CAENORHABDITIS ELEGANS AS A MODEL TO DETERMINE THE MOLECULAR EFFECTS OF PLAUSIBLE ENVIRONMENTAL RISK FACTORS OF BREAST CANCER

by

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DEDICATION

This is dedicated to family. For my dog who kept me (mostly) active when I just wanted to sit and work. For my husband who surprised me with treats when I was crunched for time and pushed me to "get it done." For my mother who always watched my children when I needed to buckle down and provided a listening ear for my frustrations. But mostly, this is for my children who are my inspiration to fulfill this dream. My time and energy became much more limited for this pipe dream once you were born, but you've put my world into clearer perspective, and I've pushed myself far beyond my comfort boundaries to get to this point. So, my loves, this is for you. However demanding and out-of-the-realm your desires, here's your proof that they're possible, and I'll be your cheerleader and sounding board through it all.

There have been many people in my life who have been afflicted with this dreaded "C." My mother, my father, my grandparents, my sister-in-law, my aunts and uncles…the list continues. However, of these, my father was particularly tickled at the thought of having a "doctor" in the family. He was an advocate for women's equality and STEM initiatives. He lost his battle to cancer a few weeks before my final defense of this work. I hope this makes him proud nonetheless. XOXO

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LIST OF ABBREVIATIONS AND SYMBOLS

ADI…… acceptable daily intake
AR…… androgen receptor
BPA……Bisphenol A
C. elegans…… *Caenorhabditis elegans*
DDE……dichlorodiphenyldichloroethylene
DDT…….....Dichlorpdiphenyltrichloroethane
DMSO……..Dimethyl sulfoxide
DNA……deoxyribonucleic acid
EPA…… Environmental Protection Agency
EPSPS…… enolpyruvylshikimate-3-phosphate synthase
ER…… estrogen receptor
EST…… expressed sequence tag
GTPases…… guanosine triphosphatases
IARC…… International Agency for Research on Cancer
IOM…… Institute of Medicine
M……Molarity
MAPK…… mitogen-activated protein kinase
MCL…… Maximum Contaminant Level
mg/L…… milligrams per liter
microgram…….ug
microliter……...ul
miRNA……microRNA
mM……millimolar
MOS…… Margin of Safety
NPDWR…… National Primary Drinking Water Regulations
PAH…… polycyclic aromatic hydrocarbons
PCBs…… polychlorinated biphenyls
PFCs…… perfluorinated compounds
PNEC…… predicted-no-effect-concentration
ppm…… parts per million
PQ…… plastoquinone
RNA……ribonucleic acid
SERM…… selective estrogen receptor modulator
STATs……Signal Transducers and Activators of Transcription
WHO……World Health Organization
Δ…….....delta

ABSTRACT

CAENORHABDITIS ELEGANS AS A MODEL TO DETERMINE THE MOLECULAR EFFECTS OF PLAUSIBLE ENVIRONMENTAL RISK FACTORS OF BREAST CANCER

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Cancer is a multi-faceted disease that may involve many different tissues of various origins. Environmental factors have been identified as agents of concern for carcinogenesis, but there is little known about the molecular interactions in the cell that lead to transformation from normal to malignant. Nematodes are among the model organisms recently being utilized to expand our understanding of oncogenesis, as 60-80% of human genes have an orthologue in the genome of *C. elegans.*

This study aimed to evaluate expression (up-regulation/down-regulation) of *C. elegans'* orthologues for genes associated with breast carcinoma progression in nematodes exposed to plausible toxins and compare that to control animals in order to develop a network of molecular interactions which connect the toxin to the observed gene expression changes. We profiled the following orthologues in *C. elegans*: AIR-1, PIG-1, CUL-1, FZR-1, CPAR-1, HCP-3, HCP-4, HCP-6, KNL-1, KNL-3, JUN-1, BRC-1, BRC-2, CEP-1, MML-1, SLO-1, and SLO-2. The following potentially carcinogenic agents were studied for their impact on gene expression: Bisphenol A, atrazine, DDT, beta-Estradiol, glyphosate, and DMSO. These toxins were diluted to 50-0.0001mM concentrations, and *C. elegans* were grown in these environments for 7 days at the LC10 concentrations of each toxin. Toxin-driven gene expression shifts were quantified using qPCR. The up-regulation of HCP-3 and BRC-1 in atrazine, the up-regulation of BRC-1 in DDT and down-regulation of CEP-1 and MML-1 in DDT, the up-regulation of HCP-3 in b-Estradiol, the down-regulation of CUL-1, FZR-1, BRC-2, CEP-1, MML-1, and PIG-1 in glyphosate, the up-regulation of HCP-3 and BRC-1 in glyphosate, and the down-regulation of JUN-1 in DMSO were evident. The connections between each toxins and each carcinogenesis-related gene responding to the toxin were analyzed using Pathway Studio. In each case, this analysis supported environmental influence studied. These results indicate that even at very small concentrations that we would expect in the natural environment, these omnipresent toxins may exert health impacts.

CHAPTER ONE - BACKGROUND

Cancer is a multi-faceted disease, which may involve many different tissues of various origins (American Cancer Society, 2016). It is among the leading causes of death in the world, and the National Cancer Institute estimates there were over 1.6 million new cancer cases in the United Stated in 2016 and an approximate 35% mortality overall (National Cancer Institute, 2016). Further, almost 40% of people will receive a cancer diagnosis at some point in their lives. In 2010 alone, cancer led to $125 billion for health care expenditures (National Cancer Institute, 2016). Although the mortality for most cancers in the United States is declining, with an increasing number of cancer survivors, the studies of the environmental root causes of cancer development remain a hot area for further research, outside of the well-known smoking (National Cancer Institute, 2016).

Introduction to Female Breast Carcinoma

The breast is a mass of glandular, fatty, and fibrous tissues positioned over the pectoral muscles. Located within the glandular tissues are the lobules, which are milk-producing glands at the ends of the lobes, and the ducts, which are the milk passages (DT Ramsay et al., 2005). A layer of fatty tissue surrounds the breast glands and extends throughout the breast. From 50 to 75% of invasive breast cancers originate in the cells that line the ducts (ductal carcinoma), while 10-15% begin in the lobules (lobular carcinoma), and a small number originate in other breast tissue (American Cancer

Society, 2011; Susan G. Komen for the Cure, 2012). The National Cancer Institute estimated that breast carcinoma comprises 32% of all cancers in American women and 15% of all cancer deaths in American women (National Cancer Institute, 2009). Furthermore, recent estimates show that an estimated 12.2% of currently living women will be diagnosed with breast cancer at some point in their lives (National Cancer Institute, 2011).

Treatment decisions for cancer are guided by the metastatic potential of tumors, which includes lymph node involvement, stage - tumor size, and histological grade - aggressiveness from cellular differentiation and replicative potential (Bloom & Richardson, 1957). The most prominent method of breast tumor grading is the Nottingham Grading System, which is based on microscopic evaluation of morphologic and cytologic features of tumor cells, including the degree of tubule formation, nuclear pleomorphism, and mitotic count (Ivshina et al., 2006). The sum of these scores grade tumors in the following three categories: Grade 1 (G1; well-differentiated, slow-growing), Grade 2 (G2; moderately differentiated), and Grade 3 (G3; poorly differentiated, highly proliferative) malignancies (Elston & Ellis, 1991).

Large patient cohorts have consistently shown that the grade of invasive breast cancer is a powerful indicator of disease recurrence and patient death, independent of lymph node status and tumor size (Elston & Ellis, 1991; Schumacher et al., 1993; Roberti, 1997; Lundlin et al., 2001). Untreated Grade 1 breast carcinoma has a 95% survival for 5 years; untreated Grade 2 breast carcinoma has a 75% survival for 5 years; untreated Grade 3 breast carcinoma has a 50% survival for 5 years (Dalton et al., 2000).

Although the histological grading of malignancy in patients with operable breast cancer plays an important role in identifying patients at high risk of recurrence, the most effective combination of breast cancer diagnostic factors is still not completely clear. In assessing a patient's overall health prognosis upon initial cancer diagnosis including the chances of survival, mortality and recurrence, the problems associated with assigning the grade of malignancy are not only related to the particular grading system being used, but also to the heterogeneity within the tumor and pathologists' experience (Komaki, Sano, and Tangoku, 2006). In fact, the gene expression signatures of Grade 2 tumors showed that this category of tumors contains a mixture of both Grade 1 and Grade 3 tumors, resulting in inaccurate grading (Ivshina et al., 2006).

In addition to tumor grade, tumor stage is also considered when determining oncological treatment options. Tumor staging is primarily used to determine the metastatic nature of the tumor and the new sites affected (National Cancer Institute, 2009b). Cancer cells may invade the surrounding normal tissue in the breast and then spread to other areas of the body by migrating through tissues themselves, through the lymph vessels, or within the blood. The presence of neoplastic cells in the axillary lymph nodes is the most important indicator of the breast cancer outcome, as this is used as a metastatic "threshold" (Gipponi et al., 2004). Breast cancer cells most often migrate to the bones, liver and brain (National Cancer Institute, 2009b).

When cancer cells distribute themselves away from the original (primary) tumor location and metastasize to other areas of the body via the blood or lymph systems, secondary (metastatic) tumors form. These secondary tumors belong to the same type of

cancer as the primary tumor (National Cancer Institute, 2009b). Although the specific type of cancer can slightly change the criteria for staging criteria, as a general rule, the tumor's stage takes into account its size and metastasis to other tissues and organs, indicating the overall advanced state of the disease (Dana-Farber Cancer Institute, 2016).

In line with these criteria, a sample of 72,367 female cases from 1973-1998 aged 21-90 years with invasive ductal breast cancer were assessed to determine the effect of patient and tumor characteristics on breast cancer survival using the Surveillance, Epidemiology, and End Results (SEER) Program (Rosenberg, 2005). Cox proportional hazards regression showed that tumor size, grade, race, and the historical year of diagnosis (due to changes in disease detection and treatment) all had significant constant effects on breast cancer survival, while the effects of age at diagnosis and disease stage had significant effects that varied depending on the patient's personal developmental changes, which may include environmental exposures and/or genetic predispositions, in relation to the onset of the disease (Rosenberg, 2005).

Cancer-contributing Mutations

Oncogenes are the genes that allow unregulated cell growth by over-producing certain proteins involved in positive regulation of cell proliferation or a negative regulation of a cell death (Darnell, 2002). Oncogene activation can result from chromosomal translocations, gene amplifications, mutations involving crucial residues that up-regulate the activity of gene products, or from abnormally high gene expression (Vogelstein and Kinzler, 2004). The latter type of oncogene deregulation may be corrected by pharmacological alteration of respective transcription factor activity, or by

suppressing the synthesis of the protein from overexpressed mRNA, thus, presenting an opportunity for development as a therapeutic approach (Emery, Ohlstein, and Jaye, 2001).

The protein products of viral and cellular oncogenes are typically found in the cytosol, plasma membrane and the extracellular space, as they encode for growth factors, growth factor receptors, protein kinases and GTPases (guanosine triphosphatases) that are involved in cellular response to extracellular signals (Varmus, 1987). Excessive amounts of cytoplasmic or extracellular growth regulating proteins disrupt the control network by sending persistent signals for cell division to the nucleus (Varmus, 1987). Moreover, certain cytoplasmic oncoproteins can relocate in the nucleus and directly or indirectly regulate the activity of various viral and cellular transcriptional promoters (Varmus, 1987). Examples of cytoplasmic oncogenes with this mode of action include Signal Transducers and Activators of Transcription (STATs), and beta-catenin (Varmus, 1987; Gavert and Ben-Zeev, 2007). In addition to cytoplasmic oncogenes, a number of oncogenic proteins reside in the nucleus. Many of these oncogenes are capable of direct regulation of transcription, including well-studied transcription factors MYC and JunB (Meyer and Penn 2008; Piechaczyk and Ferras, 2008). With the exception of nuclear receptors which may relocate into the nucleus of eukaryotic cells from the cytoplasm, oncogenic transcription factors are often indirectly regulated by signal transduction cascades, often involving phosphorylation/dephosphorylation processes (Emery, Ohlstein, and Jaye, 2001).

Another functional class of genes involved in cancer is tumor suppressors. In normal tissues, these genes slow down cell division, repair DNA replication errors and mutations, or command damaged cells to die in a process known as apoptosis or programmed cell death. When tumor suppressor genes don't work properly, for example, due to their damage by mutation or deletion, cells continue to divide, and metabolic control is removed, which can lead to cancer. In fact, the loss of these naturally growth-restraining genes, by mutation, deletion, rearrangement or down-regulation may be even more important for the formation of many kinds of human cancer cells than proto-oncogene/oncogene activation.

The most well-known tumor suppressors associated with breast cancer are *BRCA1* and *BRCA2*, which may be damaged by genetic mutations. Both of these genes were discovered in the early 1990s as the component of the machinery involved in repairing the DNA damaged during its replication (U.S. National Library of Medicine, 2013a; U.S. National Library of Medicine, 2013b; National Cancer Institute, 2009b).

In addition to the "classic" oncogene and tumor-suppressor genes, endogenous tumorigenic agents include microRNAs (miRNAs). miRNAs are small non-coding RNAs that control gene expression by targeting protein-coding mRNAs and activating either their translational repression or straightforward degradation (Dahiya et al., 2008). Evidence suggests that altered regulation of miRNAs may cause other genes to overexpress and, therefore, miRNAs may function as oncogenes by indirectly stimulating tumor growth (Dahiya et al., 2008).

The study of breast cancer patient pedigrees has allowed researchers to estimate the risk of an individual to develop this type of cancer. These risks are deduced from both the heritable characteristics and environmental determinant, for example, hormonal contraceptive exposure and parity (National Cancer Institute, 2009b). It was shown that *BRCA1* mutations contribute to a majority of heritable breast cancer cases in young women (Krainer, et al., 1997). Examples of other tumor suppressor genes involved in the process of tumorigenesis include *TP53* and *KCNRG*. *TP53* encodes the protein that connects the DNA damage response and the apoptosis (Schwartz, 2005; Poulin et al., 2004; GeneCards, 2016c). The product encoded by *KCNRG* inhibits an assembly of potassium ion channels, and, therefore, alter the membrane potential and ultimately the reactivity of the nervous system. The loss of *KCNRG* gene has been has been implicated in a number of solid tumors and in lymphomagenesis (Birerdinc at el., 2010).

Introduction to *C. elegans* as a model organism for studying cancer

Typically, a process of human tumorigenesis is studied either in cell cultures or in murine models. These models have their advantages and disadvantages, with the overall lack of the predictive power required to translate preclinical efficacy into the clinic (Gould et al., 2015).

In fact, no single model can suffice to fully inform us about all facets of tumorigenesis. Having this in mind, there is now a renewed interest in non-vertebrate organisms such as yeast, worms, and flies that already showed their potential to revolutionize our understanding of oncogenesis. In particular, the soil nematode *Caenorhabditis elegans (C. elegans)* has emerged as an important model organism for the

study of cancer. The first reports showing that the signaling cascades of *C. elegans* are homologous to that of mammalian cells were published in 1994, when the worm's *CED-9* gene and the mammalian *BCL-2* gene were compared in their ability to overrun apoptotic control (Hengartner & Horvitz, 1994).

C. elegans is a free-living nematode found globally, it is hatched at 0.25mm and grows to 1mm (Corsi et al., 2015). With an aid of dissecting or compound microscope, the transparent bodies of these nematodes allow for easy monitoring of their development, movement, digestion, and reproduction (Corsi et al., 2015). The life cycle runs for approximately 2-3 weeks at room temperature (20-25 degrees Celsius) (Corsi et al., 2015). Under adverse environmental conditions, *C. elegans* proceeds onto a stress-resistant alternative larval stage called dauer. This is the primary survival stage for *C. elegans* in nature; to acquire the resistance to harsh environments, the tissue of nematodes undergo substantial remodeling (Androwski, Flatt, and Schroeder, 2017).

With a predefined number of somatic cells, each cell fate can be tracked, including its 302 neurons, giving a glimpse into the most basic circuitry of eukaryotic nervous system (Corsi et al., 2015). As a multicellular eukaryotic model, this not-so-primitive organism can provide information about more complex eukaryotes, as 60-80% of human genes have an orthologue in genome of *C. elegans* (Corsi et al., 2015).

Most *C. elegans* are hermaphroditic, but there are 0.1-0.2% males in the population, allowing for cross-breeding (Corsi et al., 2015). As such, *C. elegans* are either XX or XO, allowing for cross-fertilization before self-fertilization takes place; however, when self-fertilization takes place, the sperm is stored until the eggs are ready

for fertilization (Corsi et al., 2015). For a geneticist, there are several benefits to nematode' self-fertilization that include: ease of stock maintenance wherein a single animal can give rise to a whole population, isogenic populations with preserved standard Mendelian inheritance, and the ability of the worms' populations to thrive despite the presence of inherent genetic defects preventing normal mating (Corsi et al., 2015).

There are a number of examples of successful transfer of the insights acquired while studying nematodes onto vertebrates and even humans. In aging and development research, after exposure to certain compounds, *C. elegans* showed an extended lifespan; the same set of compounds is often efficient in aging rodents. More specifically, Ackerman and Gems (2012) used the *C. elegans* model to show that triglyceride lipase and lipid desaturase activity might protect against aging. After nematode-based pre-screening for initial effects of healthy and unhealthy forms of lipids, researchers moved to mammalian models to determine if aging or anti-aging effects are preserved in higher-level eukaryotes. Indeed, Lin et al. (2014) showed that in Drosophila, overexpression of diacylglycerol lipase extends the lifespan and enhances response to oxidative stress. Seah et al. (2016) later showed that lysosomal lipolysis modulates the lifespan in mice. Additionally, both *C. elegans* and *M. musculus* show an improvement in mitochondrial capacity after being fed urolithnin A (UA), which is found in pomegranate, nuts, and berries (Ryu et al., 2016).

Another example came from a study of apoptosis, a cellular process that occurs for all eukaryotes. Using *C. elegans* model, Malin et al. (2016) determined that the SET-16/MLL3/4 chromatin regulation complex works along with HSF-1 to control LET-

70/UBE2D2 expression, indicating which genes play a role in its transcriptional regulation and where to target future studies in other eukaryotes. The cell cycle-propagating mechanisms are also shared between eukaryotes. Kotak et al. (2016) determined that Aurora A kinase is needed for accurate spindle positioning both in *C. elegans* and *Homo sapiens*. As nanotechnology rapidly expands, high-throughput-compatible models for verification of *in vitro* findings are in high demand. Therefore, Gonzalez-Moragas and Laromaine (2015) proposed *C. elegans* as a model to test *in vivo* effects of nanomaterials.

In addition to being already accepted as a model for molecular, cellular and physiological systems in higher order eukaryotes, *C. elegans* has been increasingly utilized as a model for toxicology studies (Kaletta, T. & M. Hengartner, 2006; Leung et al., 2008). Recently, Meier et al. (2014) exposed *C. elegans* to DNA crosslinking agents, which caused guanine substitutions and clustered genetic rearrangements, both of which are associated with carcinogenesis in humans. In nematodes, Aflatoxin B1 induced substitutions of guanines in a GpC context, similar to that in aflatoxin-induced human hepatocellular carcinomas, while DNA crosslinking agents cisplatin and mechlorethamine caused indels, substitutions, and clustered rearrangements. The study cited above demonstrates that *C. elegans*-based experimental model systems are capable of providing mechanistic insights into mutational signatures associated with human diseases.

The following are proto-oncogenes and tumor suppressor genes that can be studied in the *C. elegans* model as they have the homologues in this animal. The list

includes *TP53* (F52B5.5/CEP-1 in *C.elegans*), *BRCA1* (C36A4.8/BRC-1 in *C. elegans*), *BCL-2* (CED9 in *C. elegans*), *AURKA/STK6/STK15* (AIR-1 in *C. elegans*), *KCNRG* (functionally similar to F47D12.3, F46G10.1, and T23G5.3 in *C. elegans*) and *CUG2* (functionally similar to F20D6.9, Y47G6A.26, KNL-1, KNL-3, HCP-1, HCP-3, HCP-4, HCP-6, and CPAR-1 in *C. elegans*) (Schwartz, 2005; Poulin et al., 2004; GeneCards, 2016a; GeneCards, 2016b; GeneCards, 2016d).

CUL-1 encodes a core component of ligase complexes for proteins requiring ubiquitination that can be involved in mitosis, signal transduction, and transcription (Tan et al., 1999). As adaptor for the APC/C ubiquitin-protein ligase complex, FZR-1 promotes anaphase and telophase, working to prevent accumulation of mitotic regulators, like cyclins and cyclin-dependent kinases, that would otherwise halt the cell cycle prematurely (Fang, Yu, and Kirschner, 1998). It is also involved in the G2 checkpoint, preventing mitotic entry to when damaged DNA is present (Lafranchi et al., 2014).

AIR-1 encodes an Aurora A kinase involved in mitotic and meiotic events via microtubule formation and stabilization near the centrosome site during anaphase (GeneCards, 2016a). In a related centromere-associated function, PIG-1 encodes a kinase that interacts with the centrosome during cell divisions and promotes proper differentiated cell divisions (Chien et al., 2013).

CPAR-1 is a CENP-A-encoding gene for a Histone H3-like centromeric protein that directs kinetochore assembly in mitosis and meiosis (Monen et al., 2005). Like CPAR-1, HCP-3 is a CENP-A gene that encodes a Histone H3-like centromeric protein that directs kinetochore assembly in mitosis and meiosis (Monen et al., 2005). Following

HCP-3 utilization in the cell, HCP-4, which encodes a holocentric CENP-C-like protein, wherein the entire length of the chromosome acts as the centromere for mitosis, is utilized for sister centromere resolution and a functional kinetochore that attaches to the spindle apparatus (Moore and Roth, 2001). HCP-6 encodes a centromeric protein involved in chromosome segregation and proper condensation of chromosomes to ensure appropriate orientation in the cell cycle to prevent merotelic events wherein one kinetochore attaches to both spindle apparatuses, which may result in aneuploidy (Stear and Roth, 2002). KNL-1 encodes a scaffold protein that influences mitotic spindle assembly to create the kinetochore-microtubule (Cheeseman et al., 2008). KNL-3 is a crucial protein in the kinetochore complex (Kline et al., 2006).

BRC-1 is a tumor suppressor gene that functions in a protein complex that transduces DNA damage information in addition to interacting with RNA polymerase II to influence transcription in concert with BRCA2 via Rad51 complexing in genetic crossover events (Scully, 2000). BRCA1 has also been linked to proto-oncogene BCL-2 and mitogen-activated protein kinase (MAPK) (Frenaux et al., 2000; Gilmore et al., 2004). In addition, BRC-1 also complexes with the tumor suppressor gene p53 to halt the cell cycle when necessary (Ouchi et al., 1998; Adamo et al., 2008s). CEP-1, also known as p53, is a tumor suppressor gene and transcription factor that can activate egl-1 and ced-13 to induce apoptotic events when DNA damage occurs (Hoffman et al., 2014).

Although *C. elegans* does not possess specific orthologue for MYC, within the MYC family, MML-1 is part of a transcription factor network required for longevity of *C. elegans* through germline removal via accumulation of the selected gene in the nucleus

followed by regulation of HLH-30/TFEB, which controls autophagy and lysosome biogenesis (Nakamura et al., 2016).

SLO-1 and SLO-2 are involved in Ca++ and K+ channel regulation via neurotransmitter release to post-synaptic cells (Wang et al., 2001; Liu, Chen, and Wang, 2015).

Justification for inclusion of CUG2 as breast cancer gene candidate

Previous studies identified human *CUG2* as part of the small expression signature differentiating aggressive breast carcinomas with poor prognoses from relatively indolent Grade 1 tumors (Ivshina et al., 2006). A decade-old genome-wide study identified CUG2-encoding mRNA as an expressed sequence tag (EST) exhibiting significant overexpression in multiple human cancer types, including ovary, liver, lung, pancreas, breast, stomach, rectum, and colon (Lee et al., 2007). Recently, *CUG2*-encoded protein CENP-W has been identified as a new centromeric component that interacts with CENP-T. In a complex with CENP-T, CENP-W plays crucial roles in assembly of the functional kinetochore complex (Chun et al., 2011). Other studies showed that *CUG2* products enhance metastasis and drug resistance through STAT1 activation, which eventually contributes to tumor progression (Malilas W et al., 2013). Nematode homologs of *CUG2* are involved in the cell cycle via the maintenance of the kinetochore.

Environmental Risk Factors in Breast Cancer

Breast cancer is the most common cancer in women and the leading cause of cancer death among women 35–54 years of age (Brody, 2003). Rising incidence and poor prediction of individual risk have prompted a search for additional contributing

factors (Brody, 2003). The Institute of Medicine (IOM) published a report in December 2011 that reviewed the current understanding of associations between breast cancer and the environmental factors (Schmidt, 2012). For this report, a 15-member panel compiled evidence from scientific literature, including the International Agency for Research on Cancer (IARC) and the World Cancer Research Fund International, and defined "environment" as any factor that is not inherited through DNA (Schmidt, 2012). The panel classifies cancer risk factors into 3 groups: established, possible, and biologically plausible (Schmidt, 2012). "Established" risk factors showed positive results from animal and mechanistic studies and were supported by human epidemiological data, "possible" risk factors were assigned if the human data was conflicting in nature, and "biologically plausible" risk factors were assigned if the only relevant data were from animal and *in vitro* mechanistic studies (Schmidt, 2012). Few risk factors have been classified as "established." These factors include hormone therapy and exposure to ionizing radiation (Schmidt, 2012). "Possible" risk factors include nightshift work and exposure to secondhand smoke and benzene (Schmidt, 2012). "Biologically plausible" risk factors include exposure to industrial chemicals, many of which are found in common consumer products used on a regular basis (Schmidt, 2012).

Known risk factors for breast cancer include parity and other reproductive circumstances associated with exposures to estrogen, estrogen-like pharmaceuticals, and other hormones. Many hormonally active compounds found in commercial products and in environment, in fact, induce mammary tumors in animals (Brody, 2003). The main estrogen receptors in the body, ERα and ERβ, serve as ligand-gated transcription factors

that exert a role in the development of breast cancer. Xenoestrogens, a class of estrogen-resembling chemical compounds, which includes several natural or synthetic molecules, bind to ERs and interfere with the transmission of the estrogen signals into the nucleus. The de-regulation of estrogen/ER signaling contributes to the pathogenesis of many diseases including cancer (Acconcia, Fiochetti, and Marino, 2016). 17 beta-Estradiol is a steroid hormone that binds with an estrogen receptor (ER) in the nucleus that affects the transcription and expression of a variety of proteins in endocrine organs and glands, many of which can contribute to cancer pathways through undetermined means (National Center for Biotechnology Information, 2017). Interestingly, Bisphenol A (BPA; 4,40-dihydroxy-2,2-diphenylpropane) was initially tested as a synthetic estrogen, but found it to be orders of magnitude less efficient than estradiol. Later, this compound was further developed in the chemical industry for plastic resin polymerization (Acconcia, Pallottini, and Marino, 2015). BPA has been found to bind to both of the nuclear estrogen receptors (ERs), ERα and ERβ (Caserta et al., 2014). However, this compound can both mimic the action of estrogen and antagonize estrogen, indicating that it is either a selective estrogen receptor modulator (SERM) or partial agonist of the ER. Relatedly, at high concentrations, BPA also binds to and acts as an antagonist of the androgen receptor (AR) (Desdoits-Lethimonier et al., 2017). In line with this, BPA exposure in testes disrupts the hormone environment via steroidogenic gene activation through the JNK/c-Jun signaling pathway (Lan et al., 2017). Moreover, experiments with tissue explants showed that direct exposure to BPA or BPA-A at typical exposure concentrations can

result in endocrine disturbance in the adult human testis (Desdoits-Lethimonier et al., 2017).

Occupational studies show associations between breast cancer and exposure to certain organic solvents and polycyclic aromatic hydrocarbons (PAHs). Dimethylsulfoxide (DMSO) is an organosulfur, clear, odorless liquid used as a solvent in research fields due to its miscibility in both polar and nonpolar compounds through aprotic means, specifically without donating hydrogen to the environment, which would affect the pH of the environment. However, population-based association studies of a sample of organochlorine compounds and PAHs have returned mostly negative results (Brody, 2003). Numerous study design challenges, including a lack of exposure assessment tools, limited hypothesis-generating toxicological studies, unknown latency and periods of breast vulnerability, and individual differences in susceptibility may have contributed to lack of conclusive finding connecting incidence of breast cancer with individual exposure to various environmental contaminants (Brody, 2003).

However, some more recent studies returned interesting observations. For example, Guimaraes et al. (2009) used data from the Brazilian Mortality Information System to retrieve breast cancer mortality rates from 1980-2005 and showed a consistent linear regression in mortality rates ($y = 0.5427x + 8.5973$; $R2 = 0.8281$, $P < 0.001$) (Guimaraes et al., 2009). In this study, significant Pearson's correlations with breast carcinoma mortality were observed for indirectly assessed total pesticide exposure ($r = 0.618$, $P = 0.001$), as well as exposures to insecticide ($r = 0.550$, $P = 0.004$), herbicide ($r = 0.652$, $P < 0.001$), fungicide ($r = 0.509$, $P = 0.008$), and acaricide ($r = 0.411$, $P = 0.037$)

(Guimaraes et al., 2009). These data suggest that although pesticide exposure and breast cancer have not been causatively linked, there may be corroborating associations (Guimaraes et al., 2009). Of interest to this study include atrazine, DDT, and glyphosate. Atrazine is a triazine pesticide that binds to the D1 protein of the Photosystem II electron transport chain, disrupting its binding ability to reduce plastoquinone (PQ) located in the thylakoid membrane of photosynthesizing plant cells (Xiong, Subramanian, and Govindjee, 1998). While this pathway may not be present in human cells, the microbiota with which we interact may be impacted by this agent, resulting in indirect histological effects. DDT (dichlorodiphenyltrichloroethane) is an organochlorine insecticide that has been implicated in carcinogenesis through a variety of mechanisms. In particular, it slows the closing of Na+ channels in neurons, affecting the overall neuronal reactivity of the organism to environmental signals (Vijverberg, van der Zalm, and van den Bercken, 1982). A particularly important trait of DDT is that it bioaccumulates in lipid deposits, which is an asset when targeting insect populations, but this also causes it to persist in human tissues for an extended time. Glyphosate's (N-phosphonomethyl-glycine) active ingredient is derived from the amino acid glycine, and it is used as an herbicide by inhibiting the activity of enolpyruvylshikimate-3-phosphate synthase (EPSPS) through competitively inhibiting phosphoenol pyruvate and thereby inhibiting the biosynthesis of amino acids and other metabolites from carbohydrate precursors through the shikimic acid pathway (Amrhein et al., 1980).

Female breast cancer genetic risk factors account for less than 30% of all female breast cancer cases, and thus do not seem to solely justify continual increases in

incidence and mortality (Davis et al., 1993). Experimentation has shown that some compounds, including organochlorines, polycyclic aromatic hydrocarbons (PAHs), triazine herbicides, solvents and pharmaceuticals affect estrogen production and metabolism, and therefore, can be classified as xenoestrogens because they (Wolff et al., 1996; Davis et al. 1993; Dey et al., 2009). Significantly elevated levels of some of these products have been identified in breast fat and in serum lipids of female breast cancer patients (Davis et al., 1993). Furthermore, these products have been experimentally shown to induce carcinogenesis in breast tissue (Davis et al., 1993). However, it should be noted that organochlorine measurements in blood samples from occupationally selected populations may be one to three orders of magnitude higher than those in the general population due to workplace exposures to chemicals (Xu et al., 2010).

