Noelle Chung and Justin Lu
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Ecotoxicity of Bisphenol S Through Proteomic and Genomic Changes in *C. elegans*: Preliminary Findings

1. Introduction

1.1 BPS in the environment

Plastics have become a major global contaminant. From 1950 to 2015, the amount of plastic in water has been estimated to be at 6.3 billion tons of waste, with only 21% of plastics being recycled or incarcerated.[1] Bisphenol A (BPA), an important precursor for synthesis of polycarbonate plastics and epoxy resins, has been found to be in 2.2 million tons of plastic in 2009.[2,3] However, a number of negative correlations with BPA has caused industry to shift towards the use of bisphenol S (BPS), an analog of BPA.[4] Now present in baby bottles, thermal receipt papers, food packaging materials, and personal care items, it is increasingly disposed into the environment as through both industrial and personal object disposal. Furthermore, BPS has been determined to be in low, but noticeable amounts in bodies of water in Asia.[5] BPS therefore will affect both humans and the ecological systems that it is contaminating. Now ubiquitous in our ecosystems, it is critical that BPS is well-characterized in order to screen for potential effects on organisms as exposure and accumulation of the chemical increases.

1.2 What is BPS?

BPS is a nonpolar white colorless solid under standard temperature and pressure. Though soluble in organic solvents, it is poorly soluble in water. Its low Kow values, a measure for its tendency of a compound to move from the aqueous phase into lipids, reflects larger concerns for its presence in the water.[6] Coupled with the fact that BPS is not amenable for degradation in the environment, has a high boiling point, and has the characteristics of a strong oxidizing agent, the structure of the compound poses serious threats to the environment.[7]

1.3 C. elegans as a model organism to model ecotoxicity

Caenorhabditis elegans is an excellent model organism, being incredibly easy to handle within the laboratory with a mere three-day maturation time, body length of about 1mm, being non-parasitic, and ease of maintenance. The small worm boasts other advantages, ranging from a large brood size, transparent body, ease of genetic manipulation, a well-characterized genome. *C. elegans* has already been a critical model organism for elucidating numerous behavioral and molecular mechanisms, including the seminal discoveries in apoptosis, siRNA, and aging. Many of the features that made *C. elegans* a prime animal model in the fields it is well known for, from neurobiology, developmental biology, and genetics, the same characteristic make it a prime candidate for toxicity screening.[8]

Toxicity screening has typically been performed *in vitro* through tissue culture and *in vivo* using simple mammalian systems. However, *in vitro* remains unpredictable at the organismal level, and immortalized cell lines may trigger false-positives and false-negatives.[8] Mammalian models

are both expensive, time-consuming, and rodent models are only predictive for humans about 50% of the time.

Considering that nematodes are the most abundant animals in soil ecosystems and found in both aquatic and sediment environment, *C. elegans* can therefore play a pivotal role in providing whole-organismal data for ecotoxicology. Mounting evidence suggests that its genetic and physiological traits are able to result in predictive toxicity data for higher eukaryotes.[9] Many physiological processes within the worm are observed in higher organisms, humans included. Indeed, LC50 rankings in *C. elegans* have been as predictive of acute toxicity in mammals as rats. They particularly will be useful for this study: endocrine disruption and mitochondrial DNA mutations.

1.4 Nuclear Hormone Receptors in C. elegans

BPS's better known cousin BPA is known to have an endocrine-disrupting capacity due to its structural features, acting as an agonist and agonist for different members of the estrogen receptor family.[10,11] Nuclear hormone receptors (NHRs) are group of transcriptional regulators easily identifiable through two traits, a well-conserved DNA binding domain consisting of two zinc fingers and a ligand-bonding domain at the C-terminal.[12,13] These proteins are involved in a slew of cellular and organismal processes.[14]

C. elegans has a dramatically larger number of NRs than humans, 284 to about 50.[14] The *C. elegans* gene NHR-14 is homologous to the human estrogen receptor ERα, as evidenced by sequence similarity and confirmed binding with natural ligands.[15,16] It is therefore reasonable to think that BPS may display many of the same characteristics and may act as an agonist or antagonist for NHR-14 and thereby its homologous counterparts in other organisms. It is sensible to detect whether BPS is indeed causing endocrine disruption before proceeding onto other effects it may be causing on the worms.

