investigating possible ROS production.

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BIBLOGRAPHY


Danish Environmental Protection Agency., *Environmental and health screening profiles of phosphorous flame retardants* (Environmental Project No. 1823, 2016). 2016 Copenhagen, Denmark, government printing office.


Lu, B. Mitochondrial dynamics and neurodegeneration (pp. 17-19). 2011 Dordrecht: Springer.


Meyer, J.; Stapleton, H., shared unpublished NIEHS data that indicates mitochondria toxicity to TPP (personal communication with Mamta Behl from NIEHS, February 3, 2015)


MRIGlobal., Chemical identity and purity screen final report tert-butylphenyl diphenyl phosphate chemical identity and purity screen of tert-butylphenyl diphenyl phosphate (NTP Chemtask No.: Chem10729) 2012. Kansas City, MO.


San Francisco Estuary Institute., Characterization of organophosphorus chemicals in a PentaBDE replacement mixture and their detection in biosolids. 2009 Oakland, CA.


**SUPPLEMENTAL INFORMATION**

*Growth Assay Test Statistics*

**Table S1, Test Statistics for TPP growth assay**

<table>
<thead>
<tr>
<th>Dose of TPP</th>
<th>Global (Kruskal-Wallis test)</th>
<th>Dct-l (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Drp-l (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Eat-3 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Fzo-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Pdr-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Pink-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>X² = 9.97, df = 6, p = 0.13</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 µM</td>
<td>X² = 22.5, df = 6, p = 9.9 * 10⁻⁴</td>
<td>Z = 1.59, p = 0.6678</td>
<td>Z = 3.47, p = 3.1 * 10⁻³</td>
<td>Z = 3.16, p = 9.4 * 10⁻³</td>
<td>Z = 3.73, p = 1.1 * 10⁻³</td>
<td>Z = 2.71, p = 4.0 * 10⁻⁷</td>
<td>Z = 1.86, p = 0.37</td>
</tr>
<tr>
<td>5 µM</td>
<td>X² = 44.4, df = 6, p = 9.2 * 10⁻⁸</td>
<td>Z = 0.77, p = 1.0</td>
<td>Z = 0.48, p = 1.0</td>
<td>Z = 2.81, p = 3.0 * 10⁻²</td>
<td>Z = 4.52, p = 3.8 * 10⁻⁵</td>
<td>Z = 1.75, p = 0.48</td>
<td>Z = 2.36, p = 0.11</td>
</tr>
<tr>
<td>10 µM</td>
<td>X² = 137, df = 6, p = 2.2 * 10⁻¹⁶</td>
<td>Z = 2.86, p = 2.5 * 10⁻²</td>
<td>Z = 0.502, p = 1.0</td>
<td>Z = 4.40, p = 6.5 * 10⁻⁵</td>
<td>Z = 8.31, p = 5.4 * 10⁻¹⁰</td>
<td>Z = 3.66, p = 1.5 * 10⁻³</td>
<td>Z = 0.223, p = 1.0</td>
</tr>
<tr>
<td>20 µM</td>
<td>X² = 141, df = 6, p = 2.2 * 10⁻¹⁶</td>
<td>Z = 3.56, p = 2.2 * 10⁻³</td>
<td>Z = 4.11, p = 2.4 * 10⁻⁴</td>
<td>Z = 5.66, p = 8.9 * 10⁻⁸</td>
<td>Z = 3.62, p = 1.8 * 10⁻³</td>
<td>Z = 5.46, p = 2.8 * 10⁻⁷</td>
<td>Z = 0.810, p = 1.0</td>
</tr>
<tr>
<td>50 µM</td>
<td>X² = 186.4, df = 6, p = 2.2 * 10⁻¹⁰</td>
<td>Z = 6.41, p = 8.7 * 10⁻⁹</td>
<td>Z = 6.22, p = 3.0 * 10⁻⁵</td>
<td>Z = 4.35, p = 8.3 * 10⁻⁵</td>
<td>Z = 2.09, p = 0.22</td>
<td>Z = 6.42, p = 8.1 * 10⁻¹⁰</td>
<td>Z = 0.451, p = 1.0</td>
</tr>
</tbody>
</table>
Table S2, Test Statistics for IPP growth assay