Elserougy et al. (2012) analyzed maternal and umbilical sera, adipose tissue, placenta and breast milk and found finding significantly higher levels of the organochlorine pesticide residues of DDT in urban as compared to rural mothers (Elserougy et al., 2012). This raises the possibility that these chemicals may be transferred from mother to child through placental circulation and breast milk in addition to concern regarding the localization of toxic reservoirs (Elserougy et al., 2012). Further, Xu et al. (2010) analyzed the NHANES, National Health Nutrition Examinations Survey, data from 1999-2004 and found statistically significant associations of exposure to three pesticide residues and incidence of prostate cancer (p-value 0.02, 0.002, and 0.04, respectively). In fact, the NHANES cross-sectional data provide a unique route to study

possible associations between serum concentrations of individual pesticides and breast cancer in a representative sample from the general population (Xu et al., 2010).

Extrapolation from the population prevalence of inherited mutations in known oncogenes and tumor suppressor genes suggests that only a small quantity of women should be diagnosed with breast cancer, but in reality, a larger percentage of women develop breast cancer, which suggests that the majority of female breast cancer patients have acquired cancer-promoting mutations, possibly via interactions with environmental carcinogens (Davis et al., 1993). Perhaps a combination of genetic predisposition to cellular carcinogenesis with exposure to certain environmental factors may better explain the increase in breast carcinoma incidence among females. In their study of the U.S. ecological data, Wei, Davis and Bina (2012) uncovered a possible association between female breast cancer and air quality. They found higher breast cancer incidence in metropolitan and other high emissions areas, as compared to rural areas, in addition to statistically significant associations between breast cancer incidence and emissions of nitrogen oxides, carbon monoxide, sulfur dioxide, and volatile organic compounds ($r= 0.89, 0.82, 0.71$, and 0.68, respectively, $p<0.001$).

Pesticides are of interest in etiologic studies of breast cancer because many pesticides mimic action of estrogen, a known breast cancer risk factor, and have been known to cause mammary tumors in animal exposure experiments (Brody, 2004). Organochlorine pesticides, including DDT, persist in human tissues, especially in the stored fat, for years (Bounias 2003; Xu et al., 2010). The half-lives of these chemicals have been reported to be more than 20 years. Moreover, losing adipose tissue might result

in an increase of these chemicals in the blood and organs as with the release of adipocyte fat storages into the surrounding body environment (Xu et al., 2010).

Unscheduled DNA synthesis and gene expression, decreases in serum metabolites that normally inhibit breast cell growth, changes in signal transduction pathways, endocrine disruption, pro-carcinogen activation by detoxification enzymes, and intercellular communication impairment are thought to be possible mechanistic triggers of carcinogenesis due to environmental exposure (Bounias, 2003; Bradlow, 1995). The evidence relating to DDT and carcinogenicity has historically been uncertain and unclear. In 2003, a four-fold increase in breast cancer was connected to elevated levels of DDE (dichlorodiphenyldichloroethylene), the metabolite of DDT, in women's blood. In addition, DDT and DDE levels were previously shown to be associated with tumor aggressiveness in women diagnosed with breast cancer; in his study, aggressiveness was defined as a high metastatic potential (Aube et al., 2008; Charlier et al., 2003; Pavuk et al., 2003; Mathur et al., 2002). Shakeel et al. (2010) performed a PubMed search for literature on the use of pesticides, including organochlorines and organophosphates, in both developing and developed countries from 1990-2009. They found numerous studies indicating that the levels of exposure to pesticides like DDT, DDE, polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), and hexachlorocylohexane (HCH) were higher in the developing world than the developed world, and that DDT was found to be positively associated with breast cancer, while the connections to breast carcinoma incidence for other pesticides levels were not confirmed (Shakeel et al., 2010). Given the dramatic differences in exposure levels between developed and developing nations

indicate that the relationship between pesticides and breast cancer needs to be assessed in larger studies with a broader range of exposure levels (Shakeel et al., 2010). Cultured human cells that had been exposed to both estrogen and DDT revealed that its metabolite DDE could enhance breast cancer progression by opposing the androgen signaling pathway which normally inhibits growth in hormone-responsive breast cancer cells (Aube et al., 2008). Interestingly, it is still unproven as to whether this would correlate to an increased risk of breast carcinoma development in hormone replacement therapy patients, who willingly incur exogenous estrogen to reduce the bodily effects of menopause and the hormone-related disorders.

In a European study, high levels of DDT in serum showed a statistically significant association with 5-fold increase in risk of breast cancer development among women who were born after 1931 (Cohn et al., 2007). These women were under 14 years of age in 1945 when DDT came into widespread use, and less than 20 years of age as the use of DDT peaked; however, older women who were not exposed to DDT before 14 years of age showed no association between DDT exposure and breast cancer. This suggests that early-life exposure to DDT may increase the risk of breast cancer development later in life, and that the ultimate effect of DDT exposure may depend on the age of the individual at the time of exposure, in addition to other environmental triggers (Cohn, 2010; Li et al., 2006a). Extrapolation of this finding to other environmental exposures may imply that susceptibility to future carcinogenesis is a common theme during certain key periods in human development, such as early

childhood and puberty, in particular due to the rapid bodily changes that occur at these times (Wolff, et al., 1996).

Pan et al. (2011) performed a population-based case-control study in Canada and found statistically significant association of the development of breast cancer with living in proximity to steel mills (0.8 to 3.2 km) and thermal power plants (<0.8 km) in premenopausal women, to a proximity to petroleum refinery (0.8 to 3.2 km) and pulp mills (0.8 to 3.2 km) in postmenopausal women, and for 10 or more years of residing near thermal power plants. Bonefeld-Jorgensen et al. (2011) performed a case-control study in Greenland from 2000-2003 and were able to show a statistically significant association between serum levels of perfluorinated compounds (PFCs) and female breast cancer. These studies further support the idea of environmental toxins contributing to the carcinogenic process.

The individual's capability to detoxify herself of these chemical is an underestimated component of the risk (Bounias, 2003). Each person has a unique combination of genetic variants that determine their specific response to drugs, chemical, and carcinogenic exposures, as evidenced by the variety in a spectrum of reactions induced by common drug therapies (Miller et al., 2001). Specifically, genetic variations in cytochrome-encoding genes of CYP (P450) family, acetyltransferases (NAT), and glutathione S-transferases (GST) have shown to affect the risk of acquiring mutations and cancer (Miller et al., 2001). Detoxification occurs in two phases: Phase 1, functionalization, which uses oxygen to form a reactive site, and Phase 2, conjugation, which adds a water-soluble group to the reactive site (Liska, 1998). Most pharmaceutical

agents are metabolized in the human body during Phase 1 by enzymes from the cytochrome P450 superfamily (Liska, 1998). If reactive molecules cannot be metabolized via Phase II conjugation, they may damage DNA, RNA, and proteins within the cell (Liska, 1998). In some cases, the damage could be repaired, in some – not, depending on individual genetic constitution (Liska, 1998).

Poor sanitation and, especially, poor diet, which currently account for most of Africa's environmentally-related disease burden, may further impair resilience to toxicological challenges, leading to an increase breast cancer risk (Nweke et al., 2009). A study in China showed that age and animal fat intake were positively associated with the total serum levels of DDT, whereas leafy vegetable and fresh bean consumption was negatively associated with serum DDT levels (Lee et al., 2007b). Similarly, Masala et al. (2012) performed a prospective case-control study on 31,000 women aged 36-64 in Italy and showed an inverse association between the consumption of all vegetables, leafy vegetables, fruiting vegetables, and raw tomatoes with breast cancer ($P < 0.003$, 0.0001, 0.01, 0.03, respectively). These studies suggest that an individual's diet may have a large impact on breast cancer risk. However, consumption of vegetables, which have presumably been exposed to pesticides and then thoroughly decontaminated prior to ingestion and digestion, seems to lead to a decrease in female breast cancer; this may be either from an overall reduction in pesticide exposure from the decontamination process itself and/or the inherent molecular/chemical properties of the vegetable reacting in the human body during digestion.

In regard to the higher incidence rate of breast cancer associated with animal fat intake, it is possible that livestock are exposed to pesticides due to grazing in contaminated areas. The chemical may be stored in animal fat cells and undergo further bioaccumulation in the fat compartment of a human body. Consequently, a consistently reduced level of human body fat "storage areas" may therefore provide an effective barrier to toxic bioaccumulation of chemical agents that may lead to breast cancer. In this way, women who ingest plenty of leafy vegetables may be minimally exposed to environmental toxins that may impact their breast cancer risk both due to reduced waistlines and to lesser intake of a chemical, but women who eat high quantities of animal fats may increase their risks both directly (through ingestion) and indirectly (through increased fat mass). Moreover, rapid release of stored chemicals during periods of stress (by dieting or as a result of illness) may expose the individual to an onslaught of previously "locked" carcinogens.

The precautionary principle relies on the prevention of adverse health effects when scientific evidence is uncertain but persistent. Improved understanding of these exposures and their potential interactions with breast cancer susceptibility genes may, in the future, improve the prospects for breast cancer prevention (Brody, 2005; Bernstein, 2002).

The Level of Toxins in the Environment

The use of organic chemicals can be introduced into the environment and further organisms through a variety of means. Whether through ingestion via food residues or water sources, these chemicals have been posed to have potential direct and indirect

action on the affected organisms. One of the measurements used to assess the amount of these toxins in the environment is through the United States Environmental Protection Agency (EPA), which sets National Primary Drinking Water Regulations (NPDWR). These are standards used by public water treatment system that are enforceable by federal law (40 CFR 141) (USEPA, 2017a). The EPA has set the Maximum Contaminant Level (MCL), which is the highest level of a contaminant that is allowed in drinking water, which generally coincides with an expectation of no known or expected health risk. At these levels milligrams per liter (mg/L) is equivalent to parts per million (ppm). Under these regulations, atrazine has an MCL of 0.003 mg/L, and glyphosate has an MCL of 0.7 mg/L (USEPA, 2017a). However, not all organic chemicals have an MCL set by the EPA. BPA was evaluated in water sources and plastics assessments, and Staples et al. (2002) indicated that BPA was unlikely to cause adverse aquatic effects in surface waters with concentrations ranging from 0.001-0.10 ug/L. In the plastics industry, BPA levels are reported if they exceed 1ppm, and this was validated that suppliers who market as "BPA-free" indeed distribute plastic products at less than 1mg/L (Mathieu, 2013). The United Kingdom proposed a predicted-no-effect-concentration (PNEC) of 1ng/L for beta-Estradiol (Young et al., 2004). The Margin of Safety (MOS) for total estrogen exposure is greatly dependent on an individual's acceptable daily intake (ADI), but even so, effluent concentrations from an Illinois wastewater treatment plant ranged in concentration from 0-25.3ng/L, with an average of 3.6ng/L, a level known to cause ecological risk. Interestingly, these levels seemed to increase drastically with the population level changes at each semester in the

local university (Heffron et al., 2016). The EPA does not have an MCL defined for DDT, but the New York State Department of Health established an MCL or 5ug/L in drinking water, and found levels of 0.2ug/L in the waterways assessed (NYSDH, 1998). DMSO has been reported at concentrations of 0.05-3.7ppm in preserved food products, with less than 5,000ppm to be "safe" as a relatively innocuous Class 3 Solvent according to the FDA (Gaylord Chemical Company, 2007).

Epidemiological Studies

Observational studies, including case-controls, cohorts, and cross-sectionals, are used in medical research for analyze the benefits and harms of a particular medical condition or course of action, report new findings, and confirm/refute old claims (von Elm et al., 2007). Large blood-based observational epidemiological studies have been used to identify associations between risk factors and complex diseases and data from clinical trials have confirmed that associations in observational studies are causal by showing that treatment of specific risk factors using certain therapeutic agents reduces the risks of developing disease (Keavney, 2004). In parallel, utilizing animal models of disease to create transgenic and gene-targeted animals has also resulted in causality associations (Keavney, 2004). However, critics argue that important research information is unclear or missing in many observational studies, leading to doubts in the research overall (von Elm et al., 2007). In order to strengthen this type of research, methodologists, researchers and journal editors at the World Health Organization (WHO) developed the Consolidated Standards of Reporting Trials (CONSORT) Statement was

developed in 1996 and revised five years later as STROBE, the Strengthening the Reporting of Observational Studies in Epidemiology (von Elm et al., 2007).

The most common biostatistical models for evaluating these interactions are based on the analysis of variance in multivariate regression models, which tend to be linear and assume that the main effects of genetic and environmental factors will combine additively in affecting disease (Vineis and Kriebel, 2006). Mutations may cause a change from normal reactions, indicating that a non-linear model may be needed to describe the interaction between a change in genotype and a change in environmental conditions (Vineis and Kriebel, 2006). However, analysis of variance methods will correspond to a cause when: (a) individual environmental exposure- response relationships are linear, and (b) the study provides enough statistical power to detect an interaction over a sufficiently broad range of exposures (Vineis and Kriebel, 2006).

For this reason, four factors are necessary to consider when analyzing association and the causation: (1) interaction is an essential component of any causal process involving a series of probabilistic steps, and not a second-order phenomenon identified after first accounting for "main effects"; (2) individuals may be at different stages of development along the path to disease, and acquired susceptibility can be an important source of variation; (3) there is a distinction between individual-based and population-level models; (4) at the population level, causal components may be unknown, producing additional uncertainty in quantifying interaction and assigning etiologic fractions to different necessary causes (Vineis and Kriebel, 2006).

There are three main factors why there are difficulties in connecting associations with causalities: (1) the effects of novel risk factors are less known than those for the classical risk factors, which means that larger studies are required to produce accurately robust results; (2) the associations between novel risk factors and disease might be confounded by other inaccurately measured factors that relate to the risk of disease; (3) some diseases may experience reverse causality (Keavney, 2004).

The most common causal model is Rothman's "pies," in which a sufficient causal complex (a pie) is represented by the combination of several component causes (slices of the pie) occurring together and initiating the disease process, and one cause (slice) may appear to be stronger or weaker depending on how common the other causes (slices) are (Vineis and Kriebel, 2006). Although the above considerations target understanding disease causality at the individual level, this model is also valid at the population level for chronic diseases because there is no single necessary component, but rather many necessary components of disease among the many individuals in a population (Vineis and Kriebel, 2006). A major challenge in identifying causal sequences is to determine whether an intermediate variable belongs between exposure and disease in a causal pathway, or whether it lies on a separate pathway and is correlated in some way with exposure or disease (Vineis and Kriebel, 2006). If the intermediate is a confounder, then its effect should be controlled to produce less biased estimates of associations in the cause, but if the intermediate is on the causal pathway, then controlling for it will introduce bias (Vineis and Kriebel, 2006). Often, intermediate events are both causal

events and confounders, thereby complicating resolution of causality (Vineis and Kriebel, 2006).

If a mutation is environmentally induced, then it represents a type of genetic susceptibility acquired from an environmental exposure and not from a fixed genetic trait that may be due to epigenetic data, methylation and/or hypomethylation (Vineis and Kriebel, 2006). Standard biostatistical methods may currently underestimate the disease cases that are related to an exposure because a disease process may reach a stage at which even an unlikely exposure triggers the final disease transformation if a person has already undergone most of the required stages of predisposition (Vineis and Kriebel, 2006). Even considering this, specific mutations caused by environmental chemicals may or may not truly constitute acquired susceptibility, but this associative information may provide evidence of the agent's effects for further future causative studies and experimental trials (Vineis and Kriebel, 2006). Given this, analysis without this associative information may lead to an underestimation of the true effect of the exposure or failure to detect the risk entirely (Vineis and Kriebel, 2006). Ignorance about steps in a causal chain will minimize the identification of causal components, whether environmental or genetic, so (a) more attention needs to be paid to exposures that can induce acquired susceptibility to disease, and (b) multiple exposures and their sequence in the determination of chronic diseases need to be more thoroughly considered (Vineis and Kriebel, 2006).

SPECIFIC AIM:

Cancer is an indiscriminate disease with an increasing prevalence in older age cohorts. Epidemiological and experimental evidence supports concerted action of both genetic and environmental determinants toward the development of cancer. As genetic changes are identified as the key cancer progression, the continuing trend of cancer incidence and mortality deems further research necessary to assess the role of possible interaction between environmental and genetic determinants of cancer. Understanding of these interactions may lead to better prevention and, therefore, a reduction in cancer incidence and mortality. Plasticizers and pesticides were recently named as plausible cancer-causing agents, but the extent of their involvement in the changes in oncogene and tumor suppressor gene expression levels has not been fully assessed. This study aims to narrow this knowledge gap.

In this study, we seek to test the hypothesis that the levels of exposure to environmental toxic may be linked to the development of cancer through a toxin induced changes in expression levels of mRNA encoding tumor-suppressor genes and oncogenes. As many of these genes have their orthologues in *C. elegans,* and as conservation of these genes in evolution implies that the regulatory networks surrounding their genes are also preserved in evolution, we hypothesize that nematode *C. elegans* may serve as affordable and efficient model for the screening of plausible cancer-promoting toxins for their potential carcinogenic effects.

There was a constraint on a selection of candidate environmental toxins, which was necessitated by working with minors in a Biosafety Level 1 lab. Because of that, acutely toxic and inhalant-hazard toxins were removed from the initial screening list.

Specific Aims:

To evaluate the expression (up-regulation/down-regulation) of *C. elegans* orthologues for genes associated with breast carcinoma progression in nematodes exposed to plausible toxins and to compare that to control animals.

To develop a network of molecular interactions which connect the toxin to the observed gene expression changes.

CHAPTER TWO - METHODOLOGY

Cultivation of Bacterial Feedstock and *C. elegans* Populations

Caenorhabditis elegans N2 (wild-type) strain was purchased from Carolina Biologicals (Burlington, NC) as pre-plated on Nematode Growth Media seeded with *Escherichia coli* OP50. Upon receipt, each plate was stored at room temperature until observed to need passaging. *E. coli* OP50 was purchased from Carolina Biologicals (Burlington, NC) as pre-plated on LB agar and stored for up to 6 months at 4°C using Parafilm and inverted plate storage. When aliquots of bacteria were needed for seeding NGM plates, a small loopful of bacteria was incorporated into Nutrient Broth and grown for 48 hours at 37°C. These vials were stored at 4°C for up to 8 weeks but could have also been pelleted in a centrifuge and stored at 4°C for 4 weeks or 6 months at -70°C. As a reference point, 250mL of bacterial culture grown for 24 hours at 37°C is needed to feed 4 large plates of worms that have been moved to liquid culture.

When needed, 0.1mL of the liquid bacteria sample was evenly spread in the center of Nematode Growth Media (NGM) plates and grown for an additional 24-48 hours at 37°C. Had smaller plates been used, 0.05mL of the sample would have been evenly spread in the center of the Nematode Growth Media (NGM) plates and grown for an additional 24-48 hours at 37°C. Once bacterial seeding took place, a chunk of the older *C. elegans* plate was cut out and placed on the new plate. Dissection microscopes were used to ensure appropriate transfer of worms to the new plates. Upon review of

efficiency, it was optimal to wash the older plates with M9 Buffer, K-media, or 0.03% saline to remove as many viable worms as possible rather than use the chunk methodology. For any of the aforementioned solutions, each plate was washed with 2-5mL of liquid, transferred to a 15mL conical tube, and centrifuged at 4,400 rpm for 10 minutes. It is important to note that there was bacterial transfer in addition to worm transfer with the wash, which may have affected further testing, if overlooked. The salt solutions will remove any viable bacteria, but the cellular material is still present. The pellet formed from worm centrifugation is not fully compact, as it would be with a bacterial pellet. For this reason, when pouring off or aspirating supernatant, it is imperative that careful methodology is used or the worms intended for isolation will be lost in the supernatant waste.

When performing toxicity tests, liquid S-complete media was used for worm growth. To obtain age-synchronized larvae, M9 Buffer, K-media, or 0.03% saline were used to wash each NGM plate. Each of the three washes was followed by a centrifugation at the aforementioned speed and time, and then the supernatant was aspirated or poured off. As described in Willett et al. (2010), the worms were mixed in a minimum of 2mL sterile water, followed by cold treatment to obtain L1-L3 larvae. Cold treatment consisted of placing the tube with water and worms into a salty ice bath that was approximately -2°C. This resulted in the worms settling to the bottom of the tube in a senescence-like state. After 5 minutes, the tubes were removed and set at room temperature. After 30-60 seconds, the supernatant was removed, which contained L1-L4 larvae; the adult worms were still recovering from the cold treatment in the pellet. This

was repeated two more times. Once the tubes with L1-L4 larvae were obtained, they were placed in the aforementioned salty ice bath for 10 minutes, followed by sitting at room temperature. After 2-3 minutes, the supernatant was removed, which contained L1-L4 larvae; the L4 larvae were still recovering from the cold treatment in the pellet. The adult worms and L4 larvae were replated or stored appropriately for future use, while the L1-L3 larvae were used for further experimentation.

Once experimentation was completed, worms were stored long-term in an S Buffer:Glycerin solution at -80°C, but storage could also be in liquid nitrogen. During experimentation, short-term culture storage was used wherein soft agar freezing media was mixed with S Buffer and stored at -80°C.

24-Hour Toxicity Screening

Each plausible toxin underwent a toxicity screen using motility testing to determine optimal exposure concentrations in 24-well plate format. Since toxin exposure concentrations in *C. elegans* parallel murine models, and murine models parallel human models, a scale-down approach was sufficient to determine concentration dosage.

An age-heterogeneous mixture of worms was obtained in 150mL of M9 Buffer. After obtaining an even suspension of worms throughout the fluid, 396uL of the suspension was plated in each well of 14 24-well plates. This allowed for an additional 4uL of toxin solution per well, further diluting the toxin by a factor of 100 from the stock and dilution vials.

The worms were grown axenically during toxin exposure for 24 hours at 20°C, as was used in Ura et al. (2002). Each of the solid-state toxins were homogenized in the

given stock concentration (M) using the appropriate solvent (Table 1; Equation 1). The liquid state toxins were used as the stock vial, making the starting molarity different from the solid-state toxins. We were not able obtain pure glyphosate compound, therefore, commercial "RoundUp" preparate with 50.2% glyphosate was used in its place. The dilution factor for glyphosate was taken into account when data were analyzed.

Table 1: Selected Toxins with Their Molecular Masses, the Concentration in Each Stock Solution, and Respective Solvents

Toxin	Molecular Mass (g/mol)	Concentration Stock (mol/L) M	Solvent for Stock
Bisphenol A (BPA)	228.29	5	DMSO
Atrazine	215.68	5	DMSO
Calcium Hypochlorite	142.98	5	WATER
Dichlorodiphenyltri chloroethane (DDT)	354.49	5	DMSO
Monobutyl Phthalate (MBP)	222.24	5	DMSO
Pyrene	202.25	5	DMSO
Antharcene	178.234	5	DMSO
Acenapthene	154.21	5	DMSO
Estradiol	272.382	5	DMSO
Diethyl phthalate	1M solution (>98% pure))	1	N/A
Butyl benzyl phthalate (BBP)	1 M solution (>98% pure)	1	N/A
Dimethylsulfoxide (DMSO)	1 M solution (>98% pure)	1	N/A
RoundUp (Glyphosate Present)	In solution (50.2% Glyphosate)	2.5 (Glyphosate)	N/A

Equation 1: Calculation of the Weight of Each Selected Toxin to Prepare 1 Liter of Stock Solution in a Solvent

$$\frac{g}{mol} \times \frac{mol}{L} = \frac{g}{L}$$

Equation 2: Calculation to Make 20uL of Each Toxin Stock Solution

$$\frac{m1}{V1} = \frac{m2}{V2}$$

Each stock solution underwent a serial dilution (by a factor of 10) to produce a total of six exposure concentrations in the 24-hour study (Equation 2; Table 2). Each subsequent dilution used 2uL of the higher concentration solution homogenized in 18uL of M9 buffer. For each toxin, 4uL of each concentration were added to each well in one column of a 24-well plate, wherein each concentration was completed in quadruplicate to validate results. These values were chosen to maintain a level of <1% solvent in the media to avoid confounding factors related to excessive solvent rather than toxin exposure, and prior studies indicated that visible changes were noticed at an exposure of 1mM (Boyd et al., 2011).

Table 2: Toxin Concentrations in Each Serial Dilution used in *C. elegans* 24-hour Mortality Screen. The Starting Stock Concentration was 5M. The concentration values used in 24-mortality Screens are Shown in Green.

	Units	Dilution 5	Dilution 4	Dilution 3	Dilution 2	Dilution 1	Stock
In Solution	M	0.00005	0.0005	0.005	0.05	0.5	5
	mM	0.05	0.5	5	50	500	5000
	uM	50	500	5000	50000	500000	5000000
In Vivo	M	0.0000005	0.000005	0.00005	0.0005	0.005	0.05
	mM	0.0005	0.005	0.05	0.5	5	50
	uM	0.5	5	50	500	5000	50000

The live and dead worms were counted using a dissection microscope and a probe to evaluate motility of individual worms. Non-motile worms were considered either deceased or dauer, both of which we counted as reacted negatively to its

environment. The LC10 was determined for each chemical using the PROBIT method in IBM SPSS.

Effects of each toxin on the microbial food population to be utilized in the next phase of the project were evaluated in *E. coli* OP50. A batch of these cells was grown overnight at 37°C, then diluted in 96-we1l plates to 10,000 cells per well, according to the manual of the XTT Assay manufacturer (Biotium, Inc., Fremont, CA). Each of the environmental contaminants intended for subsequent exposure experiments in the *C. elegans* 24-hour study was tested in the volume compatible to the 96-well plates cultures. In short, 99uL of the microbe-containing media were added to each well of the 96-well plate, and then 1uL of the diluted toxin in a solvent was mixed into each well, by pipetting for homogeneity. Each toxin dilution was tested in quadruplicate, and therefore, each 96-well plate held 3 repeats of experiment with the toxin added to certain concentrations and 1 set of controls (Table 3). Each plate was incubated for 1 cell duplication period (20 minutes) at 37°C, then the 25uL of XTT reagent was added to each well followed by another incubation of 20 minutes at 37°C. The plates were read at 490nm and 630nm using a microplate reader (Biotek, Inc., Winooski, VT).

Table 3: Typical 96-well Plate Setup for the E. coli OP50 XTT Assay, Where Three Selected Toxins and Controls were Tested on the Same Plate. Each Environmental Condition is Highlighted by a Different Color.

	Toxin 1 (M)						Toxin 2 (M)					
Trial 1	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	0.0000005	0.000005	0.00005	0.0005	0.005	0.05
Trial 2	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	0.0000005	0.000005	0.00005	0.0005	0.005	0.05
Trial 3	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	0.0000005	0.000005	0.00005	0.0005	0.005	0.05
Trial 4	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	0.0000005	0.000005	0.00005	0.0005	0.005	0.05
Trial 1	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	Solvent	Broth:Microbe	Empty	Solvent: Toxin1	Solvent: Toxin2	Solvent: Toxin3
Trial 2	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	Solvent	Broth:Microbe	Empty	Solvent: Toxin1	Solvent: Toxin2	Solvent: Toxin3
Trial 3	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	Solvent	Broth:Microbe	Empty	Solvent: Toxin1	Solvent: Toxin2	Solvent: Toxin3
Trial 4	0.0000005	0.000005	0.00005	0.0005	0.005	0.05	Solvent	Broth:Microbe	Empty	Solvent: Toxin1	Solvent: Toxin2	Solvent: Toxin3
	Toxin 3 (M)						Controls					

Chronic Toxin Exposure

The Lethal Concentrations for 10% of the population (LC10) were calculated using the PROBIT analysis. The log values at p=0.10 were converted to the concentration (mM) required for inclusion in the growth media. Once the LC10 value was determined, a series of dilutions in the appropriate solvent were performed to reach that concentration, keeping in mind that the final concentration in the series only composed 0.001% of the overall media solution (10mL). This methodology was used to limit chemical waste, in addition to working within the purchased volumes and concentrations obtained for this study.

Worms were cultivated using the aforementioned techniques to obtain L1-L3 larvae from 10 large Petri dishes of heterogeneous worms. Once obtained the L1-L3 worms were mixed in 800mL of S-complete media pre-mixed with 10mL of pelleted OP50 through them methodology aforementioned. Each toxin was plated in triplicate 25mL flasks in that 0.25uL of the given concentration was added to 10mL of the inoculated media. The flasks were incubated at 25°C for 7 days while rotating to

maintain oxygenation. Given that *C. elegans* has a lifespan of 12-15 days, this incubation period was chosen to mimic that seen in human exposures over a long-term, beginning with exposure as a child up until the development of cancer at 60-65 years old. In addition, because the shaking incubator had a minimum temperature of 24°C, this is the minimum temperature allowed while still maintain adequate oxygenation. *C. elegans* also experiences a slightly faster growth rate at this higher temperature, so this was also taken into account when finalizing the time needed for a chronic exposure in this model organism (Maniatis et al., 1982). During the chronic exposure period, each flask was observed for adequate growth and movement. Upon observation of stress, additional OP50 was added to each flask. This occurred at Day 4 of the experimental phase. Behavioral changes were documented, and genetic material in the form of RNA was extracted at the conclusion of the experimental period.

RNA Extraction

After the growth and exposure period, RNA was extracted from *C. elegans* using standard TRIZol method (Burdine & Stern, 1996).

cDNA Generation

cDNA was made using standard techniques in the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor from ThermoFisher Scientific (Waltham, MA). The concentration of each cDNA samples was quantified using a NanoDrop Spectrophotometer from ThermoFisher Scientific (Waltham, MA) and diluted to 1ug/uL to maintain consistency across all samples.

Primer Generation and Optimization

Primer sets for each selected gene in *C. elegans* were chosen based on homology to human gene structure and function. In seven primer sets, there were several isoforms of the human gene with distinct activity and localization. Each isoform was chosen for the gene of interest based on genetic sequence similarity and physiological action upon activation in the chosen organism. Fzr-1 Isoform 2 is the major gene isoform, and it is localized in the nucleus to function as an adaptor in ubiquinylation processes. Isoform 1 of ISCU, the orthologue for reference gene Y45F10D.4, is located in mitochondria and functions to cluster iron in cellular respiration processes. KCNRG-related orthologues each shared homology with a different isoform, both of which have deletions from the parent form of the gene but are otherwise similar. This same pattern wherein a deletion from the longest human isoform shared the greatest homology to *C. elegans* was seen in the following genes: Isoform 5 of BRCA1 (orthlogue for BRC-1), Isoform 1 of MLX-interacting protein (orthologue for MML-1), and Isoform A of FBXW11 (orthologue for LIN-23)

Standard polymerase chain reactions were performed with the following temperatures: denaturing at 94°C, annealing at 64°C, and extending at 72°C using Taq PCR Master Mix Kit (ThermoFisher Scientific, Waltham, MA). All PCR reactions were cycled 40 times through the protocol and stored at -20°C. To confirm the correct amplification by the primers, 2uL of the resulting PCR products from wild-type non-exposed worms were analyzed with gel electrophoresis using 0.8%% agarose gel stained with GelGreen (Biotium, Fremont, CA). The gel was used to detect the correct size bands of each genetic primer pair. Primers were generated for selected genes (Table 4).

Table 4: Oncogene and Tumor Suppressor Genes Selected for this Study. Pink Indicates Oncogenes, and Purple Indicates Tumor Suppressor Genes. The Orthologues Between humans and *C. elegans* are Noted in Each Group. Reference Genes used for Normalization were Y45F10D.4 (Human Isoform 1 of Iron-sulfur cluster assembly enzyme ISCU in Mitochondria), Tba-1 (Human Tubulin alpha-4A chain), and Pmp-3 (Human ATP-binding cassette sub-family D member 4).

