STRESS RELATED CHANGES IN PURINE, TYROSINE AND TRYPTOPHAN METABOLITES OF *CAENORHABDITIS ELEGANS*

by

Neeraja Podugu
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Committee:

Dr. James. D. Willett, Dissertation Director

Dr. Jenefir Isbister, Committee Member

Dr. Jennifer Weller, Committee Member

Dr. Yuntao Wu, Committee Member

Dr. James. D. Willett, Department Chairperson

Dr. Richard Diecchio, Associate Dean for Academic and Student Affairs, College of Science

Dr.Vikas Chandhoke, Dean, College of Science

Date: NOVEMBER 1ST

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George Mason University
Fairfax, VA

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By

Neeraja Podugu
Master of Science
Gujarat Agricultural University, 2003
Bachelor of Science
Gujarat Agricultural University, 2000

Director: Dr. James. D. Willett, Professor
Department of Molecular and Microbiology

Fall Semester 2010
George Mason University
Fairfax, VA

DEDICATION

This dissertation is dedicated to my parents Podugu Vasantha Kumari and Podugu Mohan Kumar who have been a source of inspiration and to my husband Sridhar Ramachandran for his continuous support.

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LIST OF ABBREVIATIONS

3-OH Anth	-	3-hydroxy anthranilate
3-OH-kyn	-	3-hydroxy kynurenine
5-OH-tryp	-	5-hydroxy tryptophan
5-HT,Ser	-	Serotonin, 5-hyroxy tryptamine
8-OH guanine	-	8-hydroxy guanine
ACM	-	Axenic culture media
Anth	-	Anthranilic acid
As	-	Arsenic
Ba	-	Barium
BSA	-	Bovine Serum albumin
CAT	-	Catalase activity
Cd	-	Cadmium
C.elegans	-	*Caenorhabditis elegans*
CEAS	-	Coulometric electrochemical array system
Co	-	Cobalt
Con	-	Control
Cr	-	Chromium
DL-kyn	-	DL-kynurenine
E.coli	-	*Escherichia coli*
EF	-	Edema toxin
FAH	-	Fumarylacetoacetase
Guan	-	Guanine
HGA	-	Homogenistic acid
Hg	-	Mercury
HLE	-	Heated liver extract
HPLC	-	High performance liquid chromatography
HVA	-	Homovanillic acid
IAA	-	Indole acetic acid
IPA	-	Indole pyruvic acid
Kyn	-	Kynurenine
L1	-	Larval stage L1
L2	-	Larval stage L2
L3	-	Larval stage L3
L4	-	Larval stage L4
LCEC	-	HPLC coupled with electrochemical detection
LF	-	Lethal toxin

LPS	-	Lipoploysaccharide
M	-	Reproductive *C.elegans* population
MA	-	3-methoxy-4-hydroxy-mandelic acid
MAPK	-	Mitogen activated protein kinase
Mel	-	Melatonin
MPA	-	Mobile phase A
MPB	-	Mobile phase B
mV	-	milli volts
NGM	-	Nematode growth medium
ng	-	nanograms
ng/ml	-	nano gram/milliliter
NMR	-	Nuclear magnetic resonance
Norepi	-	Norepinephrine
PA	-	Protective antigen
Pb	-	Lead
PCA	-	Principal Component Analysis
pg	-	picograms
p-OH-paa	-	P-hydroxy-phenyl acetic acid
ROS	-	Reactive oxygen species
SOD	-	Super oxide dismutase
Tryp	-	Tryptophan
TSB	-	Tryptic soy broth
Tyr	-	Tyrosine
Uric	-	Uric acid
UV	-	Ultra violet
Xan	-	Xanthine
Xantho	-	Xanthosine
Y	-	Young *C.elegans* population
µg	-	microgrmas

ABSTRACT

STRESS RELATED CHANGES IN PURINE, TYROSINE AND TRYPTOPHAN METABOLITES OF *CAENORHABDITIS ELEGANS*

Neeraja Podugu, Ph.D.

George Mason University, 2010

Dissertation Director: Dr. James. D. Willett

Caenorhabditis elegans has been used as a model organism in many areas of research over the last few decades. This research investigated stress related changes in *C.elegans* following exposure to different components of *B.anthracis* and varying dosages of lead acetate. Metabolites of purine, tyrosine and tryptophan pathways of *C.elegans* were measured using high profile liquid chromatography coupled with electrochemical detection. Perturbations in metabolites of these pathways in *C.elegans* were measured and compared to the metabolites of the untreated. *C.elegans* cultures grown under axenic and monoxenic conditions responded differently to the same stressors. The results from this research support the hypothesis that a relationship exists between the initial stress response and the subsequent changes in the metabolic constituents of purine, tyrosine and/or tryptophan pathways of *C.elegans*.

The exposure of axenic mixed cultures of *C.elegans* to a *Bacillus anthracis* three-toxin combination resulted in an acute response showing perturbations in purine, tyrosine and tryptophan pathways. Increasing the concentration of lead affected growth in axenic young, reproductive processes in axenic middle aged after 1.5 days of exposure whereas the adult *C.elegans* lysed. Perturbations in analytes of purine, tyrosine and tryptophan, as well as unknown analytes specific to the life stage and dosage of lead treatment, were observed. Axenic and monoxenic *C.elegans* demonstrated time and dose-dependent responses to lead exposure. Axenic *C.elegans* cultures responded to biological stressors and lead acetate more rapidly than monoxenic cultures, suggesting that axenic cultures could provide a superior model system for measuring acute stress responses.

1. INTRODUCTION

Caenorhabditis elegans (*C.elegans*), a free-living, nematode, is an excellent organism for obtaining an integrated view of an organism's response to diverse environmental and biochemical stresses. Metabolic activity in *C.elegans* responds to differences in environmental conditions, nutritional differences, contact with pathogens etc. and is an emerging model for studying organismal stress responses (Prahlad and Morimoto, 2009). *C.elegans* has been used as a model host for study of many human pathogens (Darby, 2005). Recent studies monitored small molecule signaling in *C.elegans* at various developmental stages (pre-reproductive, reproductive and adult) and following exposure to different environmental stimuli (Kaletta & Hengartner, 2006, Fielenbach & Antebi, 2008, Pungaliya *et al*., 2009, Schroeder, 2006).

Responses in three metabolic pathways (tryptophan, tyrosine and purine) were followed in *C.elegans* after exposure to environmental and physiological initiators. Two classes of initiators were used in this research, biological and chemical. *C.elegans* was exposed to *B.anthracis* components and lipopolysaccharide (LPS) from Gram negative microbes (biological stressors) and to soluble lead, a chemical found in the environment. Tryptophan (e.g. kynurenine, melatonin), tyrosine (e.g. dopamine, norepinephrine) and purine (e.g. guanine, xanthine) pathway responses in *C. elegans* were examined with respect to changes in the relative ratios of metabolites by using high performance liquid

chromatography (HPLC) coupled with electrochemical detection. The modulations in the metabolic profiles of *C.elegans*, reflected by these small molecules were studied using HPLC coupled with electrochemical detection to obtain the complete metabolic profiles of *C.elegans* following exposure to the different initiators used in the experimental studies.

A variety of analytical approaches to detect, record and monitor changes in the small metabolites of interest, i.e. key biological processes, prior to and following a stress induced event have been used (Schroeder, 2006; Srinivasan *et al*., 2008; etc.). The analytical platform used in these studies allows the detection and recording of molecular patterns of electrochemically active metabolites representative of altered physiological activity in the cell (Rozen *et al*., 2005; Yao *et al*, 2009). These molecular patterns can, in turn, serve as powerful indicators or reporters of cellular status and functional change.

This study was designed to determine if there are significant, reproducible alterations in tryptophan, tyrosine and purine catabolic pathway metabolites initiated by, and responsive to internal and/or external sources of stress. Does a relationship exist between stress and a common metabolite that represents alterations in the metabolic fingerprint in *C.elegans*? Does a specific metabolic change correspond only to exposure to a specific stressor? The results may assist in the signaling changes that arise in the initial stages of an organism's response to stress.

HYPOTHESIS

The hypothesis for this study is based on the assumption there will be alterations in metabolic profiles, especially those involving changes in the metabolites of tryptophan,

tyrosine and purine pathways in *C.elegans* as a result of stress. Common metabolite changes in *C.elegans* will occur irrespective of the stressors used for exposure. Unique metabolic alterations to tryptophan, tyrosine and purine pathways will occur following exposure of *C.elegans* to specific stress factors.

2. REVIEW OF LITERATURE

CAENORHABDITIS ELEGANS AS A MODEL ORGANISM

Sydney Brenner chose the soil nematode, *C.elegans*, as a model for studying the fundamental aspects of developmental and neuronal biology (Brenner, 1973). A relatively small investment of equipment and time is necessary for the start up cultures and maintenance of *C.elegans*. Apart from being a simple and genetically tractable nematode, *C.elegans* offers great potential as a model organism because of its relatively short, three-day, reproductive cycle (Supplementary material), small size, hermaphroditic nature, fixed cell lineage, and ease of cultivation under laboratory conditions (www.wormbook.org). Specifically, *C.elegans* has a short generation time, from egg to larva to fertile adult, of 2.5 days at 25°C and 6 days at 15°C. The organism is transparent, thus cellular developments can be observed using light microscopy. Fluorescent reporter genes, such as green fluorescent protein (gfp), are easily employed to mark specific genes or cells in *C.elegans* (Riddle *et al*., 1997). Adult hermaphrodites of *C.elegans* contain 959 somatic cells with different tissue types, such as muscles, nerves, and intestinal cells. They have a simple yet highly conserved nervous system (Bargmann, 1998). A single hermaphrodite adult can produce approximately 300 eggs (Riddle *et al*., 1997). The organism withstands cryopreservation at -80°C, allowing for long-term storage of stocks and mutant organisms (Corsi, 2006). Substantial detailed knowledge of fundamental

aspects of *C.elegans* biology exists; the complete genome sequence is known. Data from a variety of system-wide metabolism studies exist, and techniques and reagents are available to facilitate the investigation of stress responses in *C.elegans* (The *C.elegans* sequencing consortium *et al*., 1998). Pairwise comparisons demonstrated protein similarities between *C.elegans* and *Homo sapiens* as well as *C.elegans* and *E.coli*. *C.elegans* lacked proteins that were found in both yeast and *E.coli*.

C.elegans responds to a broad range of stimuli (Bargmann & Mori, 1997) demonstrating phenotypic and developmental changes (Avery & Thomas, 1997; Riddle & Albert, 1997) and has been used as a model system for the study of host-pathogen interactions, genetic analyses, heavy metal toxicity studies etc. (Kaletta & Hengartner, 2006; Srinivasan *et al*., 2008; Xing *et al*., 2009). The following sections describe some of the pertinent literature regarding these studies.

C.elegans has been used to monitor normal stages of development, aging, senescence and specific life stage responses to acute or chronic exposure to pharmaceuticals, environmental contaminants, ROS inhibitors and other potential modulators of cellular homeostatic controls. GPCRs found in eukaryotes consist of a large protein family receptors which sense specific molecules in the environment (*e.g.* disease related molecules) and activate the signal-transduction pathway. The ligands that can bind to these GPCRs consist basically of many hormones and neurotransmitters. All G-protein-coupled receptors (GPCRs) share a common molecular architecture (with seven putative transmembrane segments) and signaling mechanisms. GPCRs regulate the synthesis of intracellular second messengers like cyclic AMP, inositol phosphates,

diacylglycerol and modulate calcium ions (Harmar, 2001). GPCRs that function as neurotransmitters, and the associated neurotransmitter synthesis and release pathways, are conserved between mammals and *C.elegans*. They are of ancient origin and homologs are present in many eukaryotes, including insects and plants (Hill et.al, 2002). The similarity has allowed mammalian bitter-taste receptors (T2R family) and TRPV (VR1) channels to be functionally expressed in the neurons of *C.elegans*. Related channels are normally present in wild-type nematodes and modified versions can be inserted using gene manipulation techniques (Bargmann, 1998; Teng *et al*., 2006).

Other advantages to this model organism include its relative low cost and the availability of large numbers of individuals for testing as well as the ease of genetic manipulation. Using mice or other mammals as model host systems to investigate virulence genes of a pathogen is often impractical and usually expensive. *C.elegans* overcomes these limitations as it is readily cultured. In this research, *C.elegans* serves as the host system for determining mechanisms of actions of toxins, and to examine the effect of alternate feeding sources. With respect to the latter, Nass and Hamza (2007) used *C.elegans* as an animal model for toxicological studies to assess molecular nutritional mechanisms and found nematode responses similar to those in humans with diseases such as Parkinson's disease. Clearly this is a limited model, since *C.elegans* does not have complete overlap of genes with humans; however 40% of genes associated with human diseases have homologs in *C.elegans* homology with human disease related genes (Corsi, 2006).

A broad range of bacterial pathogens and fungi kill *C.elegans* or produce non-lethal disease symptoms. Table 1 gives a brief description of the studied pathogens and their effects on *C.elegans* (Darby, 2005). Our selection of a particular organism was driven by the worldwide incidence of anthrax (caused by a Gram-positive organism, *Bacillus anthracis*) in animal populations, the risk of human infection associated with animal outbreaks, and the threat of *Bacillus anthracis* (*B.anthracis*) as a biological weapon. Anthrax has not been widely studied using *C.elegans* as a model, although recent reports demonstrate that *B.anthracis* spores can survive (up to 3 hours in the intestinal gut of *C.elegans* (Laaberki and Dworkin, 2007). This finding suggests that *C.elegans* can serve as a reservoir for *B.anthracis* in the environment. *B.anthracis,* as a biological stressor, provided vegetative cells, spores and bacterial products (exotoxins) for study in this project.

Table 1: Interaction of *C.elegans* with other organisms (Darby, 2005)

Organism	**Human pathogen?**	***C. elegans* pathology**	**References**
Aeromonas hydrophila	Yes	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Agrobacterium tumefaciens	Yes **(b)**	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Bacillus thuringiensis	No	Pore-forming toxin	Griffitts *et al*., 2003; Griffitts *et al*., 2001; Marroquin *et al*., 2000
Burkholderia cenocepacia	Yes	Gut infection; toxin	Coenye *et al*., 2001; Kothe *et al*., 2003
Burkholderia pseudomallei	Yes	Diminished lifespan **(a)**; toxin	Gan *et al*., 2002; O'Quinn *et al*., 2001
Cryptococcus neoformans	Yes	Gut infection; toxin	Mylonakis *et al*., 2002; Mylonakis *et al*., 2004
Drechmeria coniospora	No	Whole body parasitized	Jannson, 1994
Enterococcus faecaelis	Yes	Gut infection	Garsin *et al*., 2001; Kim *et al*., 2002; Sifri *et al*., 2002
Erwinia carotovora	No **(c)**	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Erwinia chrysanthemi	No **(c)**	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Escherichia coli	Yes	Gut infection	Garsin *et al*., 2001
Microbacterium nematophilum	No	Rectal & posterior cuticle infection	Hodgkin *et al*., 2000; Nicholas and Hodgkin, 2004
Photorhabdus luminescens	Yes **(b)**	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Pseudomonas aeruginosa	Yes	Gut infection; multiple toxins	Darby *et al*., 1999; Gallagher and Manoil, 2001; Kim *et al*., 2002; Mahajan-Miklos *et al*., 1999; Tan *et al*., 1999; Tan *et al*., 1999; Wareham *et al*., 2005

Salmonella enterica	Yes	Gut infection; germline cell death	Aballay and Ausubel, 2001; Aballay *et al*., 2000; Labrousse *et al*., 2000; Tenor *et al*., 2004
Serratia marcescens	Yes	Gut infection; tissue degradation	Kurz *et al*., 2003; Mallo *et al*., 2002
Shewanella frigidimarina	No **(c)**	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Shewanella massilia	No **(c)**	Diminished lifespan **(a)**	Couillault and Ewbank, 2002
Staphylococcus aureus	Yes	Gut infection	Bae *et al*., 2004; Begun *et al*., 2005; Garsin *et al*., 2001; Sifri *et al*., 2003
Streptococcus agalactiae (Group B)	Yes	Toxin (hydrogen peroxide)	Bolm *et al*., 2004
Streptococcus dysgalactiae	Yes	Toxin (hydrogen peroxide)	Bolm *et al*., 2004
Streptococcus mitis (Viridans group)	Yes	Toxin (hydrogen peroxide)	Bolm *et al*., 2004
Streptococcus oralis	Yes	Toxin (hydrogen peroxide)	Bolm *et al*., 2004
Streptococcus pneumoniae	Yes	Toxin (hydrogen peroxide)	Jansen *et al*., 2002
Streptococcus pyogenes (Group A)	Yes	Toxin (hydrogen peroxide)	Jansen *et al*., 2002
Xenorhabdus nematophila	No	Biofilm on cuticle; toxin	Couillault and Ewbank, 2002
Yersinia pestis	Yes	Biofilm on cuticle, feeding blocked	Darby *et al*., 2002
Yersinia pseudotuberculosis	Yes	Biofilm on cuticle, feeding blocked	Darby *et al*., 2002

(a) "Diminished lifespan" indicates that the organism's killing mode has not been determined in more detail.
(b) Rare reports of human infection by this species.
(c) Rare reports of human infection by some species in this genus.

Host-pathogen interactions

In the past few years, *C.elegans* has proven to be an effective model system for both broad host range pathogens and specific human pathogens. Several *Salmonella enterica* serovars (Aballey *et al*., 2000) including *Salmonella typhimurium* were shown to kill *C.elegans,* suggesting that the nematode is a suitable model host for at least some specific vertebrate pathogens. A high titer of *Salmonella typhimurium* persists in the intestinal lumen of the nematode, ultimately followed by the death of the nematode. Bacterial mutants with lesions in several Salmonella virulence genes, such as HilA (hyperinvasive locus A), HilD, SptP (Salmonella protein tyrosine phosphatase) from the Salmonella pathogenicity island 1 (SPI-1), all showed reduced capability for infection of epithelial cells in *in-vitro* mammalian enteropathogenesis. When *C.elegans* intestinal cells were infected with the mutants containing these virulence factors, pathogenicity of Salmonella was observed (Aballay and Ausubel, 2002; Tenor *et al*., 2004).