<table>
<thead>
<tr>
<th>Dose of IPP</th>
<th>Global (Kruskal-Wallis test)</th>
<th>Dct-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Drp-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Eat-3 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Fzo-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Pdr-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
<th>Pink-1 (Dunn’s comparison with control and Bonferroni Correction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>X² = 2.82, df = 6, p = 0.83</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>X² = 7.23, df = 6, p = 0.30</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0.25 µM</td>
<td>X² = 21.6, df = 6, p = 1.4 * 10⁻³</td>
<td>Z = 1.67, p = 0.56</td>
<td>Z = 2.86, p = 2.6 * 10⁻²</td>
<td>Z = 2.15, p = 0.19</td>
<td>Z = 0.232, p = 1.0</td>
<td>Z = 1.84, p = 0.39</td>
<td>Z = 1.60, p = 0.66</td>
</tr>
<tr>
<td>9 µM</td>
<td>X² = 101, df = 6, p = 2.2 * 10⁻¹²</td>
<td>Z = 5.60 * 10⁻² p = 1.0</td>
<td>Z = 7.26, p = 2.4 * 10⁻¹²</td>
<td>Z = 2.13, p = 0.20</td>
<td>Z = 2.09 * 10⁻³, p = 1.0</td>
<td>Z = 4.71, p = 1.5 * 10⁻⁵</td>
<td>Z = 2.85, p = 2.7 * 10⁻²</td>
</tr>
<tr>
<td>18 µM</td>
<td>X² = 66.7, df = 6, p = 1.9 * 10⁻¹²</td>
<td>Z = 0.384, p = 1.0</td>
<td>Z = 6.42, p = 8.3 * 10⁻¹⁰</td>
<td>Z = 1.80, p = 0.44</td>
<td>Z = 0.531, p = 1.0</td>
<td>Z = 2.44, p = 8.8 * 10⁻²</td>
<td>Z = 2.85, p = 1.0</td>
</tr>
<tr>
<td>28 µM</td>
<td>X² = 89.5, df = 6, p = 2.2 * 10⁻¹⁰</td>
<td>Z = 1.15, p = 1.0</td>
<td>Z = 1.67, p = 0.57</td>
<td>Z = 6.73, p = 9.9 * 10⁻¹¹</td>
<td>Z = 4.19, p = 1.7 * 10⁻⁴</td>
<td>Z = 2.89, p = 2.6 * 10⁻²</td>
<td>Z = 0.473, p = 1.0</td>
</tr>
<tr>
<td>37.5 µM</td>
<td>X² = 136, df = 6, p = 2.2 * 10⁻¹⁶</td>
<td>Z = 4.48, p = 4.5 * 10⁻⁵</td>
<td>Z = 0.814, p = 1.0</td>
<td>Z = 8.96, p = 2.2 * 10⁻¹⁰</td>
<td>Z = 3.94, p = 4.8 * 10⁻⁴</td>
<td>Z = 6.51, p = 4.5 * 10⁻⁴</td>
<td>Z = 0.353, p = 1.0</td>
</tr>
<tr>
<td>Dose of TBDP</td>
<td>Global (Kruskal-Wallis test)</td>
<td>Dct-1 (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>Drp-1 (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>Eat-3 (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>Fzo-1 (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>Pdr-1 (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>Pink-1 (Dunn’s comparison with control and Bonferroni Correction)</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>0 µM</td>
<td>X² = 1.2828, df = 6, p = 0.97</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 µM</td>
<td>X² = 29.5, df = 6, p = 4.8 * 10⁻⁵</td>
<td>Z = 1.25, p = 1.0</td>
<td>Z = 1.88, p = 0.36</td>
<td>Z = 0.814, p = 1.0</td>
<td>Z = 0.903, p = 1.0</td>
<td>Z = 3.17, p = 9.2 * 10⁻³</td>
<td>Z = 2.72, p = 3.8 * 10⁻²</td>
</tr>
<tr>
<td>5 µM</td>
<td>X² = 49.7, df = 6, p = 5.4 * 10⁻⁹</td>
<td>Z = 5.72, p = 6.3 * 10⁻⁸</td>
<td>Z = 3.26, p = 6.7 * 10⁻³</td>
<td>Z = 1.46, p = 0.87</td>
<td>Z = 0.142, p = 1.0</td>
<td>Z = 2.38, p = 0.10</td>
<td>Z = 4.50, p = 4.1 * 10⁻⁵</td>
</tr>
<tr>
<td>10 µM</td>
<td>X² = 13.5, df = 6, p = 3.6* 10⁻²</td>
<td>Z = 0.380, p = 1.0</td>
<td>Z = 08.81 8.10⁻³, p = 1.0</td>
<td>Z = 0.968, p = 1.0</td>
<td>Z = 2.34, p = 0.12</td>
<td>Z = 1.89, p = 0.35</td>
<td>Z = 4.87 * 10⁻², p = 1.0</td>
</tr>
<tr>
<td>20 µM</td>
<td>X² = 108, df = 6, p = 2.2 * 10⁻¹⁶</td>
<td>Z = 1.01, p = 1.0</td>
<td>Z = 4.58, p = 2.7 * 10⁻⁵</td>
<td>Z = 1.74, p = 0.49</td>
<td>Z = 2.04, p = 0.25</td>
<td>Z = 1.715, p = 0.52</td>
<td>Z = 9.30, p = 2.2 * 10⁻¹⁶</td>
</tr>
<tr>
<td>50 µM</td>
<td>X² = 106, df = 6, p = 2.2 * 10⁻¹⁶</td>
<td>Z = 1.77, p = 0.45</td>
<td>Z = 4.46, p = 4.7 * 10⁻⁵</td>
<td>Z = 7.11, p = 6.6 * 10⁻¹²</td>
<td>Z = 3.36, p = 4.6 * 10⁻³</td>
<td>Z = 6.30, p = 1.8 * 10⁻⁹</td>
<td>Z = 3.91, p = 5.5 * 10⁻⁴</td>
</tr>
</tbody>
</table>