Proto-oncogenes		Tumor Suppressor Genes	
C. elegans	Human	*C. elegans*	Human
CUL1/LIN19	CUL1	F52.B5.5/CEP-1	TP53
LIN23	Beta-TRCP *Isoform A*	C36A4.8/BRC-1	BRCA1 *Isoform 5*
CED9	BCL-2	T07E3.5/BRC-2	BRCA2
FZR1	FZR *Isoform 2*	LIN-35	RB1
KNL-1 KNL-3 HCP-1 HCP-3 HCP-4 HCP-6 CPAR-1	CUG2/CENP family-related/H3.3-related	SLO-1 SLO-2	KCNRG family-related (*Isoform 5 of Calcium-activated potassium channel subunit alpha-1; Isoform 2 of Potassium channel subfamily T member 1*)
AIR-1	AURKA/STK6/STK15	MML-1/MXL-2	MYC family (*Isoform 1 of MLX-interacting protein*)
PIG-1	MELK	JUN1	JUNB

Chronic Toxin Exposure

Although *C. elegans* tends to hold eggs while growing in liquid culture, in this study, this phenomenon was not observed. To avoid dauer formation and death due to overcrowding, 10-15 L1-L3 worms were observed per culture flask at the onset of the project with normal thrash patterns. Midway through the study, the worm population size had grown approximately 30 worms per flask, but their thrash counts decreased, indicating stress within the population. At this point, an additional food allotment of *E.*

coli OP50 bacteria was added to each flask. By the end of the study, there were over 100 worms per flask with normal thrash counts and no physiological effects noticeable.

qPCR with Agilent AriaMx

The aforementioned orthologues were used as the experimental genes for this study, and as such, primers were generated for qPCR output with tba-1, Y45F10D.4, and pmp-3 as the genetic controls for use in the qPCR Agilent AriaMx (Santa Clara, CA) (Zhang, 2012).

Real-Time PCR Reactions were performed in a 96-well format in the qPCR Agilent AriaMx (Santa Clara, CA). The presence of a single specific PCR product was verified for each reaction by melting curve analysis and confirmed on 0.8% agarose gels by observing primer product size for the control group. SYBR® Green PCR Master Mix from ThermoFisher Scientific (Waltham, MA) was used to setup qPCR reactions. For each gene of interest and control gene, five independent qPCR experiments from the same RNA sample were performed. Primers used for Real-Time PCR profiling are listed in Table 5.

Table 5: Primer Sequences Designed *C. elegans* Genes Selected for This Study

CUL-1/LIN19/CUL1 Forward: CGAGAGAACCGGATCCATGA Reverse: TCCTCCGTTGCTCAGGAAAT	**F52B5.5/CEP-1/TP53 (p53)** Forward: ACGCAGAATGGAACCCGATG Reverse: GTTTCCTCGCCATTGCCCAG
LIN23/Beta-TRCP Forward: TGTCTCCGGAGCATACGACG Reverse: TGTCTCCGGAGCATACGACG	**C36A4.8/BRC-1/BRCA1** Forward: AGCCAGTGGTCGTAGCATCA Reverse: GAGCTCGGCAAATTCGGCAA
CED9/BCL-2 Forward: TGACACGCGATGCAATGGTC Reverse: TGGTGTGCTAGGAGTCGGTG	**T07E3.5/BRCA2** Forward: CCGACGCCATTCAATTGCCA Reverse: ACTCGCATTGATTCGCTGCT
FZR-1/FZR Forward: TCGCCACGAGTCACACCTAC Reverse: ACCGCCCAAATGCCGATTAC	**LIN-35/RB1** Forward: TTTTCCTTCAAATTCAGGCAAGT Reverse: TCAGTAACAATAATGGCATGGGG
KNL-1/CUG2 /CENP-W Forward: CCGATGCTCTCCGTCATCCA Reverse: CCAGAATCCGGGGCAGAAGT	**SLO-1/KCNRG** Forward: TGGCTACAGTACCCCGGAGA Reverse: GCCCGATACAAAGTTGGCCG
KNL-3/CUG2 /CENP-W Forward: ATCGACGACATGGAAGCGGT Reverse: CGGCATCCTGGGTTTTGTCG	**SLO-2/KCNRG** Forward: GGCATCCGAAGCCAGCAAAA Reverse: CCATTGCAATTGACGGGCGA
HCP-1/CUG2 /CENP-W Forward: TTCCAAGCCCAATCGAAGCG Reverse: GCAGCGCGACTCCAATCAAT	**MML-1/MYC** Forward: TTCCCCAATCGAGTGCACCA Reverse: CAATCAGAACACGAGGCGGC
HCP-3/CUG2 /CENP-W Forward: GCGATGAAGTTGTGTCCTCCC Reverse: AGTAAAACGGACTGCGCACT	**JUN-1/JUNB** Forward: GGCGAGAAGGAAAGCACAGC Reverse: AGCAATTGATGGGCCGCTTG
HCP-4 Forward: CGCTGAATTTCCGGTCCGTG Reverse: CAGTTCTTGCAGGCACGCTT	**PIG-1/MELK** Forward: CGTGAAGCCAATGTCACCGG Reverse: AGACTCGCCGAATCACTGCT
HCP-6 Forward: ACAAGAATGGGTGACGAGCAG Reverse: CCGTTCCTGTGTGTTTCTGACC	**AIR-1/AURKA/STK6/STK15** Forward: TGGGATGCAACAGTCCGAGA Reverse: TTCCCAATCCACTGCGTCCA
CPAR-1 Forward: AGAATGGGAGGGTGGCAAGG Reverse: GGCCGTGGGAGTAATCGACA	**Tba-1** Forward: CGTTCCAAAGGACGTCAACA Reverse: CGGTGGTGTTGGAGAGCATA
Y45F10D.4 Forward: TGCAGATTCGAGTCGACGAT Reverse: GGCGAGCATTGAACAGTGAA	**Pmp-3** Forward: GGAGGACATCACCTCCAGATTT Reverse: TGAGCGGTCTCAAAGAATGC

Each PCR product amplified using designed primer pair was initially assessed with a 0.8% agarose gel to determine whether the size of the product is correct. In Figure 1, amplification with primers designed for HCP6 and HCP1 genes showed evidence of a double product, while for the primers intended to amplify LIN35, LIN23, and CED9 mRNAs only minimal levels of the PCR were detected. For the rest of the tested primer pairs, the PCR bands were as expected.

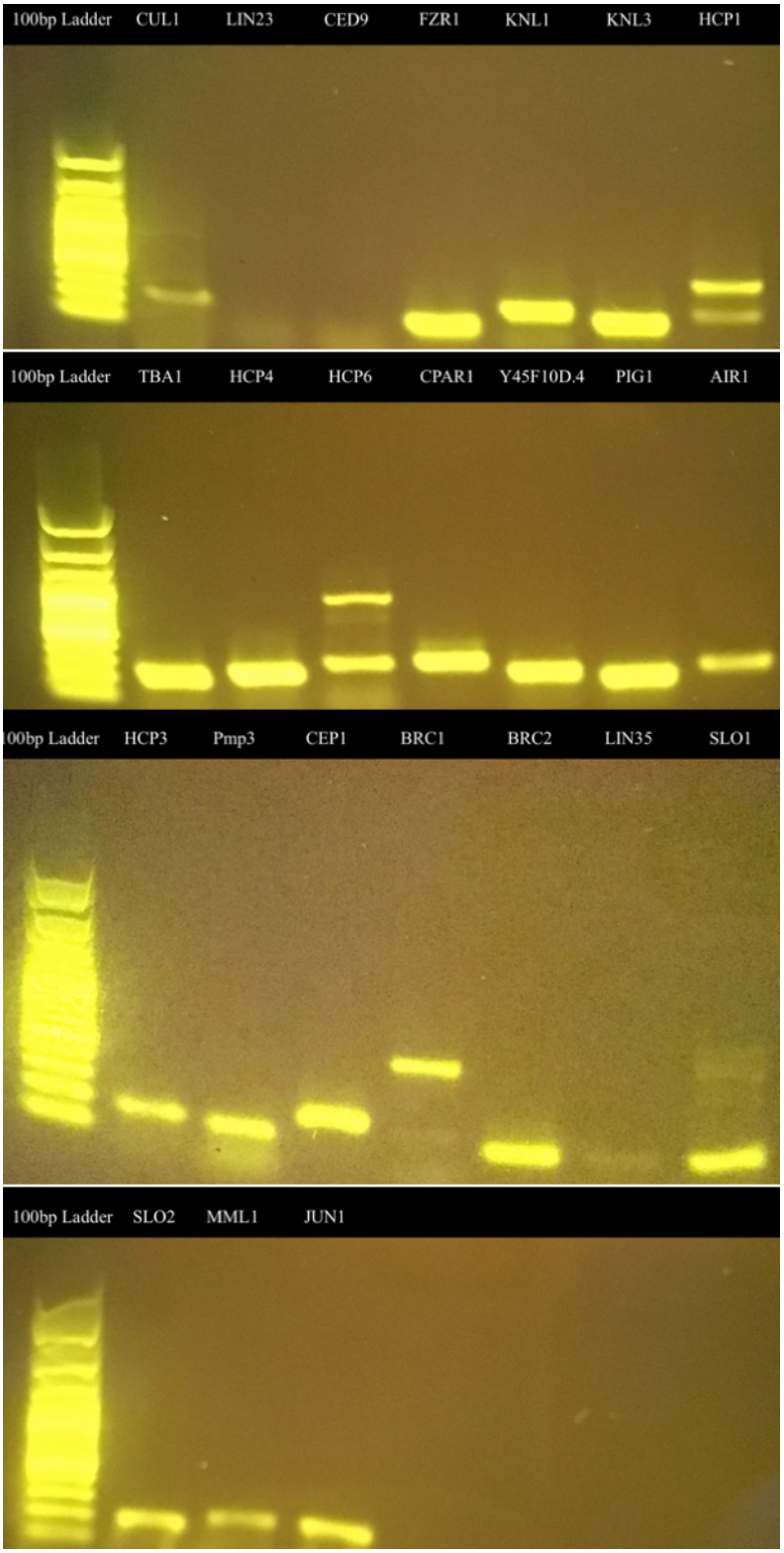

Figure 1: Product Bands Observed after Electrophoresis in 0.8% Gelgreen Stained Agarose Gels after PCR on a template of *C. elegans'* DNA with the Gene-specific Primers

To verify the presence of these products in qPCR, an analysis of Cq values, melt curves, and verification of the PCR products using a 0.8% gel electrophoresis were performed. Figure 2 shows that the products of primer pairs designed to amplify fragments of AIR1, BRCA1, CED9, LIN23, and LIN35 genes appeared as very faint bands, but none of the primer pairs generated any double band products. The primer pairs with confirmed presence of correctly sized bands were setup to be amplified on 96-well plates. Each plate setup accommodated the maximum number of primer pairs while profiling the changes in expression of genes in nematodes exposed to the same toxin.

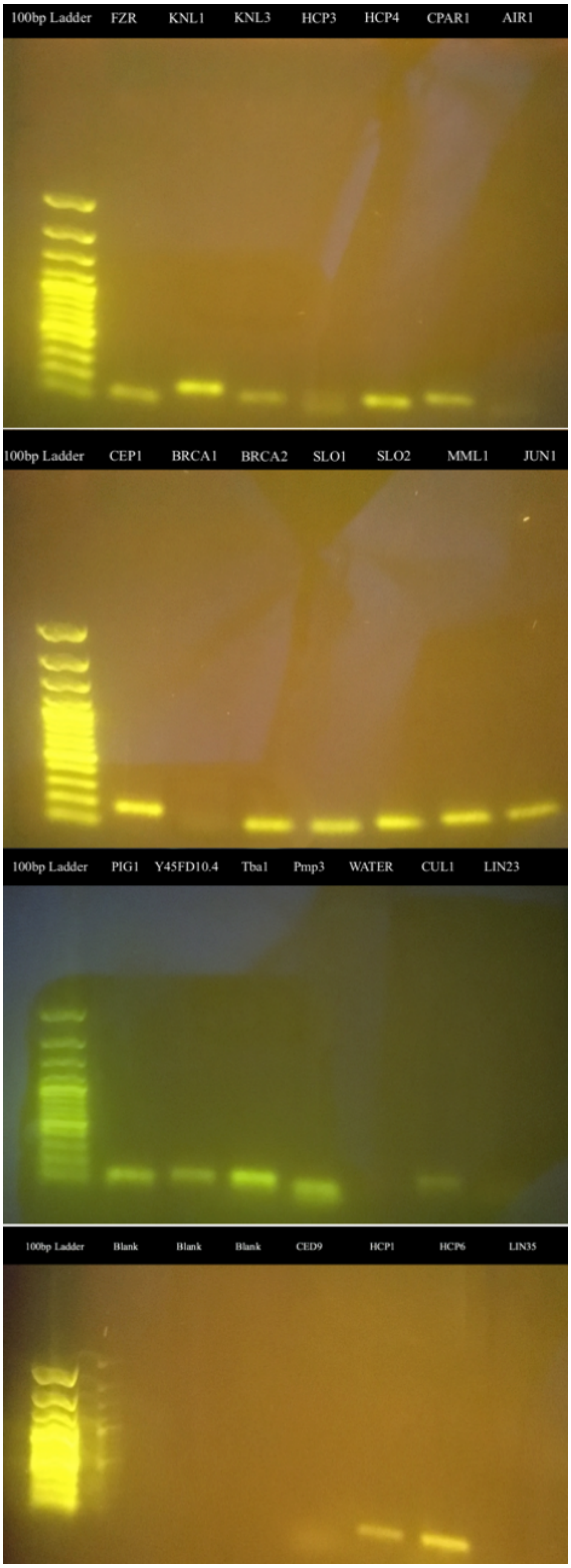

Figure 2: qPCR Product Bands Observed after Electrophoresis in 0.8% Gelgreen Stained Agarose Gels after qPCR on a template of *C. elegans'* DNA with the Gene-specific Primers

After qPCR, outputs for each gene-specific primer pair were separately normalized to each of the three reference genes, Y45F10D.4, Tba1, and Pmp3, by subtracting the raw Cq values of the reference gene from the raw Cq values of the gene-specific primer pair, thus producing the **Δ**Cq values. The three values were then averaged and used for calculation of the fold change in comparison of control nematodes to exposed ones.

The amplification plots and melt curves were used to visually analyze the incoming qPCR data and identify any potential confounding information (Figure 3; Figure 4). The plots shown are sample plots from the No Toxin Control qPCR output from Plate 1, and there is no indication of any disturbance to data collection. In addition, all analyses indicate that one product was generated, and this was confirmed with the aforementioned gel analysis. All other experimental setups were analyzed in the same manner to verify the accuracy of the data output.

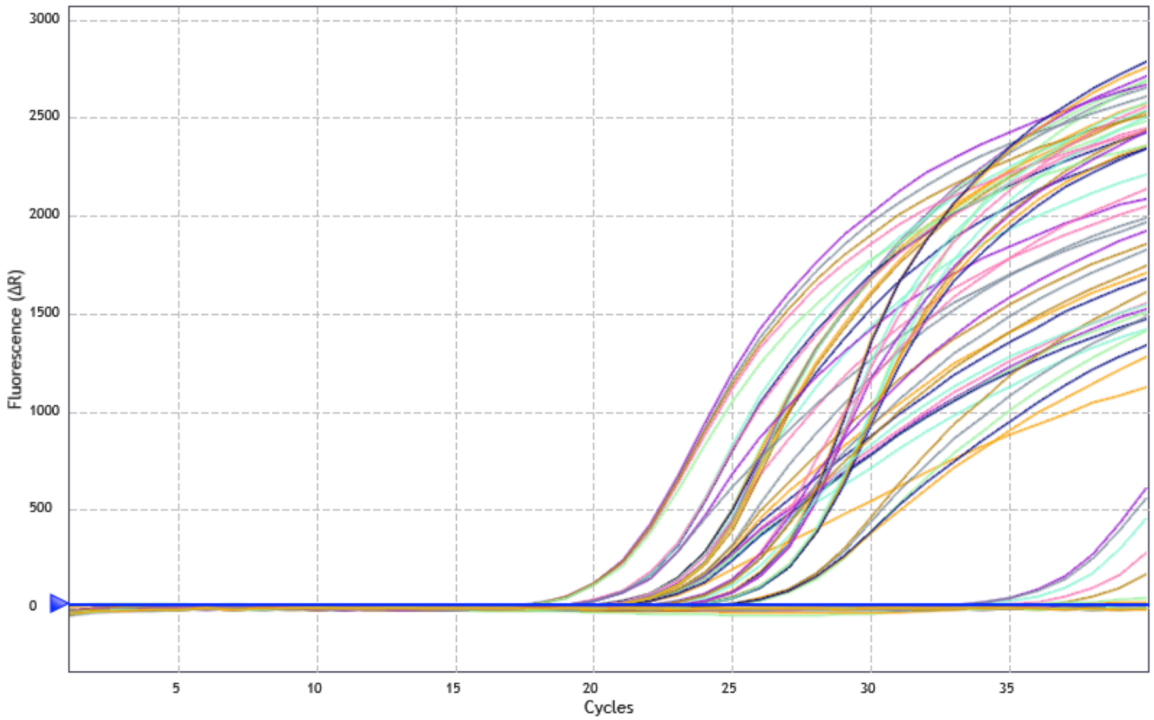

Figure 3: An example of qPCR Amplification Plot of PCR Products Observed in No Toxin Control Wells Indicates the Expression Intensities of Genes Selected for this Study. qPCR Experiments were Performed in AriaMx qPCR Thermocycler and SYBRGreen dye.

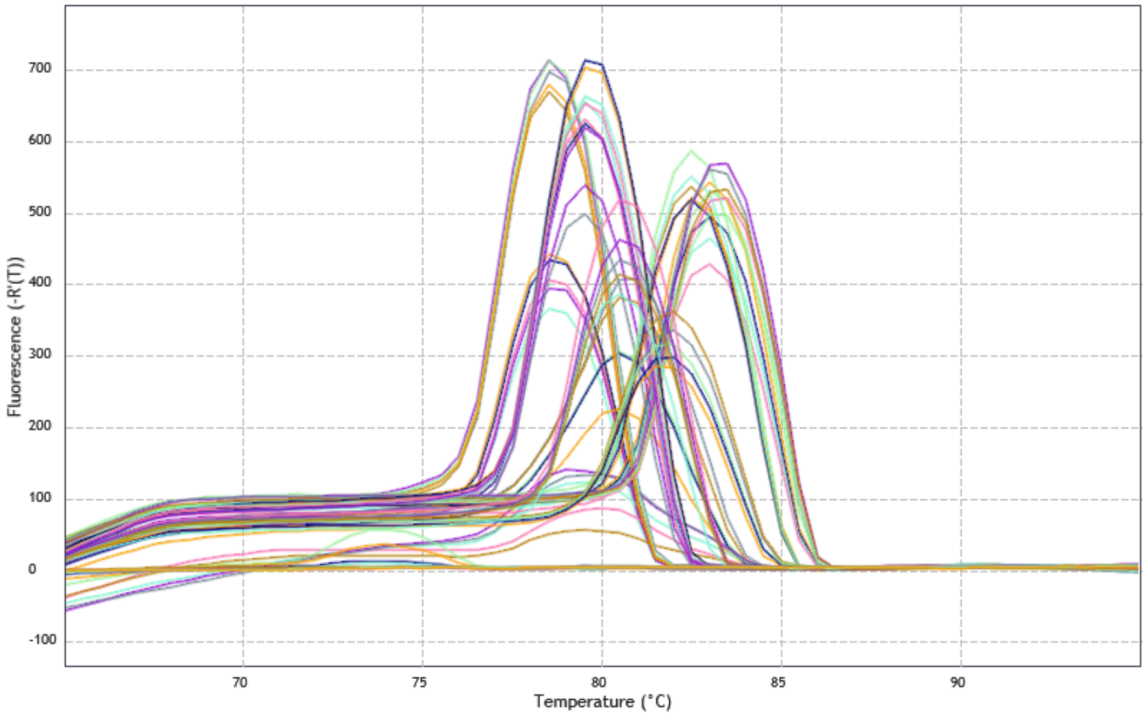

Figure 4: An example of qPCR Melt Curves of PCR Products Observed in No Toxin Control Wells Indicates that for Each of the Genes Selected for this Study, only One PCR product was observed. qPCR Experiments were Performed in AriaMx qPCR Thermocycler and SybrGreen dye.

Statistical Analysis of qPCR Data

Genes and toxins were evaluated using the ΔCq scores, partitioned by reference gene, for their difference from the control samples using non-parametric Mann Whitney U tests to determine the significance of the data output. In addition, to eliminate possible false positive data, Benjamini-Hochberg ranking was applied to the Mann Whitney p-values.

Equation 3: Calculation for False Positive Significance Using Benjamini-Hochberg Critical Value Assessment

$$Benjamini - Hochberg\ Critical\ Value - \frac{i}{m} x\ Q$$

The Benjamini-Hochberg critical value assessment utilizes the variables of i, which is the rank of the sequentially larger Mann Whitney p-values; m, which is the number of cases assessed; and Q, which is the self-assigned false discovery rate of 5% in this study. The Mann Whitney p-values were compared to the Benjamini-Hochberg critical values. The Mann Whitney p-values that were less than the Benjamini-Hochberg critical value were determined to be statistically significant (Equation 3). Three reference genes were used to standardize the data output, Y45F10D.4, Tba1, and Pmp3. Upon review of the data, normalization to Pmp3 housekeeping gene revealed a systemic target-gene down-regulation pattern, indicative that expression of this housekeeping gene itself increases in the response to at least some of the toxins. For this reason, Pmp3 data were excluded from the final analysis. While the statistical tests utilize the ΔCq scores to compare the mRNA expression levels of the selected genes from the toxin exposure samples to the control samples, the graphical output are shown on a $\log_2$ scale using the ΔΔCq values calculated using the ΔΔCq methodology (Equation 4; Equation 5; Equation 6).

Equation 4: Calculation of ΔCq Values using a Target Gene and a Reference Gene.

$$\Delta Cq = Cq\,[Target\ Gene] - Cq\,[Reference\ Gene]$$

Equation 5: Calculation of ΔΔCq Values using a Selected Toxin and a Control Sample Without Toxin Exposure.

$$\Delta\Delta Cq = \Delta Cq\ [Experimental\ Toxin] - \Delta Cq\ [No\ Toxin\ Control]$$

Equation 6: Calculation of Fold Change using the ΔΔCq values.

$$log_2\ Fold\ Change = 2^{-\Delta\Delta Cq}$$

Pathway studio -guided analysis of the connections between toxins and genes

To construct a concise network that bridges environmental toxins investigated in this study and genes, which changed their expression in response to these toxins, we used the Pathway Studio software (Elsevier, Rockville, MD) that is able to dynamically create and draw protein interaction networks and pathways. Each of the human orthologues for the genes selected in this study were automatically converted by the software to the translated protein counterpart, and the intracellular interactions were linked from the MammalPlus database. Each node represents either a molecular entity or a control mechanism of the interaction. In this study, the shortest path analysis function was utilized predominantly.

CHAPTER THREE – RESULTS

The Results of 24-Hour Toxicity Screening in *C. elegans*

The lethal effect of each of the selected toxins on *C. elegans* varied based on the concentrations tested over a 24-hour period. At the end of the 24-hour incubation period, the motile and non-motile worms were quantified to determine the mortality rate at each selected concentration. Non-motile worms were classified as either dead or dauer, depending on the age of the individual worm at the time of exposure. Both of these states were recorded as an adverse reaction in the environment, while any nematode movement was indicative of the ability to survive in the tested environmental condition. However, it should be noted that the thrash count of the worm post-exposure was noticeably decreased when compared to the pre-exposure states. The mortality rates of *C. elegans* after 24 hours of exposure to the selected toxin concentrations are visualized in Figure 5 and Figure 6. Post hoc comparison aimed at calculating the lethal concentration for 10% of the population (LC10) using IBM SPSS (Table 6). This concentration was chosen to mimic the natural environment wherein low-dose, long-term exposures have the potential for non-physiological but genetic effects that may impact carcinogenesis. There was an equivalent concentration of DMSO in the samples where it was tested as an individual agent than when it was used as a solvent for selected toxins Table 1. However, there was 5 times as much toxin in these samples as compared to the

amount of solvent present. Even so, it should be noted that there may be a synergistic effect on the mortality resulting in the combined DMSO and toxin samples.

The *E. coli* OP50 XTT assay data confirmed that no adverse effects would suppress the *C. elegans* population due to the scarcity of food. The microbes were able to withstand exposures to each of the experimental toxins while maintaining close to 80% survival ability at the highest concentration tested. Therefore, we proved that this bacterial strain could be used as the *C. elegans* food source at an even lower concentration than was tested in nematode 24-hour exposure experiments.

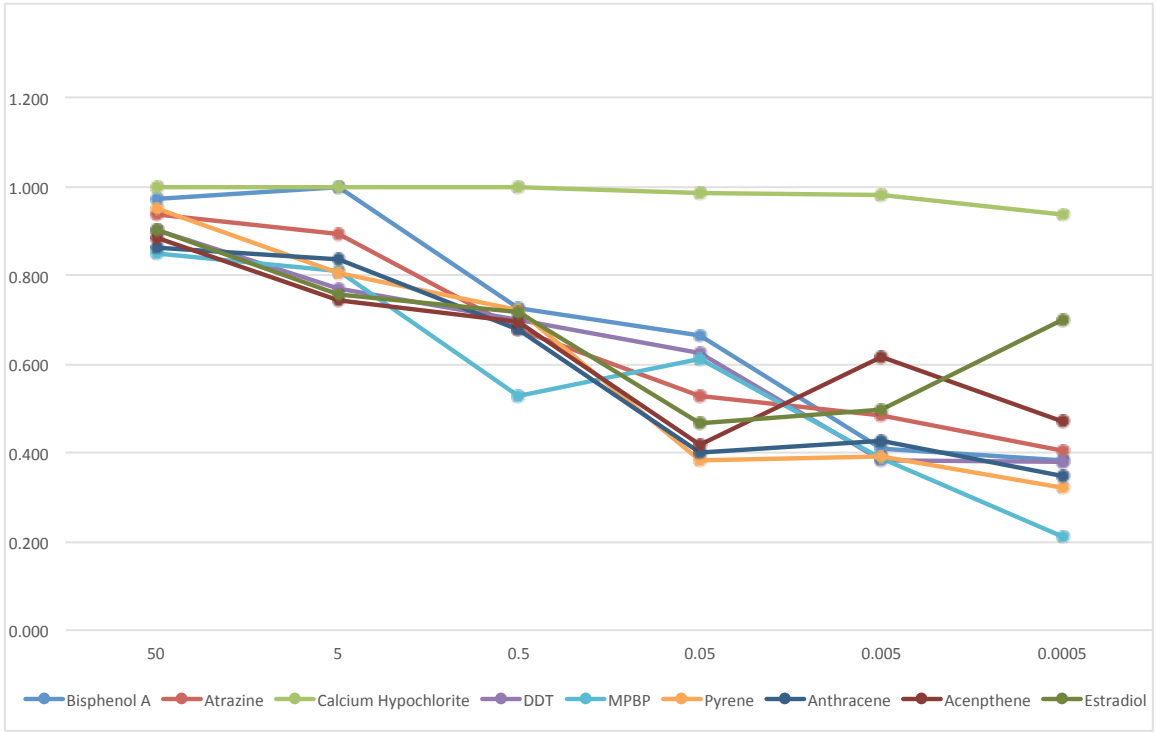

Figure 5: A Plot of Mortality Rates of *C. elegans* After 24 Hours of Exposure to Toxins at Varying Concentrations (0.00005-50mM).

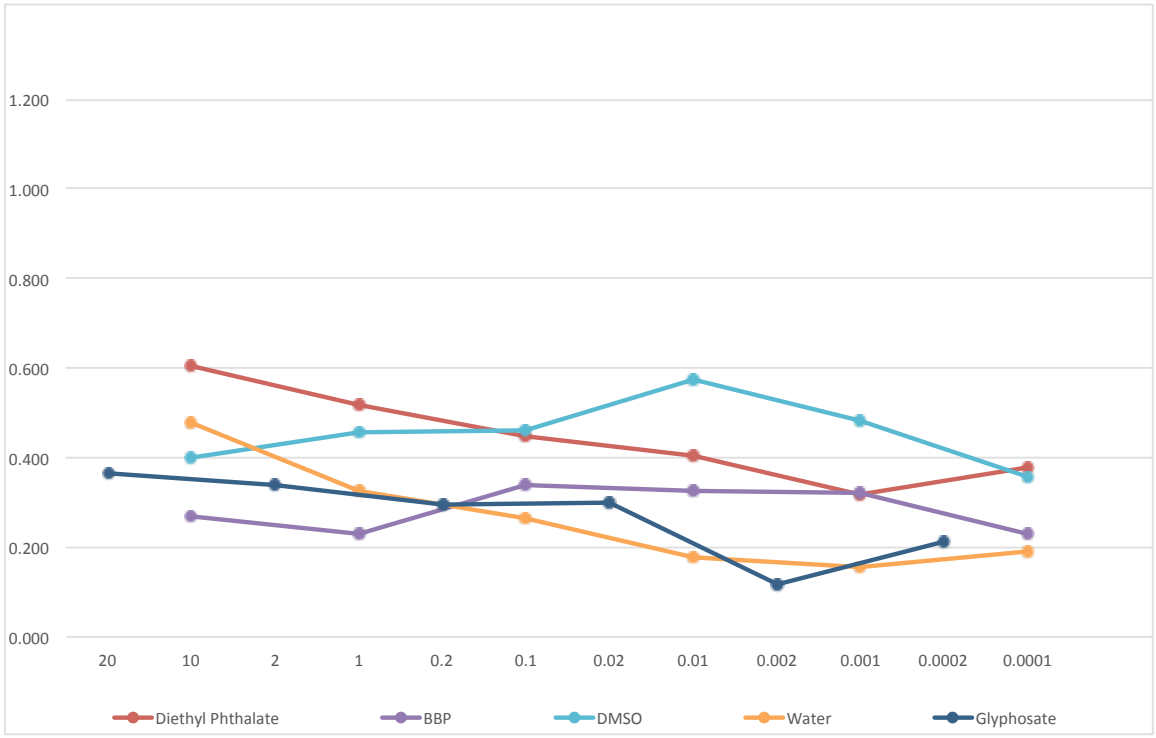

Figure 6: A Plot of Mortality Rates of *C. elegans* After 24 Hours of Exposure to Toxins at varying Concentrations (0.0001-20mM).

Table 6: Calculated Estimates for LC10 Concentrations for Each Tested Toxin

Toxin	Confidence Limit Estimate for LD10	logX LC10 Estimate	LC10 Calculated (mM) In Culture Flask	LC10 Calculated (mM) In Diluent Stock Vial	LC10 Calculated (M) In Diluent Stock Vial
BPA	<0.000	-10.186	6.516E-11	6.516E-06	6.516E-09
Atrazine	<0.000	-10.206	6.223E-11	6.223E-06	6.223E-09
Calcium Hypochlorite	<0.000	-18.852	1.406E-19	1.406E-14	1.406E-17
DDT	<0.000	-9.505	3.126E-10	3.126E-05	3.126E-08
MPBP	<0.000	-8.293	5.093E-09	5.093E-04	5.093E-07
Pyrene	<0.000	-8.765	1.718E-09	1.718E-04	1.718E-07
Anthracene	<0.000	-9.053	8.851E-10	8.851E-05	8.851E-08
Acenapthene	<0.000	-9.994	1.014E-10	1.014E-05	1.014E-08
Estradiol	<0.000	-9.594	2.547E-10	2.547E-05	2.547E-08
DEP	<0.000	-7.36	4.365E-08	4.365E-03	4.365E-06
BBP	<0.000	-4.961	1.094E-05	1.094E+00	1.094E-03
DMSO	<0.000	-7.769	1.702E-08	1.702E-03	1.702E-06
Water	<0.000	-5.055	8.810E-06	8.810E-01	8.810E-04
Glyphosate	<0.000	-5.071	8.492E-06	8.492E-01	8.492E-04

Toxin Exposure-related Changes in the Expression Levels of *C. elegans'* Orthologues of Human Genes Involved in Tumorigenesis

Bisphenol A (BPA; 4,40-dihydroxy-2,2-diphenylpropane)

In our experiments, *C. elegans* nematodes were incubated at 6.516×10^{-11} mM concentration of BPA for 7 days with Bisphenol A, a plastic polymerization reagent that can both mimic the action of estrogen and antagonize estrogen by interacting with the androgen receptor (AR). When expression levels of tested mRNAs were normalized to reference gene Tba1 and exposed nematodes where compared to control animals, genes CPAR-1 ($p<0.000$) and HCP-3 ($p<0.002$) displayed significant differences in their expression levels in exposed nematodes as compared to control animals. When

expression levels of tested mRNAs were normalized to reference gene Y45F10D.4 and exposed nematodes where compared to control animals, genes HCP-6 ($p<0.000$), JUN-1 ($p<0.016$), and SLO-1 ($p<0.003$) displayed significant differences when their expression values.