1.5 mtDNA damage and mitochondria as a biomarker for ecotoxity

Though adult *C. elegans* somatic cells are post-mitotic, cellular machinery for DNA replication and repair is highly conserved between *C. elegans and* mammals, from proteins involved in nucleotide excision repair, nonhomologous end joining, and DNA nucleases. Other assays for detection of DNA damage in *C. elegans* includes DNA sequencing, reporter assays, and gene expression. In spite of this, there has been relatively few studies of genotoxicity in *C. elegans* with the exception of UV radiation.

While mitochondria are most known for generation of ATP and apoptotic induction through oxidative phosphorylation, they also have their own homeostatic processes such as mitochondrial DNA (mtDNA) replication, mitochondrial biogenesis, and mitochondrial fusion and fission. They also play other critical roles in maintaining health. For example, mitochondrial dysfunction and mitochondrial DNA (mtDNA) mutations are associated with a number of diseases.[17]

Mitochondria and mtDNA furthermore may be particularly susceptible to environmental stressors and lack some repair mechanisms present in the nucleus. Furthermore, the compact sequence, lacking new introns and non-coding regions, means that any damage to the DNA may be even more impactful upon the organelle.[18] For example, mtDNA is particularly sensitive to

damage from reactive oxidative species and the effects are more harsh and long-lasting, therefore more likely to induce metabolic illness. mtDNA integrity and general mitochondrial function is therefore important for organisms. Investigating mtDNA damage therefore may serve as a particularly sensitive biomarker for ecotoxicity.

1.6 Role of Type 1 NADH Dehydrogenase respiratory complex I

Complex 1 is the largest and least understood component of the mitochondrial oxidative phosphorylation (OXPHOS) system and yet is the entry point for most electrons into the chain. The number of OXPHOS complex includes NADH: ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol–cytochrome c oxidoreductase (complex III, or cytochrome bc1 complex), cytochrome c oxidase (complex IV), and ATP synthase (complex V).

Complex I, composed of seven hydrophilic and seven hydrophobic subunits, is an L-shaped trans-membrane protein that oxidizes NADH, using its two electrons to reduce ubiquinone to ubiquinol, which is consequently reoxidized. Redox energy is used to transfer protons from the mitochondrial matrix to the periplasmic space and generates a proton-motive force across the inner mitochondrial membrane and complexes I, III, and IV. Complex V uses this force to produce ATP from ADP and inorganic phosphate. As complex I is the major entry point for electrons, it is suggested to be the rate-limiting step of overall respiration and plays a central role in energy metabolism.[19]

2. Research Questions and Objective

2.1 Research Questions

- Is BPS a mutagen?
- Can Type I NADH dehydrogenase respiratory complex I be used as a marker for mtDNA damage and oxidative stress?

2.2 Specific Aims

- Determine LC50 doses of BPS for *C. elegans* and establish acute toxicity dosage concentration and interval
- Determine whether BPS is indeed an endocrine disruptor
- Investigate correlations between mutations in the sequence encoding for mitochondrial complex and mitochondrial homeostasis

2.3 Hypothesis and Predictions

- Acute BPS exposure will induce mutations in *C. elegans* mtDNA, thereby disturbing the worm's metabolism, ATP levels and normal ratio of NAD/NADH
- BPS interacts with NHR-14 receptors, therefore impacting the brood sizes and maturation rates

3. Methods

3.1 Assays

<u>BPS Treatment</u>

BPS in absolute ethanol will be diluted at 0, .1, 1, and 10 mM in NGM agar. 1 ml of treatment agar will be pipetted into each well of a 12-well plate. After inoculating plates with OP50, worms synchronised to the L1 stage will be picked onto the plate for appropriate exposure times.