Table S3, Test Statistics for TBDP growth assay
ATP Assay Test Statistics

Table S4, TPP Test Statistics for ATP growth assay

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>LUM</th>
<th>LUM/GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Global Test (Kruskal-Wallis test)</strong></td>
<td>$X^2 = 14.3$, df = 4, $p = 6.3 \times 10^{-3}$</td>
<td>$X^2 = 31.8$, df = 4, $p = 2.1 \times 10^{-6}$</td>
<td>$X^2 = 24.35$, df = 4, $p = 6.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>0.25 $\mu$M (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>$Z = 0.982$, $p = 1.0$</td>
<td>$Z = 1.47$, $p = 0.56$</td>
<td>$Z = 1.53$, $p = 0.50$</td>
</tr>
<tr>
<td>1 $\mu$M (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>$Z = 1.90$, $p = 0.23$</td>
<td>$Z = 2.02$, $p = 0.17$</td>
<td>$Z = 1.90$, $p = 0.23$</td>
</tr>
<tr>
<td>5 $\mu$M (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>$Z = 0.314$, $p = 1.0$</td>
<td>$Z = 0.682$, $p = 1.0$</td>
<td>$Z = 0.742$, $p = 1.0$</td>
</tr>
<tr>
<td>20 $\mu$M (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>$Z = 1.64$, $p = 0.41$</td>
<td>$Z = 3.03$, $p = 9.8 \times 10^{-3}$</td>
<td>$Z = 2.39$, $p = 6.8 \times 10^{-2}$</td>
</tr>
</tbody>
</table>
Table S5, IPP Test Statistics for ATP growth assay

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>LUM</th>
<th>LUM/GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Global Test (Kruskal-Wallis test)</strong></td>
<td>( X^2 = 28.8, ) ( \text{df} = 4, ) ( p = 8.5 \times 10^{-6} )</td>
<td>( X^2 = 37.8, ) ( \text{df} = 4, ) ( p = 1.2 \times 10^{-7} )</td>
<td>( X^2 = 28.5, ) ( \text{df} = 4, ) ( p = 9.8 \times 10^{-6} )</td>
</tr>
<tr>
<td>0.25 µM (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>( Z = 3.66, ) ( p = 1 \times 10^{-3} )</td>
<td>( Z = 3.03, ) ( p = 9.9 \times 10^{-3} )</td>
<td>( Z = 1.54, ) ( p = 0.49 )</td>
</tr>
<tr>
<td>1 µM (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>( Z = 2.54, ) ( p = 4.6 \times 10^{-2} )</td>
<td>( Z = 2.65, ) ( p = 0.03 )</td>
<td>( Z = 1.33, ) ( p = 0.73 )</td>
</tr>
<tr>
<td>10 µM (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>( Z = 2.79, ) ( p = 2.1 \times 10^{-2} )</td>
<td>( Z = 3.10, ) ( p = 7.8 \times 10^{-3} )</td>
<td>( Z = 2.33, ) ( p = 8 \times 10^{-2} )</td>
</tr>
<tr>
<td>40 µM (Dunn’s comparison with control and Bonferroni Correction)</td>
<td>( Z = 5.20, ) ( p = 7.9 \times 10^{-7} )</td>
<td>( Z = 6.12, ) ( p = 3.6 \times 10^{-9} )</td>
<td>( Z = 5.08, ) ( p = 1.5 \times 10^{-6} )</td>
</tr>
<tr>
<td>Table S6, TBDP Test Statistics for ATP growth assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Global Test (Kruskal-Wallis test)</strong></td>
<td><strong>GFP</strong></td>
<td><strong>LUM</strong></td>
<td><strong>LUM/GFP</strong></td>
</tr>
<tr>
<td><strong>X^2 = 7.52, df = 4, p = 0.11</strong></td>
<td><strong>X^2 = 12.1, df = 4, p = 1.7 \times 10^{-2}</strong></td>
<td><strong>X^2 = 21.3, df = 4, p = 12.7 \times 10^{-4}</strong></td>
<td></td>
</tr>
<tr>
<td><strong>0.25 µM (Dunn’s comparison with control and Bonferroni Correction)</strong></td>
<td>NA</td>
<td>Z = 1.76, p = 0.31</td>
<td>Z = 0.491, p = 1.0</td>
</tr>
<tr>
<td><strong>1 µM (Dunn’s comparison with control and Bonferroni Correction)</strong></td>
<td>NA</td>
<td>Z = 0.508, p = 1.0</td>
<td>Z = 2.34, p = 7.8 \times 10^{-2}</td>
</tr>
<tr>
<td><strong>5 µM (Dunn’s comparison with control and Bonferroni Correction)</strong></td>
<td>NA</td>
<td>Z = 1.54, p = 0.5</td>
<td>Z = 3.45, p = 2.3 \times 10^{-3}</td>
</tr>
<tr>
<td><strong>20 µM (Dunn’s comparison with control and Bonferroni Correction)</strong></td>
<td>NA</td>
<td>Z = 0.573, p = 1.0</td>
<td>Z = 1.02, p = 1.0</td>
</tr>
</tbody>
</table>