When the mRNA levels from CEP-1, an orthologue for TP53 that encodes a transcription factor to induce pro-apoptotic events after DNA damage, in BPA-exposed nematodes were compared to that in controls, normalization with either Y45F10D.4 or Tba1 reference genes resulted in observation that the differences in its expression were significant, with $p<0.001$ and $p<0.029$, respectively (Figure 7; Figure 8; Table 7).

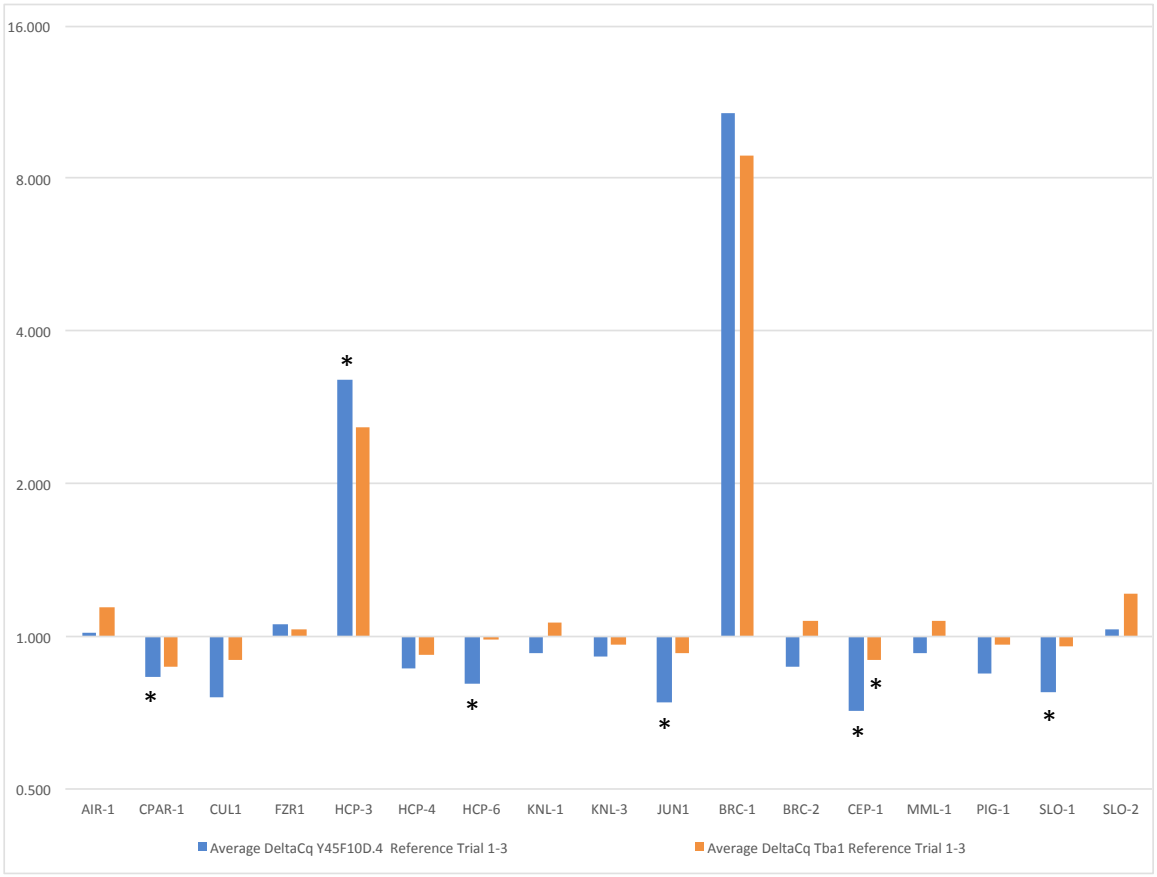

Figure 7: Average Fold Change in Expression Levels of Orthologues of Human Oncogenes and Tumor Suppressor Genes in C.elegans Exposed to LD10 (6.516x10-11 mM) Concentration of BPA for 7 Days

Table 7: Orthologous Genes in *C. elegans* and Humans with their Associated Functions.

C. elegans	Human	Functional Description
AIR-1	AURKA	aurora kinase A
C36A4.8/BRC-1	BRCA1	breast cancer 1
T07E3.5	BRCA2	breast cancer 2
KNL-1	CASC5	cancer susceptibility candidate 5
CPAR-1	CENPA	centromere protein A
HCP-3	CENPA	centromere protein A
HCP-4	CENPC	centromere protein C
CUL1/LIN19	CUL1	cullin 1
KNL-3	DSN1	DSN1 homolog, MIS12 kinetochore complex component
FZR1	FZR1	fizzy/cell division cycle 20 related 1
JUN-1	JUNB	jun B proto-oncogene
SLO-1	KCNMA1	potassium calcium-activated channel subfamily M alpha 1
SLO-2	KCNT1	potassium sodium-activated channel subfamily T member 1
PIG-1	MELK	maternal embryonic leucine zipper kinase
MML-1	MLX	MLX, MAX dimerization protein
HCP-6	NCAPD3	non-SMC condensin II complex subunit D3
F52.B5.5/CEP-1	TP53	tumor protein p53

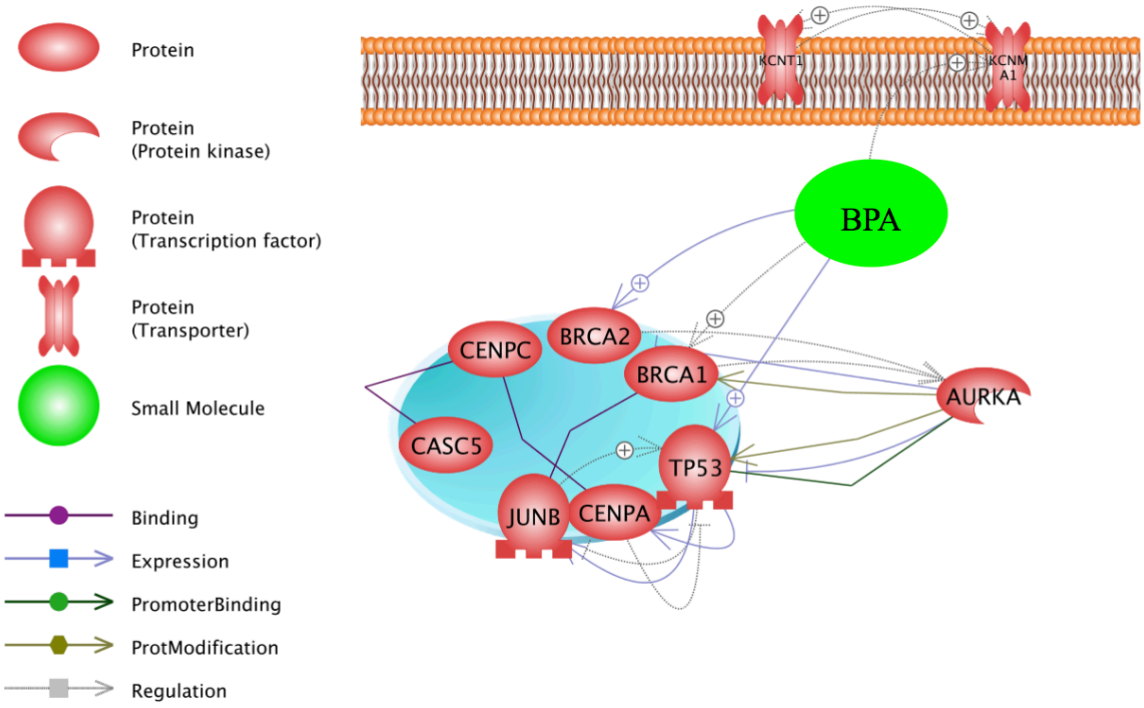

Figure 8: Pathway Studio-guided Visualization of Interactions of BPA and Human Orthologues of the Genes that Changed Their expression in *C. elegans* After Chronic Low-Dose Exposure to this Environmental Toxin.

Atrazine

C. elegans nematodes were incubated at 6.223×10^{-11} mM concentration for 7 days in atrazine, a triazine pesticide that negatively affects the electron transport chain in photosynthetic cells. When expression levels of tested mRNAs were normalized to reference gene Tba1, HCP-4 ($p<0.007$) gene displayed significant change in its expression in exposed nematodes as compared to control animals. When the levels of mRNA from genes HCP-3 ($p<0.000$; $p<0.000$), a gene involved in proper kinetochore assembly, and BRC-1 ($p<0.000$; $p<0.000$), a gene involved in activating cell cycle checkpoints and DNA repair, in atrazine-exposed nematodes were compared to that in controls, normalization with either Y45F10D.4 or Tba1 reference genes, respectively,

resulted in observation that the differences in its expression were significant (Figure 9; Figure 10). These results indicate that atrazine may affect microtubule formation and induce DNA damage, which can contribute to the carcinogenic process.

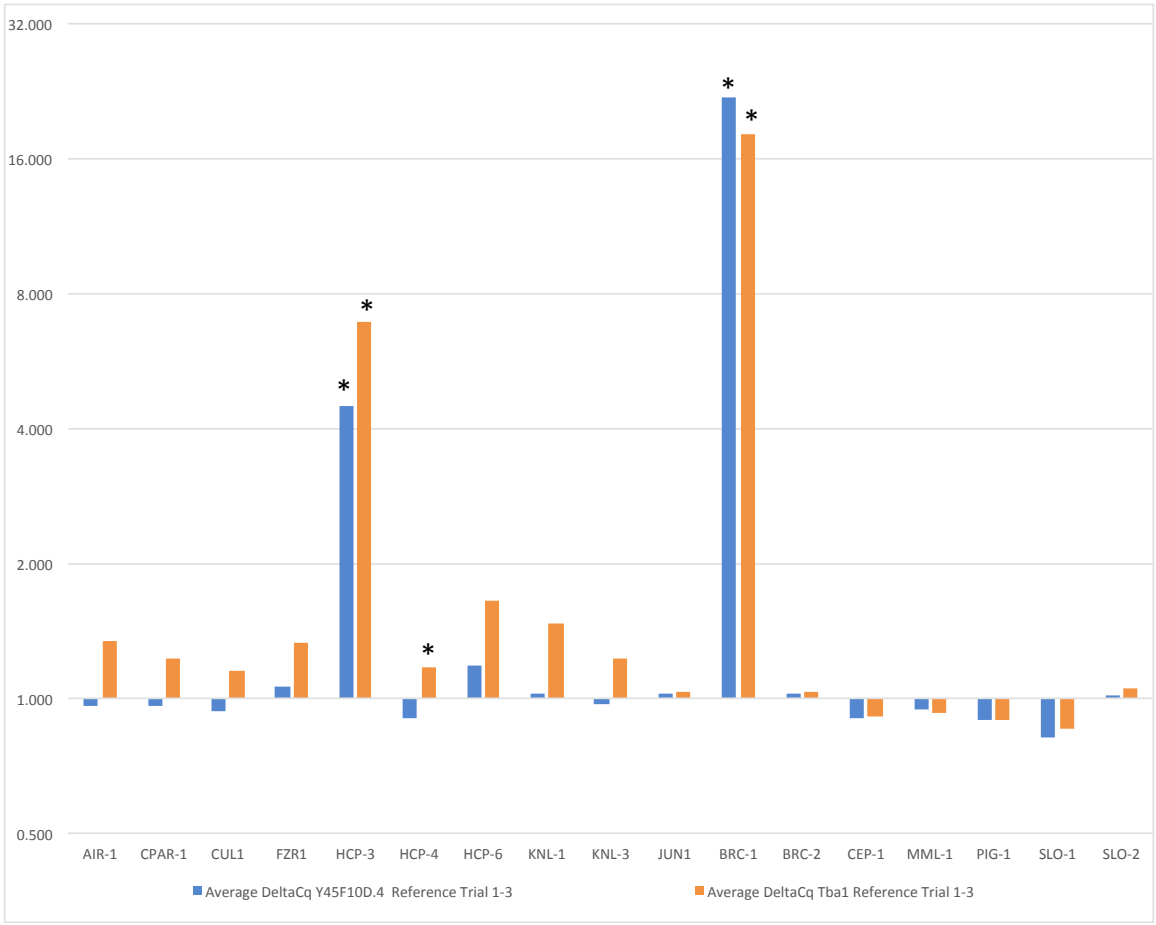

Figure 9: Average Fold Change in Expression Levels of Orthologues of Human Oncogenes and Tumor Suppressor Genes in C.elegans Exposed to LD10 (6.223x10-11 mM) Concentration of Atrazine for 7 Days

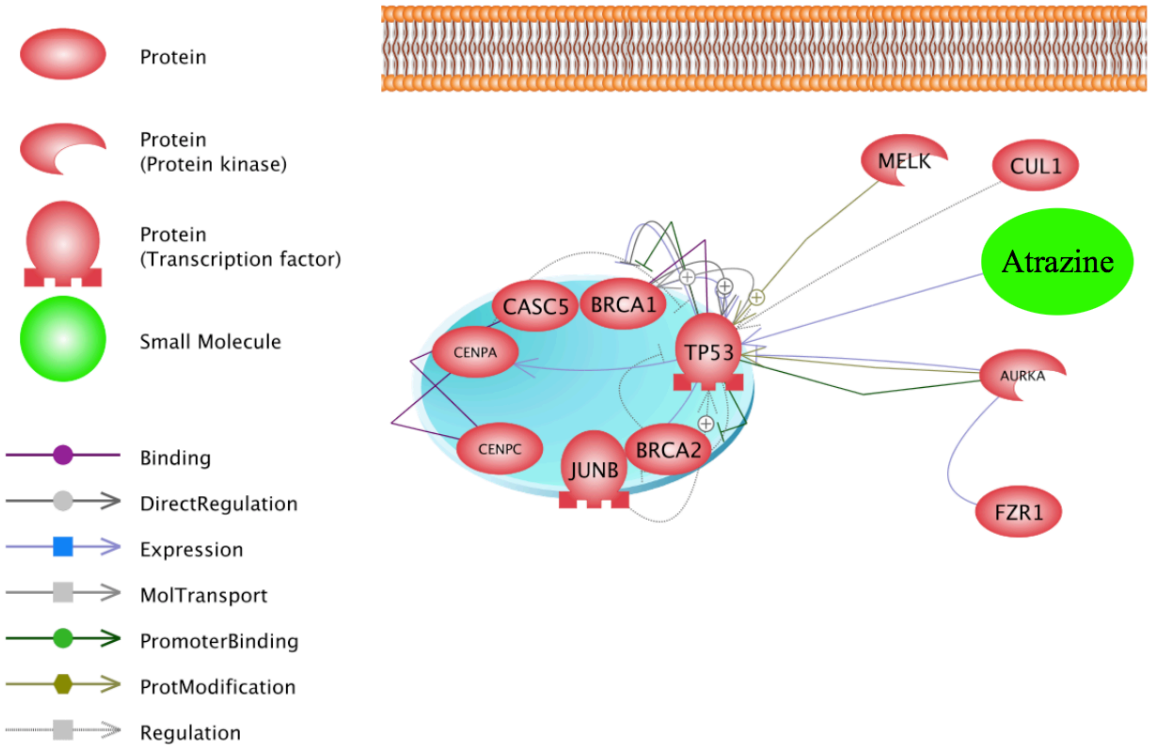

Figure 10: Pathway Studio-guided Visualization of Interactions of Atrazine and Human Orthologues of the Genes that Changed Their expression in *C. elegans* After Chronic Low-Dose Exposure to this Environmental Toxin.

Dichlorodiphenyltrichloroethane (DDT)

C. elegans nematodes were incubated at 3.126×10^{-10} mM concentration for 7 days with DDT, an organochlorine insecticide that slows the closing of Na+ channels in neurons and affects the overall reactivity of the organism to environmental signals. When expression levels of tested mRNAs were normalized to reference gene Tba1, CUL1 ($p<0.002$) and BRC-2 ($p<0.000$) genes displayed significant differences in their expression when exposed nematodes were compared to control animals. When expression levels of tested mRNAs were normalized to reference gene Y45F10D.4, expression levels of JUN1 ($p<0.013$) significantly changed in exposed nematodes as

compared to control animals. When mRNA levels for genes BRC-1 ($p<0.001$; $p<0.000$), a gene involved in DNA repair and MAPK activation, CEP-1 ($p<0.001$; $p<0.000$), a pro-apoptotic gene, and MML-1 ($p<0.000$; $p<0.000$), a pro-autophagy gene, in DDT-exposed nematodes were compared to that in controls, normalization with either Y45F10D.4 or Tba1 reference genes, respectively, resulted in observation that the differences in expression levels of respective genes were significant (Figure 11; Figure 12). These results indicate that DDT may have cause a genetic disruption with a reduction in apoptosis and autophagy.

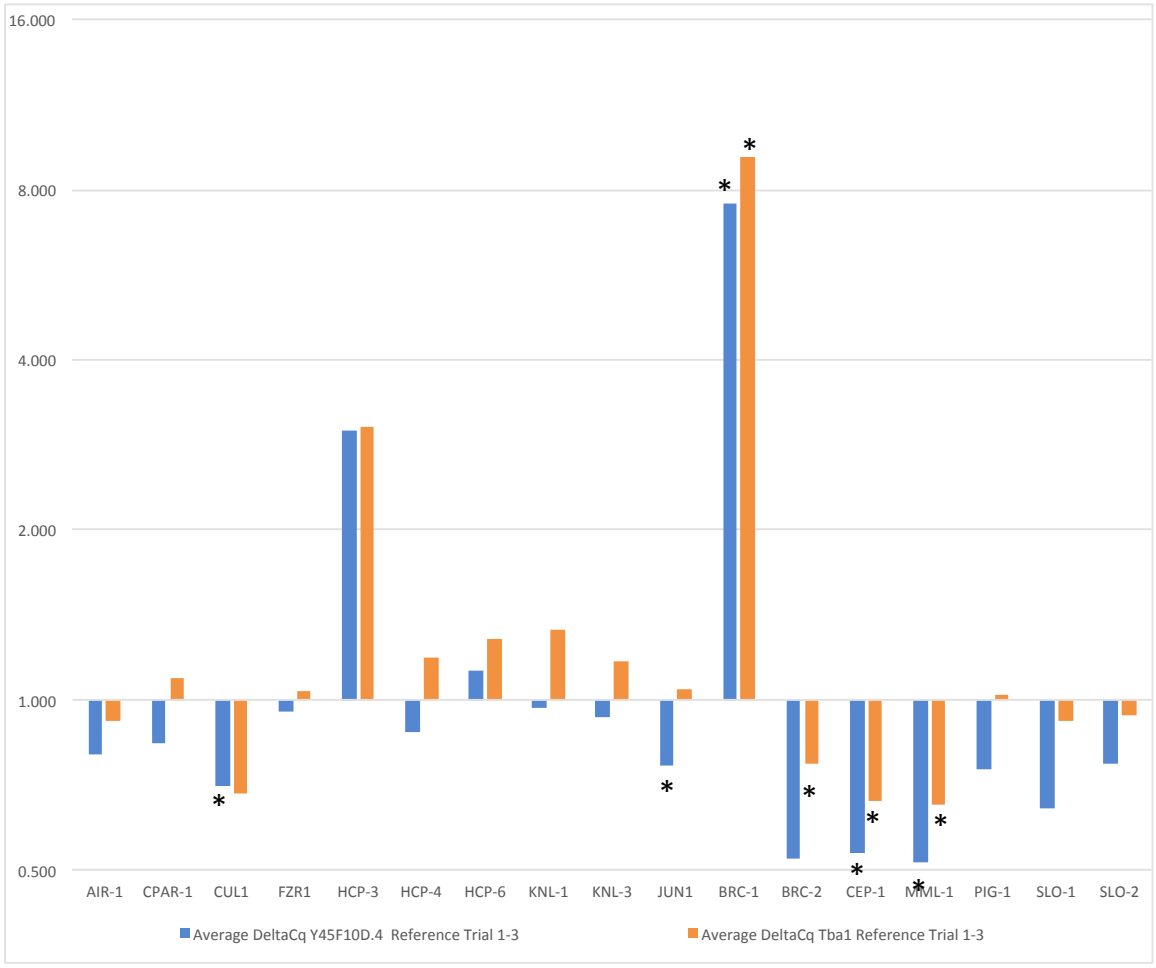

Figure 11: Average Fold Change in Expression Levels of Orthologues of Human Oncogenes and Tumor Suppressor Genes in *C. elegans* Exposed to LD10 (3.126x10-10 mM) Concentration of DDT for 7 Days

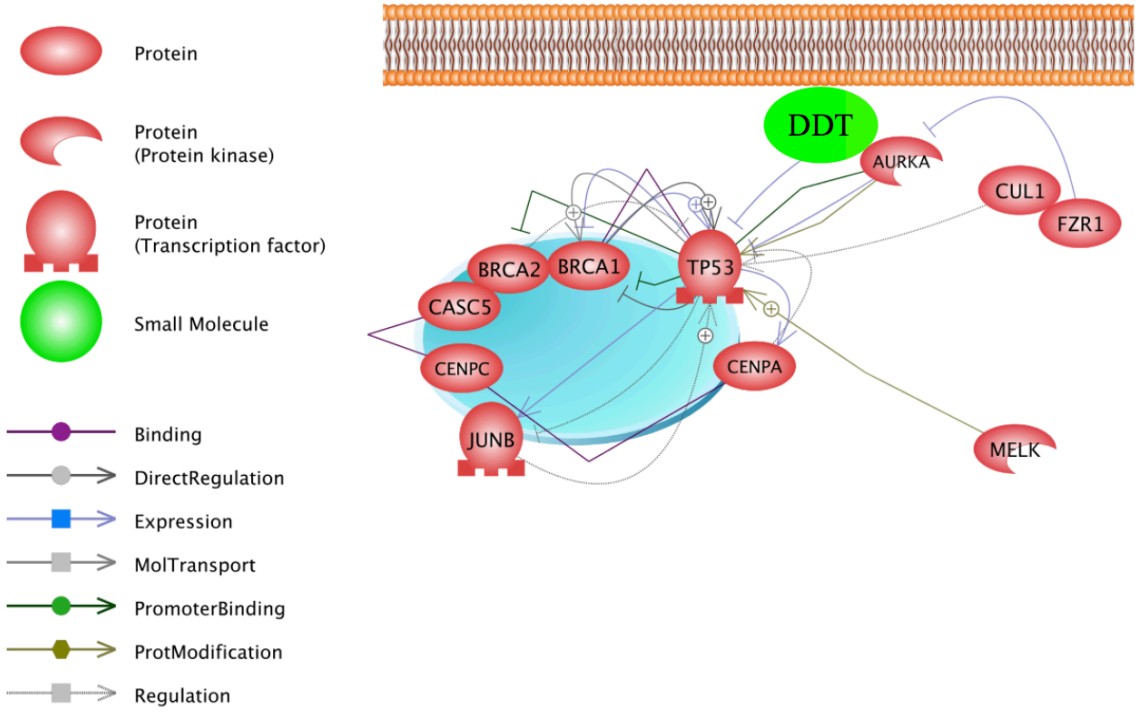

Figure 12: Pathway Studio-guided Visualization of Interactions of DDT and Human Orthologues of the Genes that Changed Their expression in *C. elegans* After Chronic Low-Dose Exposure to this Environmental Toxin.

17 beta-Estradiol

C. elegans nematodes were incubated at 2.547×10^{-10} mM concentration for 7 days with beta-Estradiol, a steroid hormone that binds with an estrogen receptor (ER) in the nucleus and, therefore, affects the expression of a variety of proteins in endocrine glands and other organs. When expression levels of tested mRNAs were normalized to reference gene Tba1, three genes: CPAR-1 ($p<0.000$), CUL1 ($p<0.023$), HCP-4 ($p<0.000$), and KNL-3 ($p<0.004$) displayed significant differences in their expression when exposed nematodes were compared to control animals. When mRNA levels of HCP-3 ($p<0.000$; $p<0.000$), a gene involved in appropriate kinetochore assembly, in beta-Estradiol-exposed nematodes were compared to that in controls, normalization with either Y45F10D.4 or

Tba1 reference genes resulted in observation that the differences in its expression were significant (Figure 13; Figure 14). These results indicate there may be impact on microtubule formation following exposure to beta-Estradiol.

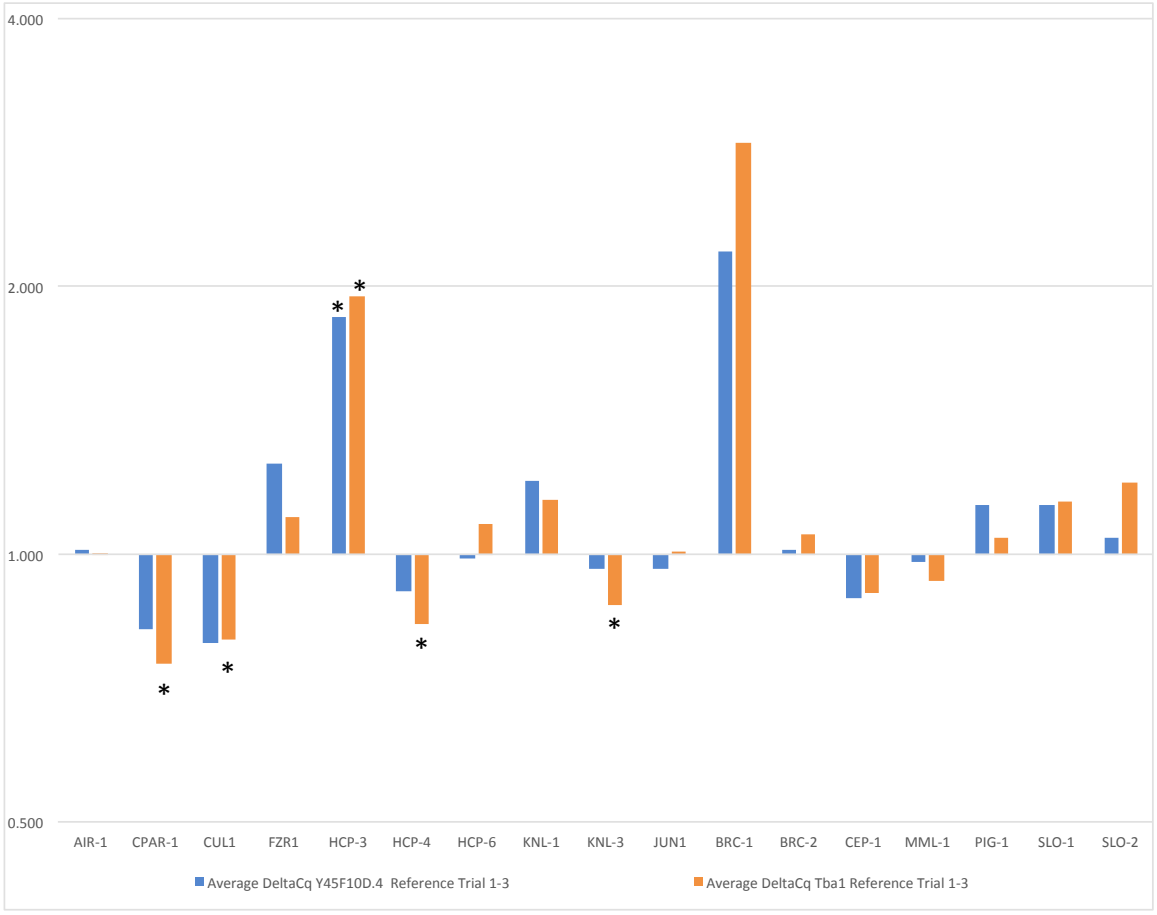

Figure 13: Average Fold Change in Expression Levels of Orthologues of Human Oncogenes and Tumor Suppressor Genes in C.elegans Exposed to LD10 (2.547x10-10 mM) Concentration of b-Estradiol for 7 Days

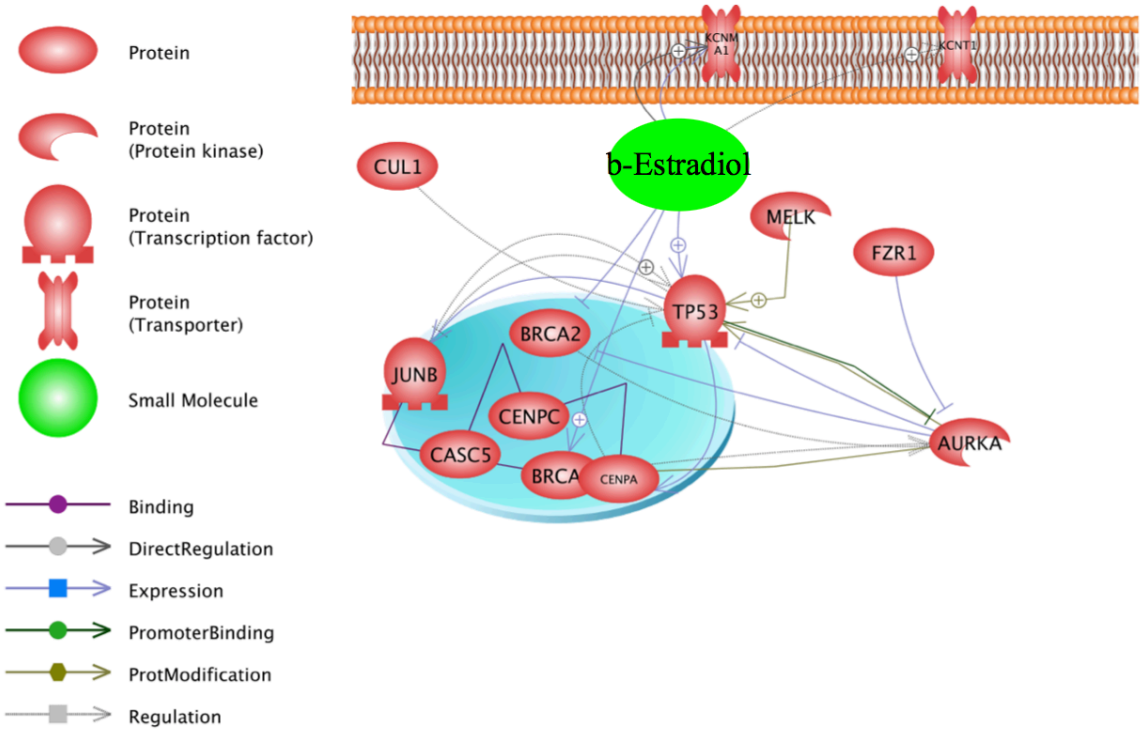

Figure 14: Pathway Studio-guided Visualization of Interactions of b-Estradiol and Human Orthologues of the Genes that Changed Their expression in *C. elegans* After Chronic Low-Dose Exposure to this Environmental Toxin.