Worm Viability

Treated worms will be collected in 100 µl of M9 wash buffer and suspended homogeneously in the solution. 10 µl of the subsequent solution will be pipetted onto a glass slide. Thrashing worms will be considered live.

Brood size and maturation rate

While an equal number of worms will be treated per experimental group, the ratio of mature *C. elegans* to eggs on the plate will determine brood size. Similarly, the number of mature to juvenile, L1-L3, worms will be counted in order to investigate if BPS potentially retards growth.

ATP Luciferase Assay

Levels of ATP are an indicator for general organismal health (and therefore can be used to titer BPS) and for potentially metabolic disturbances. Worm lysates will first be normalized using a BCA assay followed by analysis by BioVision's ATP Colorimetric Assay Kit, which relies on the phosphorylation of glycerol to give a reading of ATP concentrations.

Colorimetric NAD+/NADH Quantification

Like ATP levels, the ratio of NAD+ to NADH can help titer BPS and indicate metabolic disturbances. Biovision's colorimetric ELISA will be performed on age-synchronized *C. elegans* to quantitatively compare NAD+/NADH ratios.

Determining mutagenesis

DNA will be isolated using Nemametrix' *C. elegans* Worm Lysis Kit. Each worm will be picked into a mixture of the kit's solutions followed by a fifteen minute boil. After a ten-fold dilution, DNA extracts will amplified via PCR (using reagents from Nemametrix' PCR Master Mix) before being sent out for sequencing. Primer design was taken from Claudia P. Gonzalez-Hunt et al.[20]

Mitochondrial gene

Forward primer sequence: 5′-CAC ACC GGT GAG GTC TTT GGT TC-3′
Reverse primer sequence: 5′-TGT CCT CAA GGC TAC CAC CTT CTT CA-3′

Act1

Forward primer sequence: 5'-GCT GGA CGT GAT ACT GAT TAC C-3'
Reverse primer sequence: 5'-GTA GCA GAG CTT CTC CTT GAT GTC-3'