Garsin and his co-workers demonstrated the suitability of using *C.elegans* as a model host for Gram-positive infection. The *Enterococcus faecalis* virulence factor, sucrose-6-phosphate hydrolases (ScrB), required for virulence in mammals, is also required for *C.elegans* killing. Mutation in the ScrB gene (regulated by ScrR) encoding for sucrose-6-hydrolase caused attenuation of *C. elegans* killing (Garsin et al, 2001)

Pseudomonas aeruginosa, a ubiquitous Gram-negative environmental organism and important opportunistic pathogen, kills *C.elegans,* depending on the pathogen strain and the medium on which *P.aeruginosa* is grown. The infected worm exhibits phenotypic changes such as inhibition of pharyngeal pumping, sluggish locomotion, and

hypercontraction of body wall muscles before death, showing that the toxic effect of *P.aeruginosa* is targeted towards neuromuscular function (Darby *et al*., 1999).

Staphylococcus aureus is a Gram-positive coccus causing a wide variety of diseases, including skin infections, food poisoning, toxic shock syndrome and septic shock in humans. Alpha-hemolysin, is a potent cytolytic pore-forming toxin that acts as a *S.aureus* virulence factor in many mammalian model systems, and is also important for pathogenicity in *C.elegans.* All developmental stages of *C.elegans* are killed by *S.aureus* infection. Nematode locomotion, pharyngeal pumping and foraging are reduced after 24 to 48 hours exposure to *S.aureus*. The nematodes become immobile and finally die **(**Sifri *et al*., 2003).

Effect of heavy metals on *C.elegans*

Another research area in which *C.elegans* has been used extensively as a model is heavy metal toxicity studies. *C.elegans* responds to trace amounts of heavy metals and other toxic compounds in soils and rivers (David *et al*., 2003). Heavy metal exposure can affect organismal endpoints such as fecundity, life-cycle and development (Swain *et al*., 2004).

Cobalt is found in the environment and excess dietary exposure to cobalt can lead to toxic effects in many organisms. Although it was known to cause toxicity, it was unknown if these effects were stably passed from parent to progeny. Wang and his co-workers analyzed the multiple toxicities of Co in *C.elegans* populations (approximately 100 animals) and the progeny of the exposed nematodes. Phenotypic changes such as endpoints of life span, body size, vulval development, brood size, generation time, body

bend, head thrash, and chemotaxis plasticity were evaluated as indicators of toxicity. They found that cobalt induced multi-biological defects in *C.elegans* which were transferred from parent to filial progeny.

C.elegans stress responses to barium (Ba) exposure (24 hours) were monitored with respect to phenotypic defects (Wang and Wang, 2007). High concentrations of Ba, 75 µM and 200 µM caused several phenotypic defects (body size, head thrash) in *C.elegans* and affected the reproductive ability of the nematode. Life span defects reduced life span by 4 days compared to controls; other effects included impaired growth, reproductive defects and altered development of the nervous system. These multiple, biological defects could provide a new evaluation system to monitor toxicity following Ba exposure. Toxicity levels from Ba exposure were measured by comparison of the activity of superoxide dismutase (SOD) and catalase activity (CAT) in controls and Ba exposed nematodes. The treated nematodes showed reduced levels of SOD and CAT activities at all treatment concentrations suggesting severe oxidative stress in Ba exposed nematodes.

From an evolutionary point of view, *C.elegans* genes and signal transduction pathways are conserved and these genes could be used to study Cd related human diseases. Pawlak *et al* (2004) demonstrated that tryptophan metabolites (such as kynurenine and kynurenic acid) can be used to monitor for Cd toxicity as Cd exposure resulted in dose-dependent decreases in kynurenine and kynurenic acid.

Wang and Yang (2007) monitored *C.elegans* to analyze for multiple toxicities from lead exposure and the possible transfer of these properties to progeny. Lead

exposure caused a series of severe multi-biological defects in a concentration-dependent manner including the endpoints of life span, development, reproduction and locomotion behavior in nematodes. Moreover, most of these properties could be transferred to progeny from lead exposed animals and some of the defects in progeny (body size, mean life span) appeared even more severe than in their parents (Wang & Yang, 2007).

In attempts to identify a sensitive biomarker for environmental monitoring and risk assessment, transgenic *C.elegans* was used to test for the toxicity of cadmium (Cd), lead (Pb), chromium (Cr) and arsenic (As) (Roh *et al*., 2006). Cadmium exposure resulted in a three fold increase in heat shock protein 16.2, heat shock protein 70, metallothionein 2, cytochrome P450 family protein 35A2, glutathione-S-transferase 4, superoxide dismutase 1, catalase 2, *C.elegans* p53-like protein 1, and apoptosis enhancer 1 genes as compared to controls. This was determined by gene expression profiling of young adult *C.elegans*. The LC50 acute toxicity order for different metals in this study was Pb> As > Cr> Cd. The nematodes showed a high level of tolerance to Cd (cdr-1 corresponds to *C.elegans'* tolerance to Cd exposure).

A neurotoxicity study in *C.elegans* was performed by Williams and Dusenbery in which the effects of four metals – copper (Cu), beryllium (Be), mercury (Hg), and lead (Pb) - and two organophosphate pesticides - malathion and vapona, were investigated. Cu and Be were chosen as controls since they do not have a neurotoxic effect. The results indicated that rate of movement of exposed nematodes could be used as an indicator of neurotoxicity (Williams and Dusenbery, 1990).

These references clearly reveal the existence of a significant “stress” response in *C.elegans* to a number of heavy metals. Previous work in our laboratory (Troast et.al, 2006) evaluated mixed cultures of *C.elegans* following exposure to lead. The results of these studies suggested that lead as a heavy-metal stressor would be appropriate for our studies.

HPLC coupled with electrochemical detection (Coularray) as a detection tool

Metabolites have typically been studied using high performance liquid chromatography (HPLC) and columns with specific separation properties, in combination with specialized solubilization and concentration steps. This has sensitivity limitations. Matson *et al*., (1984) described the coulometric electrochemical-detector array system (CEAS) HPLC, Inc. (Chelmsford, MA, USA). CEAS has four cells with four electrodes in each; generating sixteen electrochemical detectors set from 0 to 900. CEAS consists of coulometric electrodes in series to increase the selectivity and resolution for the direct analysis of electroactive compounds. The potentials applied to these cells are set from 0 to 900 millivolts, in 60 millivolts increments. Detection involves redox reactions occurring at the voltage of the channel. The instrument is sensitive in detecting signals in the picoAmpere ranges (corresponding to femtogram or femtomole concentration levels). Any compound compatible with the extraction and separation system employed, and having a redox potential of between -1000 and + 1000mv, is detected with this system. The signals produced from the cells are saved as chromatograms. In the chromatograms, the retention time represents the time when a particular constituent of the eluant is detected. The peaks arise from the response of each of the electrodes as measured by the

electrochemical potential at which the constituent is detected, in this case oxidized, as it is being eluted.

HPLC separations coupled with coulometric array detectors have multiple advantages:

- Permits study of redox metabolites in complex biological matrices
- Allows for detection of analytes having oxidation potential with 100% efficiency
- Increases analyte resolution by adding electrochemical potential to time and current

Purine, tyrosine and tryptophan metabolites detected by HPLC coupled with electrochemical detection

Three classes of metabolites (Purine, tyrosine and tryptophan) were our focus for this research. Numerous of the metabolites in these pathways are detected using Coularray. These metabolites have been detected in nematode extracts using HPLC coupled with electrochemical detection (Matson *et al*., 1984; Kristal *et al.*, 2007, Patkar *et al*., 2009). Purines are widely distributed molecules that are responsible for a diverse range of effects in a number of tissues by acting as important extracellular signaling molecules in addition to their more established roles in cellular metabolism (energy production, antioxidants, and nucleic acid structural components) (Barsotti et al., 2002; Barsotti & Ipata, 2004). Purine nucleotides are degraded to free purines, salvaged and used to make nucleotides. Purine molecules act as messengers and assist in cellular

signaling (nerve conduction, muscle contraction), help in elimination of excess nitrogen from the cells and act as antioxidants. Purine metabolites also have been found to indicate oxidative stress in different biological systems. For example, uric acid exhibited neuroprotective as well as neurotoxic properties. Under conditions of oxidative stress, uric acid shifts its properties between between pro-oxidant and anti-oxidant depending on the tissue protection (Proctor, 2009). Guanosine has been shown to reduce the CD40 expression, thereby, reducing the inflammation which is considered important in numereous neurodegenrative diseases e.g. Alzheimers (Alimonte *et al*., 2007). In addition to this, guanosine shows protective effects in several neurological cell diseases (e.g. cerbral palsy, epilepsy) (Litsky *et al*., 1999; Moretto *et al*., 2005; Tomaselli, 2005). Gout and Lesch-Nyhan disease are two examples of metabolic diseases with neurological abnormalities in which purine molecules show perturbations. Understanding the metabolism of purine metabolites may help us get a better picture of the processes in the central nervous system in terms of physiology and pathology. Human pigment retinitis results in perturbations in purine metabolism causing increased concentrations of uric acid, a product of purine metabolism (Etingof, 2001).

Tryptophan and tyrosine are the amino acids that represent the first level of two signal systems, catecholamines and indolealkylamines, and are the building blocks for proteins in all the organisms. Neurotransmitters and hormones have widespread effects as chemical regulators of coordinated physiological activity within intact viable cellular systems. Neurotransmitters, such as the cateocholamines and their related compounds, act as messengers, hormones, and modulators of key control enzymes. Tryptophan and

tyrosine metabolites are multigene products providing signals with feedback capabilities to control genomic expression. Catabolic metabolism of tryptophan generates indolealkyl amines, neurotransmitters which are produced by reactions involving oxidation, hydroxylation, decarboxylation, methylation and acetylation. Tryptophan is an essential amino acid which is the precursor of serotonin. Serotonin, an indolealkylamine, is a brain neurotransmitter, platelet clotting factor and neurohormone found in organs throughout the body (Pineyro & Blier, 1999). Alterations in the metabolite profiles of the tryptophan, tyrosine and purine degradation pathways are thought to reflect life cycle states of the organism as well as changes in response to exposure to both internal and external sources and types of stress. Two endogenous neuroactive metabolites, quinolinate and kynurenate of the kynurenine pathway of tryptophan degradation, have been proposed as modulators of excitotoxic neuronal death in Huntington's disease (HD) (Cross *et al*., 1986). Serotonin is present in *C.elegans* (Horvitz *et al*., 1982) along with other neurotransmitters known to have important functions in mammalian systems such as dopamine (Sulston *et al*., 1975), octapamine (Horvitz *et al*., 1982), and tyramine (Alkema *et al*., 2005).

Tyrosine is converted to the catecholamine hormones dopamine, epinephrine and norepinephrine. These catecholamines and their related compounds act as messengers for cell signals, hormones and key control enzymes. The compounds with neurological functions play a major role in normal homeostasis processes, regulation of blood pressure, and the onset of diseases, such as, Parkinson's and, Schizophrenia (Flyckt *et al*., 2001). The alterations in the metabolites give rise to signals that are a commonality shared by the biochemistry of many organisms. In this research, by measuring the change

in these metabolites, we can measure the change/perturbation in the purine, tyrosine and tryptophan pathways at an organismal level in *C.elegans*.

Biogenic amines (serotonin, norepinephrine, dopamine etc.) serve an important function in human as well as *C.elegans* metabolism. These biogenic amines, octopamine, tyramine, dopamine and serotonin are known to act in *C.elegans* to modulate behavior in response to changing environmental cues (Butcher, 2008). These neurotransmitters act on both neurons and muscles to affect egg laying, pharyngeal pumping, locomotion and learning (Horvitz *et al*., 1982; Sanyal *et al*., 2004). Dopamine and serotonin act through membrane receptors with downstream signaling mechanisms that are homologous to those in the mammalian brain. Thus, *C.elegans* has been suggested as a useful model for elucidation of biogenic amine signaling studies in the brain. The catecholamines and indole alkylamines are of particular interest in this project as these tryptophan and tyrosine metabolites serve as important ligands, neurotransmitters and hormones that are key to the establishment and maintenance of cellular physiologic responses and homeostatic controls in *C.elegans*.

Polarographic, voltametric and coulometric measurements collectively constitute "Electrochemical Detection". These measurements help quantify conductometric and high frequency impedance measurements and aid in the monitoring of electrokinetic phenomena during electrochemical detection in liquids. HPLC with electrochemical detection (LCEC) is widely used in both clinical laboratories and physiological research to monitor catecholamines and related compounds. It separates and detects electrogenic compounds. High redox potential and rapid electrochemical reactions make the

catecholamines and their metabolites perfect for electrochemical detection. Even with a low value of background current and noise (where very few other substances interfere electrochemically) high output signals are obtained.

There are many different types of metabolomics techniques in use to analyze samples. NMR (Nuclear magnetic resonance) is a powerful metabolomics tool which studies the presence of low molecular weight compounds (mostly below 1000 Da) in biological fluids such as urine. A spectrum of lines will be obtained where some of the lines may be identified. The NMR-spectrum can be analyzed to provide valuable information as to the status of the cell, animal or subject that provided the sample (Shaw, 2006). Besides NMR, other high-throughput techniques involve the use of Mass Spectrometry (MS). MS can be coupled to Gas Chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE), which ensures the inclusion of compounds of diverse structures and physiochemical properties. Data from these systems can provide more qualitative and quantitative information about biological samples (Zhang *et al*., 2009). Data produced from the Coularray HPLC, with electrochemical detectors (ECD HPLC) provides valuable information about the profiles of redox active metabolites in biological samples, and is used in our study.

The HPLC coulometric electrochemical detector array system (CEAS), (ESA Inc., Chelmsford, MA) used in this study has four cells with sixteen electrochemical detectors set from 0 to 900, incrementing in 60mVolts. After 30 μl sample injection the sample is pushed through the column by the dual pumps, where the analytes are separated via reverse phase chromatography. The analytes get oxidized at specific voltages as they

pass through the electrochemical cells. The signals produced by the cells (the current generated which is dependent upon the redox potential of the compound and its concentration) are saved in chromatograms. In the chromatograms the retention time represents the time when that particular sample is eluted and, the channels represent electrochemical potential at which the sample is detected, in this case oxidized as it is being eluted. The instrument is sensitive in detecting signals at picoAmps ranges (femtogram or femtomol concentration levels). The elution buffer, which is called the Mobile Phase, contains electrolytes and organic solvents. The Coularray system used two mobile phases A and B (MPA and MPB) which were run together in relative percentages varying in time to increase elution and separation. A lithium phosphate buffer solution was the major buffer for the mobile phases and methanol was the organic solvent. The methods are used to separate the analytes based on their solubility, partition coefficient using the C-18 reversed phase chromatography, oxidation-reduction potentials and size. This in turn depends on the constituents used in the mobile phases. The mobile phases, samples as well as the standards are made using HPLC grade reagents and finally filtering with the 0.22µm filters. Mobile phases and methods were chosen depending on the polarity of metabolites selected for separation. The methods developed and used in the research are listed in Appendix 1. A solvent gradient with fluctuating flow rate is used to elute the metabolites. The methods used were tweaked adjusted as needed as and when possible to optimize get the best separation of the metabolites according to their retention times. The cells potentials are set in increments of 60 mV, starting with 0 mV in channel 1 (or cell 1) and ending with 900 mV in channel 16 (or cell 16). A Carbon18 column

(HR-80 {RP-C18}, 4.6 mm ID, from ESA Inc.) is used in separating the metabolites. Phase composition, column packing and the temperature of the chamber decide the flow rate of the mobile phases. The temperature chamber was maintained at 25°C in the system containing the C-18 column.

The advantages of Coularray are many. Even at low solute concentrations, the Coularray gave high sensitivity in quantitation. Many background substances present in samples do not interfere, and therefore, the sample preparation is much simpler. The sample size required is small, ranging from 10 µl to 50 µl with analyte concentrations from the femtogram to microgram range.

The Coularray technique has its pitfalls. The most serious drawback lies in the fact that the sensor, the working electrode, is in direct contact with the test solution which results in clogging of the column and sensor. Another drawback is the dependence of the electrochemical signal on the liquid flow-rate, which makes the detection susceptible to fluctuations in the pumping rate (the data experience an accordion effect of about a minute at most since it is gradient flow). The electrochemical signal also depends on temperature, but not critically. As the number of samples analyzed increases and there are changes to the environment, such as temperature, the retention time of samples may shift forward or backward creating a window of error. Therefore, it is important to run the known standards frequently during a run (about every nine to 12 sample, as controls), using the same batch of mobile phases and using the same column for all the analyses. All of these variables affect where the analyte is detected in the time line of the

chromatogram, but do not affect which electrodes set at which potentials will see the molecule and respond.

Advantages of *C.elegans* as a model system in metabolic profiling

- Analytically stable, reproducible biochemical profile patterns are produced in response to physiologic state changes.
- There are measurable, reversible physiologic states which depend upon, intracellular, extracellular and neurochemical signaling.
- *C.elegans* is a suitable model to determine the mechanism of action of neurotoxins.
- Metabolite patterns produced identify responsible genomic elements.

The effect of stress on biogenic amines levels has been studied in insects as well. Levels of tyrosine, tyramine were increased in *Periplanata Americana* L. (American cockroach) as a result of stress. Other biogenic amines such as dopamine, serotonin, epinephrine and norepinephrine were found to decrease with stress. Thus, these biogenic amines were shown to function as neuromediators and to be precursors in the biosynthetic pathways in the American cockroach (Hirashima & Eto, 1993). In the red flour beetle, *Trobolium castaneum* Herbst, larval growth was reduced following stress resulting from mechanical, thermal, optical or starvation stressors. Tyrosine, octapamine, tryptophan and 3,4-dihydroxymandelic acid were found to be in higher concentrations as measured using high-performance liquid chromatography coupled with electrochemical detection in this organism (Hirashima *et al*., 1993).