Glyphosate (N-phosphonomethyl-glycine)

In our experiments, *C. elegans* nematodes were incubated with glyphosate at 8.492×10^{-6} mM concentration for 7 days. Glyphosate is an herbicide that disrupts the biosynthesis of amino acids and other metabolites from carbohydrate precursors by competitively inhibiting phosphenol pyruvate (PEP), an intermediate of glycolysis, in the biosynthetic shikitimic pathway in plants. When expression levels of tested mRNAs were normalized to reference gene Y45F10D.4, CPAR-1 ($p<0.000$), HCP4 ($p<0.007$), KNL-3 ($p<0.000$), JUN1 ($p<0.000$), and SLO-2 ($p<0.000$) genes displayed significant differences in their expression when exposed nematodes were compared to control

animals. When expression levels of tested mRNAs were normalized to reference gene Tba1, both KNL-1 ($p<0.000$) and SLO-1 ($p<0.000$) genes displayed significant differences in their expression when exposed nematodes were compared to control animals. When CUL1 ($p<0.000$; $p<0.000$), FZR1 ($p<0.001$; $p<0.000$), HCP-3 ($p<0.000$; $p<0.000$), BRC-1 ($p<0.000$; $p<0.000$), BRC-2 ($p<0.000$; $p<0.001$), CEP-1 ($p<0.000$; $p<0.000$), MML-1 ($p<0.000$; $p<0.001$), and PIG-1 ($p<0.000$; $p<0.001$) mRNA levels in glyphosate-exposed nematodes were compared to that in controls, normalization with either Y45F10D.4 or Tba1 reference genes, respectively, resulted in observation that the differences in its expression were significant (Figure 15; Figure 16). HCP-3 is a pro-mitotic gene involved in proper kinetochore assembly and PIG-1 encodes a kinase involved in centromere function. While BRC-1 is involved in halting the cell cycle after DNA damage, BRC-2 works in conjunction to repair damaged DNA. CUL-1 encodes a core component in ligating ubiquitin complexes, while FZR-1 works in concert to limit regulatory molecules that would halt the cell cycle upon increases in ubiquitin complexes. CEP-1 is in apoptosis after DNA damage and MML-1 is involved in controlled autophagy. Collectively, these results indicate glyphosate has a role in contributing to DNA damage, interferes with microtubules, affects the ubiquinylation, and disrupts apoptosis and autophagy actions.

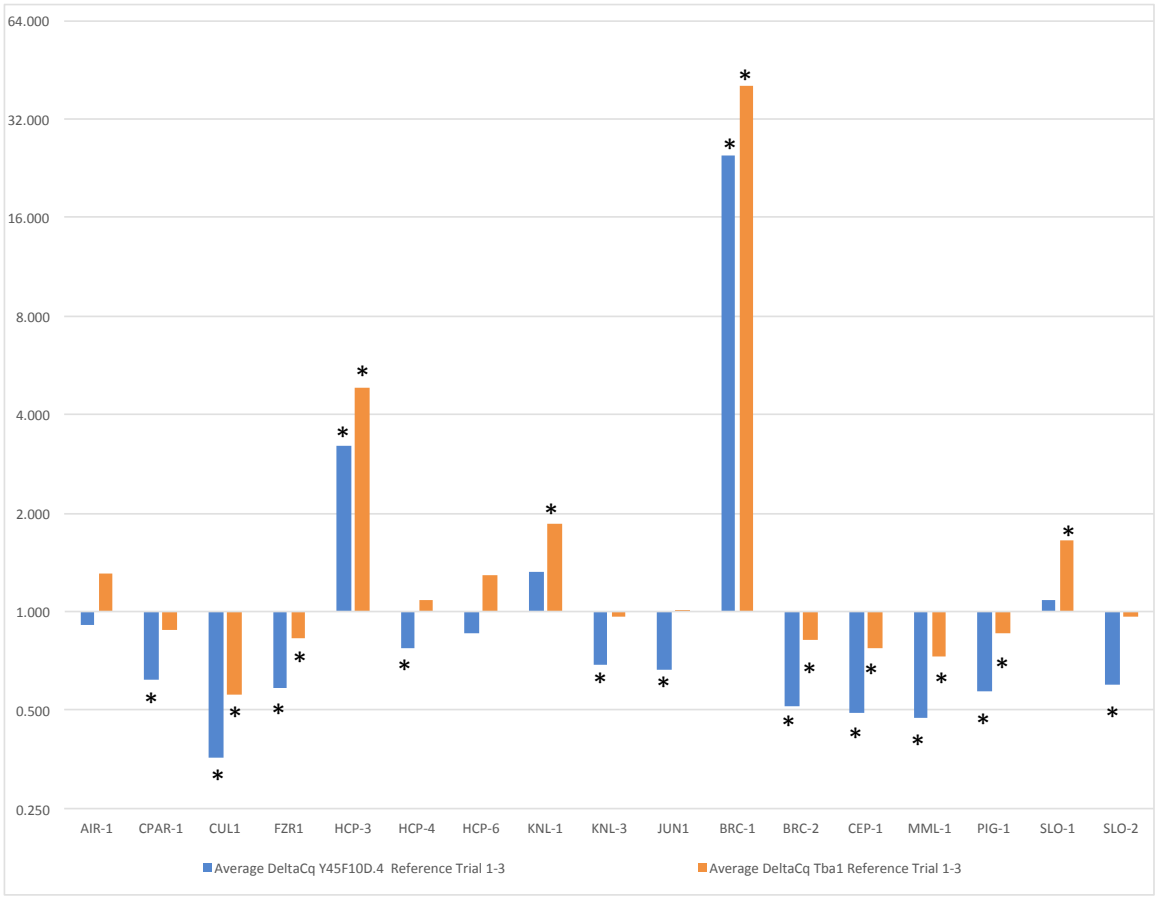

Figure 15: Average Fold Change in Expression Levels of Orthologues of Human Oncogenes and Tumor Suppressor Genes in *C. elegans* Exposed to LD10 (8.492x10-06 mM) Concentration of Glyphosate for 7 Days

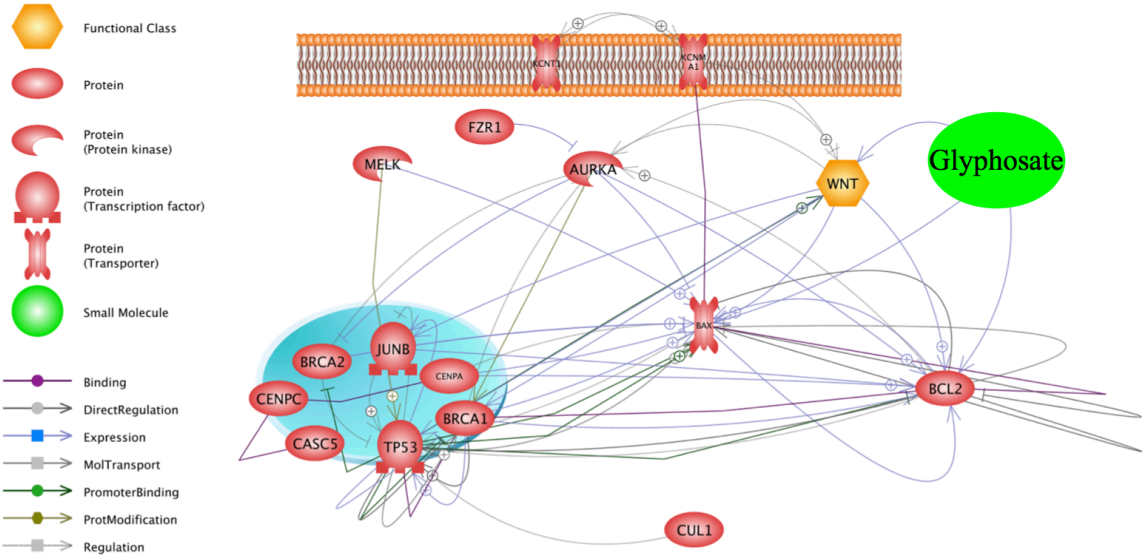

Figure 16: Pathway Studio-guided Visualization of Interactions of Glyphosate and Human Orthologues of the Genes that Changed Their expression in *C. elegans* After Chronic Low-Dose Exposure to this Environmental Toxin.

Dimethylsulfoxide (DMSO)

DMSO was used as a solvent for other toxins in this study and was assessed as a toxin in its own right to establish any confounding effects on the nematodes.

It should be noted that DMSO was used only as the initial solvent for the stock solutions (1mL) of BPA, atrazine, DDT, and beta-Estradiol; further dilutions to obtain the final concentration for each of these toxins was performed in molecular-grade water. Glyphosate was obtained in its pure form, and was dissolved in molecular-grade water directly, rather than in DMSO. The concentration of DMSO included in the BPA and atrazine culture flasks was 1000 times lower than the concentrations tested on *C. elegans* to assess the gene expression changes after exposure. The concentration of DMSO included in the DDT and beta-Estradiol culture flasks was 100 times lower than the concentrations tested on *C. elegans* to assess the gene expression changes after

exposure . Even so, a limitation of this study is that the nematodes may be influenced by synergistic action of the residual amounts of the solvent and the toxin.

In our experiments, *C. elegans* nematodes were incubated at $1.702x10^{-8}$ mM concentration of DMSO for 7 days. When mRNA levels of JUN-1 ($p<0.019$; $p<0.002$), a gene the encodes a transcription factor, in DMSO-exposed nematodes were compared to that in controls, normalization with either Y45F10D.4 or Tba1 reference genes, respectively, resulted in observation that the differences in its expression were significant. When expression levels of tested mRNAs were normalized to reference gene Tba1, AIR-1 ($p<0.006$), CPAR-1 ($p<0.000$), CUL-1 ($p<0.002$), HCP-4 ($p<0.004$), KNL-1 ($p<0.015$), KNL-3 ($p<0.001$), BRC-2 ($p<0.002$), CEP-1 ($p<0.000$), MML-1 ($p<0.003$), PIG-1 ($p<0.006$), and SLO-1 ($p<0.003$) genes displayed significant differences in their expression when exposed nematodes were compared to control animals (Figure 17, Figure 18).

For each of the other toxins, the impacts of DMSO were assessed using Spearman's non-parametric regression analysis, with no significant findings to be reported. Therefore, the DMSO solvent did not have a significant impact on the gene expression levels reported for each of the other toxins for which it was used as a solvent (Table 8). DMSO specific effects includes down-regulation of the expression of JUN-1, which can also impact transcription of the targets downstream of JUN-1 by modifying their own expression.

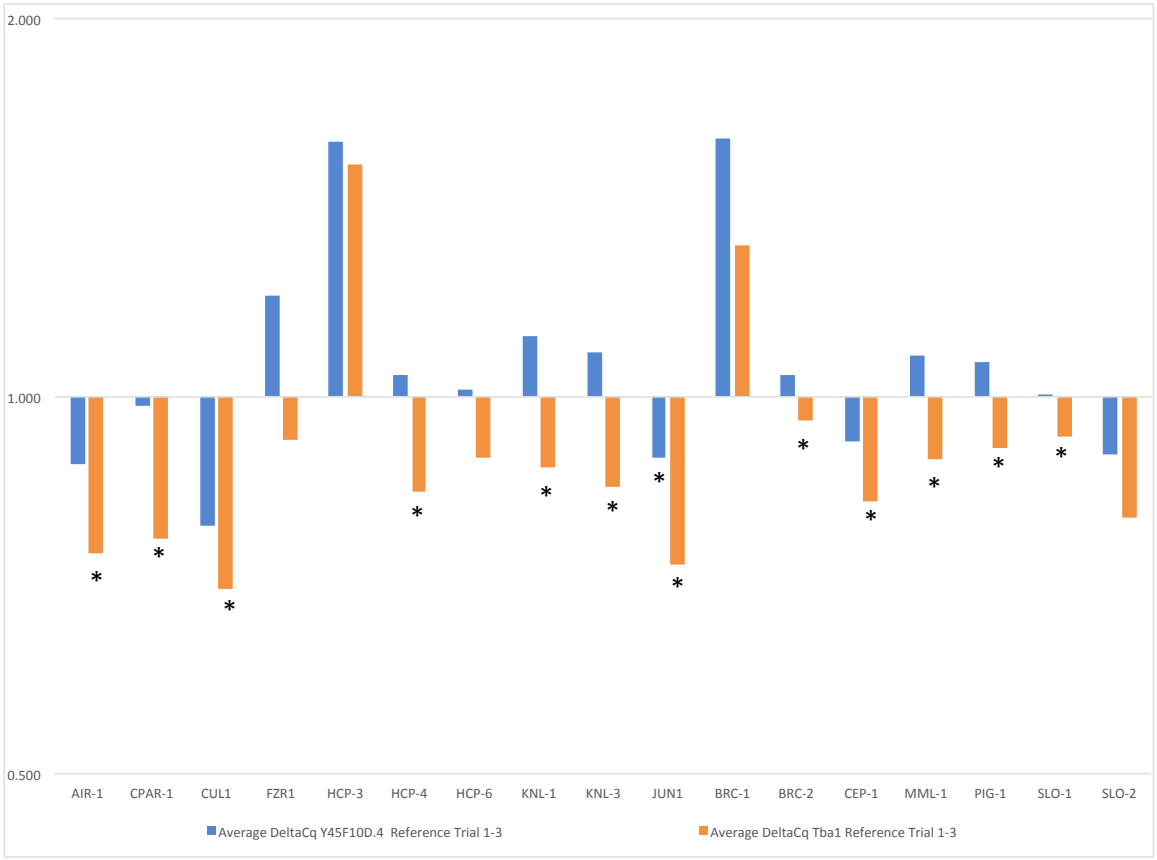

Figure 17: Average Fold Change in Expression Levels of Orthologues of Human Oncogenes and Tumor Suppressor Genes in C.elegans Exposed to LD10 (1.702x10-08 mM) Concentration of DMSO for 7 Days

Table 8: An Assessment on DMSO's Impact on the Gene Expression Changes Produced by Exposure to Each Toxin using Spearman Calculation.

	BPA	Atrazine	DDT	B-Estradiol
DMSO	<0.108	<0.125	<0.502	<0.257

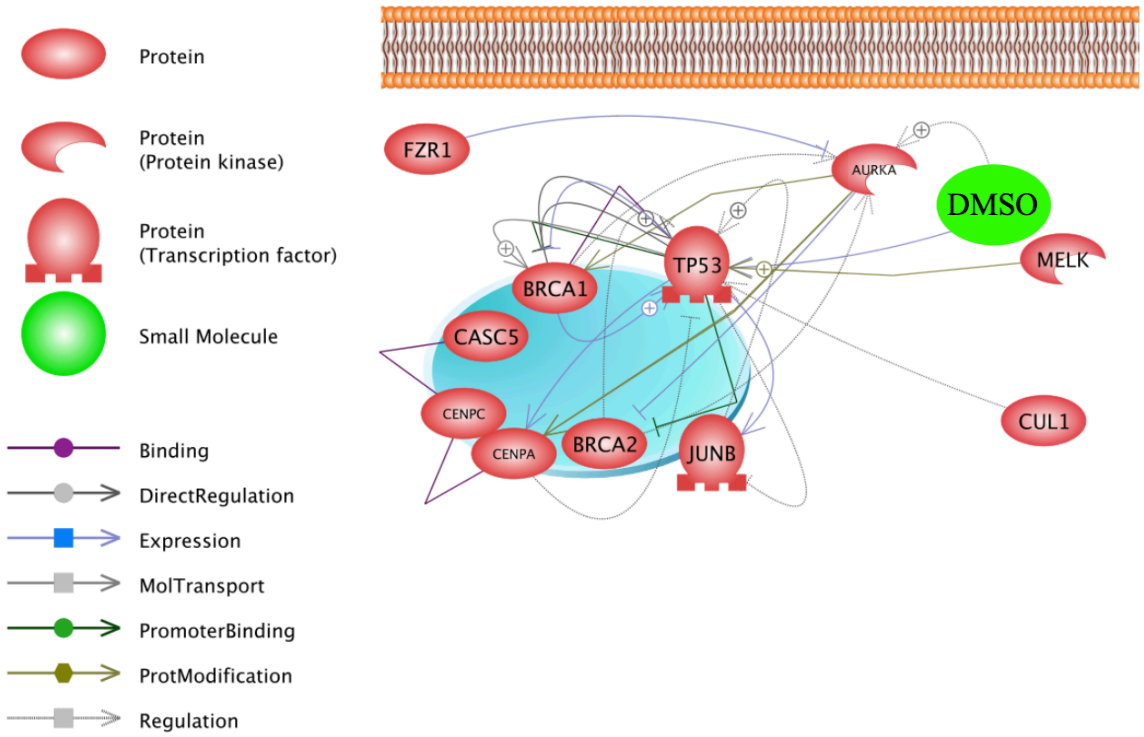

Figure 18: Pathway Studio-guided Visualization of Interactions of DMSO and Human Orthologues of the Genes that Changed Their expression in *C. elegans* After Chronic Low-Dose Exposure to this Environmental Toxin.

CHAPTER FOUR: DISCUSSION

The environment plays a significant role in causing various human cancers. Apart from the well-known "voluntary" contributors of environmental carcinogenesis, for example, use of tobacco products and exposure to PAHs in fried foods, other environmental factors such as herbicides and plasticizers also pose significant risk for cancer.

There are a number of environmental carcinogens with known carcinogenic potential, which may be enhanced or alleviated depending on several variables. The most prominent variable is the set of gene variants present in somatic cells of the particular person exposed to the toxin. Additionally, the developmental timeframe when the exposure occurred, the duration of exposure and the concentration at which the environmental agent was introduced to the organism play significant role. Given these very complex dynamics into account, it is imperative that the changes in signal transduction across intracellular and intercellular pathways and the biochemical events generated from exposure to these agents are elucidated, and their contributions to the carcinogenic process is assessed. As technology expands, an advent of high-throughput screenings, computational biology and knowledge-based algorithms gradually advances the stepwise dissection of these gene-environment interactions.

This study combines the use of *Caenorhabditis elegans*, a well-characterized soil nematode that is becoming a useful model in the assessment of various toxins, and the Pathway Studio, one of the knowledge-based tools of post-genomic biology that facilitates network analysis in humans and model organisms. Pathway Studio (Elsevier, Rockville, MD) is a software which builds networks and pathways from literature- and database-extracted relationships between biological molecules and processes specified by the researcher. As network discovery and analysis gain an importance for thorough understanding of gene signaling and molecular communication in the fields of biomedical and environmental research, it is expected that Pathway Studio and other knowledge-based algorithms would become as common an instrument as the PCR thermocycler or the electrophoresis box in the very near future.

Pathway Studio was instrumental in this study to comprehensively link each selected toxin with its downstream targets. By utilizing the Pathway Studio databases, we visualized potential genomic impacts of each exposure.

In our experiments, toxin-induced changes in gene expression were studied using quantitative reverse transcriptase PCR, which is considered as a mature discovery and validation tool. Importantly, the interpretation of the resulting gene expression values requires the normalization to reliable internal controls, namely, stably expressed genes non-modifiable by exposure. In this study, three previously suggested genes, Y45F10D.4, Tba1 and Pmp3 were used. Hoogewijs et al. (2008) validated several reference gene candidates for quantifying sod gene expression in *C. elegans*, with the most stable pattern from the use of reference genes cdc-42, pmp-3, and Y45F10D.4. Zhang et al. (2012)

further evaluated several candidate reference genes in *C. elegans* for nanotoxicity assessment, and the most reliable expression patterns were found from reference genes tba-1, Y45F10D.4, and pmp-3. Later, Taki and Zhang (2014) assessed a variety of potential reference genes for multigenerational gene expression changes in *C. elegans* after exposure to nicotine, and the most reliable reference genes were shown to be tba-1, cdc-42, eif3.c, arp-6, and Y45F10D.4. After normalizing the Cq output from the qPCR experiments to each reference gene individually, a noticeable systemic down-regulation in the targets normalized to Pmp-3 was present, indicating the reference gene itself experienced a direct gene expression change from the selected toxins. For this reason, Pmp-3 normalized data were excluded from the final analysis in this study.

To consider observed gene expression change impactful, a statistically significant up-regulation or down-regulation in expression after normalization with both reference genes, Y45F10D.4 and Tba1 was considered. As such, the following key expression changes that were statistically significant in this study deserve the most detailed discussion: the down-regulation of CEP-1 in exposure to BPA, the up-regulation of HCP-3 and BRC-1 in exposure to atrazine, the up-regulation of BRC-1 and simultaneous down-regulation of CEP-1 and MML-1 in exposure to DDT, the up-regulation of HCP-3 in exposure to b-Estradiol, the down-regulation of CUL1, FZR1, BRC-2, CEP-1, MML-1, and PIG-1 in exposure to glyphosate, the up-regulation of HCP-3 and BRC-1 in exposure to glyphosate, and the down-regulation of JUN-1 in exposure to DMSO.

Bisphenol A (BPA)

Bisphenol A is a chemical used to make polycarbonate, a common plastic in food packaging; it is also used as a resin to coat the interior of food containers and similar industrial products (USFDA, 2016). Since its adoption for these means, questions regarding its human health impact have been raised since it can affect estrogen receptors, and in 2010, the Food and Drug Administration evaluated the potential impacts of BPA through their own independent evaluations and those conducted by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction under the National Institutes of Health. Murine gestational models were used to orally expose unborn offspring to BPA at 100-1000 times higher levels than is found in human food, and there was no measureable active BPA found 8 hours after exposure (Doerge et al., 2010; Doerge et al., 2011). This may be due to the rapid conversion of active BPA to its inactive form through ingestion exposures, which is even more prominent in humans as to compared to rodents (Fisher et al., 2011). Although testing didn't indicate a required modification for the use of BPA in human products, the FDA amended its regulations to no longer include BPA-derived materials in infant bottles, sippy cups, and formula packaging. However, this was due to social pressure within the industry and subsequent abandonment of use of this chemical for these products rather than safety concerns at the federal level (FDA, 2016).

BPA is associated with a higher presence of reactive oxygen species (ROS) and an increase in c-Myc, which is also linked to these ROS and overall breast cancer development (Pfeifer et al., 2015). Human mammary epithelial cells (HMEC) show an increase in a senescence-related heterochromatin protein-1y, in addition to an increase in

p16 and cyclin E, both also known to increase cellular proliferation and senescence in the cell, and methylating genes related to cancer development, like BRCA1 (Qin et al., 2012). In our study, nematodes exposed to BPA showed significant down-regulation in the mRNA encoded by CEP-1.

CEP-1 is known for its human orthologue p53. p53 protein is a nuclear transcription factor that is normally found in low levels through ubiquitin and proteasome protein degradation (Haupt et al., 1997). p53 can induce the cell cycle to halt at G1 through up-regulation of p21 that binds to Cdk2 (Harper et al., 1993). Further, when DNA is damaged, p53 serves as a transcriptional control agent for apoptotic functions via phosphorylation and acetylation (Vousen and Lu, 2002). As CEP-1 is directly involved in controlled apoptosis, a down-regulation of this gene may induce the uncontrolled apoptotic events. Our studies indicate that exposure to the synthetic estrogen-like BPA results in the reduction of the expression of apoptosis-related genes, which may lead to uninhibited cell growth, resulting in a malignization.

In *C. elegans*, CEP-1 is a tumor suppressor gene, which encodes a transcription factor that can activate egl-1 and ced-13 to induce apoptotic events when DNA damage occurs (Hoffman et al., 2014). Chen et al. (2016) found that both BPA and its newer substitute BPS induce germline apoptosis and embryonic lethality in *C. elegans*, indicating that damage occurs in mitotic and meiotic means. Additionally, the exposure level for embryos may differ from older individuals, thereby requiring further study to determine the potential differential concentrations that may induce the gene expression changes from BPA.

Atrazine

Atrazine is a triazine herbicide, affected the electron transport chain in photosynthetic cells, commonly used to control broadleaf and grass-type weeds that have a negative impact on agricultural like products corn, sorghum, and sugarcane, but it can also be used in residential area and golf courses for aesthetic means (USEPA, 2017b). The Office of Water in the Environmental Protection Agency monitors public water systems on a regular basis with more concentrated efforts in areas where triazine levels are >2.6ppb or regularly high levels of the herbicide have been tracked (USEPA, 2017b). Most of these increases come from agricultural and residential runoff, but health assessments in 2006 revealed that cumulative exposures to atrazine through food and water were innocuous and met the level required in the Food Quality Protection Act (USEPA, 2006). However, this did not include the risk of atrazine to other organisms, and as such, it is still considered a potential hazard to amphibians, birds, and other aquatic organisms (USEPA, 2017b). In our studies, atrazine-exposed nematodes showed a significant increase in the expression of mRNAs encoded by HCP-3 and BRC-1.

HCP-3 is a CENP-A gene that encodes a Histone H3-like centromeric protein that directs kinetochore assembly in mitosis and meiosis (Monen et al., 2005). Atrazine was shown to inhibit the release of lytic granules in Natural Killer cells, a process that likely involves microtubules (Rowe, Brundage, and Barnett, 2007; Pathak and Dikshit, 2011). Yet another study used the analysis of sister chromatid exchanges (SCEs) directly in G2-phase to show that exposure to atrazine causes appearance of prematurely condensed chromosomes in cultured lymphocytes (Malik, Terzoudi, & Pantelias, 2004).

These observations, together with our own findings, indicate that the atrazine may modulate the formation of mitotic spindles, and, possibly, contribute to carcinogenesis.

BRC-1 is an orthologue of tumor suppressor gene BRCA1 that functions in a protein complex that responds to DNA damage signal and induces cell cycle checkpoint activation and DNA repair (Wu, Lu, & Yu, 2010). In addition, it interacts with BRCA2 via Rad51 complexes during in genetic crossover events (Scully, 2000). Atrazine was found to elevate ATM- and RAD- cell cycle checkpoint proteins in response to chemically-induced DNA damage (Huang et al., 2015). The data presented in this study support these previous works. Exposure to atrazine up-regulates BRC-1 expression, possibly, in order to mitigate DNA damage. In this manner, the action of atrazine is similar to that of estrogen, which is known to alter the DNA damage response and DNA repair through the regulation of key effector proteins including ATM, ATR, CHK1, BRCA1, and p53 (Caldon, 2014).

Atrazine has been found to induce the gene expression of CYP, a group of genes involved in lipid synthesis and metabolism, predominantly present to a larger degree in mitochondria (Menzel, Bogaert, & Achazi, 2001). Atrazine exposure to adult male zebrafish resulted in impaired cellular transport and abnormal steroid hormone production (Wirbisky et al., 2016). Along the same lines, in male mice, atrazine exposure interferes with spermatozoa production, interrupts normal meiotic events, enhanced histone associations in areas with high degrees of recombination, and abnormal steroid hormone metabolism (Gely-Pernot et al., 2015). These results indicate atrazine plays a role in lipid-based hormonal control and meiotic mechanisms. Combined with our

results, this chemical should be further investigated for its molecular effects to further elucidate potential mechanisms of carcinogenesis.

Dichlorodiphenyltrichloroethane (DDT)

DDT is an organochlorine insecticide that has been shown to slow the closing of Na+ channels, thereby affecting the ability to generate action potentials in the nervous system. DDT is an extremely effective synthetic chemical that used to combat vector-based diseases including malaria and typhus, but it can be used for agricultural and residential means also (USEPA, 2017c). Within 1-2 decades after its inception, insect populations developed a resistance to this chemical and unintended species were being negatively impacted. Combined with its ability to environmentally-persist and bioaccumulate in fatty tissues, DDT was banned from use in the United States in 1972 when it was classified as a human carcinogen, and only areas where insect-borne diseases are prominent can it still be used (USEPA, 2017c).

In our study, nematodes exposed to DDT resulted in a significant decrease in the expression of mRNAs encoded by CEP-1 and MML-1 and simultaneous significant increase in the expression of mRNAs encoded by BRC-1.

CEP-1, an orthologue known as p53, is a pro-apoptotic tumor suppressor gene and transcription factor that is induced to activate upon DNA damage occurs, while MML-1 is a tumor suppressor gene involved in controlled autophagy. Treatment with DDT promotes phosphorylation of mitogen-activated protein kinase (MAPK), which is involved in regulating both apoptosis and autophagy (Sui et al., 2014; Narayanan et al.,

2015). As such, a down-regulation in both of these gene expressions may cause uncontrolled cell maintenance activities that could lead to malignization. Additionally, BRCA1 has been also functionally linked to apoptosis related gene network that include proto-oncogene BCL-2 and MAPK (Frenaux et al., 2000; Gilmore et al., 2004). The changes in expression of multiple apoptosis and cell-survival related genes observed in DDT-exposed nematodes indicates possible deregulation of these tightly controlled processes, which may contribute to tumorigenesis.

As the genome of *C. elegans* does not possess specific orthologue for MYC, for this study, we selected MML-1, which is part of the transcriptional activation arm of a simplified Myc-like network in this nematode. MML-1 plays a predominant role in cell migration, cooperates with other pro-migratory pathways and is required for longevity of *C. elegans* through participation in the control of autophagy and lysosome biogenesis (Nakamura et al., 2016). CEP-1 and BRC-1 have both been linked to DNA repair upon detected genetic damage in *C. elegans*. Phosphorylation resulting from DDT exposure has the potential to induce abnormal gene expression in the MAPK pathway, and even at low levels of exposure, DDT's biomagnification effect poses a direct harm to cells. As such, exploration of how to mitigate the levels of DDT persisting in the environment warrants further investigation in order to limit the potential harm it poses to organisms. In addition, the molecular mechanisms that it affects can be assessed more specifically using Pathway Studio software and genomic methodology.

beta-Estradiol

The natural mammalian hormone 17 beta-estradiol (E2) is the primary female sex hormone which is essential for the development and maintenance of female reproductive tissues as well as bone, fat, skin, liver, and the brain. While estrogen levels in men are lower compared to those in women, estrogens have essential functions in men, as well. Nevertheless, it is known to induce tumors in various rodent models. In humans, even slightly elevated circulating estrogen levels increase breast or uterine cancer risk (Liehr, 2000). With urine and other biological fluids and solids, the world's human population of about 3.5 billion females discharges approximately 30,000 kg/yr. of natural steroidal estrogens (E1, E2, and E3) and an additional 700 kg/yr. of synthetic beta-estradiol, which comes solely from birth control pill practices. Even larger amounts of estrogens are released into the environment from sheep, cattle and swine industry. Moreover, due to widespread use of livestock manure as agricultural fertilizer environmental estrogens are directionally transferred to human food chain (Adeel et al., 2017). At polluting levels, largest levels of beta-estradiol have been detected at sites close to wastewater treatment facilities and in groundwater. In our study, nematodes exposed to 17 beta-Estradiol resulted in a significant increase in the expression of mRNAs encoded by HCP-3.

The mechanisms for the mutagenic and carcinogenic action of beta-estradiol are multiple. One of the major tumorigenic effects is exerted through formation of the depurinating estrogen-DNA adducts (Cavalieri & Rogan, 2014) and another - through direct stimulation of estrogen receptor alpha (ERα), which pushes cell to proliferate, while actively favoring cells harboring mutations (Yue et al., 2014).

Our findings indicate yet another possible mechanisms for carcinogenic action of beta-estradiol through the gene HCP-3. HCP-3/CENP-A gene encodes a Histone H3-like centromeric protein that directs kinetochore assembly in mitosis and meiosis (Monen et al., 2005). Our data are well-aligned with Aizu-Yokota, Susaki, and Sato (1995), who found that natural estrogen compounds could disrupt microtubule structures. Interestingly, they used hamster V79 cells to determine that beta-Estradiol and other natural estrogen compounds indeed disrupt microtubules, but they perform this action outside of genomic means, indicating a cytoplasmic pathway response after exposure. Further research may illustrate intracellular receptors and pathways affected by these agents to induce the changes observed in gene expression. These data might be used to monitor the changes associated with 17 beta-Estradiol concentrations in the environment so that regulations might be enacted to limit its baseline levels and reduce unintended exposures.