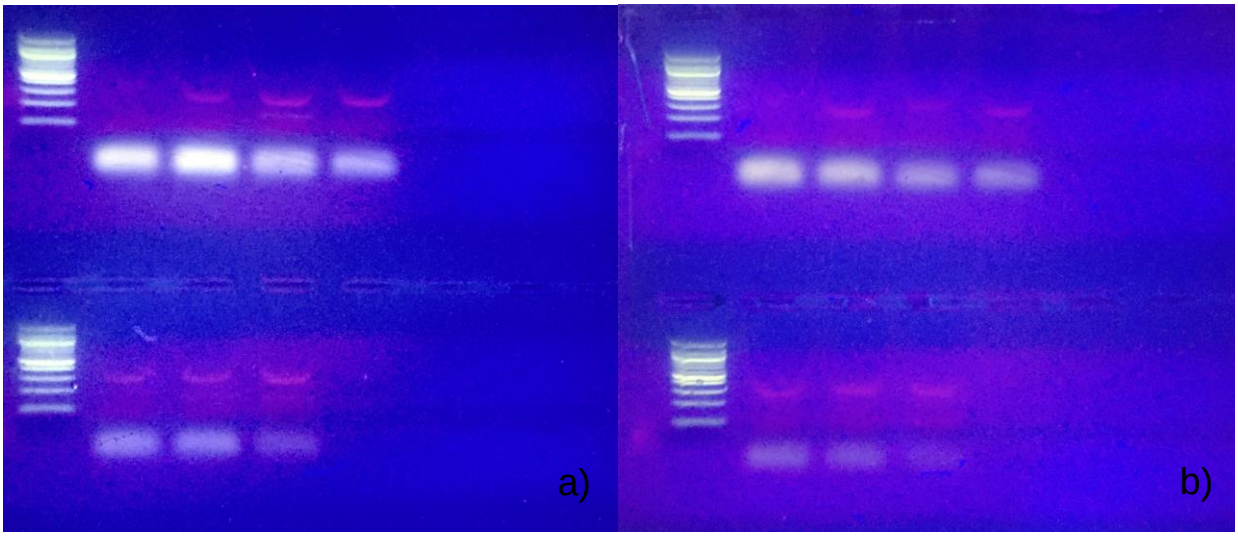
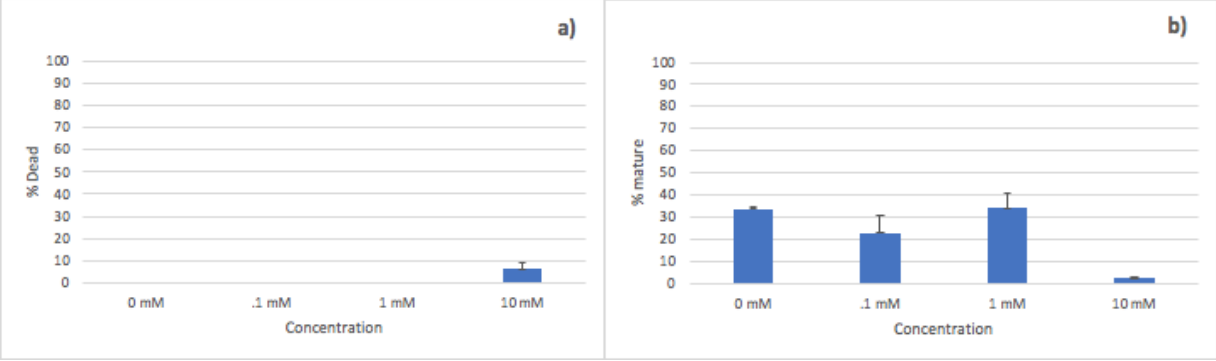


Figure 1. a. PCR on a mitochondrial gene from DNA isolated from four worms using decreasing primer concentrations (8.5, 8.5, 6 5, 3, 2.5, 1.25mM). **b.** PCR on an actin gene from the same DNA isolates using decreasing primer concentrations (8.5, 8.5, 6 5, 3, 2.5, 1.25mM)..

Co-Immunoprecipitation (Co-IP)
Co-IP will be used to isolate the proteins BPS is interacting with. The worm protein lysate will be incubated with anti-BPS antibodies, which will attach to the BPS in the BPS-protein complex. The immune complex will be precipitated by beads with antibody-binding protein and subsequently centrifuged to pull the beads down. Because the beads isolate the BPS and its interacting proteins, any other proteins not interacting with BPS can be washed away.[21]

Western Blotting
After boiling the isolated protein complex to denature the proteins, the pulled-down proteins from the co-IP will be run through a polyacrylamide gel to separate the samples. The proteins will then be eluted, the membrane blocked, and BPS and NHR-14 targeted through their respective anti-BPS and anti-NHR-14 antibodies before staining with a secondary antibody to visualize.[22,23]

4. Preliminary Data



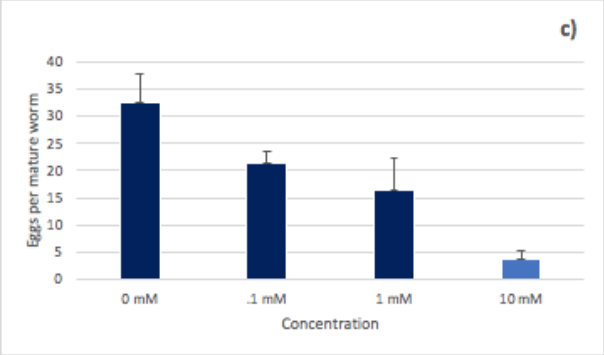


Figure 2. a. L1 worms were picked onto agar plates with varying concentrations for 3 days. No mortality was found among worm exposed to BPS at concentrations of 0, .1, and 1 mM in NGM agar while 10 mM displayed slight toxicity. **b.** A three day exposure to 10 mM agar displays distinct retardation of worm maturity. **c.** Worms exposed for three days also display distinctly different brood sizes, with an inverse relationship between concentration and eggs per mature worm.