In earlier studies, Kristal *et al*., (1999) used complex chromatographic separations coupled with Coularray detectors and assayed the redox-active compounds in the mitochondrial fractions isolated from rat liver. The rationale to do this was that the liver abnormalities are a result of oxidative stress and altered antioxidant defenses which in turn affect the respiration rates and respiratory control ratios. Kristal *et al*., (1999) found that respiration was related to decreases in various purine catabolites. Using a diabetes model, they found that elevated levels of purine catabolites were not sustained due to increases in serum ketones, oxidant stress, changes in antioxidant defense systems. In moderately diabetic rats, uric acid increased threefold where as its precursor, xanthine, increased sixfold. The purine catabolites measured (urate, xanthine, hypoxanthine, guanine, guanosine and xanthosine) were decreased by approximately 30% (Kristal *et al*., 1999). If our hypothesis is correct, similar responses in the purine pathway would be expected following exposure of *C. elegans* to various stressors.

Rozen et al., used HPLC coupled with electrochemical detection to provide insights into changes in the biochemical pathways of patients with motor neuron disease (Rozen, 2009). Pertubations were found in the metabolomes of diseased patients compared to controls. Metabolic profiles comprising of 300 metabolites showed a down regulation of metabolites in the patients with motor neuron disease. Metabolic profiles of stressed neamtodes are different than profiles of the control/untreated nematodes (Sudama, 2008).

Perturbations in tyrosine, tryptophan and purine pathways were measured in cocaine dependent subjects and healthy controls using HPLC coupled with

electrochemical detection system. N-methylserotonin and purine pathway metabolites (guanosine, guanine, xanthine, xanthosine, hypoxanthine and uric acid) were perturbed in the cocaine dependent subjects compared to the healthy controls (Patkar *et al*., 2009).

The data obtained is much richer than just the quantitative behavior of the pathway metabolites followed because these metabolites are known to function as key agents essential to homeostatic control and are compounds for which we have a set of standards allowing for identification and quatitation of these compounds in the datasets we obtain. There are other treatment- specific unknown peaks, some of which have been shown in the different sections below.

The data generated in this research allows for detecting and tracking any significant alterations in metabolic flux. This particular approach allows the detection and representation of significant alterations in three key fundamental metabolic pathways- purine, tryptophan and tyrosine.

Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg) contains 16 main data bases divided into systems, genomic and chemical information. KEGG pathways are manually prepared metabolic pathways which are continuously updated using the latest published data.

3. MATERIALS AND METHODS

C.elegans culture

1. Axenic culture (Pure, sterile cultures of *C.elegans* not contaminated by or associated with any other living organisms**)**

The initial culture of *C.elegans* was acquired from Dr.Zuckerman in 1989 (wild type-N2 Bristol strain), University of Massachusetts, East Wareham, Massachusetts. Successful culture of *C.elegans* requires a large surface to volume ratio for optimal growth. The culturing process is described in detail below:

- 1000 ml media was made using 40 g of soy peptone (BD, catalog #243620) and 10 g of Bacto yeast extract (BD, catalog # 212750) dissolved in 900 ml of distilled water and sterilized by autoclaving.
- HLE nutrient preparation is described in section "Preparation of Heated Liver Extract (HLE)".
- 15 ml aliquots of the prepared media were pipetted into ten 125 ml Erlenmeyer flasks. The flask mouths were covered with aluminum foil and placed in an autoclave to be sterilized for 20 mins at 120°C.
- Class 2 biohood interior was subjected to ultraviolet light and surfaces were cleaned with 95% ethanol; this was used for the whole process of *C.elegans* subculturing.

- Sterile disposable 10 ml and 1 ml pipettes, pipettors, an ethanol lamp and the cooled sterile media aliquots were then placed under the hood. The hood interior and its contents were sterilized with ultraviolet light for at least five minutes.
- The exterior of a 14 day old culture flask of *C.elegans* and a container of thawed sterile, heated liver extract (HLE) was cleaned with ethanol and placed into the sterile hood. Hands were washed thoroughly with ethanol.
- The sealed flasks containing the sterilized media were uncovered and 0.45 ml aliquots of HLE (Description and protocol as given below) were pipetted into the 10 flasks.
- 1 ml aliquot of the *C.elegans* in media was pipetted into these 10 flasks. All the flask tops were flamed with the ethanol burner to prevent contamination before resealing. On each subculture the flasks was labeled with the date of the subculture and the persons initials.
- All flasks with *C.elegans* were placed in an incubator in the dark at 21° C. The remaining uncontaminated sterile HLE was placed in the freezer for future use. The subcultures were allowed to grow for 14-16 days before harvesting (The cultures were periodically checked every 3-4 days to make sure that no contamination was present.)

<u>Preparation of Heated Liver Extract (HLE) (Sayre *et al*., 1963)</u>

- Freshly excised lamb, horse, calf or pig liver was obtained from a recently slaughtered animal and placed on ice. The connective tissues and fat were removed and the liver was cut into 1 inch cubes. It was maintained at 4° C for 24 hours.

- 200 g of liver with 200 ml of distilled water were homogenized in a Waring Blender for 2 minutes.
- Aliquots of up to 1000 ml of the homogenized liver were heated in a water bath maintained at 53° C for exactly 6 minutes with constant stirring.
- The heated and coagulated homogenate was placed in an ice bath and stirred till the temperature fell to 10° C. The liver homogenate lost its homogeneity and became granular with red streaks when it was ready to be cooled. The material was allowed to stand in an ice bath until it was centrifuged.
- In a high-speed refrigerated centrifuge (Sorvall SS-34) the homogenate was centrifuged at 10° C for 30 mins at 18000 rpm. The supernatant was decanted into 20 ml vials and frozen at -20° C for subsequent centrifugation and sterilization.
- The supernatant was vacuum filtered through a 0.45µm filter and then filter sterilized through a 0.22 µm filter. This HLE was frozen until needed at about -20° C for subculturing purposes.
- 10ml HLE was added to each 90ml of cooled basal media.

<u>Basal Media</u>

For 1000ml of basal medium, 40g of Bacto-Soytone and 10g Bacto-Yeast extract were dissolved in 900ml super-Q water (18mOhm) and autoclaved at 15psi for 15 minutes.

2. **Monoxenic culture** (Wild type *C.elegans* grown on *E.coli* OP50)

Initial monoxenic cultures were acquired from the Caenorhabditis Genetics Center (CGC), Minneapolis, Minnesota. Stocks were maintained on Nematode growth medium (NGM) plates seeded with 100ul of OP50, a uracil- requiring mutant of *E.coli*, and

incubated at 15°C in an oven. Petri dishes (9 cm) were used and cultures were subcultured every 7 days (Brenner, 1974). Use of the uracil requiring mutant of *E.coli* (OP 50) prevented overgrowth of the bacterial lawn. The medium contained limited uracil, thus the bacteria were unable to grow into a thick lawn obscuring the worms. Harvesting and age-fractionation of 14 day old *C.elegans* cultures was achieved following the protocol of Willett et.al, 2010.

Photographs

Photographs were taken of the age fractions using a Leica M2 IGFA (10445930) with software NIS elements (D2.20). Each sample was diluted with water (as needed), and a 100 ul volume placed onto a depression slide. Before the photographs were taken, the slides were placed in the refrigerator, at 4°C, for 3 minutes (to slow down the motility of the *C.elegans* resulting in better photographs). An ocular of 40X and exposure time of 1millisec was used. Subsequently the contrast and darkness of the photographs were evaluated and modified using Microsoft Office Picture Manager, 2006.

Biological protocols

This section includes a summary of the all the biological protocols used. The effect of *B.anthracis* vegetative cells, spores, different toxins of *B.anthracis*, LPS and soluble lead were assessed for early stress responses within tryptophan, tyrosine and purine pathways in *C.elegans*. All the treatments and controls were performed with five replications. At the start of each study the counts were made for the mixed population to establish the numbers for young, middle and adult nematodes. Similarly, at the end of every study the mixed population of nematodes was counted to account for the number of

young, middle age and adult nematodes. Three counts were performed by making 1: 10 dilution of the mixed population and performing a 10 µl count on a light microscope (Olympus Tokyo CK light microscope).

Study 1

a. *C.elegans* exposed to *B.anthracis* vegetative cells

Stress related responses of *C.elegans* were assessed after exposure to vegetative cells of *B.anthracis* (Sterne strain). Vegetative cells of *B.anthracis* were grown on TSB (tryptic soy broth) (grown on heat block at 37°C) till the turbidity reached 0.222 at 640 nm. Both axenic and monoxenic cultures were tested. The treatments included 2ml of fresh axenic culture media (soy peptone+HLE), 50 µl of vegetative cells and approximately 2000 worms (14 day old mixed population). Harvesting and age-fractionation of 14 day old *C.elegans* cultures was achieved following Willett et.al, 2010. It was determined by trial that 2000 individual gave strong signals and full metabolite profiles with on the ESA coularray. Fresh axenic culture media was used for all treatments to minimize further growth of bacteria on the plates, and to avoid clumping of worms. Controls were set as mixed population of worms in fresh axenic culture media with no vegetative cells. *B.anthracis* treated worms were harvested after 2.5 hours of exposure using 10 µm nylon filters (Spectrum Labs, Rancho Dominguez, CA; the filters did not have to be pre-treated). The plates were given 2X washes with 0.03% saline to harvest the remaining worms on the plates. These washes were necessary in order to wash the media (OP50 in case of monoxenic cultures) off of the worms' outer surface/cuticle.

b. *C.elegans* exposed to *B.anthracis* spores

Stress related responses in *C.elegans* were assessed after exposure to spores of *B.anthracis* (Sterne strain) (Colorado Serum, Denver, CO, USA). 50 µl (1.3 X 10^9 /ml concentration of spores) spores of *B.anthracis* were added to 2ml of axenic culture media (soy peptone + HLE) and approximately 2000 worms (14 day old mixed population). Controls were set as mixed population of worms in fresh axenic culture media with no spores. Controls and treated worms, exposed to B. anthracis for 2.5 hours, were harvested using 10 µm nylon filters. The plates were given 2X washes with 0.03% saline to harvest the remaining worms on the plates.

c. *C.elegans* exposed to *B.anthracis* culture supernatant

Stress related responses in *C.elegans* were assessed after exposure to *B.anthracis* (Sterne strain) culture supernatant (overnight culture). A loop of spores (1.3 X 10^9 /ml concentration of spores, ~50 ul/loop 6.5x10-8mL) were mixed in 3 mls of tryptic soy broth in a glass test tube at 37°C to germinate and grown overnight. The overnight culture was spun at 15000 rpm and the pellet was discarded. A 50 µl sample of culture supernatant was added to 2ml of axenic culture medium (soy peptone + HLE) and approximately 2000 worms (14 day old mixed population). Controls were set as mixed population of worms in fresh axenic culture media with no culture supernatant added. Treated worms were harvested after exposure for 2.5 hours using 10 µm nylon filters.

Study 2 - *C.elegans* exposed to *B.anthracis* toxins Protective Antigen (PA), Edema Toxin (EF) and Lethal Toxin (LF)

Stress related metabolic profile responses of *C.elegans* following exposure to individual toxins of *B.anthracis* (Sterne strain) individually and to toxin combinations were explored using the Coularray analytic system. The toxins were ordered from LIST Biological laboratories Inc. 1 µg of each individual toxin [(Protective antigen (PA), Edema toxin (EF) and Lethal toxin (LF)] was added to approximately 2000 worms (14 day old mixed population) in 2ml of fresh axenic culture media. The dual combination treatments were (also) tested with PA+EF, PA+LF and EF+LF. The final treatment included the three toxins in combination (each toxin was provided at 1ug/mL) Controls were set as mixed population of worms in fresh axenic culture media with no toxins added. These treated cultures were harvested using 10 µm nylon filters after exposure for 2.5 hours. The plates were given 2X washes with 0.03% saline to harvest the remaining worms on the plates.

Study 3 - *C.elegans* exposure to Lipopolysaccharides from *Escherichia coli* O111:B4

Stress related responses in tryptophan, tyrosine and purine pathways in *C.elegans* were assessed after exposure to lipopolysaccharides from *Escherichia coli* O111:B4. The lipopolysaccharides were ordered from LIST Biological laboratories Inc. (Campbell, CA). Lipopolysaccharides were tested at different concentrations, 5 µg/ml, 1 µg/ml and 0.2 µg/ml on approximately 2000 worms (14 day old mixed population) in 2ml of fresh axenic culture media. Controls were set as mixed population of worms in fresh axenic culture media with no lipopolysaccharides

added. Treated worms were harvested after exposure for 2.5 hours using 10 µm nylon filters. The plates were given 2X washes with 0.03% saline to harvest the remaining worms on the plates.

Study 4 – *C.elegans* exposure to soluble Lead (Pb)

Axenic culture: Stress related responses in *C.elegans* were assessed after exposure to Pb at different concentrations, 250 ppm, 500 ppm and 1000 ppm in the form of lead acetate (Fisher Scientific, Fairlawn, NJ). Mass cultures were grown in four 150 cm^2 Corning cell culture flasks from Corning for this experiment with fresh axenic culture media. After 15 days, the cultures from four flasks were subjected to age-fractionation to separate adults (A), middle age (M) and young nematodes (Y). Following age-fractionation, the individual age groups (Y, M and A) were pooled and then divided into five replicates. These replicates were grown in axenic culture media with different concentrations of lead in 125ml Corning flasks. One stock solution of 1000ppm was prepared and appropriate aliquot was added to achieve 250 ppm, 500 ppm or 1000 ppm in the fresh axenic medium. Approximately 500 adults and 500 middle-age worms were used for each treatment and 1000 young nematodes were used. Controls in these cases were age-fractionated worms with no lead added. The treated worms were harvested after growing for one week using 10 µm nylon filters. The plates were given 2X washes with 0.03% saline to harvest the remaining worms on the plates.

Monoxenic cultures: Monoxenic cultures were grown in 60mm plates with OP50 (*E.coli*) lawns, harvested and age fractionated for use in the lead treatments as described above for axenic cultures.

Sample preparation and analysis

Sample preparation

- *C.elegans* samples were removed from the freezer and homogenized with an ultrasonicator (60 Sonic Dismembrator from Fisher Scientific) with a microtip at level 4 (400 watts) for 30 sec. The samples always were kept chilled in an ice bath. The micro tip was washed after processing each sample with 50% methanol to prevent cross-contamination. The sonicated samples were centrifuged for 15 min at 14000 rpm using a centrifuge (Eppendorf Centrifuge 5415 C, Brinkmann). The supernatant was pipetted into a small syringe fitted with 0.22 µm filter (with Cameo 3N Syringe Filter, Nylon, 0.45 Micron, from Micron Separations Inc., Westborough, NY) and filtered into an HPLC vial (0.25 ml vials from Sun Inc., Rockwood, TN) and appropriately labeled.
- Mobile phases A and B were prepared with the lithium buffer stock solutions. Lithium buffer stock solution was prepared by adding 84 g of lithium hydroxide to 300 ml of 18.2 mega ohm water. 160 ml of phosphoric acid was added to it. After the lithium hydroxide dissolved, the volume was brought upto 900 ml with distilled water. Adjust the pH to 3.0 with phosphoric acid. The solution was then vacuum filter sterilized with 0.22 µm nylon filter paper. Mobile phase A (1 liter) is made by combining 40 ml of stock buffer, 10 mg of lauryl sulfate (dodecyl lithium sulfate) and

960 ml of double distilled water. Mobile phase B (1 liter) was made by combining 40 ml of stock buffer, 100 mg of lauryl sulfate and 600 ml of methanol B. Mobile phase was vacuum filtered with 0.22 µm nylon filter paper.

Chemical standard mixtures were prepared from standards of known concentrations, (50 µg/ml for dopac, tyrosine, norepinephrine, 5-hydroxy tryptophan, kynurenine, dopamine, tryptophan, melatonin, 3-hydroxy kynurenine, L-dopa and the other related metabolites, dissolved in mobile phase A. Majority of the individual standards (HPLC grade) were obtained from Sigma. They were dissolved in water and/or 0.1% formic acid, stored and frozen in smaller subsets of 1mL at -80°C. Standard concentrations as individuals and in cocktails were not greater than 1µg/mL. The vials of samples, standards and the mobile phases were placed in the respective positions on the HPLC machine. The HPLC run was comprised of known concentrations of standards, and the samples to be analyzed. On the CEAS HPLC system the sample injection, the mobile phase flow rate and the gradient were programmed into the computer connected to the Coularray system. The potentials of electrochemical detectors were always set at 0-900 mv at 60 mv intervals. Analysis of each sample approximately took 140 min. The Coularray HPLC system is comprised of two ESA solvent delivery systems (Model 582), ESA autosampler (Model 540), and Waters Bondapak C_{18} 3.9x150 mm Column, Coularray Detector with Sensor Model 6210, ESA 16 channel detectors (4 cells) and Coularray for Windows (version 1.12) software.

A lithium buffer stock was used in both mobile phases A and B. Under the fume hood, 300 ml of double distilled water (using an ElgaStat Maxima Ultra Pure Water System,

with the water purified at 18.2 ohms) was added to 84 g of lithium hydroxide (Fisher Scientific, L128-500), in a one liter beaker. Using a Thermolyne Nuova II Stir Plate with a magnetic stirrer, 160 ml of phosphoric acid (o-Phosphoric acid 85 %, A365-4, from Fisher Scientific) was slowly added to dissolve the lithium hydroxide. The volume was made up to 900 ml using double distilled water. The pH was adjusted to 3.0 with phosphoric acid using a pH meter (Beckman φ720 pH meter) and the pH was again checked the next morning. Lithium stock buffer solution pH was maintained between the ranges of 3.0 ±0.3. Using a 0.22 µm filter paper (Whatman GF filter paper, catalog no. 1001 090) lithium stock was vacuum filtered. One liter of MPA was made by dissolving 10 mg of lauryl sulfate (dodecyl lithium sulfate from Sigma, L-4632) in 40 ml of lithium stock buffer and the final volume was made upto one liter by adding (approximately 960ml) of double distilled water. The pH for MPA was adjusted between 4.3 and 4.4. MPB was made by dissolving 100 mg lauryl sulfate in 40 ml of lithium stock buffer and 360 ml double distilled water before adding methanol (approximately 600 ml, HPLC grade from Fisher Scientific, A452-4). Both mobile phases MPA and MPB were filtered using 0.22 µm nylon filter paper. Standards (known compounds of interest) were prepared as stock solutions of 1 mg/ml. For the runs in HPLC 1µg/ml concentration aliquots were made using the stock solutions. The HPLC vials containing the samples were placed on the autosampler with methods and series programmed for running the samples. The ‘method’ within the autosampler controls the volume of the sample to be pipetted for analysis. A 25 µl sample size was selected for analysis because it gives highly reproducible results. Controls were set to wash the pipetters with 50% methanol

solution after every new sample was injected into the system. Each “series” covered one method type. An experiment can be programmed with several series which incorporates several methods. Within the “method” one determines how many injections from the sample are needed. After the autosampler was programmed, the software method developed was then loaded and run from the Coularray software, on the computer. This software method controls and communicates with the autosampler.