Glyphosate

Glyphosate is an herbicide that disrupts the biosynthesis of amino acids and other metabolites from carbohydrate precursors by competitively inhibiting phosphenol pyruvate (PEP), an intermediate of glycolysis, in the biosynthetic shikitimic pathway in plants and other organisms that utilize this pathway. Glyphosate is the active ingredient in RoundUp and used for both commercial and residential vegetation control (EPA, 2016). Although eye protection and gloves are recommended for application, entry into sprayed areas is allowed after 12 hours of plant exposure (EPA, 2016). The majority (67%) of the total volume of glyphosate applied in the United States has been sprayed in

the last decade, and crops have been genetically-modified to withstand its application in order to generate enough food stock for the global population (Benbrook, 2016). With its predicted continued widespread use, questions regarding its impact on human health have yet to be quantified.

In our experiments, nematodes exposed to glyphosate showed a significant increase in the expression of mRNAs encoded by HCP-3 and BRC-1, while simultaneously showing a significant decrease in the expression of mRNAs encoded by CUL1, FZR1, BRC-2, CEP-1, MML-1, and PIG-1.

HCP-3/CENP-A gene encodes a Histone H3-like centromeric protein that directs kinetochore assembly in mitosis and meiosis (Monen et al., 2005). Glyphosate's mode of action has been shown in *Xenopus laevis* to disassemble the cytoskeleton microtubules and actin filaments using melanophores (Hedberg and Wallin, 2010). When the genotoxic potential of glyphosate was tested in anaphase-telophase Allium test, a significant increase in chromosome aberrations appeared, with the most frequent aberrations observed could be characterized as disturbances of the spindle (Rank et al., 1993). Our findings that glyphosate influence the expression of mitotic spindle protein encoding gene HCP-3 aligns well with these previous studies.

BRC-1 complexes with the tumor suppressor protein p53 have been reported to halt the cell cycle during times of cellular damage through the cell checkpoint modifications (Ouchi et al., 1998; Adamo et al., 2008s). Marc et al. (2004) determined that 10mM of glyphosate inhibited Tyr15 dephosporylation of CDK1/cyclin B in the cell cycle, resulting in halting the cell cycle at the G2/M checkpoint in sea urchins. The data

presented in this study align with these reported data in that expression of known checkpoint gene BRC-1 was up-regulated after exposure to glyphosate, possibly as the cell attempts to mitigate the DNA damage by stalling the cell cycle. Theses data indicate that the environmental exposure to glyphosate may lead to DNA damage and an induction of DNA repair.

Orthologues of proto-oncogenes CUL1, FZR1, PIG-1 were down-regulated in nematodes exposed to glyphosate, along with the orthologue of tumor suppressor gene BRC-2. PIG-1 encodes a kinase that interacts with the centrosome during cell divisions and promotes proper differentiated cell divisions (Chien et al., 2013). BRCA2 interacts with Rad51 in genetic crossover events (Scully, 2000). CUL-1 is a prototypic member and a core component of ligase complexes for proteins requiring ubiquitination. Cullin (CUL)-RING E3 ubiquitin ligases (CRLs) regulate diverse biological processes involved in cancer cell survival by conferring substrate selectivity for ubiquitination and degradation (Tan et al., 1999; Shafique et al., 2017). FZR-1 encodes an adaptor for APC/C ubiquitin-protein ligase complex that promotes anaphase and telophase, working to prevent accumulation of mitotic regulators, like cyclins and cyclin-dependent kinases, which would otherwise halt the cell cycle prematurely (Fang, Yu, and Kirschner, 1998). It is also involved in the G2 checkpoint, preventing mitotic entry to when damaged DNA is present (Lafranchi et al., 2014). In short, CUL1 produces a ligase for ubiquinylating compounds to aid in the cell cycle, and FZR1 generates an adaptor protein for the ubiquinylation process to promote completion of the cell cycle through prevention of cyclin and CDK buildup, which would otherwise halt the cell cycle; this protein also

works at the G2/M checkpoint to prevent transition into the cell cycle with damage DNA. Even at LC10, treatment with glyphosate showed most prominent changes in nematode gene expression as compared to other tested environmental toxins. It is possible that the protein products of the genes with dramatically increased expression level, like BRCA1 and HCP-3, drive subsequent changes in expression of other genes participating in same set of cellular processes linked to DNA damage-activated checkpoints.

Kostka et al. (2014) showed that glyphosate induced a methylated state and down-regulation of p53 in rat liver, and this was further supported by Kwiatkowska et al. (2017) via induction of p53 methylation caused by the treatment of peripheral blood mononuclear cells with glyphosate at 0.25-0.5mM. Nematode orthologue for tumor suppressor genes TP53 (CEP-1), involved in apoptosis after DNA damage, and MML-1, involved in controlled autophagy, were down-regulated in worms exposed to glyphosate. Although *C. elegans* does not possess a specific orthologue for MYC, as a part of the MYC family, MML-1 is key component of a transcription factor network required for longevity of *C. elegans* through germline removal via regulation of HLH-30/TFEB, which controls autophagy and lysosome biogenesis (Nakamura et al., 2016). As glyphosate has the ability to disrupt mitotic spindles and reduce the presence of cell cycle regulators, and as both CEP-1 and MML-1 are directly involved in control of apoptosis, an exposure to glyphosate is likely to disrupt apoptotic control and, therefore, lead to uninhibited cell growth.

Glyphosate use has expanded over the past few decades, and it is readily available at any home improvement store. Even though it doesn't seem to accumulate in lipid like

DDT, its increased level in the environment and the multitude of gene expression changes it induces it alarming. Given these data, it is imperative that the molecular mechanisms of exposure to this compound be further investigated in order to understand the harm it may be causing to an uninformed population.

Dimethylsulfoxide (DMSO)

DMSO, or dimethyl sulfoxide, is a by-product of paper making, which has been used as an industrial solvent since the mid-1800s. Interestingly, its use as an anti-inflammatory agent has been explored, with the FDA approval of DMSO as a prescription medication for treating symptoms of painful bladder syndrome. As DMSO is easily absorbed by the skin, it's sometimes used to increase the body's absorption of other medications and to treat several other conditions, including shingles and arthritis-related chronic pain. In our experiments, in nematodes exposed to DMSO, a down-regulation of mRNA encoded by nematode orthologue of JUN1 was noted.

JUN is a part of the AP1 network of transcription factors that orchestrate a number of important expression programs both under normal circumstances and during the course of tumor development and progression. Human gene JUN was originally identified as the normal cellular orthologue of the avian sarcoma (ASV17) viral Jun oncoprotein (v-jun) (Maki et al., 1987). Now it is known that human genome contains three paralogues of the Jun family: JUN, JUNB and JUND, and each protein has distinct characteristics. In particular, human JUN is a proto-oncogene, which plays a role in cell proliferation, survival and apoptosis, and is often amplified aggressive, undifferentiated human sarcomas where it enhances invasiveness (Sioletic et al., 2014). Specifically, in

breast carcinomas c-Jun is predominantly expressed at the invasive front and is associated with proliferation and angiogenesis (Vieugel et al., 2006). Moreover, in the most aggressive triple-negative breast cancer associated with poor clinical outcome c-Jun-regulated pro-invasion gene program is expressed at a very high level. Using ChIP-seq, Quao I e al. demonstrated that transcription factor JUN binds to 13,800 sites within a large number of the promoter regions, while regulating nearly a third of the TNFα-regulated transcriptome (Qiao et al., 2016).

Our study indicates that anti-inflammatory properties of DMSO may be related to down-regulating of JUN and subsequent suppression of TNF-alpha related inflammatory responses. According to our observations, exposure to DMSO is unlikely to contribute to human cancer morbidity.

However, in *C. elegans*, the action of DMSO may be disruptive. In particular, nematode JUN-1 gene is involved in inhibiting the dilation of distal spermathecal cells, which is required for fertilization (Hiatt et al., 2009). JUN-1 also enhances the partial functionality loss of nhr-6 to allow for proper spermathecal organ development through the growth of a normal number of cells (Gissendanner et al., 2013). Even more importantly, JUN-1 protein of *C. elegans* is involved in nematode life-span extension by intermittent fasting and is activated in response to fasting (Uno et al., 2013). It is, therefore, possible that DMSO-induced suppression of the transcription of JUN1 may limit the fertility of nematodes. Importantly, both core components of AP1, JUN and FOS exert a regulatory role in human steroidogenesis and spermatogenesis (Shalini & Bansal, 2005). In particulars, members of AP-1 networks define optimal expression of connexin-

43 and nectin-2 encoding genes a the Sertoli and Leydig cells' interphase in the mammalian testis (Ghouili & Martin, 2017; Lui, Sze, & Lee, 2006). Given that, the findings that DMSO suppresses nematode orthologue of human JUN are worrisome and warrants further investigations.

Potential Aims for Further Study

To expand the analysis of environmental toxins presented in this study, the set of toxins analyzed in qRT-PCR assays should be expanded to include all the molecules pre-screened in the 24-hour exposure study (Calcium hypochlorite, butyl benzyl phthalate and its metabolite mono-n-butyl phthalate and Diethyl phthalate, Pyrene, Anthracene, Acenapthene) in addition to other plausible toxins and varying concentrations of mixtures of these agents. The latter will serve to better replicate the natural environmental conditions that are faced on a regular basis in our society. This larger set of toxins should be assessed in assays profiling larger number of oncogene and tumor suppressor gene orthologues and their variant gene forms in high-throughput mode via RNAseq or microarray methodology rather than qPCR.

In parallel, the concentration of each toxin introduced should be incrementally increased in dilution experiments covering more concentrations (LC10, LC20, LC30, LC40, LC50), thus, leading to uncovering possible dose-dependent effects. Lastly, studying gene expression over incrementally increasing time frames of exposure (3 days, 6 days, 9 days, 12 days) over the lifetime of the nematodes might provide better quantitative estimates of the genetic and phenotypic effects.

Another interesting avenue would be to repeat the same experimental design in various mutant strains of *C. elegans* to determine what effect these already introduced modifications would have on the gene expression and phenotypic effects of toxic exposures. The latter experiments may provide a window into understanding differential effects of toxic exposures observed in genetically heterogeneous human populations.

APPENDIX

Table 9: Nematode Mortality Rates in 24-Hour Exposure Experiments.

	1 Mortality Rate						
BPA	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	1.0000	1.0000	0.6000	0.6429	0.2308	0.5714
	B	0.8889	1.0000	0.6000	0.4167	0.2500	0.1667
	C	1.0000	1.0000	0.8571	0.8000	0.4000	0.2143
	D	1.0000	1.0000	0.8462	0.8000	0.7500	0.5714
	Average	0.9722	1.0000	0.7258	0.6649	0.4077	0.3810

	2 Mortality Rate						
Atrazine	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	1.0000	0.9286	0.6000	0.4706	0.4167	0.2857
	B	1.0000	0.7857	0.6364	0.3846	0.3636	0.4444
	C	0.7500	0.9500	0.7273	0.6000	0.5500	0.4615
	D	1.0000	0.9167	0.7500	0.6667	0.6000	0.4286
	Average	0.9375	0.8952	0.6784	0.5305	0.4826	0.4051

	3 Mortality Rate						
Calcium	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
Hypochlorite	A	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	B	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	C	1.0000	1.0000	1.0000	0.9375	0.9286	1.0000
	D	1.0000	1.0000	1.0000	1.0000	1.0000	0.7500
	Average	1.0000	1.0000	1.0000	0.9844	0.9821	0.9375

	4 Mortality Rate						
DDT	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.8571	0.7500	0.8000	0.4000	0.3333	0.4000
	B	1.0000	0.6923	0.6667	0.6667	0.4444	0.5000
	C	0.7500	0.7333	0.5714	0.7000	0.3571	0.3333
	D	1.0000	0.9000	0.7619	0.7333	0.3889	0.2857
	Average	0.9018	0.7689	0.7000	0.6250	0.3810	0.3798

	5 Mortality Rate						
MBP	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.7857	0.7692	0.5500	0.5833	0.2667	0.2059
	B	0.8571	0.6923	0.5714	0.7308	0.3571	0.1429
	C	0.8636	0.8824	0.4000	0.6842	0.3750	0.2381
	D	0.8889	0.9000	0.6000	0.4444	0.5556	0.2500
	Average	0.8488	0.8110	0.5304	0.6107	0.3886	0.2092

	6 Mortality Rate						
Pyrene	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.9167	0.8333	0.6364	0.3333	0.3000	0.3571
	B	1.0000	0.6923	0.8571	0.3750	0.2778	0.2174
	C	0.8824	0.9000	0.6923	0.2941	0.2143	0.2667
	D	1.0000	0.8000	0.7059	0.5385	0.7778	0.4444
	Average	0.9498	0.8064	0.7229	0.3852	0.3925	0.3214

	7 Mortality Rate						
Anthracene	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.8889	0.8182	0.6667	0.4167	0.4000	0.3333
	B	0.9091	0.8889	0.5882	0.2308	0.2857	0.4667
	C	0.7222	0.7778	0.7500	0.3000	0.4000	0.3333
	D	0.9231	0.8571	0.7059	0.6471	0.6250	0.2500
	Average	0.8608	0.8355	0.6777	0.3986	0.4277	0.3458

	8 Mortality Rate						
Acenapthene	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.8571	0.7143	0.7500	0.4000	0.8462	0.6250
	B	0.9231	0.6875	0.8000	0.2727	0.6667	0.4286
	C	0.8889	0.8000	0.6667	0.4545	0.5714	0.5200
	D	0.8750	0.7778	0.5714	0.5455	0.3750	0.3158
	Average	0.8860	0.7449	0.6970	0.4182	0.6148	0.4723

	9 Mortality Rate						
Estradiol	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.8750	0.8500	0.5714	0.2143	0.6000	0.3333
	B	0.9091	0.7143	0.7500	0.4286	0.3125	0.6250
	C	0.9231	0.5833	0.8182	0.5833	0.4167	0.8333
	D	0.8947	0.8889	0.7333	0.6364	0.6667	1.0000
	Average	0.9005	0.7591	0.7182	0.4656	0.4990	0.6979

	10 Mortality Rate						
DEP	(mM)	10.0000	1.0000	0.1000	0.0100	0.0010	0.0001
	A	0.5217	0.6957	0.5484	0.8125	0.4583	0.4375
	B	0.6429	0.4706	0.6154	0.4167	0.2941	0.3500
	C	0.7407	0.5714	0.3500	0.1111	0.2500	0.1702
	D	0.5200	0.3333	0.2766	0.2857	0.2692	0.5500
	Average	0.6063	0.5178	0.4476	0.4065	0.3179	0.3769

	11 Mortality Rate						
BBP	(mM)	10.0000	1.0000	0.1000	0.0100	0.0010	0.0001
	A	0.1579	0.2903	0.4063	0.1304	0.2083	0.2759
	B	0.3333	0.2292	0.3103	0.4783	0.2857	0.1429
	C	0.2059	0.1591	0.4333	0.3810	0.3056	0.4333
	D	0.3750	0.2439	0.2059	0.3077	0.4828	0.0625
	Average	0.2680	0.2306	0.3390	0.3243	0.3206	0.2286

	12 Mortality Rate						
DMSO	(mM)	10.0000	1.0000	0.1000	0.0100	0.0010	0.0001
	A	0.4444	0.6410	0.3333	0.6207	0.5909	0.5333
	B	0.4412	0.4595	0.2759	0.5625	0.5417	0.2647
	C	0.3704	0.4848	0.5429	0.5714	0.3704	0.3750
	D	0.3438	0.2424	0.6857	0.5517	0.4231	0.2571
	Average	0.3999	0.4569	0.4594	0.5766	0.4815	0.3575

	13 Mortality Rate						
Water	Well	1	2	3	4	5	6
	A	0.3881	0.3333	0.2143	0.2273	0.1429	0.2727
	B	0.5676	0.3158	0.3158	0.1250	0.1667	0.1111
	C						
	D						
	Average	0.4778	0.3246	0.2650	0.1761	0.1548	0.1919

	14 Mortality Rate						
Glyphosate	(mM)	25.00000	2.5000	0.2500	0.0250	0.0025	0.00025
in RoundUp	A	0.2800	0.5294	0.3333	0.2857	0.0725	0.2239
	B	0.2778	0.2258	0.2292	0.1212	0.1081	0.1458
	C	0.4643	0.3333	0.1556	0.5500	0.1186	0.2326
	D	0.4375	0.2703	0.4706	0.2333	0.1702	0.2500
	Average	0.3649	0.3397	0.2972	0.2976	0.1174	0.2131

Table 10: The Results of XTT Assays Performed in E. coli OP50 Cultures Exposed to Toxins for 20 Minutes. For each Concentration of the Toxin, the Mortality Rates are Shown.

1	Mortality Rate						
BPA	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.833	0.806	0.865	0.786	0.727	0.223
	B	0.871	0.835	0.804	0.838	0.811	0.225
	C	0.913	0.880	0.855	0.856	0.869	0.153
	D	0.929	0.864	0.888	0.857	0.815	0.147
	Average	**0.886**	**0.846**	**0.853**	**0.834**	**0.805**	**0.187**

2	Mortality Rate						
Atrazine	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.833	0.822	0.800	0.724	0.663	0.499
	B	0.822	0.860	0.731	0.853	0.691	0.531
	C	0.825	0.877	0.791	0.786	0.697	0.580
	D	0.787	0.868	0.853	0.825	0.714	0.515
	Average	**0.817**	**0.857**	**0.794**	**0.797**	**0.691**	**0.531**

3	Mortality Rate						
Calcium Hypochlorite	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.532	0.494	0.475	0.334	0.129	0.172
	B	0.500	0.484	0.414	0.306	0.128	0.152
	C	0.516	0.450	0.435	0.312	0.131	0.166
	D	0.491	0.468	0.397	0.297	0.092	0.170
	Average	**0.510**	**0.474**	**0.431**	**0.313**	**0.120**	**0.165**

4	Mortality Rate						
DDT	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.923	0.890	0.900	0.928	0.965	0.865
	B	0.704	0.919	0.845	0.937	0.901	0.729
	C	0.124	0.792	0.769	0.750	0.781	0.547
	D	0.534	0.584	0.636	0.642	0.647	0.535
	Average	**0.571**	**0.796**	**0.787**	**0.814**	**0.823**	**0.669**

5	Mortality Rate						
MBP	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.745	0.952	0.876	0.960	0.776	0.082
	B	0.739	0.905	0.829	0.981	0.764	0.078
	C	0.784	0.839	0.830	0.837	0.762	0.089
	D	0.804	0.826	0.844	0.823	0.722	0.085
	Average	**0.768**	**0.881**	**0.845**	**0.901**	**0.756**	**0.084**

6	Mortality Rate						
Pyrene	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.735	0.904	0.764	0.766	0.775	0.662
	B	0.770	0.960	0.593	0.765	0.768	0.635
	C	0.780	0.938	0.734	0.753	0.855	0.769
	D	0.771	0.926	0.698	0.760	0.934	0.674
	Average	**0.764**	**0.932**	**0.698**	**0.761**	**0.833**	**0.685**

7	Mortality Rate						
Anthracene	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.831	0.863	0.822	0.804	0.791	0.770
	B	0.872	0.797	0.771	0.786	0.779	0.595
	C	0.944	0.556	0.881	0.616	0.616	0.368
	D	0.651	0.677	0.669	0.654	0.614	0.366
	Average	**0.825**	**0.724**	**0.786**	**0.715**	**0.700**	**0.525**

8	Mortality Rate						
Acenapthene	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.799	0.829	0.663	0.715	0.797	0.596
	B	0.874	0.767	0.567	0.727	0.633	0.644
	C	0.818	0.769	0.631	0.712	0.776	0.538
	D	0.817	0.767	0.704	0.763	0.843	0.670
	Average	**0.827**	**0.783**	**0.641**	**0.729**	**0.762**	**0.612**

9	Mortality Rate						
Estradiol	(mM)	50.0000	5.0000	0.5000	0.0500	0.0050	0.0005
	A	0.697	0.792	0.705	0.750	0.778	0.681
	B	0.771	0.776	0.685	0.773	0.774	0.735
	C	0.756	0.782	0.756	0.820	0.792	0.660
	D	0.720	0.803	0.899	0.900	0.917	0.802
	Average	**0.736**	**0.788**	**0.761**	**0.811**	**0.815**	**0.719**

10	Mortality Rate						
DEP	(mM)	10.0000	1.0000	0.1000	0.0100	0.0010	0.0001
	A	0.840	0.784	0.665	0.704	0.494	0.227
	B	0.741	0.737	0.771	0.714	0.600	0.159
	C	0.590	0.620	0.561	0.543	0.465	0.146
	D	0.600	0.589	0.511	0.540	0.492	0.126
	Average	**0.693**	**0.682**	**0.627**	**0.625**	**0.513**	**0.164**

11	Mortality Rate						
BBP	(mM)	10.0000	1.0000	0.1000	0.0100	0.0010	0.0001
	A	0.737	0.714	0.728	0.674	0.758	0.695
	B	0.701	0.716	0.765	0.690	0.834	0.682
	C	0.753	0.722	0.736	0.731	0.761	0.621
	D	0.739	0.760	0.824	0.686	0.853	0.766
	Average	**0.732**	**0.728**	**0.763**	**0.695**	**0.801**	**0.691**

12	Mortality Rate						
DMSO	(mM)	10.0000	1.0000	0.1000	0.0100	0.0010	0.0001
	A	0.690	0.663	0.653	0.767	0.640	0.608
	B	0.673	0.712	0.737	0.762	0.717	0.645
	C	0.796	0.703	0.684	0.801	0.733	0.594
	D	0.712	0.670	0.744	0.854	0.766	0.599
	Average	**0.717**	**0.687**	**0.704**	**0.796**	**0.714**	**0.611**

13	Mortality Rate						
Water	Well	1					
	A	0.400					
	B	0.412					
	C	0.367					
	D	0.362					
	Average	**0.386**					

14	Mortality Rate						
Glyphosate in RoundUp	(mM)	25.00000	2.5000	0.2500	0.0250	0.0025	0.00025
	A	0.532	0.494	0.475	0.334	0.129	0.172
	B	0.500	0.484	0.414	0.306	0.128	0.152
	C	0.516	0.450	0.435	0.312	0.131	0.166
	D	0.491	0.468	0.397	0.297	0.092	0.170
	Average	**0.510**	**0.474**	**0.431**	**0.313**	**0.120**	**0.165**

Table 11: Average Fold Change In Expression Levels Per Each Candidate Gene Profiled. Blue Cells Highlight Orthologues of The Tumor Suppressor Genes, While Green Highlights Orthologues of Oncogenes.

Toxin	AR1	CPAR1	CUL1	FZR	HCP3	HCP4	HCP6	KNL1	KNL3	JUN1	BRCA1	BRCA2	CEP1	MML1	PIG1	SLO1	SLO2
BPA	.050	.309	.457	.128	.568	.240	.201	.087	.178	.490	.394	.161	.408	.184	.309	.310	.046
Atrazine	.174	.219	.417	.103	.275	1.233	0.940	.072	1.153	1.152	0.125	1.120	1.283	1.285	1.302	1.375	1.162
DDT	.340	.194	.742	.189	.719	.113	.996	.028	.065	.341	.171	.981	.454	.329	.362	.555	.100
b-Estradiol	.068	.376	.718	.952	.579	.234	.079	.917	.168	.132	.676	.081	.268	.273	.096	.012	.021
Glyphosate	.036	.527	.451	.591	.299	.207	.081	.716	.363	.410	.061	.767	.907	.946	.650	.872	.566
DMSO	1.336	1.201	1.954	0.993	.767	.096	.108	.068	.078	.442	.061	.105	.297	.238	.098	.121	.305

Table 12: The Outputs of the Expression Level Comparisons for Gene JUN1, an Orthologue to the Human Tumor Suppressor Gene JUNB After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.743	0.087	15.000	<0.016	0.927	0.092	15	NS	0.680	0.089	15	<0.019
Atrazine	6.223E-09	1.022	0.090	15.000	NS	1.031	0.065	15	NS	0.751	0.068	15	<0.000
DDT	3.126E-08	0.766	0.095	15.000	<0.013	1.043	0.050	15	NS	0.635	0.041	15	<0.000
b-Estradiol	2.547E-08	0.962	0.083	15.000	NS	1.005	0.060	15	NS	0.806	0.050	15	<0.004
Glyphosate	8.492E-04	0.661	0.058	15.000	<0.000	1.013	0.070	15	NS	0.650	0.039	14	<0.000
DMSO	1.702E-06	0.893	0.151	15.000	<0.019	0.735	0.091	15	<0.002	0.749	0.055	14	<0.002

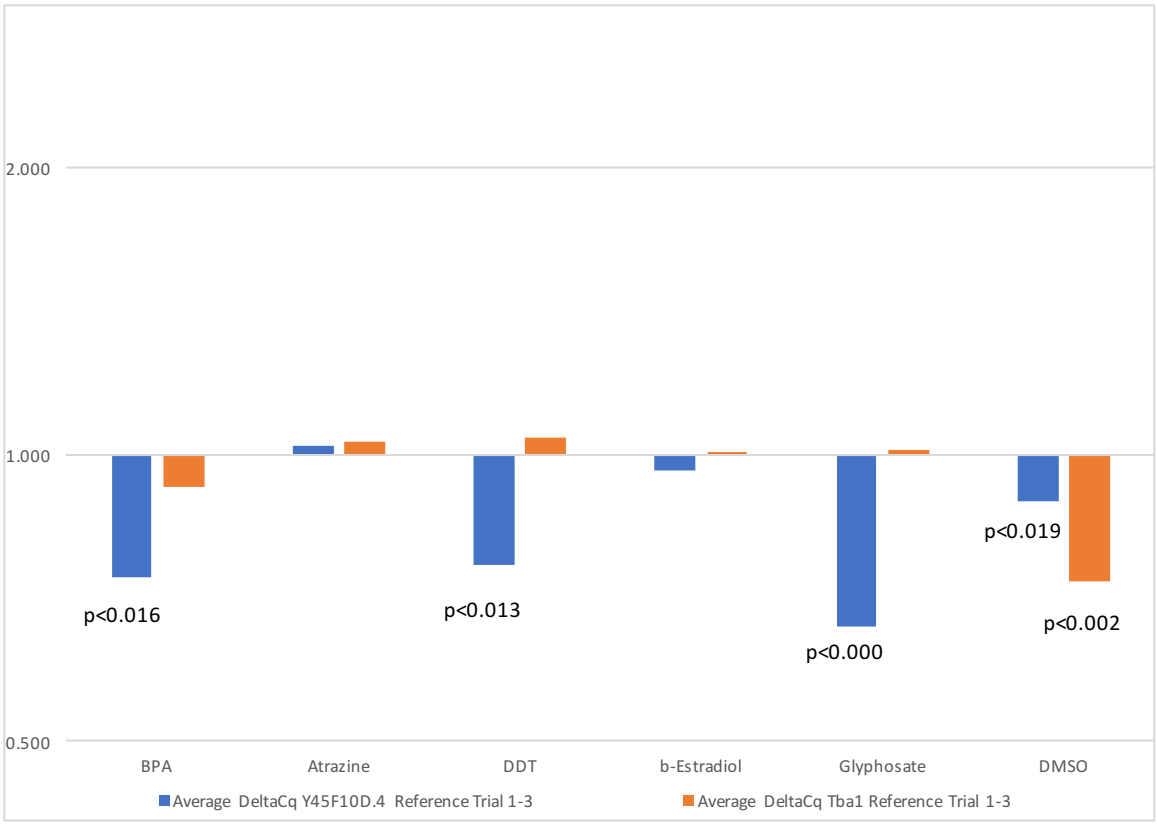

Figure 19: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene JUN1, an Orthologue to Human Tumor Suppressor Gene JUNB

Table 13: The Outputs of the Expression Level Comparisons for Gene AIR-1, an Orthologue to the Human Oncogene AURKA After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	1.014	0.126	13.000	NS	1.140	0.122	13	NS	1.019	0.175	13	NS
Atrazine	6.223E-09	0.958	0.069	14.000	NS	1.343	0.449	14	NS	0.710	0.042	14	<0.002
DDT	3.126E-08	0.803	0.089	14.000	NS	0.919	0.046	14	NS	0.719	0.039	14	<0.002
b-Estradiol	2.547E-08	1.010	0.101	14.000	NS	1.002	0.083	14	NS	0.933	0.091	14	NS
Glyphosate	8.492E-04	0.907	0.057	14.000	NS	1.305	0.067	14	NS	0.900	0.073	14	NS
DMSO	1.702E-06	0.883	0.097	14.000	NS	0.750	0.065	14	<0.006	0.835	0.061	14	NS

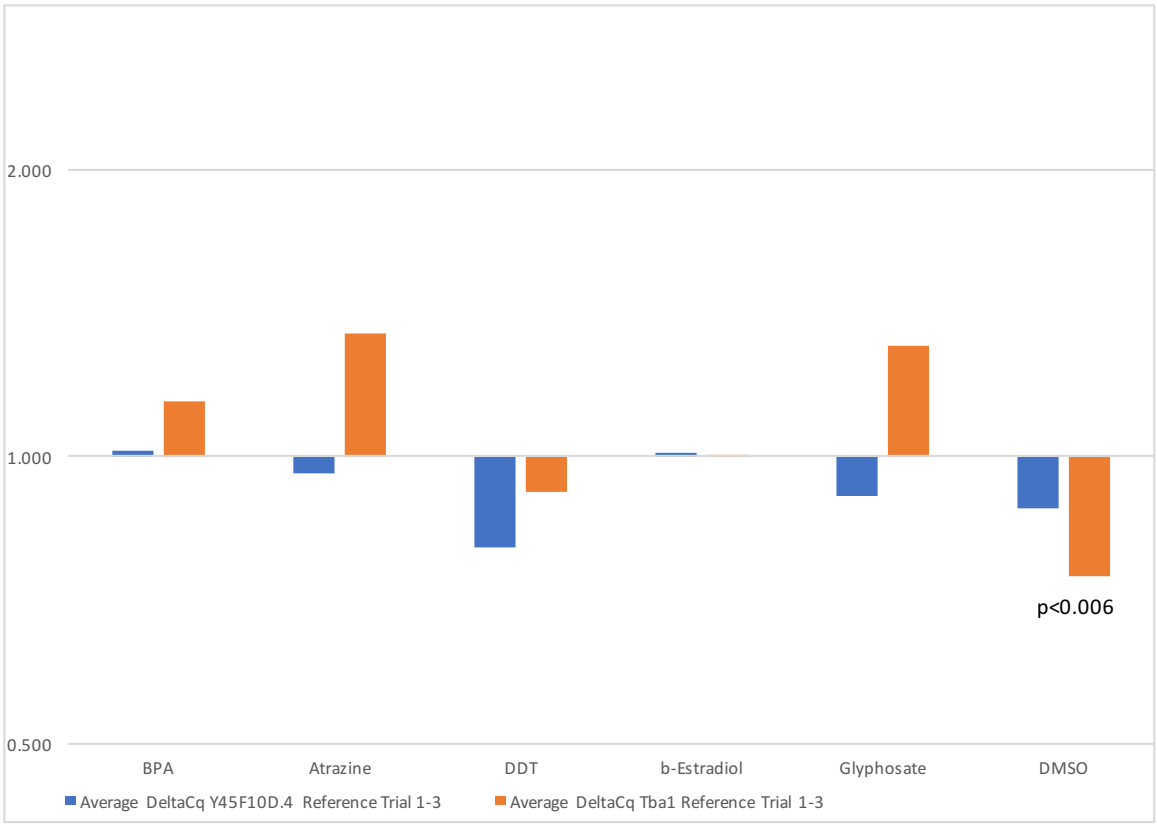

Figure 20: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene AIR-1, an Orthologue to Human Oncogene AURKA