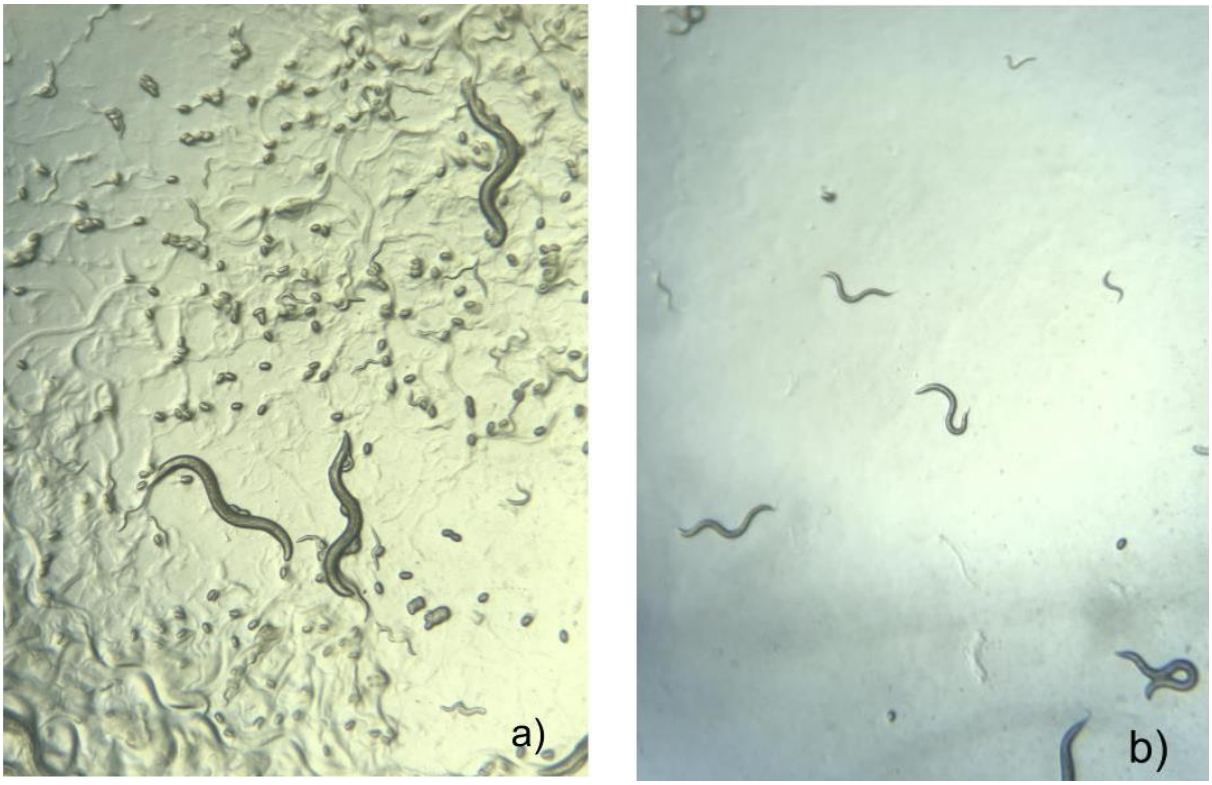


Figure 3. Representative images of worms treated for three days at 0 mM and 10 mM at 8X magnification. **a**. Control worms are not only matured, but also laying copious numbers of eggs versus **b.** worms treated with 10mM BPS, which have retarded growth and diminished brood size.