Data Analysis

The software developed for Coularray for Windows “Data Processing Module Version 2.00” uses the raw data generated from the LCEC run and converts it into useful information. The raw data was captured as files meant to be viewed as chromatograms; the files were initially saved as study groups within the software. Under the section, “work with collected data”, the data was stored and analyzed. The analysis processes provided in the Coularray software include: filter method, peak detection method, peak table method, calibration method and report method. An active filter method was chosen first, wherein baseline subtraction of the raw data is carried out. For the analysis, chromatograms processed using the baseline reduction to achieve noise reduction in the raw data. Four choices are available to assist in smoothing of the data: none, low, medium and high. The medium data subtraction filtration method was used based on the software manual suggestion for the gradient separated data. Peak name tables were created using a standards sample consisting of mixtures of the desired metabolites. Each known metabolite has a specific retention time, set of channel responses, and peak characteristics. The limits for base width, minimum shoulder, the minimum peak height,

the minimum half width and the maximum half width were selected manually for the peak name table. Here the minimum peak height was set to 2.0 nA. The steps to create the peak name table were guided by a software wizard. An active calibration method was then developed to analyze the standards cocktails at various known concentrations using the wizard in the software. The chromatograms of the samples were analyzed against the active peak name table to identify the known peaks. The chromatograms also show unknown peaks. The peak information meeting our peak identification criteria from each chromatogram was then exported into Excel (a matrix of 16800 X 16 data points of which this research used 16800 X 15 data points). This data was then standardized using the protein concentration of the samples as a proxy for the number of cells.

Subtraction Algorithm

Involved in the external data manipulation is the conversion of the collected unprocessed chromatographic data set to matrix data by first importing the data into CEAS512 software, which has the capabilities of transferring the chromatographs into text files, in matrix format. This data is in a tabulated matrix format, of 16 columns and over 9000 rows. Each column represents a data channel, the voltage at which the measurement is made. There are 16 channels with voltage increments of 60 mVolts. The captured chromatographic data is done approximately every 0.008 minute. The time line is the row. This matrix data is then read and processed by the subtraction algorithm.

Embedded in the subtraction algorithm is a normalization factor that uses the soluble protein data (from the protein assay) for each sample to normalize the chromatographic matrix data against gram of soluble protein. The purpose of the subtraction algorithm is

to find out if there are unique compounds with certain conditions, hence metabolic signature patterns per condition. In the subtracting algorithm the two text (matrix A and B) files are read, then normalized. The wobbling is removed and then subtraction is performed. The subtracted data is stored into file C in the matrix format. Finally one decides if to capture data for certain channel(s) and time frame and save all three files (A, B and C) in one file. This file can then be viewed in Microsoft Excel.

Protein Assay

The samples in MPA fractions were analyzed for their soluble protein content, using the standardized Microassay Procedure available from (Bio-Rad, 1069), according to the supplier's instructions. The standard curve was generated using known concentrations of Bovine Serum Albumin (BSA), diluted in MPA. The procedure was carried out in 96 well ELISA plates and the absorbencies were measured at 595 nm, using a spectrophotometer plate reader (μQuant Biotek Instrument Inc., Winooski, VT).

Samples (*C.elegans* cell lysate) were assayed for total protein content using the Bradford protein assay procedure (BIO-RAD, PI23236). The amount of soluble protein measured in *C.elegans* samples was used to normalize the chromatographic data, obtained from CoulArray HPLC analysis. The CoulArray generated chromatograms are a representation of the electrochemical signals of the redox active compounds (peaks), separated by HPLC. The chromatograms were converted into text files using a module of the CoulArray software (Data Module Version 2.0), a Perl script and CEAS_511 software provided by ESA. Each chromatogram was converted into a one-dimensional matrix using a script written in Python (Version 2.4). The chromatographs of interest were

normalized (corresponding Bradford protein assays) and merged into one file using the Python software (Sudama, 2008; Willett et.al, 2010). This dataset was then imported into Array Studio software (Version 3.6, Omicsoft Corporation., Research Triangle Park, NC, USA), where Principle Component Analysis (PCA) was performed to extract relevant information from the data sets or sample types.

Metabolic profile analysis of each age fraction obtained was performed in five replicates and p values obtained from the statistical analysis of the resulting data sets were <0.05. The Student *t*-test (unequal variances) was used to calculate the significance of different treatments for the p-values of the respective treatment. In applying PCA to the data, the p value was set to <0.05.

The text files generated from the CoulArray chromatographic data (from cohorts **Y**, **M** and **A**) were used to generate PCA and line graphs. Data from two different cohorts (*e.g*. Cohorts **Y** and **A**) were subjected to the subtraction algorithm written in Python. These data demonstrated differences in the age fractionated cohorts using the subtraction algorithm (Sudama, 2008). The peaks from the second cohort (*e.g.* Cohort **A**) were aligned to the peaks of the first cohort (*e.g.* Cohort **Y**) and the data was normalized against soluble protein data for the respective cohorts. Differences were obtained and displayed by subtracting cohort **Y** data from cohort **A** data.

The KEGG pathway databases were used to trace the metabolite flux within the three key pathways for which quantitation of specific member metabolites was possible using the analytic system described.

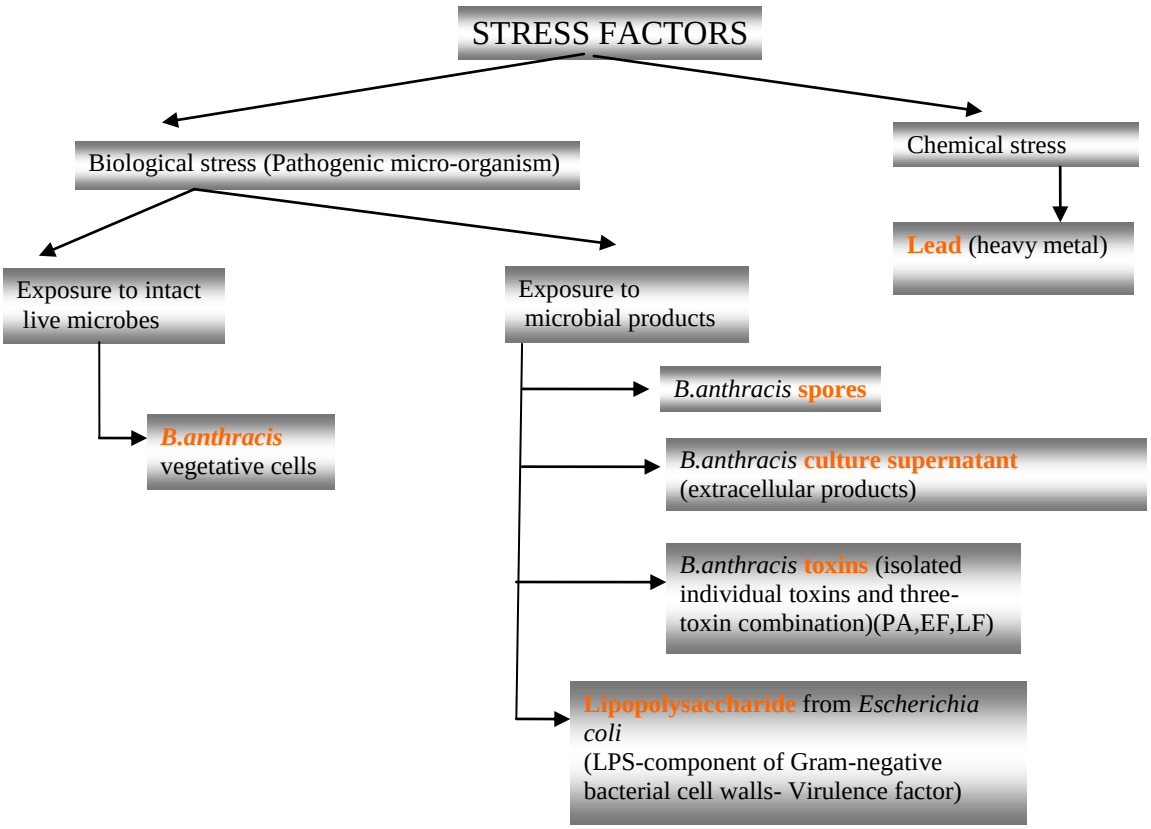


Figure 1: Flowchart of stressors used in this research

4. RESULTS AND DISCUSSION

Following the complete sequencing of the *C.elegans* genome in 1998, (CE sequencing Consortium *et.al*, 1998) many of the known biological components including physiological processes and signaling pathways were shown to be conserved in *C.elegans* and mammals (USNRC, 2000). A large number of *C.elegans* genes have been found to have homology with mammalian genes (homologues for 60-80% human disease genes have been found) (Kaletta and Hengartner, 2006). *C.elegans* has proved to be a good model for assessing the potential for neurological and developmental toxicity in diseases such as Huntington's, Parkinson's and Alzheimer's disease. This organism also has been used as a model for the toxicity of pesticides, heavy metals and pathogenesis of different diseases (Helmcke et. al., 2008, Nass and Blakely, 2003). The nematode is sensitive to many substances that function as human toxins and to pharmaceutical compounds which activate molecules and pathways found in mammals. In many of the cases, these toxic compounds were found to induce similar physiological and pathological responses in *C.elegans* as in mammals. *C.elegans* had also proven to be a good model to illustrate the metabolic phenotype resulting from the interaction of the genome and the environment. This has been well represented by showing the difference between non-frozen and frozen nematodes using NMR (Bollard *et.al*, 2005; Blaise *et .al*, 2009). This is in accordance with the current project where the metabolic profile of the nematode was specific

depending on the perturbing agent, the specific protocol performed or the nutritional medium the nematode was grown in.

This research was designed to investigate the perturbations in three of the metabolic pathways (purine, tyrosine and tryptophan) within the nematode as a result of application of different stress factors. Our assumption was that metabolites derived from the amino acids tryptophan and tyrosine are among the most sensitive indicators of toxicity to *C.elegans* based on the fact that many of these metabolites act as key regulatory control elements in cellular homeostasis. These homeostatic control agents regulate catabolic pathways in *C.elegans* such as glycolysis, energetics etc. and the metabolites produce from these pathways essential to biosynthesis of the molecular constructs required in the nematode life cycle (Johnson *et.al*, 1981; Horvitz, 1982, Alkema *et.al*, 2005). The research presented here focused on rapid metabolic changes generated by exposure of *C.elegans* to various environmental and biological stress factors. Emphasis was placed on monitoring of tryptophan, tyrosine and purine metabolites indicative of early stage cell-signaling in *C.elegans* (Bargmann, 1998). In addition, the effect of nutritional differences on *C.elegans* metabolic profiles was addressed as most studies reported in the literature employed monoxenic cultures (*C.elegans* grown with *E.coli*) whereas these studies used both axenic (*C.elegans* grown on laboratory medium-ACM) and monoxenic cultures. Coularray was used to obtain metabolite profiles and for detection and quantitative analysis of metabolites of the purine, tyrosine and tryptophan pathways. All profiling experiments were performed in triplicate.

Since this research involved two different cultures, it is important to discuss the advantages and disadvantages of axenic culture and monoxenic cultures. Metabolic profiling was obtained from the genetically identical but nutritionally different nematodes, exposed to the same type of perturbing agents in this research. Most of the *C.elegans* research has been done with monoxenic worms, so there will be many results to contrast and compare. However, it requires balancing two organisms in a readily perturbed environment. Growing the worms in monoxenic conditions required that the culture be transferred frequently leading to problems such as asynchronous cultures, different growth rates and culture saturation. Ion, pH and oxygen concentrations are affected by both the worms and the bacteria in the growth medium and can alter in the required experimental conditions increasing the potential for false inferences to be drawn (Nass and Hazma, 2007). A primary issue in conducting biochemical analytic experiments with monoxenic nematodes is dealing with the fact that the biochemical machinery of two organisms is at work in the experimental system. Axenic culture avoids many of these problems, but there is far less published data so a comparative baseline was needed. The data generated from this research shows significant differences between organisms derived from different environmental (nutritional) conditions. This research also demonstrates that these organisms respond to similar treatments in a markedly dissimilar manner when grown in different culture conditions. This data is readily accessible using *C.elegans* as the organism of interest but is less accessible using almost any other model system. Axenic cultures demonstrated metabolic differences as a result of exposure to *B.anthracis* spores and three-toxin combination. Monoxenic cultures did

not exhibit any observable metabolic changes with exposure to any biological stressors. With lead exposure axenic culture analysis showed alterations to many more metabolites in the three pathways studied than from the monoxenic culture analysis.

Therefore, in this research *C.elegans* from both axenic and monoxenic cultures were exposed to different stress factors to study their effects on *C.elegans* as well as to determine the effect, if any, of the nutritional differences the separate culture conditions present to *C.elegans*.

Biological Stress Factors

Study 1 - Effects of *B.anthracis*; vegetative cells (Vc), spores (Sp) and culture supernatant (SN) on *C.elegans*

Recent studies show that *C.elegans* is host to some members of the Bacillus genus (Anderson *et al*., 2003). *C.elegans* can serve as a potential host and vector for pathogenic bacteria like *Bacillus cereus* and *B.anthracis* (belonging to Bacillus genus) (Hall and Abel-Santos, 2008). Laaberki and Dworkin (2008) tested the vegetative cells of five Bacillus species for interaction with *C.elegans*. No difference in the *C.elegans* feeding behavior of cultures grown on different bacterial lawns was noted. This is of importance because it has been shown that the metabolic activity of gut bacteria is important for the intestinal activity of the worms. In natural conditions, most Bacillus species are found in the wild as spores (Nicholson, 2002). It is likely that there is interaction of *C.elegans* and bacterial spores in the rhizosphere as the density for nematodes (>100/gm of soil) (Tate, 2000) and for spore-forming bacteria, (104–106/gm of soil) are sufficiently large to permit interaction (Martin and Travers, 1989). This research focused on subjecting *C.elegans* to one Gram-positive organism and its components, *B.anthracis*, and the lipopolysaccharide toxin of Gram-negative bacteria, derived from E. coli. *B.anthracis* was used to determine the impact of stressors such as intact, live microbial pathogens and secreted microbial products on *C.elegans*. Metabolites from the selected pathways were monitored as potential indicators of modulation of cellular processes.

PCA is a simple, unbiased and non-parametric method which is widely used in many fields to simplify a complex dataset to understand the data globally. PCA allows

one to view the high-dimensional space into a lower dimensional space (Shlens, 2009). Principal components are defined as the smaller number of uncorrelated variables derived from the large number of possibly correlated variables. The first principal component accounts for the largest variability in the data, and each succeeding component accounts for as much of the remaining variability as possible, defined here as components 1, 2 and 3.

Figure 2 represents the PCA of the normalized chromatographic data for the mixed population, axenic *C.elegans* treated with vegetative cells, spores and culture supernatant of *B.anthracis*. The PCA data for the *C.elegans* treated with spores is shown as purple spheres encircled in red. The data was analyzed through PCA to determine the number of subgroups (including controls and the different treatments) using ~ 2000 detectable analytes through Coularray. Using PCA, we found a three-dimensional display in which the spore treated nematodes were separated into four groups ($p \leq 0.05$, Figure 2). In Figure 2, component 1, 2 and 3 captured 83.03% of the metabolic differences in the data (spores treatment compared to the control).

Some of these metabolites are known compounds and some are unknowns that show marked qualitative differences. Tracking the changes in these knowns as well as unknown compounds defines the changes as a result of the different stressors. These chromatographic profiles provide patterns which reflect differences in biochemical metabolite functions inherent to axenic *C.elegans* with each treatment. This shows that the metabolic activity (measured through the electrochemical signals from Coularray) in the spore-treated axenic *C.elegans* is much different than the control or the vegetative

cell-treated axenic *C.elegans* or the culture supernatant treated axenic *C.elegans*. Some of the differences in the PCA were measured for the unknowns compounds (since our Standards cocktails lacked the compounds corresponding to those peaks) using the subtraction algorithm and the known compounds in the purine, tyrosine and tryptophan pathways. Figure 3 presents an example of differences between control *C.elegans* and spore treated *C.elegans*, using a subtraction algorithm (see chapter Materials and Methods), for a portion of the data generated from chromatographic data at 540 mV (Channel 10), between 16.67 and 41.67 minutes. Both qualitative and quantitative information on the analytes detected can be obtained using this approach. Peaks unique to control axenic *C.elegans* and others unique to spore-treated *C.elegans* were observed. The arrows in the figure 3 display the differences between control (untreated *C.elegans*) and spores treated *C.elegans* after the peaks were aligned against each other and normalized against soluble protein using a subtraction algorithm.