Table 14: The Outputs of the Expression Level Comparisons for Gene CPAR-1, an Orthologue to the Human Oncogene Family CENP After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.830	0.098	15.000	NS	0.874	0.038	15	<0.007	0.740	0.065	15	<0.000
Atrazine	6.223E-09	0.962	0.094	15.000	NS	1.227	0.357	15	NS	0.703	0.059	15	<0.000
DDT	3.126E-08	0.839	0.069	15.000	NS	1.094	0.127	15	NS	0.799	0.077	15	<0.002
b-Estradiol	2.547E-08	0.824	0.088	15.000	NS	0.753	0.042	15	<0.000	0.713	0.044	15	<0.000
Glyphosate	8.492E-04	0.619	0.057	15.000	<0.000	0.875	0.061	15	NS	0.606	0.034	15	<0.000
DMSO	1.702E-06	0.984	0.111	15.000	NS	0.772	0.046	15	<0.000	0.894	0.058	15	NS

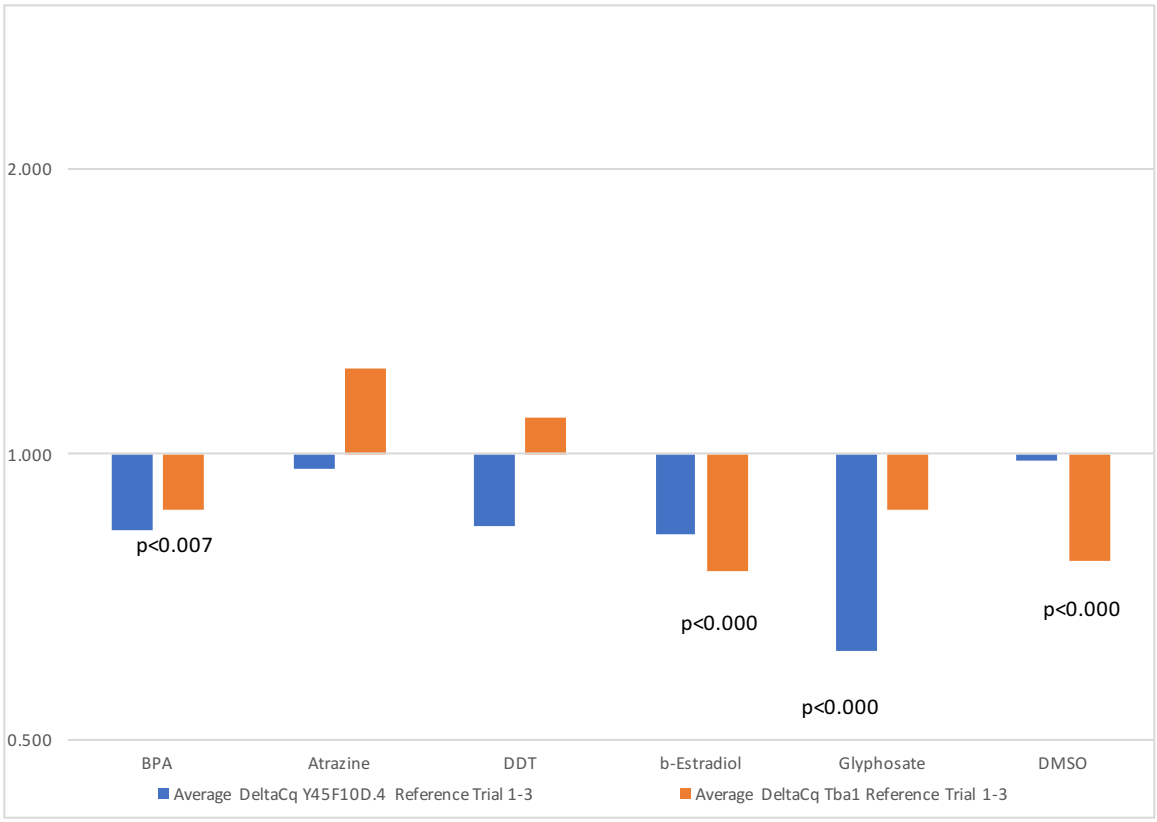

Figure 21: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene CPAR-1, an Orthologue to Human Oncogene Family CENP

Table 15: The Outputs of the Expression Level Comparisons for Gene CUL-1, an Orthologue to the Human Oncogene CUL1 After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.757	0.108	15.000	NS	0.898	0.168	15	NS	0.908	0.170	15	NS
Atrazine	6.223E-09	0.933	0.164	15.000	NS	1.149	0.311	15	<0.023	0.712	0.129	15	<0.005
DDT	3.126E-08	0.702	0.163	14.000	NS	0.683	0.151	14	<0.002	0.555	0.126	14	<0.002
b-Estradiol	2.547E-08	0.796	0.142	15.000	NS	0.800	0.149	15	NS	0.754	0.121	15	NS
Glyphosate	8.492E-04	0.358	0.085	15.000	<0.000	0.556	0.150	15	<0.000	0.372	0.092	15	<0.000
DMSO	1.702E-06	0.790	0.156	15.000	NS	0.702	0.178	15	<0.002	0.771	0.167	15	NS

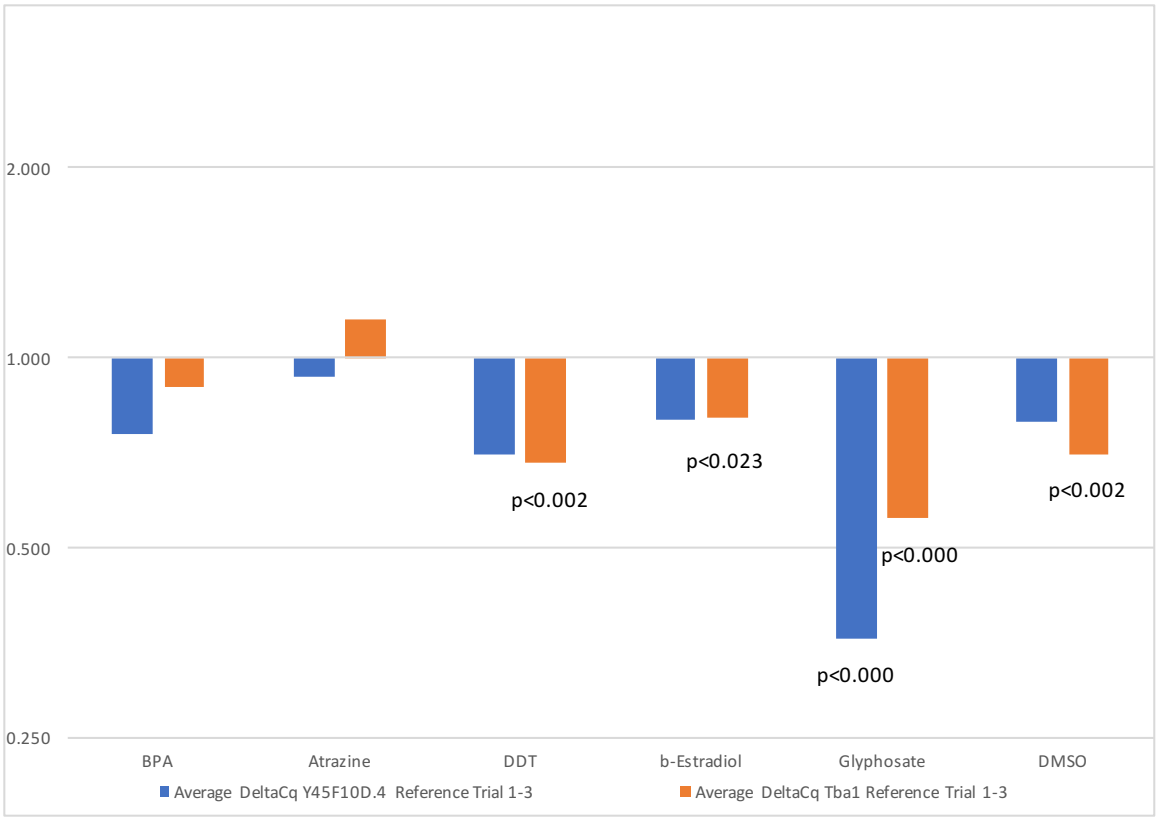

Figure 22: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene CUL1, an Orthologue to Human Oncogene CUL1

Table 16: The Outputs of the Expression Level Comparisons for Gene FZR1, an Orthologue to the Human Oncogene FZR After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	1.056	0.179	15.000	NS	1.032	0.069	15	NS	0.840	0.058	15	NS
Atrazine	6.223E-09	1.065	0.112	15.000	NS	1.335	0.374	15	NS	0.757	0.041	15	<0.001
DDT	3.126E-08	0.955	0.149	15.000	NS	1.033	0.065	15	NS	0.784	0.054	15	NS
b-Estradiol	2.547E-08	1.267	0.158	15.000	NS	1.101	0.070	15	NS	1.074	0.097	15	NS
Glyphosate	8.492E-04	0.581	0.039	15.000	<0.001	0.826	0.040	15	<0.004	0.592	0.048	15	<0.000
DMSO	1.702E-06	1.206	0.136	15.000	NS	0.924	0.045	15	NS	1.070	0.055	15	NS

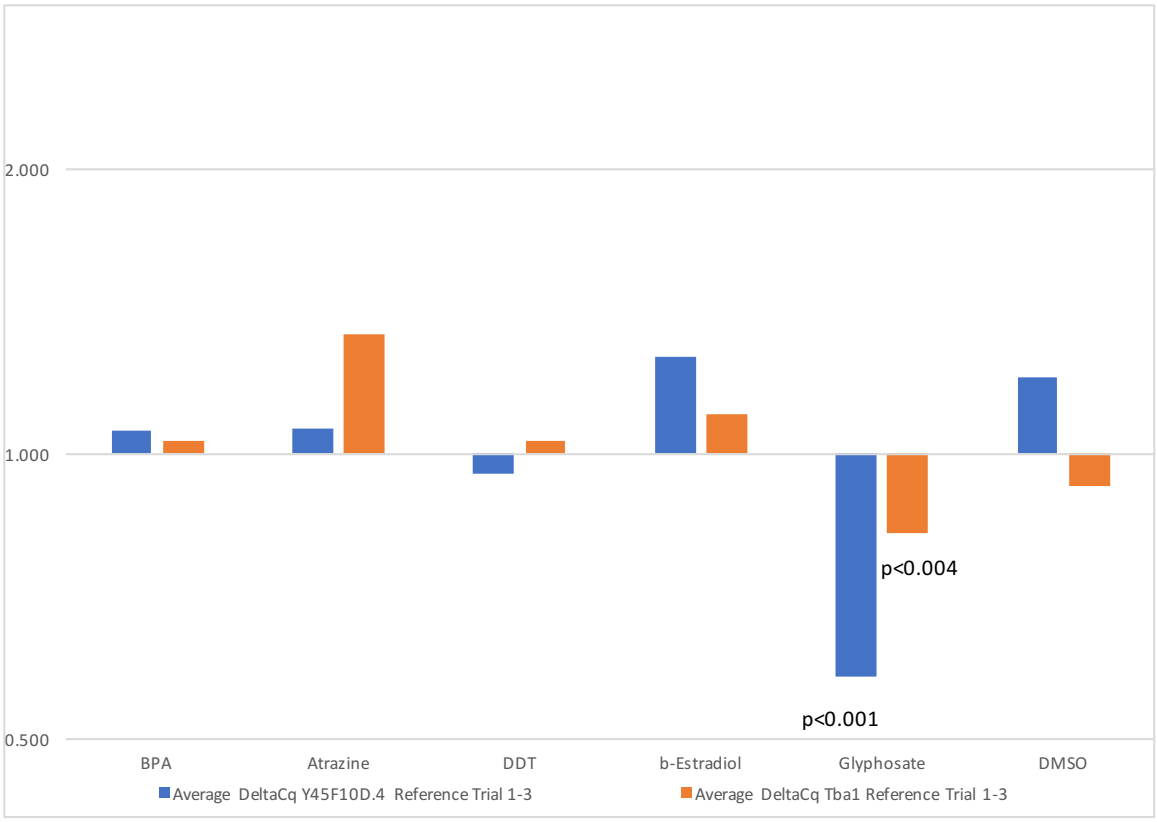

Figure 23: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene FZR1, an Orthologue to Human Oncogene FZR

Table 17: The Outputs of the Expression Level Comparisons for Gene HCP-3, an Orthologue to the Human Oncogene Family CENP After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	3.215	0.918	15.000	NS	2.580	0.516	15	<0.002	1.845	0.263	15	<0.006
Atrazine	6.223E-09	4.480	0.597	15.000	<0.000	6.922	2.958	15	<0.000	3.085	0.237	15	<0.000
DDT	3.126E-08	3.002	0.585	15.000	NS	3.034	0.569	15	NS	2.414	0.456	15	NS
b-Estradiol	2.547E-08	1.853	0.130	15.000	<0.000	1.948	0.244	15	<0.000	1.733	0.145	15	<0.000
Glyphosate	8.492E-04	3.222	0.332	15.000	<0.000	4.805	0.588	15	<0.000	3.183	0.281	15	<0.000
DMSO	1.702E-06	1.597	0.213	15.000	NS	1.534	0.286	15	NS	1.749	0.365	15	NS

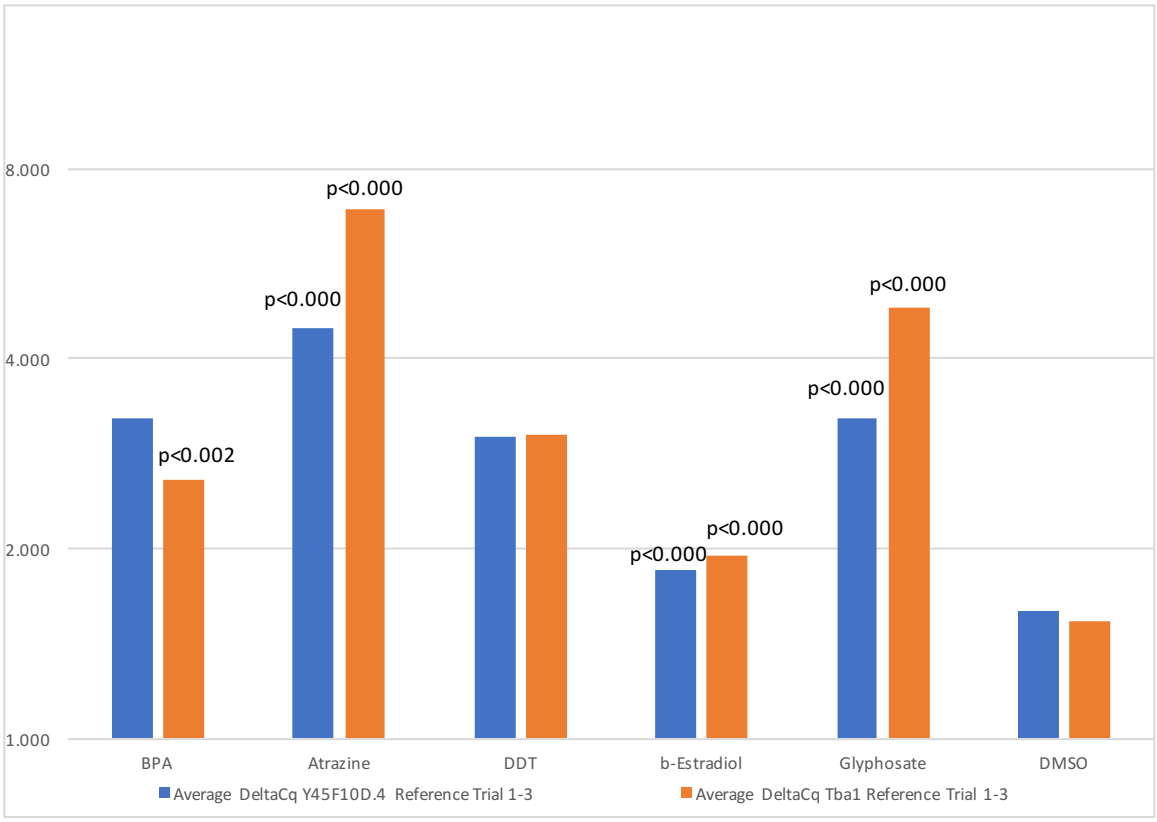

Figure 24: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene HCP-3, an Orthologue to Human Oncogene Family CENP

Table 18: The Outputs of the Expression Level Comparisons for Gene HCP-4, an Orthologue to the Human Oncogene Family CENP After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.867	0.104	15.000	NS	0.920	0.038	15	NS	0.767	0.056	15	<0.000
Atrazine	6.223E-09	0.904	0.058	15.000	NS	1.178	0.331	15	<0.007	0.700	0.059	15	<0.000
DDT	3.126E-08	0.877	0.050	15.000	NS	1.190	0.151	15	NS	0.869	0.090	15	<0.026
b-Estradiol	2.547E-08	0.908	0.093	15.000	NS	0.833	0.035	15	<0.000	0.802	0.059	15	<0.000
Glyphosate	8.492E-04	0.769	0.055	15.000	<0.007	1.088	0.049	15	NS	0.796	0.078	15	<0.000
DMSO	1.702E-06	1.040	0.092	15.000	NS	0.840	0.044	15	<0.004	0.995	0.076	15	NS

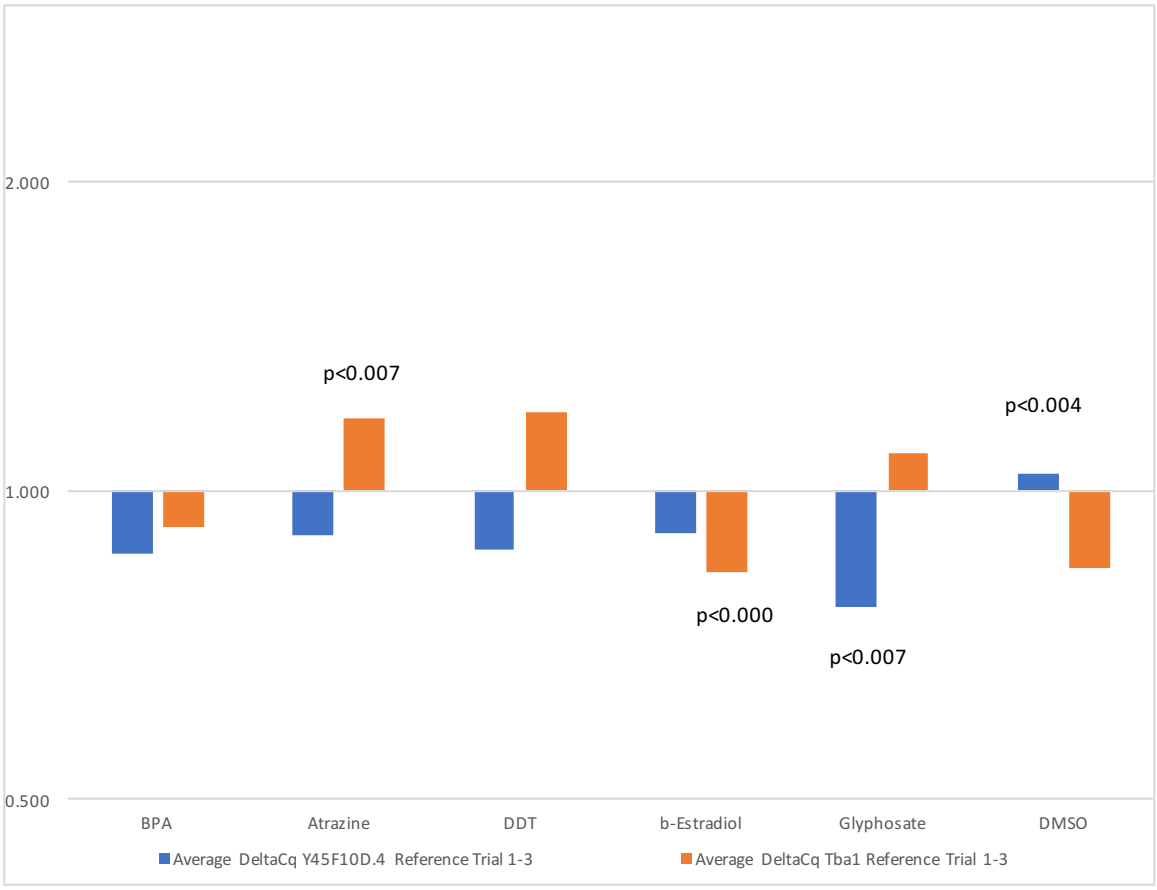

Figure 25: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene HCP-4, an Orthologue to Human Oncogene Family CENP

Table 19: The Outputs of the Expression Level Comparisons for Gene HCP-6, an Orthologue to the Human Oncogene Family CENP After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.808	0.061	15.000	<0.000	0.989	0.123	15	NS	1.075	0.213	15	NS
Atrazine	6.223E-09	1.184	0.092	15.000	NS	1.653	0.588	15	NS	0.977	0.087	15	NS
DDT	3.126E-08	1.127	0.159	15.000	NS	1.283	0.176	15	NS	1.115	0.155	15	NS
b-Estradiol	2.547E-08	0.989	0.074	15.000	NS	1.079	0.177	15	NS	1.068	0.139	15	NS
Glyphosate	8.492E-04	0.854	0.068	15.000	NS	1.290	0.185	15	NS	0.953	0.086	15	NS
DMSO	1.702E-06	1.014	0.083	15.000	NS	0.894	0.112	15	NS	1.111	0.123	15	NS

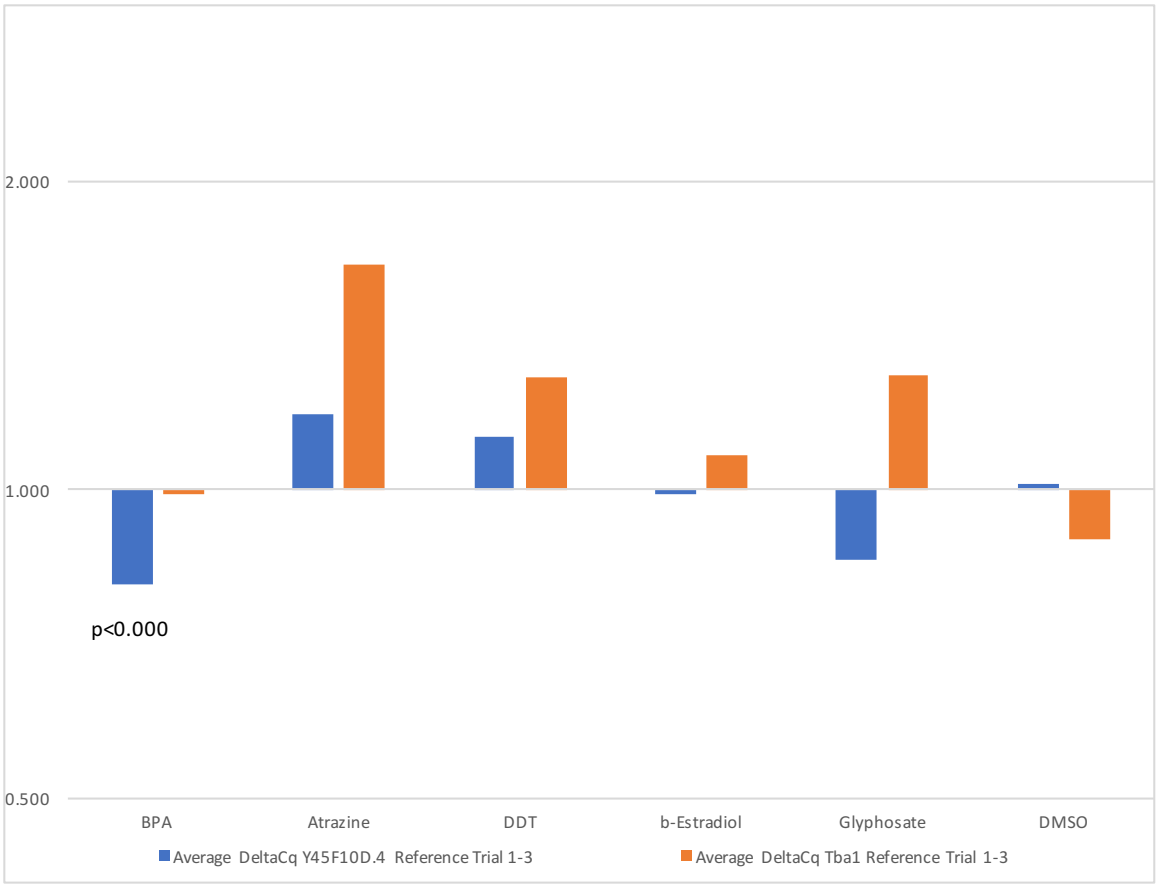

Figure 26: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene HCP-6, an Orthologue to Human Oncogene Family CENP

Table 20: The Outputs of the Expression Level Comparisons for Gene KNL-1, an Orthologue to the Human Oncogene Family CENP After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.928	0.065	15.000	NS	1.064	0.061	15	NS	0.914	0.089	15	NS
Atrazine	6.223E-09	1.028	0.051	15.000	NS	1.472	0.514	15	NS	0.810	0.071	15	<0.003
DDT	3.126E-08	0.966	0.074	15.000	NS	1.326	0.188	15	NS	0.968	0.121	15	NS
b-Estradiol	2.547E-08	1.209	0.125	15.000	NS	1.151	0.081	15	NS	1.097	0.097	15	NS
Glyphosate	8.492E-04	1.321	0.123	15.000	NS	1.847	0.111	15	<0.000	1.362	0.148	15	<0.014
DMSO	1.702E-06	1.117	0.138	15.000	NS	0.879	0.065	15	<0.015	1.067	0.116	15	NS

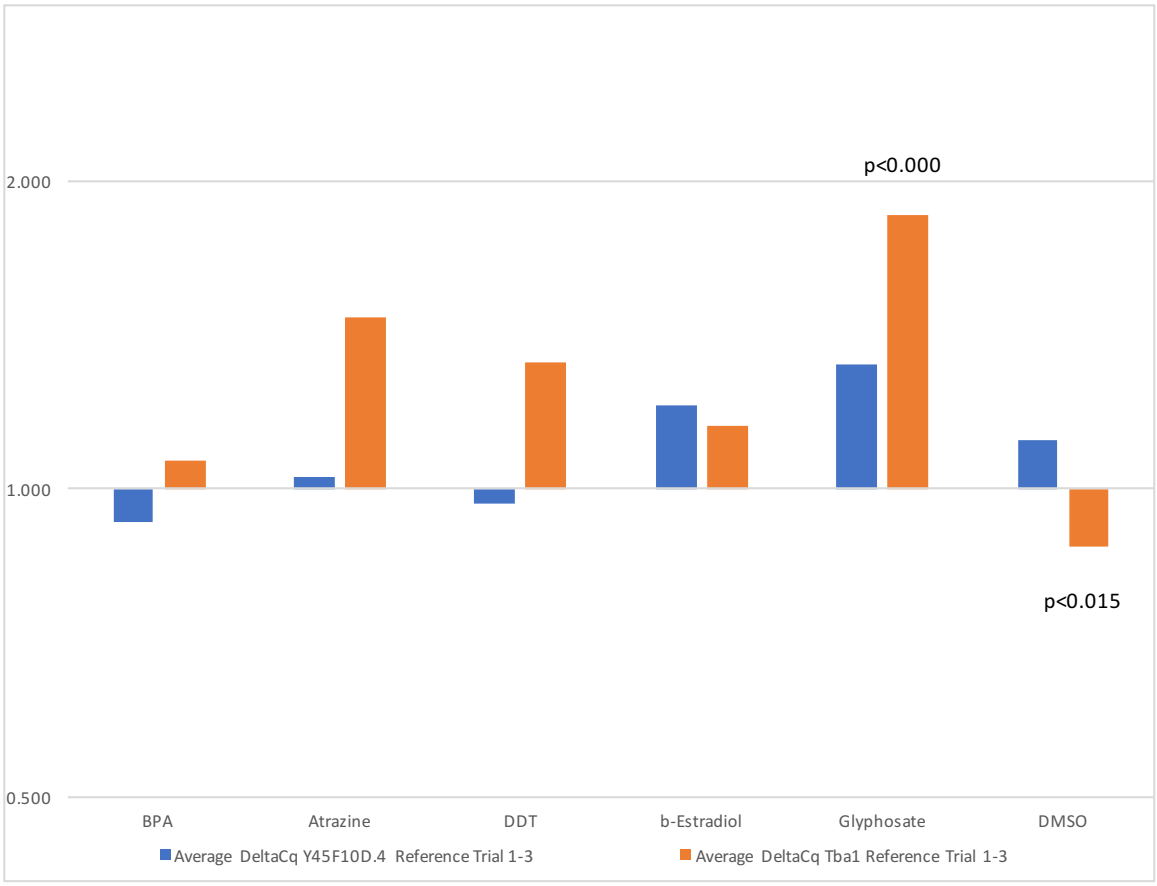

Figure 27: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene KNL-1, an Orthologue to Human Oncogene Family CENP

Table 21: The Outputs of the Expression Level Comparisons for Gene KNL-3, an Orthologue to the Human Oncogene Family CENP After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.911	0.107	15.000	NS	0.964	0.030	15	NS	0.810	0.061	15	<0.005
Atrazine	6.223E-09	0.971	0.068	15.000	NS	1.232	0.317	15	NS	0.731	0.048	15	<0.000
DDT	3.126E-08	0.934	0.077	15.000	NS	1.167	0.088	15	NS	0.864	0.049	15	<0.029
b-Estradiol	2.547E-08	0.965	0.094	15.000	NS	0.877	0.032	15	<0.004	0.843	0.053	15	<0.003
Glyphosate	8.492E-04	0.685	0.051	15.000	<0.000	0.965	0.047	15	NS	0.697	0.060	15	<0.000
DMSO	1.702E-06	1.086	0.113	15.000	NS	0.848	0.036	15	<0.001	0.991	0.057	15	NS

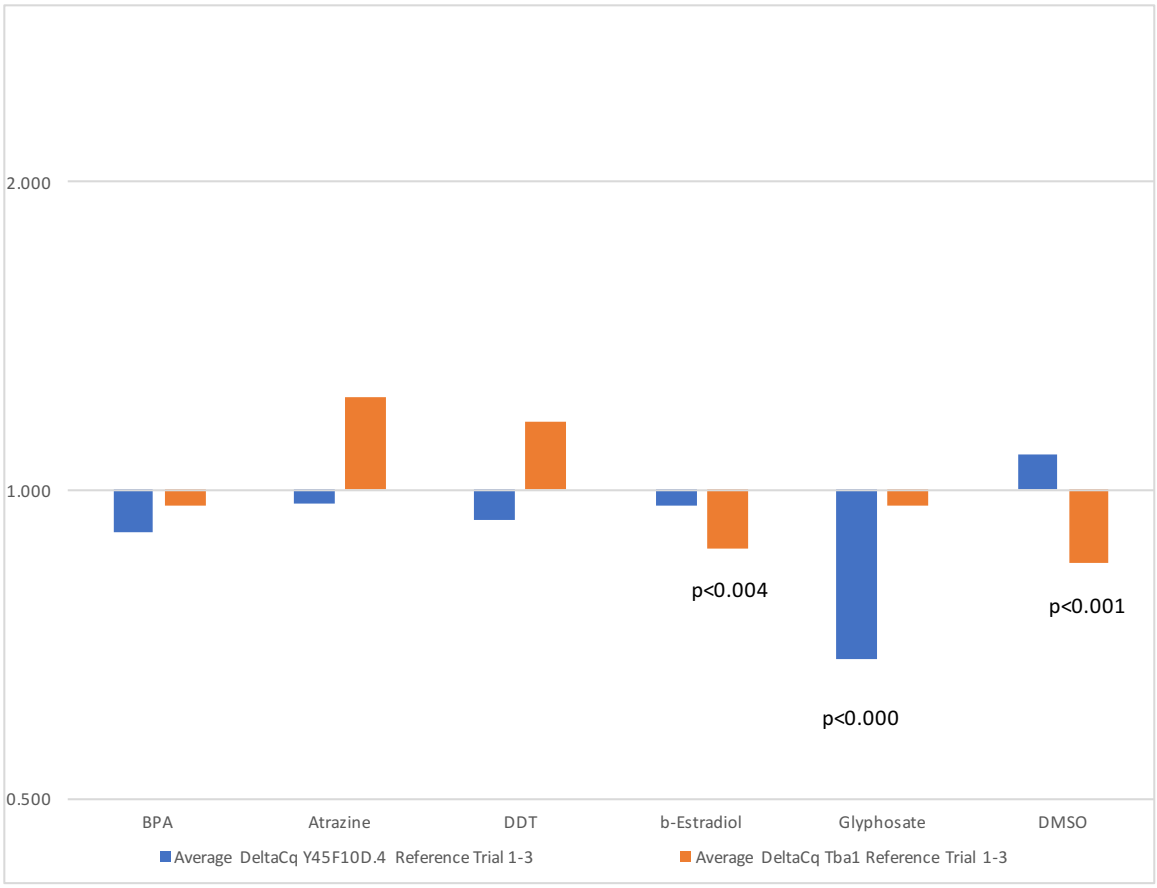

Figure 28: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene KNL-3, an Orthologue to Human Oncogene Family CENP