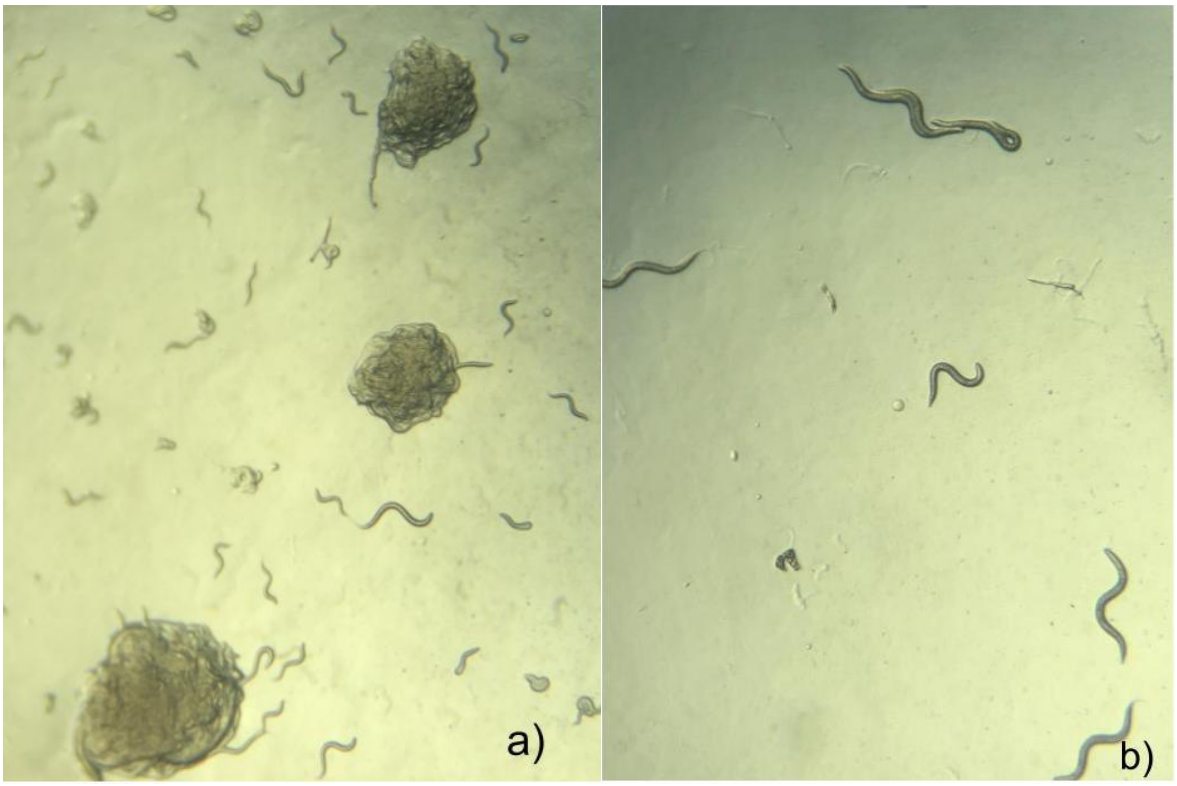


Figure 4. a. The same control worms treated for two more days, five total, display distinct phenotypic differences. Not only have the eggs hatched, the copious numbers of worms have clustered together, a behavior typical of low food and high worm density, an indication of the control worms' successful maturation from L1s and reproduction. **b.** 10 mM dosed worms still have not all matured, and little to no eggs have been laid.

5. Timeline

November:

- Acquire toxicants
- Begin LC50 screening for BPS

December:

- Continue LC50 screening
- Optimize and begin PCR analysis of Type I NADH dehydrogenase respiratory complex I

January:

- Co-IP and Western blotting analysis of worm lysates

February:

- ATP Luciferase Assay and Colorimetric NAD+/NADH Quantification
- Data analysis
- Finalize poster

6. References

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