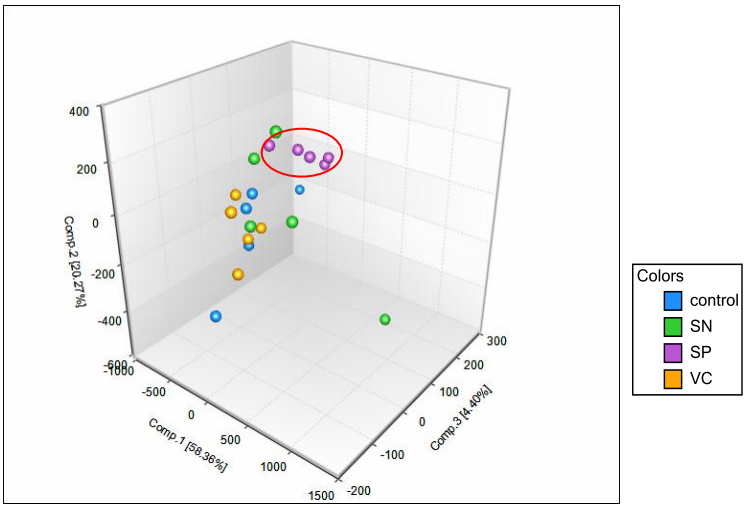


Figure 2: PCA of normalized chromatographic data obtained from axenic mixed population *C.elegans* with stressor shown. SN: supernatant. SP: spores. VC: vegetative cells

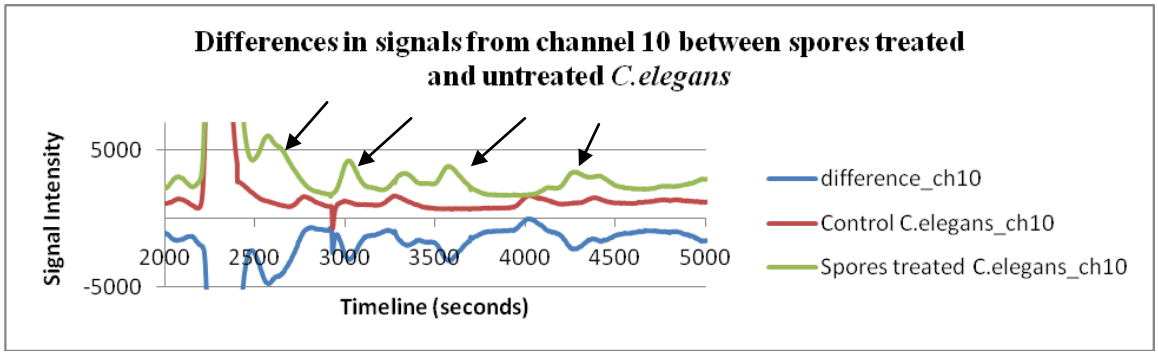


Figure 3: A line graph of the chromatographic data at 540 mV (Channel 10), between 16.67 and 41.67 minutes. Control *C.elegans* was subtracted from spores treated *C.elegans*. The difference (blue line) can be positive and/or negative depending on the presence and/or absence of peaks and their concentrations in the cohorts being compared

Metabolic pathways have evolved in a strictly regulated fashion so that the metabolites move through pathways in the correct direction and rate to maintain normal

“homeostasis” under the given conditions (Lehninger, 2008). The result of these changes in the metabolic flow in the pathways is generation of a new steady state for the organism (Lehninger, 2005; Weckwerth, 2007; Yao et.al, 2009). Therefore, the ratio of analytes along a specific pathway is a statement about metabolic flow in that pathway. Any metabolic pathway consists of a series of biochemical reactions containing precursors which generate different products. Metabolic ratios reported in this research were calculated using the product to precursor ratio in a contiguous or divergent pathway. In comparing alterations in components of separate branches of a pathway, ratios between specific branch metabolites were taken. The values for some of the metabolites which are detectable but were not measurable in the samples were replaced with the lowest detection limits of the detection system (Coularray) (see supplementary material 1). It is possible to detect hypoxanthine but with the detector system used, the variances were such that the quantitation was erratic.

Purine nucleotides and cogerminants like amino acids (histidine, tryptophan, tyrosine etc.) are required for the successful germination of *B.anthracis* endospores (Ireland and Hanna, 2002). Some perturbations were found in the purine and tryptophan pathways in *C.elegans* as a result of exposure to *B.anthracis* and its bacterial products. Guanine was found in elevated concentrations ($p<0.003$) in axenic *C.elegans* samples treated with *B.anthracis* spores compared to the control axenic *C.elegans* samples. In terms of the ratio of analytes (Tables 2, 3 and 4, $p\leq0.05$) in the purine catabolic pathway, uric acid/xanthine and guanine/guanosine ratios increased ($p\leq0.05$) while the xanthine/guanine ratio decreased ($p\leq0.05$) in axenic *C.elegans* treated with *B.anthracis*

spores. These three ratios account for the increase in guanine concentration in axenic *C.elegans* treated with *B.anthracis* spores. It can be speculated that as a result of spore treatment, the perturbation in the pathway occurs in the purine branch guanosine→ guanine→ xanthine→ uric acid (Figure 4). It was demonstrated that with cells treated with guanine (50 and 500µM concentration), the dopamine concentration was decreased in phoechromocytoma rat PC12 cells. Loeffler et al. (2000) reported that increased purine metabolite concentrations affects dopamine metabolism phoechromocytoma rat PC12 cells. No changes in the metabolite ratios of tyrosine and tryptophan pathways were observed in *C.elegans* exposed to *B.anthracis* spores at this particular exposure time period. No death or change in locomotion behavior was recorded for spore treated *C.elegans*. It is possible to detect hypoxanthine, but with the detector system used, the variances were such that quantitation was not possible.

Table 2: Effect of different biological stressors on purine ratios ($p \leq 0.05$) of axenic mixed cultures

Metabolites	*B.anthracis* spores	PA+EF+LF	*E.coli* LPS
Uric/Xan	↑	↓	-
Xan/Guan	↓	↑	-
Guan/guanosine	↑	↓	-

Table 3: Effect of different biological stressors on tyrosine ratios ($p \leq 0.05$) of axenic mixed cultures

Metabolites	*B.anthracis* spores	PA+EF+LF	*E.coli* LPS
Norepi/tyr	-	↓	-
Dopamine/tyr	-	↓	-
L-dopa/tyr	-	↓	-
HVA/tyr	-	↓	-
HGA/tyr	-	↓	-
p-OH-paa/tyr	-	↓	-

Table 4: Effect of different biological stressors on tryptophan ratios ($p \leq 0.05$) of axenic mixed cultures

Metabolites	*B.anthracis* spores	PA+EF+LF	*E.coli* LPS
Mel/Tryp	-	↓	↓
Ser/tryp	-	↓	↓
5-OH-tryp/tryp	-	↓	↓
IAA/tryp	-	↓	-
IPA/tryp	-	↓	↓
3-OH-anth/tryp	-	↓	↓
Anth/tryp	-	↓	-
3-OH-kyn/tryp	-	↓	-
Kyn/tryp	-	↓	-

Similar studies were conducted where mixed population of monoxenic *C.elegans* were exposed to *B.anthracis* and its bacterial products. With the exposure time of 2.5 hours, neither significant separation of the treatments, in PCA display nor any change in metabolites/metabolic flux in purine, tyrosine and tryptophan pathways was found. Since

monoxenic worms have been exposed to bacteria during culture, this may explain why little/no effect is seen upon exposure to *B.anthracis* cells.

Recent attempts to spread anthrax spores in the United States have brought attention to the use of *B. anthracis* as a weapon of bio-terrorism. Currently, antibiotic therapy against inhalation anthrax is administered in the early stages of infection. For the late stages of systemic dissemination or CNS invasion, no effective therapy is available. This study demonstrates that the exposure of *B.anthracis* and its bacterial products affects some of the purine metabolites in axenic *C.elegans* in the early stages of exposure. The increase in guanine and its derivatives observed here supports the proposal that it has the neuroprotective action (Proctor, 2009; Alimonte *et al*., 2007; Litsky *et al*., 1999; Moretto *et al*., 2005; Tomaselli, 2005). Similarly in this case, increase in guanine may contribute to the protective action of *C.elegans* against *B.anthracis* spores.

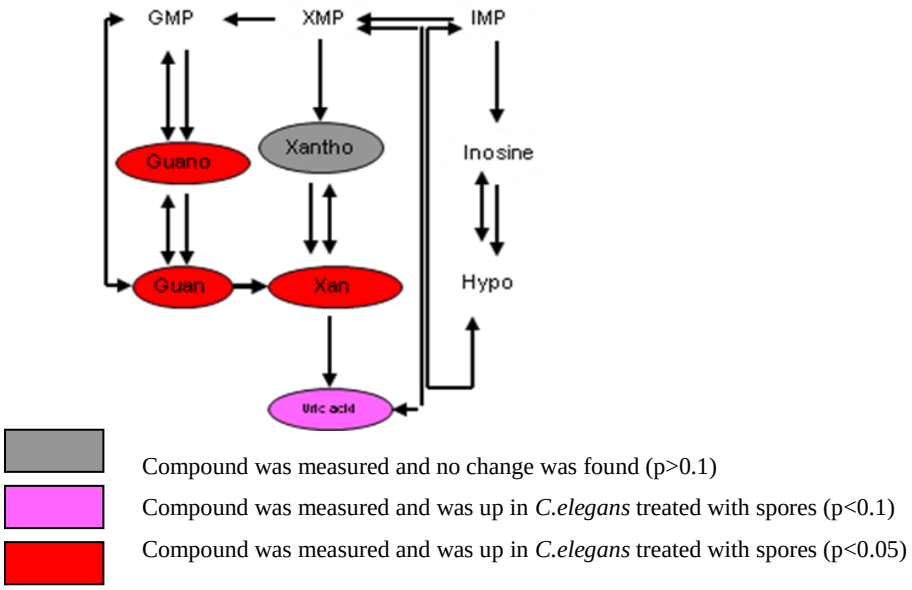


Figure 4: Effect on purine pathway of *C.elegans* after exposure to *B.anthracis* spores for 2.5 hours. Letters – metabolites, ovals – metabolites detected, arrows - flow

Study 2 – *C.elegans* exposed to *B.anthracis* toxins: Protective Antigen (PA), Edema Toxin (EF) and Lethal Toxin (LF)

In study 2, *C.elegans* responses after exposure to secreted, bacterial exotoxins were monitored. Along with vegetative cells, spores and culture supernatant as stress factors discussed in study 1, specific toxins of *B.anthracis* were used, namely protective antigen (PA), edema toxin (EF) and lethal toxin (LF). The purpose of testing these toxins individually (PA, EF, LF) as well as in combination (PA+EF, PA+LF, EF+LF and PA+EF+LF) was to study how *C.elegans* responds to these different toxins and their combinations with respect to the purine, tyrosine and tryptophan pathways and whether the nutritional differences of axenic and monoxenic cultures play an important role in triggering the same or different metabolic response to this kind of biological stressor.

Time of exposure was 2.5 hours. In the literature survey done, previous studies on the exposure of *C.elegans* to anthrax toxins were not found.

Figure 5 represents the PCA of the normalized (against the protein data) chromatographic data for the mixed population axenic *C.elegans* treated with individual toxins (PA, EF and LF) and the combination of PA, EF and LF. The PCA analysis demonstrates how metabolic profiles (as defined by Coularray) of *C.elegans* treated with individual toxins (PA-orange, EF-purple, LF- dark blue) separate spatially from the control (blue spheres) and other treatments. The data was analyzed through PCA to determine if it was possible to distinguish different subgroups (including controls and the different treatments) using ~ 2000 detectable analytes through Coularray. Using PCA, we found a three-dimensional display in which the PAEFLF (red) treated nematodes were separated ($p \leq 0.05$, Figure 5) from the control and rest of the treatments.

The arrows in the figure 6 display the differences between control (untreated *C.elegans* and PAEFLF treated *C.elegans* after the peaks were aligned against each other and normalized against soluble protein using a subtraction algorithm. In Figure 2, component 1, 2 and 3 captured 93.09% of the metabolic differences in the data (spores treatment compared to the control). Some of the examples of the unknown peaks shown in figure 6 are at timeline 2729 (corresponding to 22.74 minutes for channel 10 in metabolic profile obtained from Coularray) and at timeline 3830 (corresponding to 31.91 minutes for channel 10 in metabolic profile obtained from Coularray) increased with PAEFLF treatment.

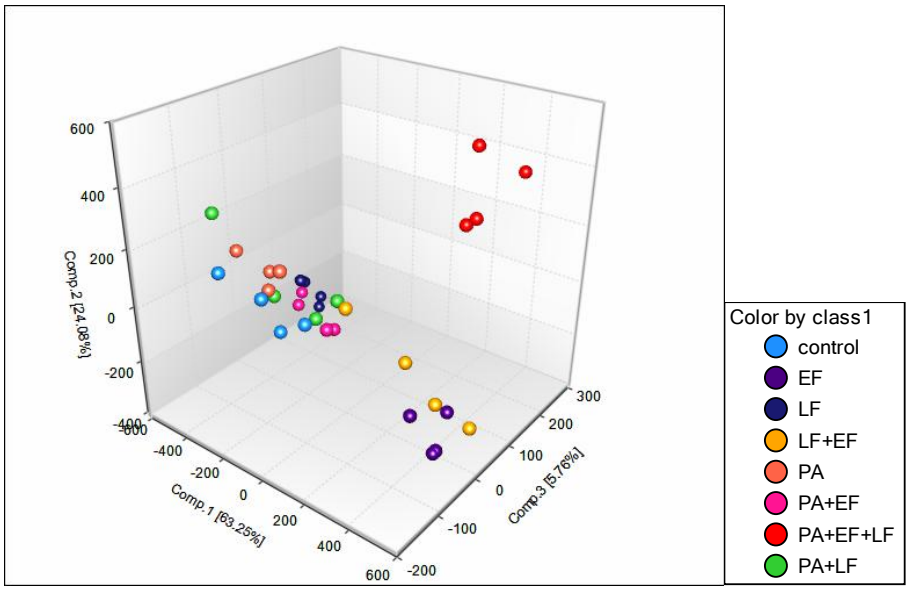


Figure 5: PCA of normalized chromatographic data obtained from mixed population axenic *C.elegans* treated with different *B.anthracis* toxins individually and in combinations

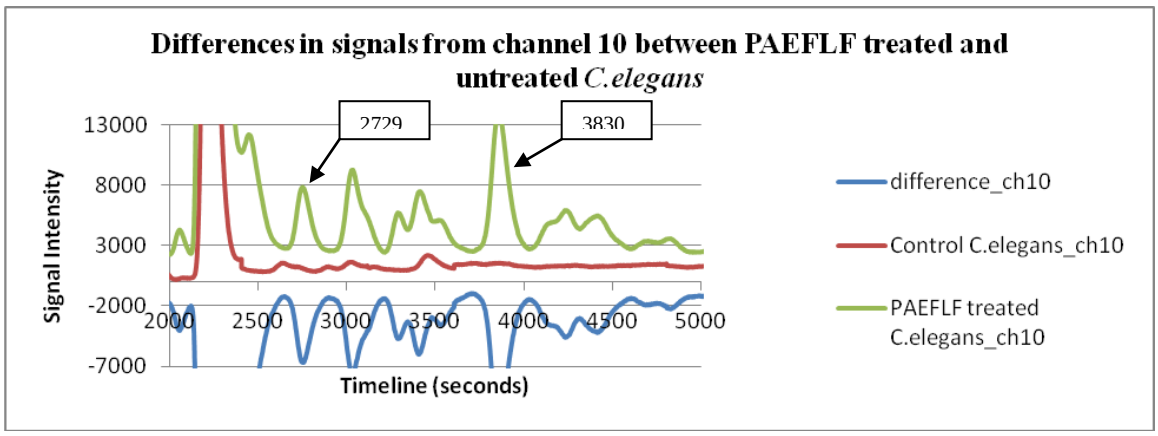


Figure 6: A line graph of the chromatographic data at 540 mV (Channel 10), between 16.67 and 41.67 minutes. Control axenic *C.elegans* from PAEFLF treated *C.elegans*

This shows that the metabolic profile of ~2000 electrochemically active metabolites (measured through the electrochemical signals from Coularray) in the three

toxin combination (PA+EF+LF) in treated axenic *C.elegans* is distinct from the control or the other toxin- treated axenic *C.elegans* (individually or in combination). These individual proteins are not toxic in *C. elegans* or humans although the three toxin combination produces toxic responses in humans and metabolic perturbations in *C.elegans*. EF acts as a calcium ion and calmodulin dependent adenylate cyclase, increasing the level of cAMP in the cell. The presence of bacteria severely affects the mammalian immune system by increasing cAMP, thereby disrupting the intracellular signaling pathways, which impairs macrophage function and finally homeostasis (Baldari et.al, 2006). Differences were found in the purine pathway of *C.elegans* following exposure to the toxin combination, PA+EF+LF. Guanosine was detected in elevated concentrations ($p \leq 0.05$) in axenic *C.elegans* samples treated with the *B.anthracis* three-toxin combination (PA+EF+LF) compared to the control axenic *C.elegans* samples. In terms of the ratio of analytes (Table 2) in the purine catabolic pathway, the uric acid/xanthine and guanine/guanosine ratios decreased ($p \leq 0.05$) and the xanthine/guanine ratio increased ($p \leq 0.05$) in axenic *C.elegans* treated with *B.anthracis* three-toxin combination (PA+EF+LF). These three ratios confirm the increase in guanosine concentration in axenic *C.elegans* treated with *B.anthracis* three-toxin combination (PA+EF+LF). It is noteworthy that, in axenic *C.elegans* treated with PA+EF, guanine increased compared to the axenic control *C.elegans* ($p \leq 0.05$). An increase in guanine concentration also was seen in axenic *C.elegans* treated with *B.anthracis* spores. It can be speculated that as a result of *B.anthracis* three-toxin combination (PA+EF+LF) treatment, the perturbation in the pathway occurs through the purine branch guanosine→

guanine→ xanthine→ uric acid. Dopamine was found at very low levels (at 0.1 nA) in the *C.elegans* samples treated with *B.anthracis* three-toxin combination (PA+EF+LF). Guanine derivatives have multiple intracellular roles in modulation of signal transduction processes. Guanine derivatives also have extracellular effects as intercellular messengers and neuromodulators in the central nervous system. Guanine derivatives also were found to protect against glutamate receptor-mediated neurotoxicity (Oleskovicz et.al, 2008). It is possible that it provides similar function in *C. elegans*.

Figure 7 represents the change in ratios of various tryptophan metabolites with respect to tryptophan whereas Figure 8 does the same with tyrosine metabolites with respect to tyrosine. All the tryptophan and tyrosine metabolite ratios (Tables 3 and 4) investigated were significantly reduced ($p \leq 0.03$). In the tryptophan pathway, the ratios demonstrate that metabolic flux is perturbed in branches leading to melatonin, indole acetate and anthranilate with PAEFLF when compared to control *C.elegans*. PAEFLF treatment in *C.elegans* also resulted in the flux change in products from the tyrosine pathway. Ratios of norepinephrine, homogenistic acid and homovanillic acid with respect to tyrosine concentration were found to be reduced ($p \leq 0.03$).