Table 22: The Outputs of the Expression Level Comparisons for Gene BRC-1, an Orthologue to the Human Suppressor Gene BRCA1 After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	10.740	4.854	10.000	NS	8.902	3.743	10	NS	4.608	1.816	10	NS
Atrazine	6.223E-09	21.846	8.638	12.000	<0.000	18.140	5.623	12	<0.000	12.230	3.272	12	<0.000
DDT	3.126E-08	7.585	2.687	9.000	<0.001	9.158	3.248	9	<0.000	6.310	2.609	9	<0.002
b-Estradiol	2.547E-08	2.190	0.526	12.000	NS	2.908	1.083	12	NS	1.922	0.542	12	NS
Glyphosate	8.492E-04	24.638	7.174	11.000	<0.000	40.347	11.868	11	<0.000	22.302	7.835	10	<0.000
DMSO	1.702E-06	1.608	0.542	11.000	NS	1.320	0.364	11	NS	1.259	0.370	10	NS

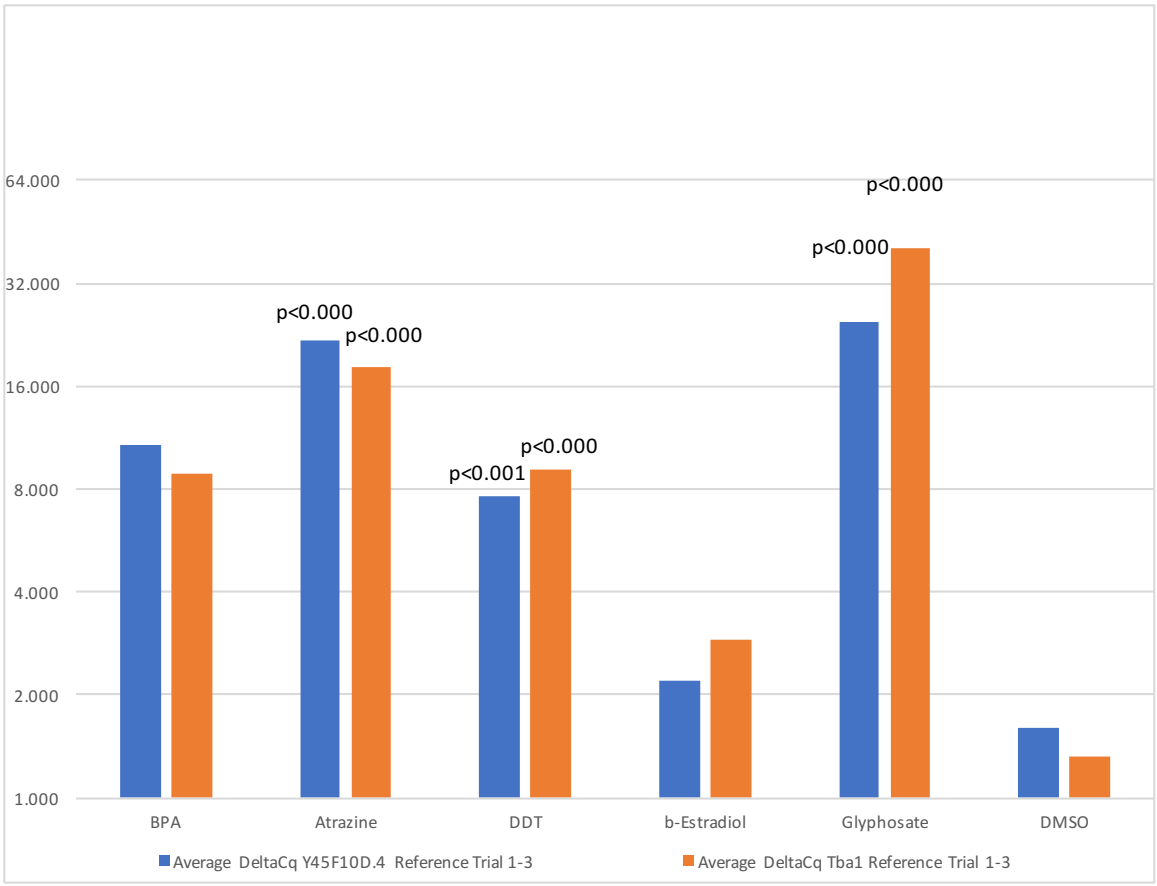

Figure 29: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene BRC-1, an Orthologue to Human Tumor Suppressor Gene BRCA1

Table 23: The Outputs of the Expression Level Comparisons for Gene BRC-2, an Orthologue to the Human Suppressor Gene BRCA2 After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.872	0.081	15.000	NS	1.072	0.040	15	NS	0.822	0.084	15	<0.011
Atrazine	6.223E-09	1.022	0.070	15.000	NS	1.038	0.038	15	NS	0.781	0.085	15	<0.000
DDT	3.126E-08	0.525	0.088	11.000	NS	0.769	0.081	11	<0.000	0.456	0.051	11	<0.000
b-Estradiol	2.547E-08	1.013	0.091	15.000	NS	1.051	0.060	15	NS	0.839	0.045	15	<0.017
Glyphosate	8.492E-04	0.511	0.026	14.000	<0.000	0.816	0.054	14	<0.001	0.496	0.033	13	<0.000
DMSO	1.702E-06	1.039	0.124	15.000	NS	0.958	0.151	15	<0.002	0.940	0.061	14	NS

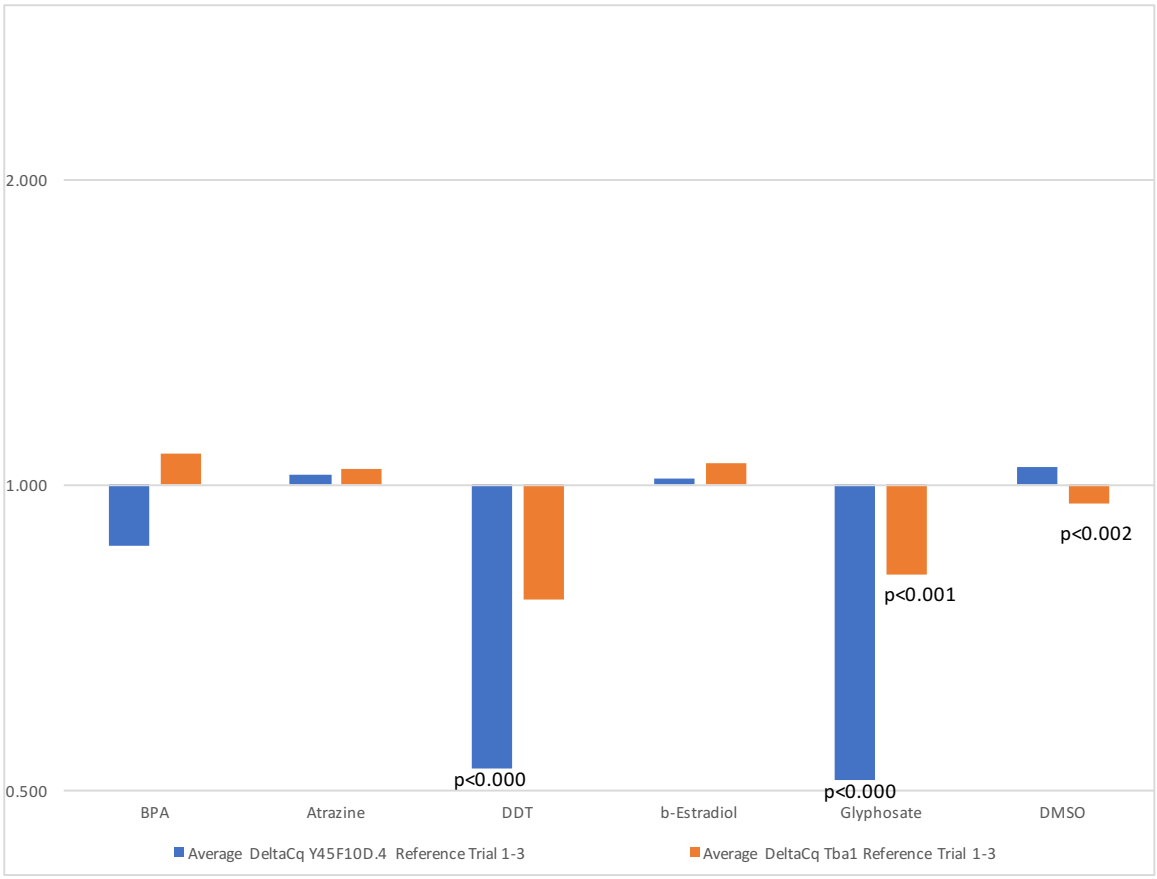

Figure 30: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene BRC-2, an Orthologue to Human Tumor Suppressor Gene BRCA2

Table 24: The Outputs of the Expression Level Comparisons for Gene CEP-1, an Orthologue to the Human Suppressor Gene TP53 After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.715	0.062	15.000	<0.001	0.898	0.054	15	<0.029	0.681	0.070	15	<0.001
Atrazine	6.223E-09	0.906	0.071	15.000	NS	0.912	0.044	15	NS	0.671	0.063	15	<0.000
DDT	3.126E-08	0.535	0.097	15.000	<0.001	0.661	0.088	15	<0.000	0.401	0.055	15	<0.000
b-Estradiol	2.547E-08	0.894	0.103	15.000	NS	0.903	0.059	15	NS	0.722	0.047	15	<0.001
Glyphosate	8.492E-04	0.493	0.040	15.000	<0.000	0.773	0.063	15	<0.000	0.462	0.028	14	<0.000
DMSO	1.702E-06	0.920	0.128	15.000	NS	0.826	0.135	15	<0.000	0.792	0.039	14	<0.004

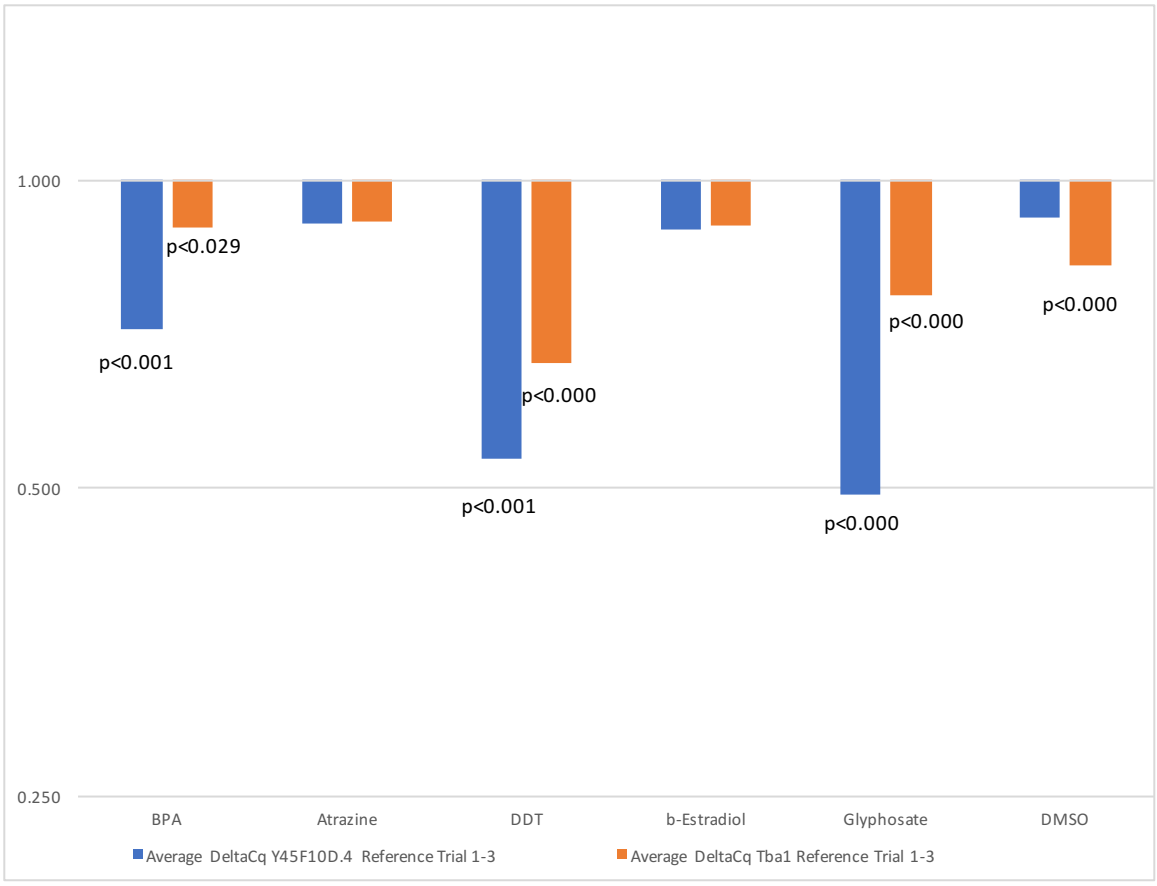

Figure 31: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene CEP-1, an Orthologue to Human Tumor Suppressor Gene TP53

Table 25: The Outputs of the Expression Level Comparisons for Gene MML-1, an Orthologue to the Human Tumor Suppressor Gene Family MYC After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.928	0.129	15.000	NS	1.070	0.062	15	NS	0.807	0.098	15	NS
Atrazine	6.223E-09	0.942	0.102	15.000	NS	0.930	0.073	15	NS	0.678	0.070	15	<0.000
DDT	3.126E-08	0.518	0.095	15.000	<0.000	0.652	0.079	15	<0.000	0.399	0.052	15	<0.000
b-Estradiol	2.547E-08	0.979	0.160	15.000	NS	0.932	0.095	15	NS	0.757	0.078	15	<0.004
Glyphosate	8.492E-04	0.473	0.035	15.000	<0.000	0.729	0.044	15	<0.001	0.464	0.032	14	<0.000
DMSO	1.702E-06	1.078	0.180	15.000	NS	0.893	0.151	15	<0.003	0.874d	0.082	14	NS

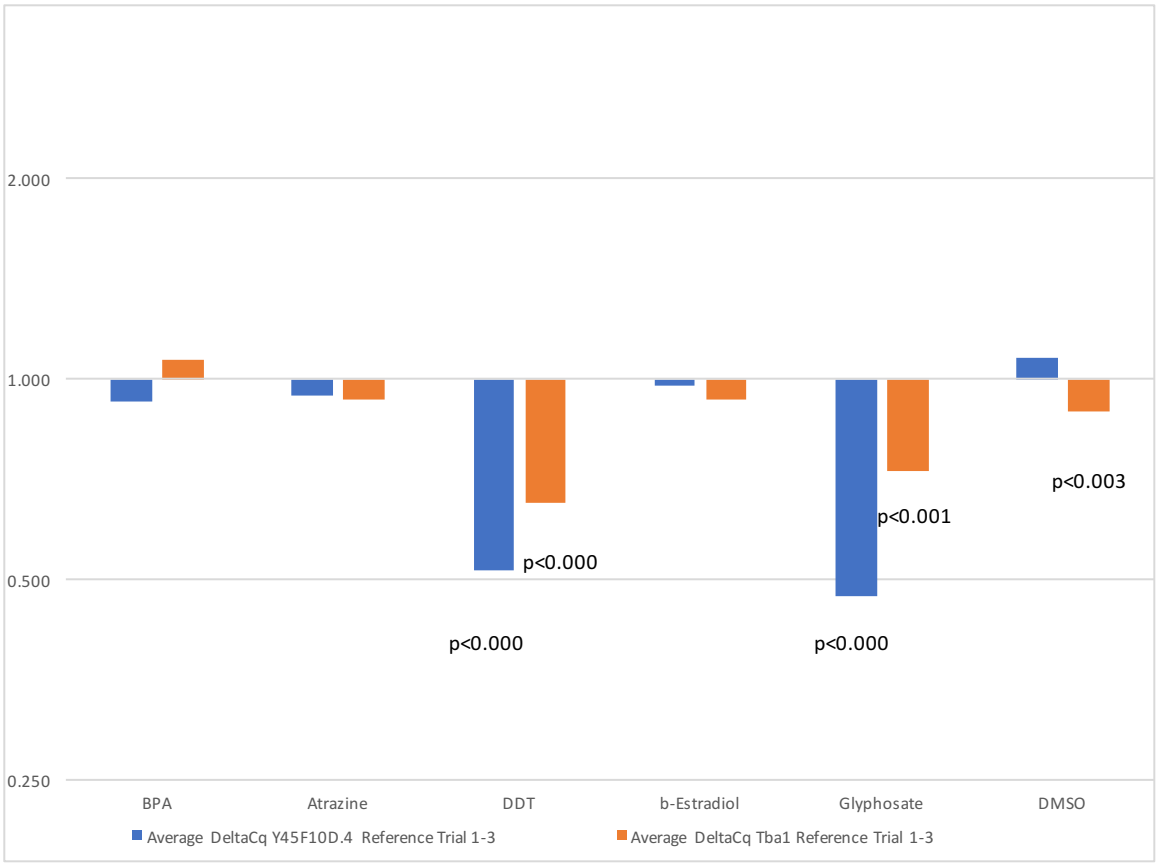

Figure 32: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene MML-1, an Orthologue to Human Tumor Suppressor Gene Family MYC

Table 26: The Outputs of the Expression Level Comparisons for Gene PIG-1, an Orthologue to the Human Oncogene MELK After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.847	0.121	15.000	NS	0.963	0.052	15	NS	0.715	0.073	15	<0.005
Atrazine	6.223E-09	0.892	0.073	15.000	NS	0.898	0.044	15	NS	0.667	0.066	15	<0.001
DDT	3.126E-08	0.751	0.088	15.000	NS	1.022	0.042	15	NS	0.623	0.036	15	<0.000
b-Estradiol	2.547E-08	1.137	0.153	15.000	NS	1.042	0.065	15	NS	0.914	0.107	15	NS
Glyphosate	8.492E-04	0.572	0.048	15.000	<0.000	0.863	0.050	15	<0.010	0.572	0.069	14	<0.000
DMSO	1.702E-06	1.066	0.124	15.000		0.911	0.093	15	<0.006	0.991	0.095	14	NS

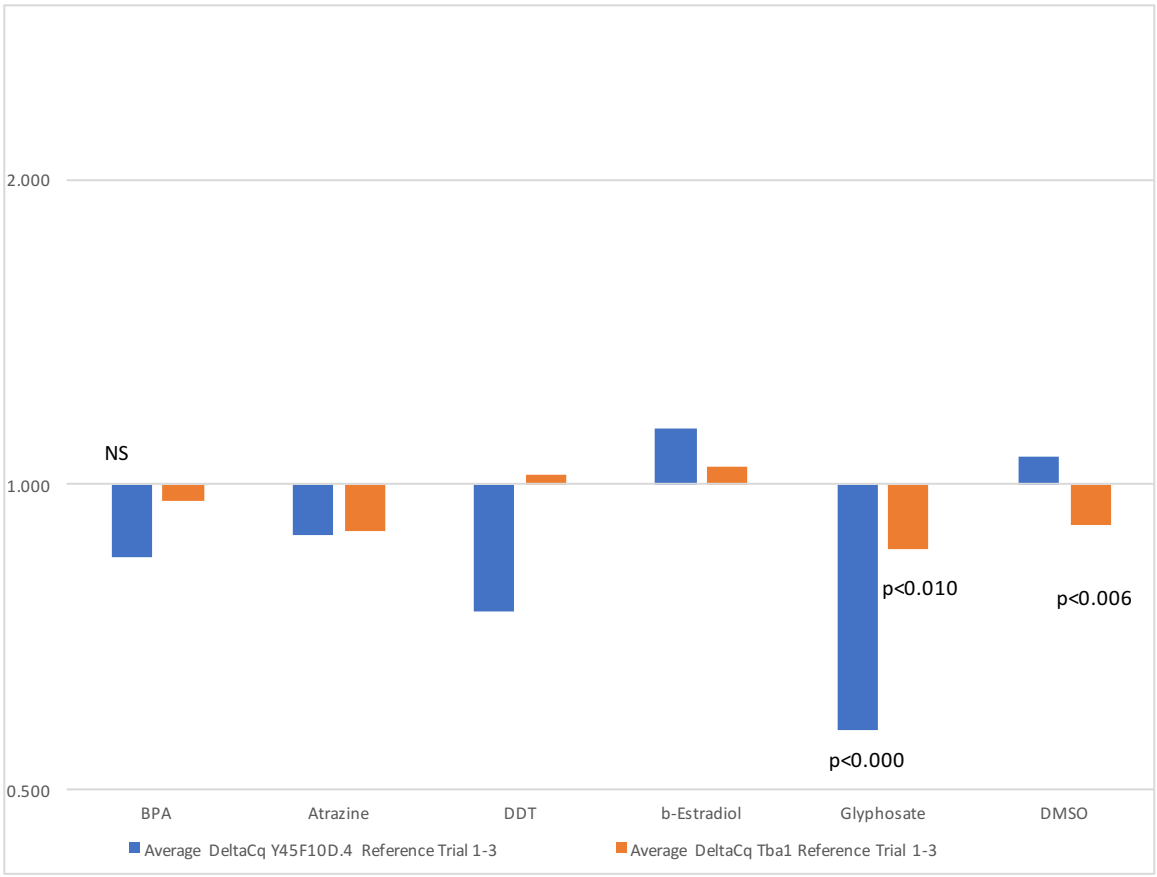

Figure 33: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene PIG-1, an Orthologue to Human Oncogene MELK

Table 27: The Outputs of the Expression Level Comparisons for Gene SLO-1, an Orthologue to the Human Tumor Suppressor Gene KCNRG After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	0.775	0.067	15.000	<0.003	0.959	0.049	15	NS	0.774	0.112	15	<0.007
Atrazine	6.223E-09	0.818	0.037	15.000	NS	0.857	0.052	15	NS	0.636	0.061	15	<0.000
DDT	3.126E-08	0.643	0.062	15.000	NS	0.916	0.063	15	NS	0.554	0.042	15	<0.000
b-Estradiol	2.547E-08	1.138	0.146	15.000	NS	1.148	0.087	15	NS	0.996	0.139	15	NS
Glyphosate	8.492E-04	1.082	0.102	15.000	NS	1.658	0.134	15	<0.000	1.144	0.187	14	NS
DMSO	1.702E-06	1.003	0.116	15.000	NS	0.929	0.137	15	<0.003	0.967	0.108	14	NS

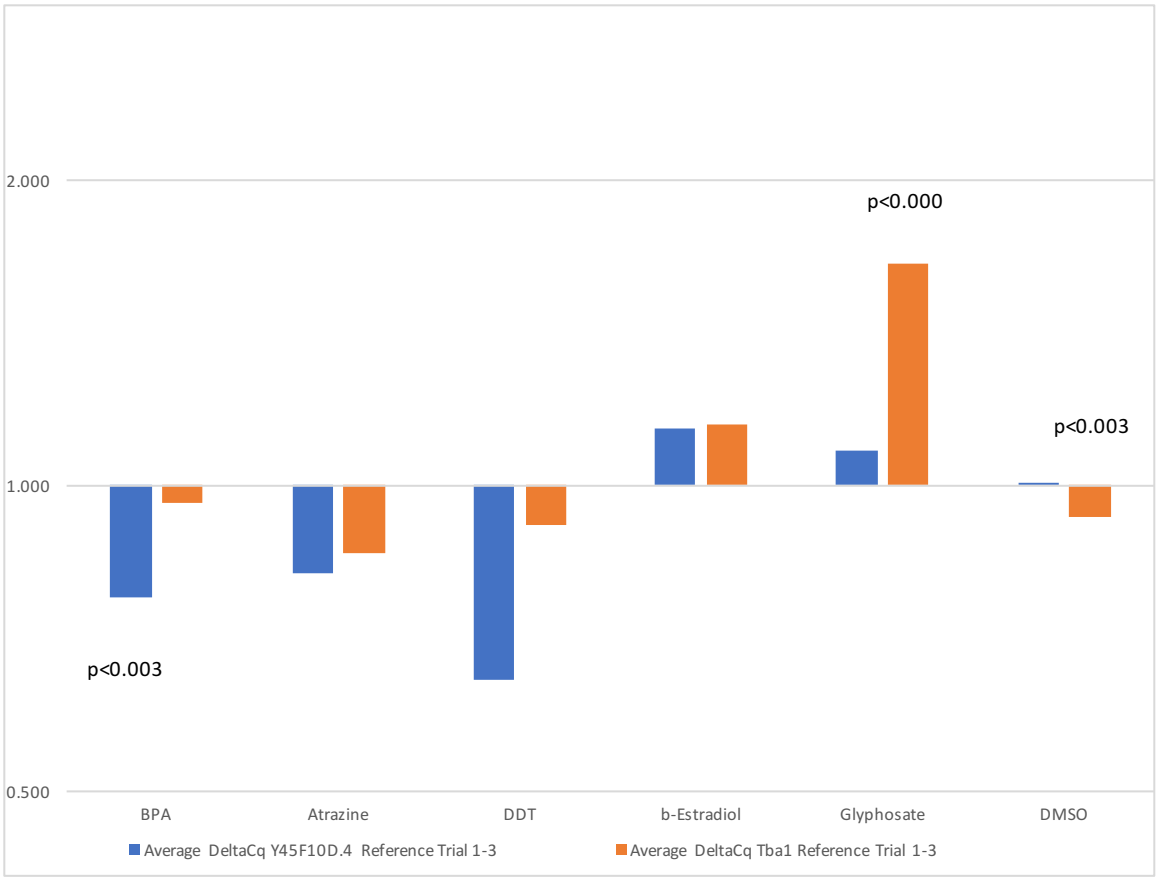

Figure 34: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene SLO-1, an Orthologue to Human Tumor Suppressor Gene KCNRG

Table 28: The Outputs of the Expression Level Comparisons for Gene SLO-2, an Orthologue to the Human Tumor Suppressor Gene KCNRG After Exposure to Each of the Tested Toxins. Each comparison was performed using Mann-Whitney U Test Statistics, with Subsequent Adjustment of the p-value for Multiple Testing Correcting using Benjamini-Hochberg Procedure.

		Y45F10D.4 Trials 1-3				Tba1 Trials 1-3				Pmp3 Trials 1-3			
Toxin	Toxin Concentration (M)	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?	Average DeltaCq	StErr	N	Significant?
BPA	6.516E-09	1.033	0.139	15.000	NS	1.217	0.079	15	NS	0.885	0.079	15	NS
Atrazine	6.223E-09	1.012	0.095	15.000	NS	1.053	0.092	15	NS	0.722	0.047	15	<0.00
DDT	3.126E-08	0.774	0.145	15.000	NS	0.938	0.152	15	NS	0.571	0.094	15	<0.000
b-Estradiol	2.547E-08	1.046	0.079	15.000	NS	1.203	0.144	15	NS	0.891	0.050	15	NS
Glyphosate	8.492E-04	0.598	0.049	15.000	<0.000	0.961	0.100	15	NS	0.606	0.056	14	<0.000
DMSO	1.702E-06	0.899	0.123	15.000	NS	0.801	0.083	15	NS	0.823	0.048	14	<0.012

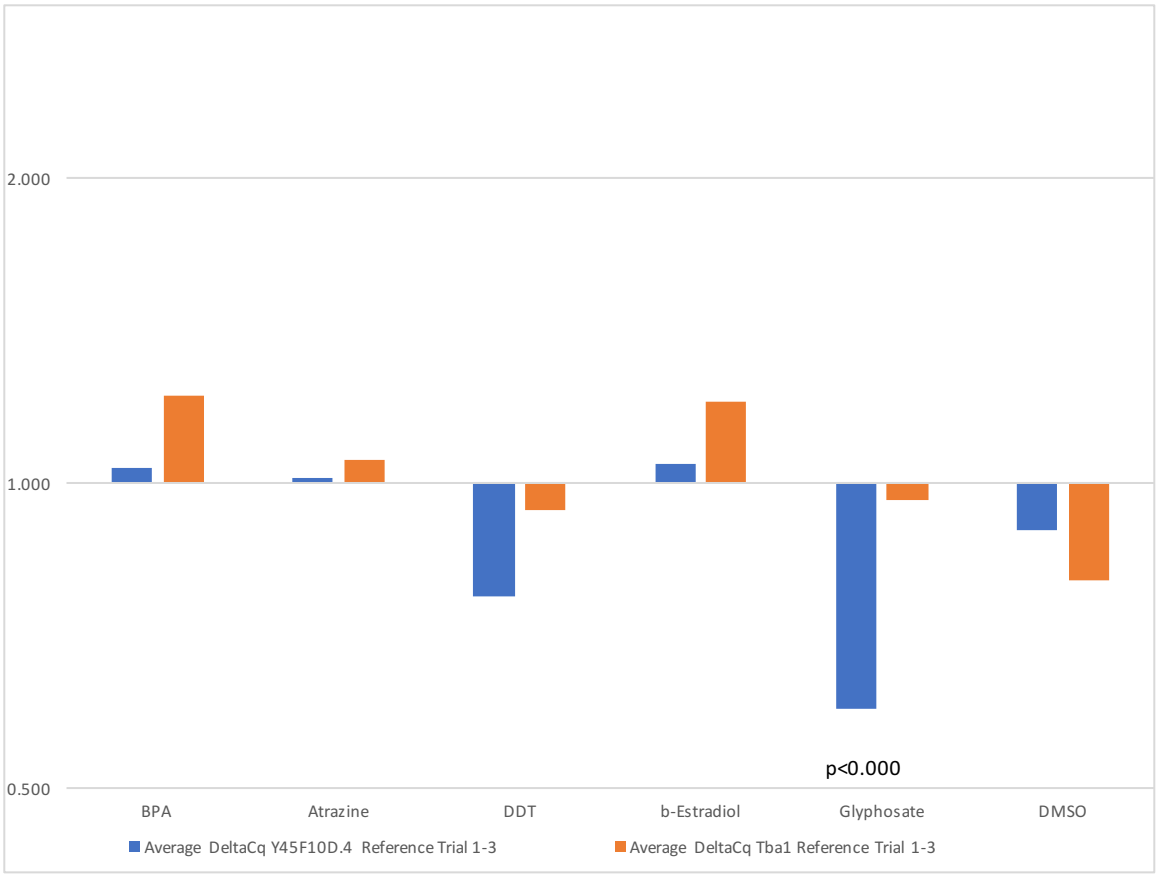

Figure 35: Average Fold Change in Expression Levels of *C. elegans* after 7 Days of Exposure to LD10 Concentration of Selected Toxins Using Gene SLO-2, an Orthologue to Human Tumor Suppressor Gene KCNRG

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