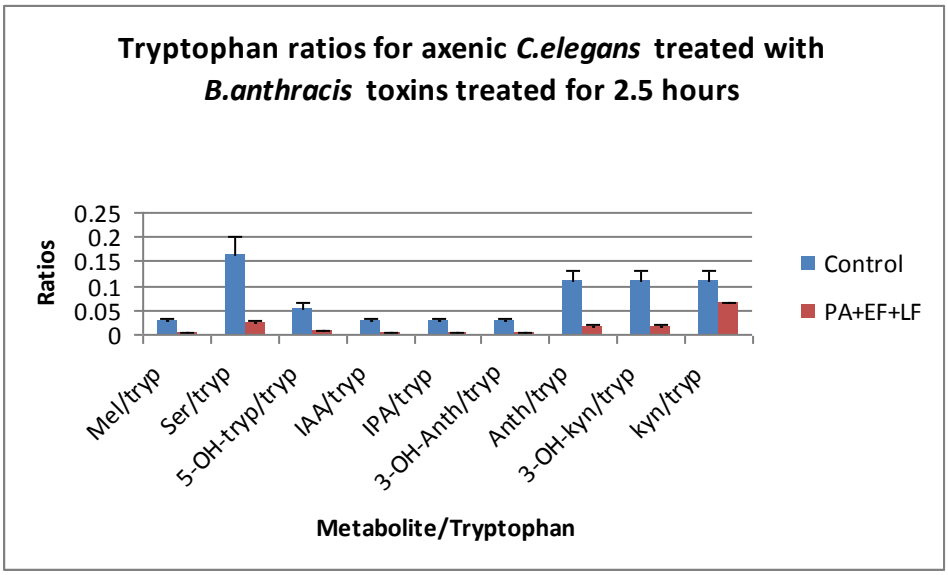


Figure 7: Bar graph representing tryptophan metabolite /tryptophan in PA+EF+LF treated *C.elegans* for 2.5 hours

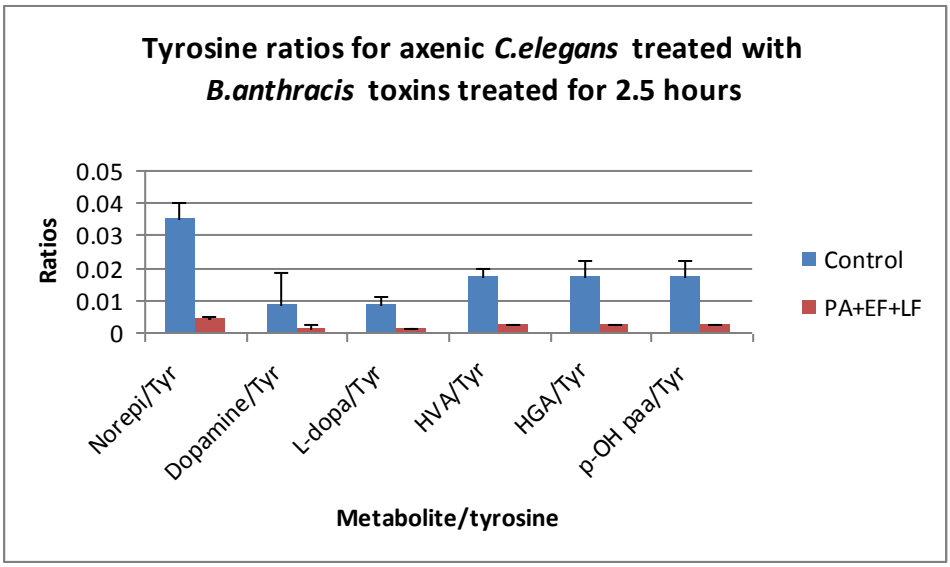


Figure 8: Bar graph representing tyrosine metabolite /tryptophan in PA+EF+LF treated *C.elegans* for 2.5 hours

Figure 9 summarizes the changes in different purine, tyrosine and tryptophan metabolites in axenic *C.elegans* samples treated with *B.anthracis* three toxins combination (PA+EF+LF) respectively. It can be speculated that as a result of *B.anthracis* three-toxin combination (PA+EF+LF) treatment, the tryptophan concentration increase, leading to the shunt in the tryptophan pathway through tryptophan→ kynurenine and tryptophan→ indole pyruvate.

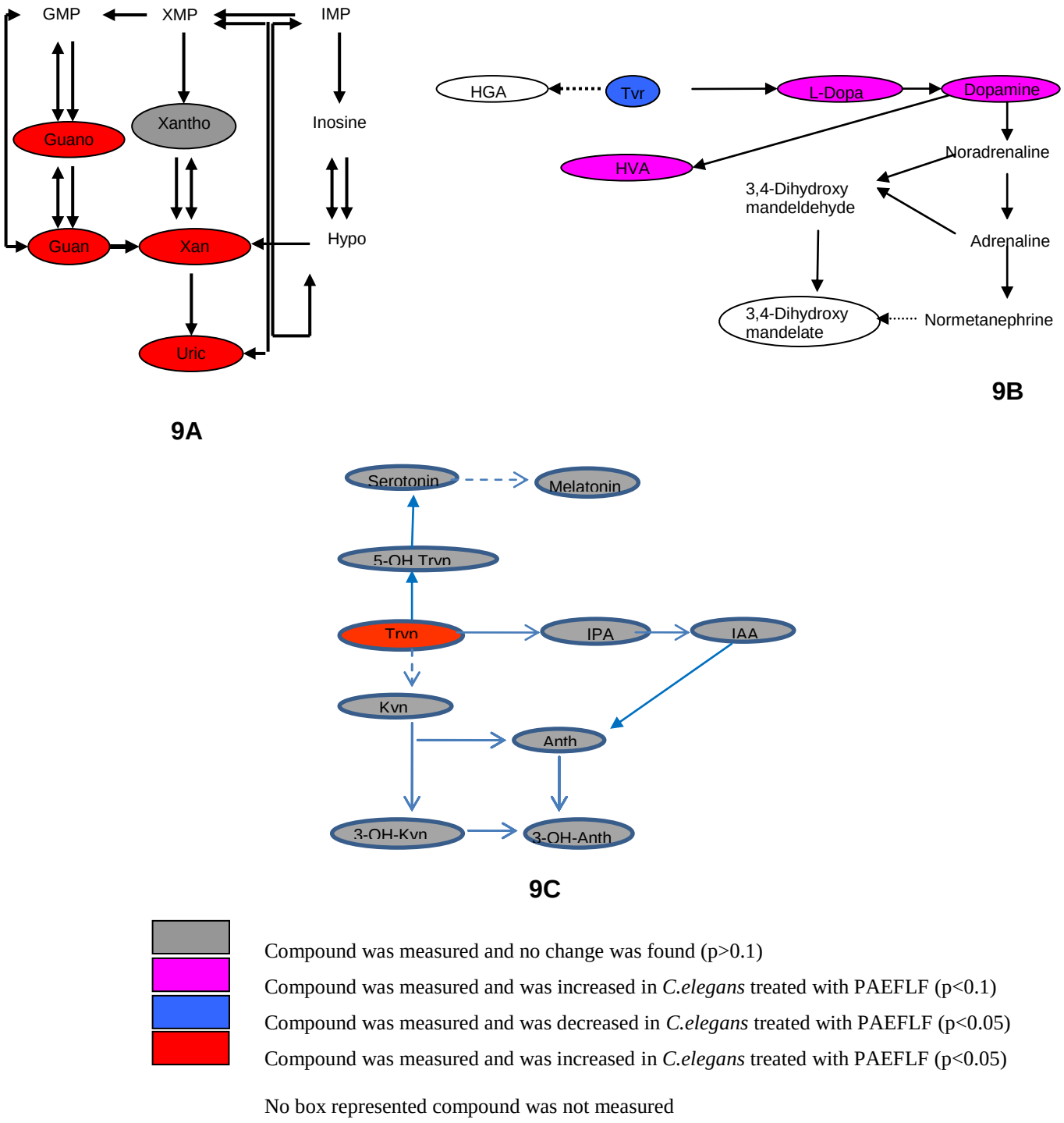


Figure 9: Changes in purine (9A), tyrosine (9B) and tryptophan (9C) in toxin challenged *C.elegans*. Letters – metabolites, icons – compound detected, arrows - flux

These data were obtained from cultures containing ~80% cohort Y, ~7% cohort M and ~13% cohort A nematodes in the mixed population used for the experiment (Appendix 3). The actual response in nematodes cultured under different nutrient culture conditions proved markedly different on exposure to the same effector agents. However, analysis of the entire analyte profile using PCA indicated that the differences from controls were not of significance. *B.anthracis* three-toxin combination (PA+EF+LF) did not affect monoxenic *C.elegans* in PCA analysis for an exposure time of 2.5 hours.

With a short exposure time of 2.5 hours, axenic and monoxenic *C.elegans* show different response to *B.anthracis* toxins in terms of the electrochemical signals detected by Coularray. Axenic mixed population *C.elegans* was affected by the *B.anthracis* PA+EF+LF treatment where there was an increase in purine metabolites guanine, guanosine, uric acid and xanthine. Axenic *C.elegans* showed similar metabolite toxicity response to anthrax as has been shown in mammals.

Serotonin has been shown to modulate locomotion of the worm as it senses and then responds to changing cues in the environment (Sawin et.al 2000), pharyngeal pumping and egg-laying in *C.elegans* (Olde and McCombie, 1997; Ranganathan et.al, 2000). Melatonin acts as a neuromodulator and regulates the locomotion in *C.elegans* and also acts as a ligand in specific receptor pathways for homeostatic control (Tanaka et.al, 2007). We speculate that treatment with *B.anthracis*, and its bacterial products, in *C.elegans* leads to a decrease in many tryptophan metabolites, a number of which are known to be responsible for locomotion, pharyngeal pumping and the egg-laying. One would then predict that treatment of *C.elegans* with *B. anthracis* spores would affect one

of these phenotypes. However after a 2.5 hours exposure time no change in locomotory behavior was observed.

Study 3 - Exposure of *C.elegans* to Lipopolysaccharides from *Escherichia coli* O111:B4

A component of the cell wall of most Gram-negative bacteria is associated with endotoxic activity, resulting in the pyrogenic effects of Gram-negative infections. Gram negative bacteria are considered generally pathogenic because of the lipopolysaccharide component of the bacterial cell wall. Lipopolysaccharide (LPS) assembly has been demonstrated as the virulence factor in many Gram-negative bacteria causing human or plant diseases (Raetz and Whitfield, 2002). Systemic administration of LPS, a pro-inflammatory cytokine inducer, has been used as one of the models of inflammation (Tilders, 1994). LPS also has been considered as a stressor that affects central neurotransmitter release: research in this area over the past few decades' shows that systemic administration of LPS involves serotonergic, dopaminergic and nonadrenergic alterations (Anisman et.al, 1993; Connor et.al, 1999). Various purine metabolites including hypoxanthine and inosine have been reported to have cytoprotective effects in response to poly (ADP-ribose) polymerase enzymes in mouse macrophages treated with interferon-γ+LPS. Poly (ADP-ribose) polymerase enzymes are found to be elevated as a result of DNA injury and oxidant-induced cell death (de Murcia, 1994; Virág and Szobó, 2001). Tryptophan and tyrosine hydroxylase enzymes were reported to be higher upon systemic administration of lipopolysaccharide by monitoring the tryptophan and tyrosine intermediates 5-hydroxy tryptophan and L-dopa (Nolan, 2000). To explore the response of *C.elegans*' to endotoxins from Gram negative organisms, mixed axenic and

monoxenic cultures were exposed to LPS; O111:B4 from *Escherichia coli*. Of particular interest was worms conditioned to the presence of a Gram negative bacterial food source which could potentially give additional protection to the worms.

LPS challenged *C.elegans* from the two genetically identical, nutritionally different cultures (axenic and monoxenic mixed population *C.elegans*) were exposed to three concentrations of LPS (0.2µg/ml, 1µg/ml and 5µg/ml). Samples were analyzed for full metabolic profile data as well as specific data on the metabolic changes in purine, tryptophan and tyrosine pathways.

Anyanful et.al (2009) demonstrated that a brief exposure of *C.elegans* to virulent or avirulent strains of enteropathogenic *E.coli* provides protection to *C.elegans* to survive a further exposure to that pathogen. They have termed this phenomenon "Conditioning", and it has been shown to require functional dopaminergic neurons and the p38 MAP kinase pathway (Anyanful et.al, 2009). Appendix 4 contains the population data for axenic and monoxenic cultures treated with LPS. This data was obtained from cultures containing ~76% cohort Y, ~6.5% cohort M and ~17.5% cohort A. PCA analysis was performed for control axenic and monoxenic *C.elegans* and *C.elegans* treated with three concentrations of LPS (0.2µg/ml, 1µg/ml and 5µg/ml) for an exposure time of 2.5 hours. PCA analysis of the complete data set did not separate cultures based on treatment status either in axenic or the monoxenic cultures. However, increased tryptophan and tyrosine metabolites were found with mixed cultures of axenic *C.elegans* treated with the highest dose of LPS (5µg/ml). This was confirmed by calculating and comparing ratios (Tables 3 and 4) to determine the metabolic flux. Ratios for melatonin, serotonin and 5-OH

tryptophan to tryptophan were decreased ($p \leq 0.03$) compared to controls (Figure 10). Similarly, ratios for dopamine, L-dopa, and norepinephrine to tyrosine decreased ($p \leq 0.03$) with exposure at 5µg/ml LPS. This is in accordance with the studies done using in-vivo microdialysis and post-mortem tissue analysis showing increases in tyrosine and tryptophan hydroxylase in rat brain cells (Connor et.al, 1999; MohanKumar et.al, 1999).

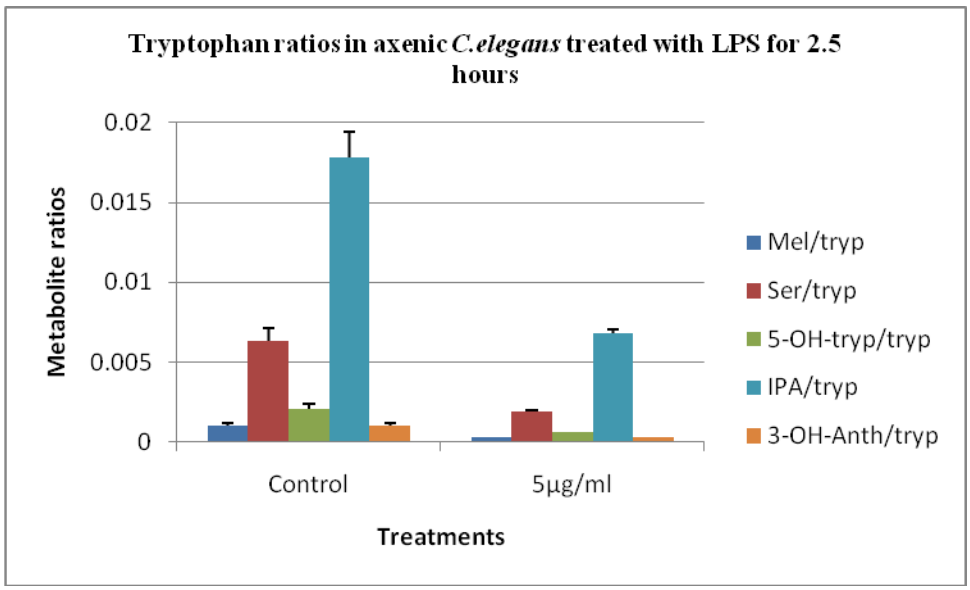


Figure 10: Ratios of tryptophan metabolites to tryptophan in LPS challenged *C.elegans*

Lead toxicity

C.elegans research in recent decades has been used to study the normal life stages of development, senescence, acute or chronic exposure to pharmaceuticals, environmental contaminants, ROS inhibitors and other potential modulators of cellular homeostatic controls (Carroll, 2003; Houthoofd, 2005; Johnson, 2007; Meyer, 2007; Leung, 2008). Studies 1, 2 and 3 investigated the effect of biological stress factors on *C.elegans*. In this section, the research focused on rapid metabolic changes generated by exposure of *C.elegans* to lead.

The effect of soluble inorganic lead in the form of lead acetate was tested on axenic and monoxenic *C.elegans* for three different time periods. Lead toxicity used in this section has been proposed as a negative effector in *C.elegans* (Guo et.al, 2009; Leung et.al, 2008). Full electrochemically active metabolite profiles of *C.elegans* were generated using Coularray which include the three particular metabolic pathways followed in the other studies. Alterations, which can be described as 'stress related', were found in the different life stages of *C.elegans* exposed to three concentrations of lead acetate. The measurables in all cases were alterations in the metabolic profiles obtained in response to treatments as opposed to controls. Thus, the entire pattern of analytes within the data set is compared and particular pathways where the specific metabolites are known are followed quantitatively in detail,; this includes metabolites of the purine, tyrosine and tryptophan pathways.

Coularray was used with five replicates for detection and analysis of metabolites of interest. In the sections discussed below, *C.elegans* samples were separated into the

three main groups (Figure 11): *C.elegans* with length <500 µm (L1, L2 and early L3) – **cohort Y** (young); *C.elegans* with length between 500 and 1000 µm (older L3, L4 and young adults) – **cohort M** (middle-age) and, *C.elegans* with length >1000 µm (adults and senescent adults) – **cohort A** (adults).

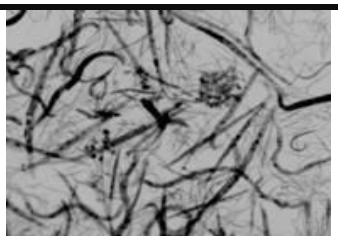
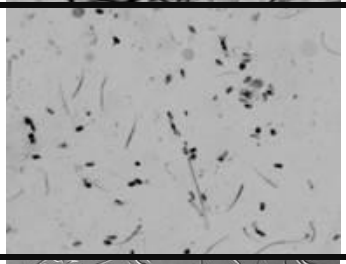
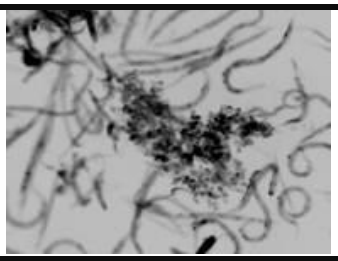
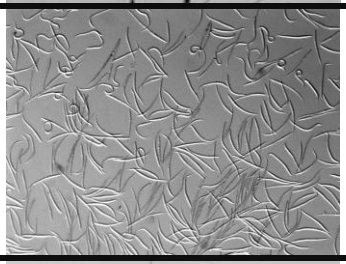
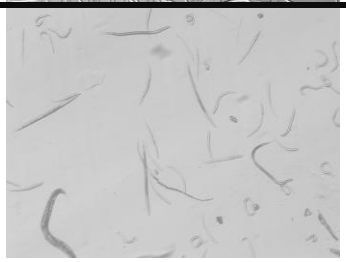
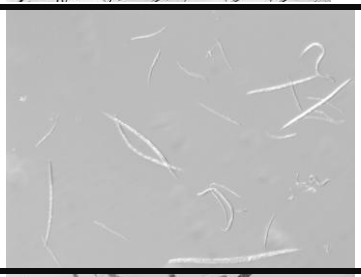
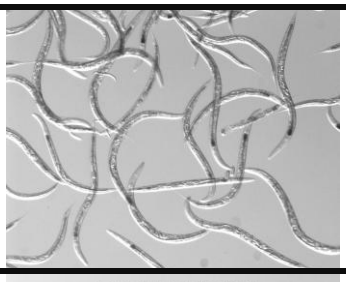
	Monoxenic-1	**Axenic-2**
A. Harvested **Mixed Population**		
B. Eggs After harvesting and age fractionation		
C. Age Fractionated **L1, L2** and some **L3** – cohort **Y**		
D. Age Fractionated **L3, L4** and **Young Adults –** cohort **M**		
E. Age Fractionated **Reproductive** and **Senescent Adults** – cohort		
F. Scale_40X		

Figure 11: Photographs of mixed populations and age fractionated *C.elegans* cohorts from both axenic and monoxenic populations. These representations presented were taken using the Leica Microscope and presented at 40X. The microscope used to obtain the photographs of the nematodes was Leica M2 IGFA (10445930) with software NIS elements (D2.20).Each sample was diluted with water onto a depression slide and then

cover-slipped. The slides were cooled on an ice-pack until photographs were taken. An ocular of 40X was used.

Study 4 – Age dependent exposure to lead (Pb)

4.1.1. Age fractionated axenic *C.elegans* cultures exposed to lead acetate for 2.5 hours

Axenic mixed populations of *C.elegans* were exposed to the "soluble inorganic lead" to determine the response to this stress by *C.elegans.* Emphasis was placed on analysis of tryptophan, tyrosine and purine metabolites, within the full, complex, multicomponent metabolite profile of *C.elegans*. Within these three pathways are metabolites having key functions in homeostatic controls. Three time periods were chosen, 2.5 hours to test early responses, 1.5 days to test the short term responses and ten days in axenic and six days in monoxenic to test the longer-term effects of lead acetate on *C.elegans.* Since the life cycle of *C.elegans* is well documented, we know where the organism should be at each of these time points. The 2.5 hrs exposure catches a particular window within the *C.elegans* developmental cycle. The earliest tested time point established for behavior assessments has been reported to be 4 hours (Anderson et al., 2004). The 2.5 hrs time point tested in this research was to study how metabolites associated with early neurological effects can be measured in *C.elegans* using high performance liquid chromatography coupled with electrochemical detection. The l.5 day's exposure captures data relevant to population profile response and reproductive toxicity. The 10 days exposure provides the longer term summation of lead's effect on the organism in terms of acute toxicity, reproductive toxicity, developmental toxicity, and genotoxicity which may contain carry-over epigenetic effects as well.

The axenic cultures of *C.elegans* were grown for 14 days and subjected to age fractionation for separation of the population into three cohorts; young (Y), middle age (M) and adult (A) nematodes. The age fractionated nematodes then were grown in fresh axenic media containing 250ppm, 500ppm or 1000ppm of lead acetate and were tested in triplicate at three time periods i.e. 2.5 hours, 1.5 days and ten days. This sub-section (section 4.1.1) describes the results for the 2.5 hours time period. This data was obtained from axenic mixed cultures containing ~77.5% cohort 1, ~6.5% cohort 2 and ~16% cohort 3. The age fractionation resulted in fraction 1 (100% Y), fraction 2 ~ (66% Y, 30% M, 4% A) and fraction 3 ~ (13% Y, 5% M, 82% A) (Appendix 5). The population distribution did not change during the experimental periods.

Figure 12 represents the PCA of the normalized chromatographic data for the age fractionated axenic *C.elegans* treated with 250ppm, 500ppm or 1000ppm of lead acetate for an exposure time of 2.5 hours. Figures 12 (a) and (b) represent the young (component 1, 2 and 3 captured 79.36% of the metabolic differences in the data) and adult (component 1, 2 and 3 captured 82.7% of the metabolic differences in the data) cohorts treated with different dosages of lead acetate respectively. The data was analyzed by PCA to determine if it was possible to distinguish different subgroups (including controls and the different treatments) using ~ 2000 detectable analytes through Coularray. Using PCA, we demonstrated a three-dimensional display in which the 1000ppm treated young nematodes were separated from the controls. The lowest lead treatment 250ppm clustered separately from the control and the 250ppm clustered close to 500ppm ($p \leq 0.05$, Figure 12 (a)). This shows that the shortest exposure time of 2.5 hours affects the treated young

C.elegans cohort significantly. Given the population distribution (100%) in the young cohort from the process of age fractionation, the treated *C.elegans* separates well from the control young cohort. Analysis by PCA of the data generated from this treatment of the middle age population did not result in clean separation of sub groups. This could be because the middle age consists of individuals from different cohorts (Y, M and A) and eggs. Control and 250ppm treated adults clustered together as did the 500 and 1000ppm treated adults ($p \leq 0.05$, Figure 12 (b)). Differences in unknown as well as known compounds were confirmed in the different treatments showing age and dose dependent responses.

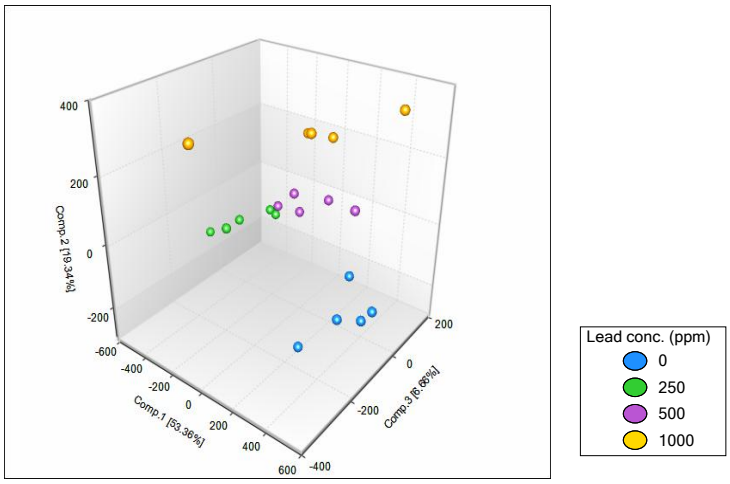


Figure 12(a): PCA for the axenic control young *C.elegans* samples (blue) and the axenic young *C.elegans* samples treated with different concentrations of lead acetate (250ppm-green, 500ppm-purple, 1000ppm-yellow)

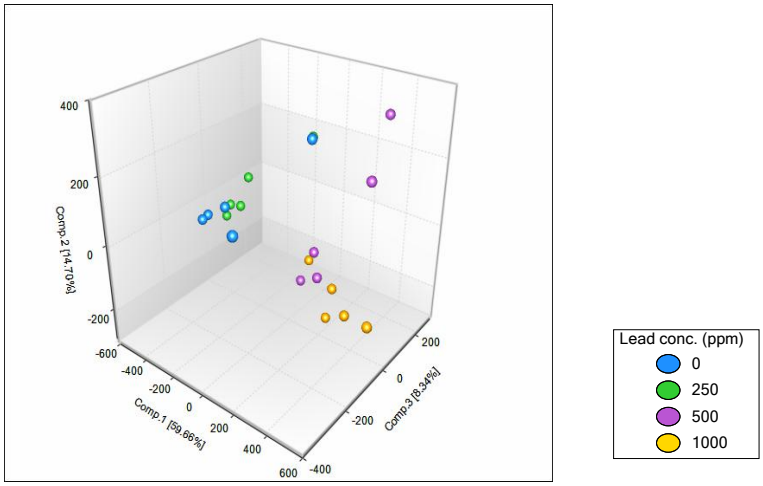


Figure 12(b): PCA for the axenic control adult *C.elegans* samples (blue) and the axenic adult *C.elegans* samples treated with different concentrations of lead acetate

Figure 13 shows the chromatograms for the complete metabolic profiles of the control and lead-treated young *C.elegans* using the Coularray software. These chromatograms demonstrate that each specific metabolic profile is a unique fingerprint for the specific perturbation in *C.elegans*. The red arrows point to the peak at around 11minutes which is only present in nematodes exposed to the different lead treatments (250, 500 and 1000ppm) and is not present in young *C.elegans* controls. Similarily, the peak present at around 24 minutes (black arrow) increases with age and dose of lead acetate. There are dose and age dependent peaks that consistently appear in the lead treated *C.elegans* chromatograms. Figure 14 represents the metafile (A metafile selects files for simultaneous display and compares in the same chromatogram plot) showing the unique peaks present at 19.47 and 24.97 minutes in young as a result of lead treatment (Appendix 7 shows the metafiles for middle age and adult). For the clarity of representing

peaks in the age groups, three replications of control and 1000ppm treatment samples have been used. Since there were no known compound peaks eluted close to these two unknown peaks (‘a’ and ‘b’), the ratios were calculated as ‘a/b’. The ratios calculated (Figure 15) for these unknown peaks for all age groups were significant compared to their respective controls ($p \leq 0.05$) for channel 6 (360 mV).

Figure 13: Screenshot capture of analysis software display of complete metabolic profiles from control young and young treated with varying concentrations of lead acetate for 2.5 hours. The line colors represent the different channels

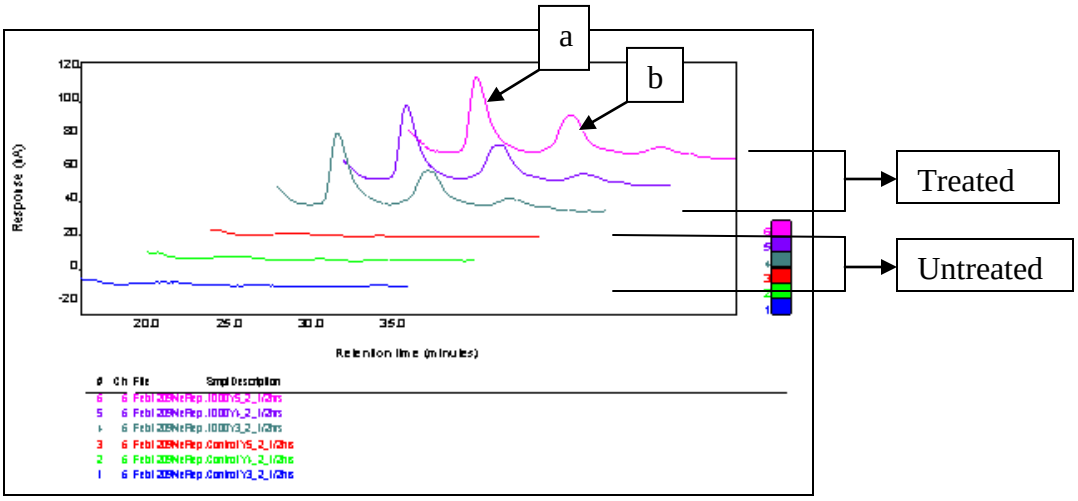


Figure 14: Metafile showing differences for channel 6 between control and 1000ppm lead treated young *C.elegans*. Blue, green and red represent three replications of untreated young; sea green, purple and pink represent three replications of 1000ppm lead treated young

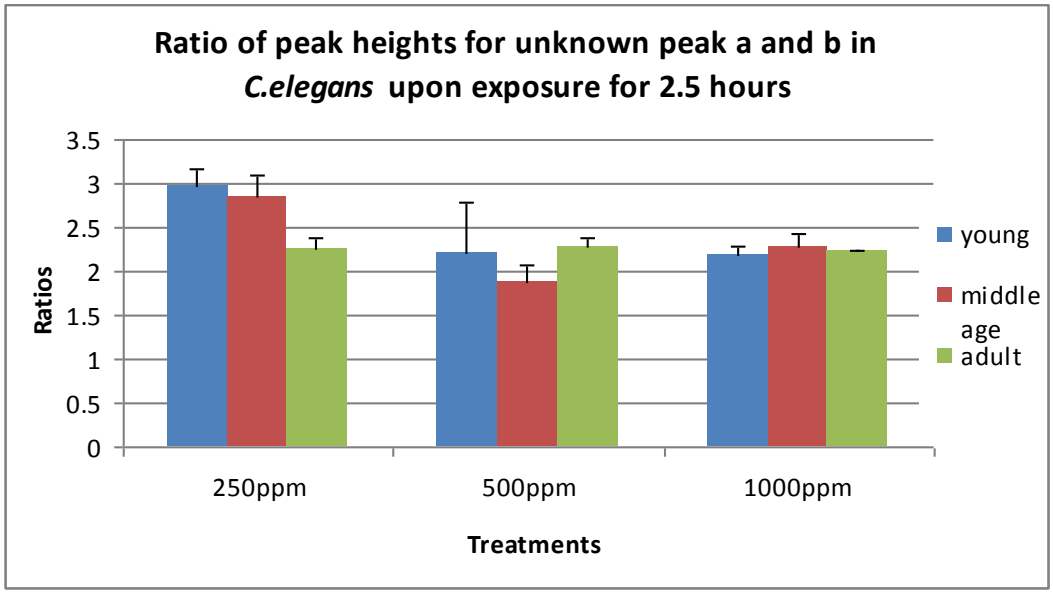


Figure 15: Ratios of two unknown peaks 'a' and 'b' in channel 6 in *C.elegans* upon exposure for 2.5 hours

Figure 16 represents the metafile to show the unique peak ‘c’ present at 25.81 minutes in young as a result of lead treatment. The peak was present in all dosages of lead treated age groups (Appendix 8). The known compound peak eluting closest to this unknown compound ‘c’ was guanosine. This shows that as a result of lead treatment, this unknown compound, labeled here as ‘c’, is present in all young, middle age and adult cohorts. The ratios were calculated (Figure 17) with respect to guanosine for the unknown peak ‘c’ from all age groups and were significant compared to their respective controls ($p \leq 0.05$) for channel 13 (720mV).

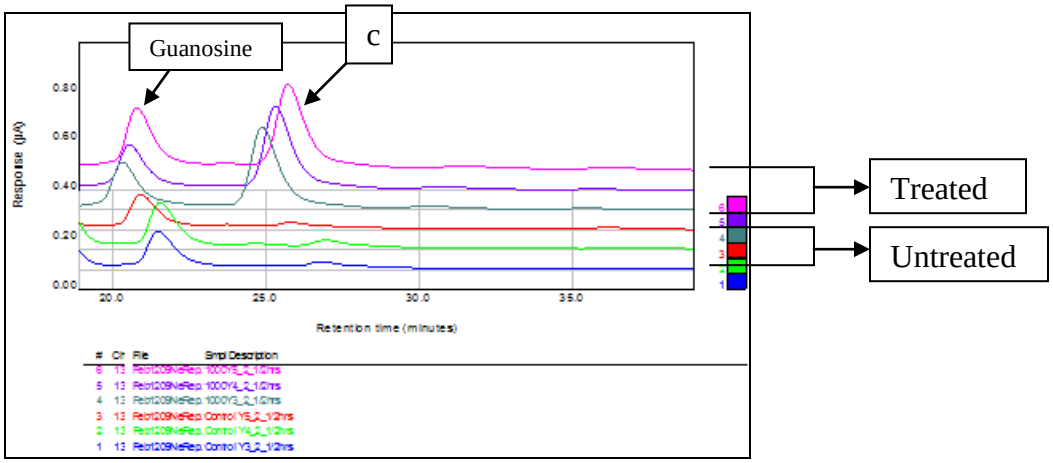


Figure 16: Metafile showing differences for channel 13 between control and 1000ppm lead treated young *C.elegans*. Blue, green and red represent three replications of untreated young; sea green, purple and pink represent three replications of 1000ppm lead treated young

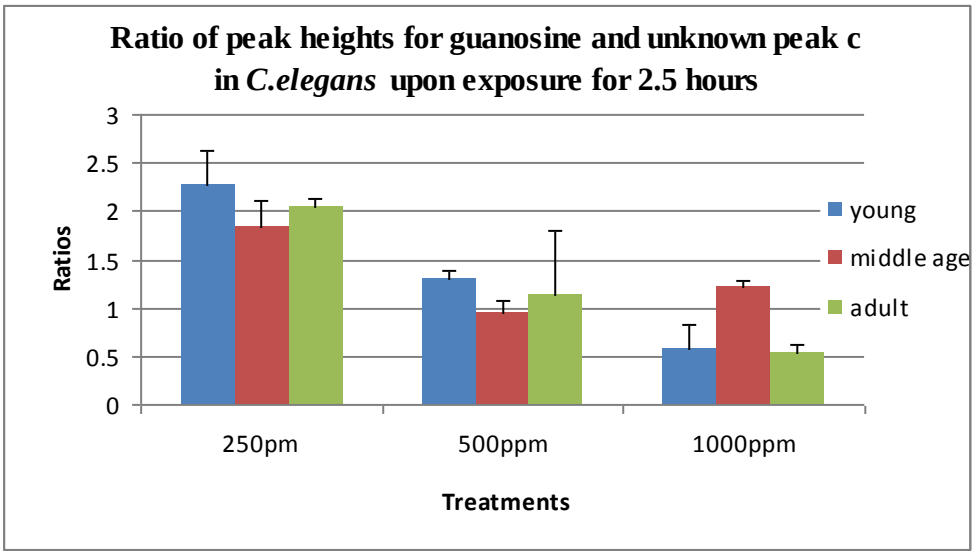


Figure 17: Ratios of guanosine and unknown peak 'c' in channel 13 in *C.elegans* upon exposure for 2.5 hours

Irrespective of the different age cohorts, the treated *C.elegans* fractions were clearly separated from the control *C.elegans*. Differences were found in purine, tryptophan and tyrosine metabolites in the different age-fractionated cohorts treated with different concentrations of lead compared to their respective controls. In the purine pathway, guanine and uric acid were significantly increased in all three treatments in the three cohorts ($p \leq 0.001$). Comparing the ratios (Table 5, $p \leq 0.05$), uric acid/xanthine ($p \leq 0.05$) increased 2.5 fold in the 1000ppm treatment compared to the control in all the young and adult treated cohorts. No significant increase was observed in the middle age cohort. The xanthine/guanine ($p \leq 0.05$) ratio decreased (2.5 fold) following the 1000ppm treatment for all cohorts. It can be speculated that, as a result of 2.5 hours lead treatment in the three cohorts, the shunt in the purine pathway occurs through the branch

guanosine→ guanine→ xanthine→ uric acid. The signal reduction in xanthine levels is likely due to reduction in production of xanthine via xanthosine.

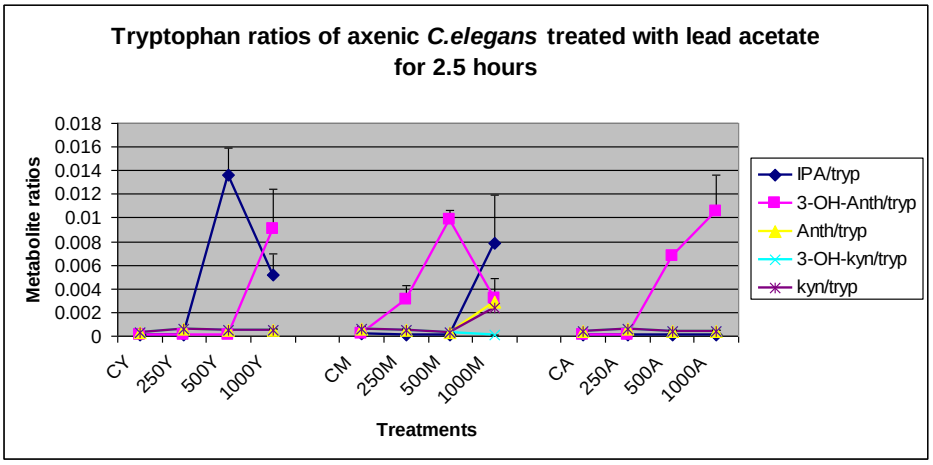


Figure 18 (a): Metabolite concentration to tryptophan concentration ratios of *C.elegans* treated with varying concentrations of lead acetate for 2.5 hours. Y-young, M-middle-age, A-adult, IPA-Indole pyruvic acid, tryp-trytophan, 3-OH-anth-3-hydroxy anthranilate, 3-OH-kyn-3-hydroxy kynurenine

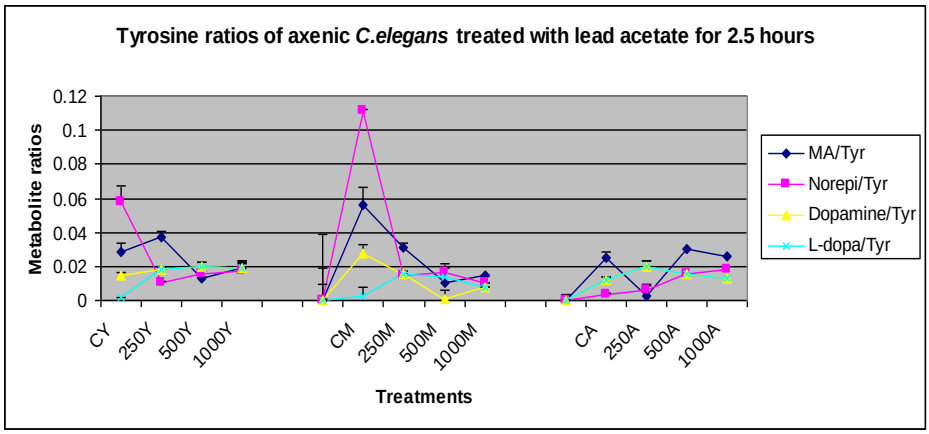


Figure 18 (b): Metabolite to tyrosine ratios of *C.elegans* treated with varying concentrations of lead acetate for 2.5 hours. MA-3-Methoxy-4-hydroxy-mandelic acid, tyr-tyrosine, norepi-norepinephrine

Table 5: Effect of lead acetate for 2.5 hours on purine ratios ($p \leq 0.05$) of axenic age fractionated cohorts

Metabolites	250 ppm			500 ppm			1000 ppm		
	Y	M	A	Y	M	A	Y	M	A
Uric/Xan	↑	-	↑	↑	-	↑	↑	-	↑
Xan/Guan	↓	↓	↓	↓	↓	↓	↓	↓	↓
Guan/guanosine	↑	-	-	↑	-	-	↑	↑	↑

Table 6: Effect of lead acetate for 2.5 hours on tyrosine ratios ($p \leq 0.05$) of axenic age fractionated cohorts

Metabolites	250 ppm			500 ppm			1000 ppm		
	Y	M	A	Y	M	A	Y	M	A
MA/tyr	↑	↓	↓	-	↓	↑	-	↓	-
Norepi/tyr	↓	↓	↑	↓	↓	↑	↓	↓	↑
Dopamine/tyr	↑	↓	↑	↑	↓	-	↑	↑	-
L-dopa/tyr	↑	↑	↑	↑	↑	-	↑	↑	-

Table 7: Effect of lead acetate for 2.5 hours on tryptophan ratios ($p \leq 0.05$) of axenic age fractionated cohorts

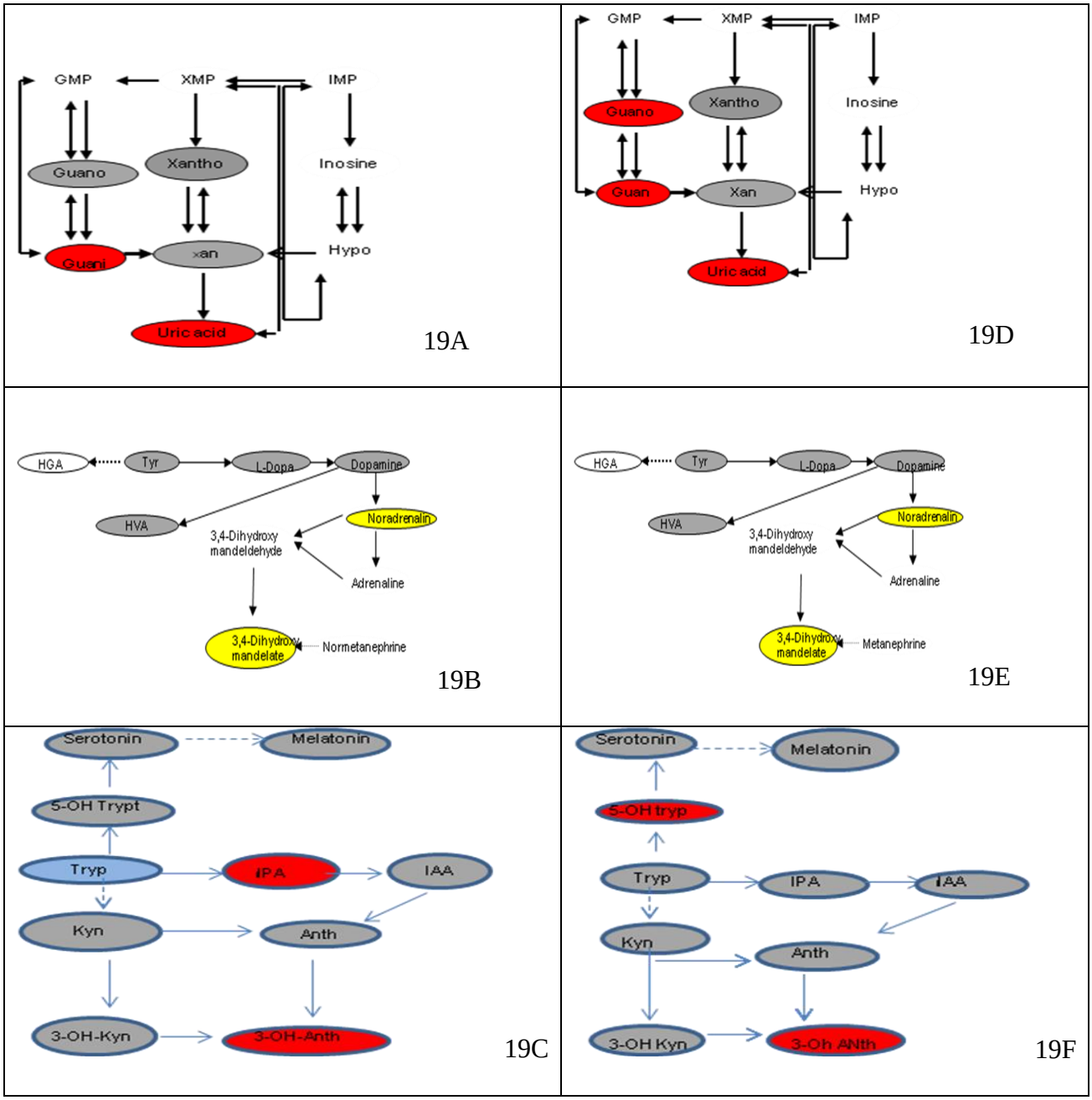
Metabolites	250 ppm			500 ppm			1000 ppm		
	Y	M	A	Y	M	A	Y	M	A
IPA/tryp	-	-	-	↑	-	-	↑	↑	-
3-OH-anth/tryp	-	↑	-	-	↑	↑	↑	↑	↑
Anth/tryp	-	↑	-	-	↑	↑	↑	↑	↑
3-OH-kyn/tryp	-	-	-	-	-	-	-	-	-
Kyn/tryp	-	-	-	-	-	-	-	-	-

Figure 19 represents the purine, tyrosine and tryptophan pathway in axenic young and adult *C.elegans* treated with 1000ppm lead acetate. Examining the pathway for tryptophan, the following metabolites, derived from tryptophan, were studied- kynurenine, 3-OH-kynurenine, anthranilate, 3-OH anthranilate, indole pyruvate, 5-OH

tryptophan, melatonin and serotonin. These metabolites affect different functions in *C.elegans* including pharyngeal pumping, locomotion, and egg-laying. Serotonin, 5-OH tryptophan and melatonin were not found to be affected by the short term lead treatment of any of the cohorts. In this study, anthranilate (with a detection limit of 1ng/ml) was found in all treated cohorts whereas kynurenine compounds showed up only as a result of 1000 ppm treatment in the middle age group. As we know from the KEGG pathway, one of the ways anthranilate is formed is from N-formyl kynurenine through kynurenine. Anthranilate was not found in the control cohorts. From the analytes measured in this study, these results suggest that anthranilate was expressed as a result of lead treatment. Earlier studies in our laboratory found that anthranilate could be a potential biomarker for lead toxicity (Troast, 2006). 3-hydroxy anthranilic acid and indole 3-pyruvic acid were detected in 500 ppm and 1000 ppm treatments in all three treated cohorts- young, middle age and the adult (detection limits for these compounds in supplementary materials). For controls and 250 ppm treated nematodes, these compounds were below detection limits. Tryptophan concentrations decreased with increasing lead concentration in treated nematodes compared to controls in all cohorts. This was confirmed by the metabolite ratio changes to show the metabolic flux in *C.elegans* as a result of lead exposure for 2.5 hours (Figure 16). Indole pyruvate and 3-hydroxy anthranilate increased in young and middle age cohorts whereas only 3-hydroxy anthranilate increased in adult nematodes. 3-hydroxy anthranilate was significantly increased with treatment- young ($p \leq 0.03$ for 250ppm, $p \leq 0.0009$ for 500ppm, $p \leq 0.02$ for 1000ppm) (Figure 18a). As a result of lead

treatment, the ratios support our conclusion that the shunt in the tryptophan pathway occurs in tryptophan→kynurenine→anthranilate→3-OH anthranilate in adults.

Our data shows that the concentration of tyrosine did not change as a result of short term lead exposure. Norepinephrine and 3-Methoxy-4-hydroxy-mandelic acid concentrations demonstrated a dose-dependent decrease ($p \leq 0.05$) in young and an increase in adult cohorts with treatment. 3-Methoxy-4-hydroxy-mandelic acid forms from tyrosine through L-dopa and dopamine. Norepinephrine to tyrosine and 3-Methoxy-4-hydroxy-mandelic acid to tyrosine ratios were decreased with lead exposure ($p \leq 0.03$) suggesting that there was decrease in concentrations of norepinephrine and 3-Methoxy-4-hydroxy-mandelic acid (Figure 18b). Epinephrine has been found in mammalian plasma as a consequence of insult to the cells, e.g. injury to neurons (Woolf et.al, 1992; Young et.al, 1998), bacterial infection (Groves et.al, 1973) and specifically in respiratory infections (Gruchow, 1979). In this case the presence of epinephrine might be suggesting that 3-Methoxy-4-hydroxy-mandelic acid and norepinephrine may play protective roles in the initial hours of lead exposure in all age groups of *C.elegans*. This is interesting since blood lead and 24-hour urinary excretions have shown increased vanillyl mandelic acid, a metabolite of norepinephrine, and homovanillic acid, - a metabolite of dopamine (Blum & Manzo, 1985). This supposition could be supported by the observation that no change in locomotory behavior was observed under microscope after 2.5 hours of exposure in any cohorts of *C.elegans*



Compound was measured and no change was found (p>0.1)

Compound was measured and was increased in *C.elegans* (p<0.03)

Compound was measured and was decreased in *C.elegans* (p<0.05)

Compound was measured and was increased in *C.elegans* (p<0.05)
No box represented compound was not measured

Figure 19: Changes in purine (19A for young and 19D for adult), tyrosine (19B for young and 19E for adult) and tryptophan (19C for young and 19F for adult) in 1000ppm lead acetate treated *C.elegans* for 2.5 hours

The tryptophan/ tyrosine ratios (Table 6 & 7) remained relatively tight in all three age fractions and in all the treatments. These amino acids are not synthesized by the nematode, therefore, it can be speculated that the tryptophan and tyrosine concentrations are maintained as one essential component of *C.elegans* homeostatic control. The real impact as a result of the external stimuli/stresses can be assumed to be the changes in concentrations of the metabolites synthesized from tryptophan and tyrosine that were detected by Coularray in this research.

4.1.2. Age fractionated monoxenic *C.elegans* cultures exposed to lead acetate for 2.5 hours

Most of the published studies dealing with metal toxicity in *C.elegans* are performed using monoxenic cultures of *C.elegans*. The results from biological stress factor response tested in this research were found to differ with the source of nutrition for *C.elegans*. The hypothesis behind the following studies was that nutritional differences will affect the response of *C.elegans* to lead toxicity. This was tested at three different time points- 2.5 hours, 1.5 days and six days. The final time point could not be sampled after ten days (unlike the axenic study) because of the growth of bacteria in the cultures. Thus this study was terminated at the end of six days. This sub-section (4.1.2.) discusses the results for the response of *C.elegans* at the first time point- 2.5 hours.

This data was obtained from monoxenic mixed cultures containing ~78% cohort 1, ~3% cohort 2 and ~19% cohort 3. The age fractionation resulted in cohort 1 (100% Y), cohort 2 ~ (80% Y, 17% M, 3% A) and cohort 3 ~ (25% Y, 1% M, 74% A). Appendix 6 presents the population distribution of the monoxenic cultures for different treatments at the end of the treatment time period i.e. 2.5 hours. The population distribution change was minimal in the experiment after 2.5 hours exposure.

There are striking differences between the monoxenic and axenic cultures (Table 8). The monoxenic *C.elegans* population peaked at 7 days while the axenic *C.elegans* population peaked at 14 days (Croll et, al, 1977). The overall development rate and population profile at the point the maximum population density was reached was different for axenic and monoxenic *C.elegans* populations. Axenic cultures contained relatively more L3 and early reproductive L4 (size 500-1000µm) *C.elegans* than did monoxenic cultured populations. Eggs of axenic *C.elegans* were laid in clumps (Figure 11, 2 AA) while, those of monoxenic worms were found singly (Figure 11, 2 MM) on the agar plates. The population distribution as well as density for axenic and monoxenic populations was found to be different (there were fewer numbers of middle aged individuals found in the monoxenic cultures compared to the axenic). The differences in the population density and egg laying may be due to the difference in the medium and nutrient for culture: liquid ACM for the axenic worms and NGM agar plates with *E. coli* for the monoxenic worms.

Table 8: Differences in characteristics of axenic and monoxenic *C.elegans*

Characteristics	**Axenic *C.elegans***	**Monoxenic *C.elegans***
Subculture interval	Every 14 days	Every 7 days
Population distribution at the end of subculture	Mixture of all stages with eggs- Figure 11-1(A)	L1, L2, Adults (very few middle age) - Figure 11-2(A)
Egg laying pattern	Big clumps- Figure 11-1(B)	Laid sparsely- Figure 11-2(B)
Size	Young is <500µm Middle age is 500-1000 µm Adult is 900-1250 µm	Young is <500µm Middle age is 500-1000 µm Adult is 900-1150 µm
Transparency of worm	Highly pigmented- Figure 11-1(E)	Lightly pigmented- Figure 11-2(E)
Harvested pellet color	Yellowish brown	Dull white

Profile data did not show significant changes measured in purine pathway metabolites as a result of lead treatment for 2.5 hours in monoxenically cultured *C.elegans* treated with lead. Uric acid significantly increased in the middle age cohort (p<_0.05) at 1000ppm treatment and in the adult cohort treated at 500 ppm and 1000 ppm. It can be speculated that *C.elegans* grown in different nutrient media responds differently to the same stress factor at a given exposure time. The *E.coli* (food source of monoxenic *C.elegans*) might alter the effect of lead exposure on monoxenic *C.elegans*. Due to the different growth conditions of the same strain of *C.elegans*, purine ratios

remained unchanged for the treated nematodes in this data set compared to the treated axenic nematodes.

In the tryptophan pathway, 3-hydroxy kynurenine, kynurenine and indole 3-pyruvic acid were expressed in increased concentrations in the 1000ppm treated young monoxenic population. 3-OH kynurenine is formed from tryptophan though kynurenine. Treatments of the young population with 500ppm and 1000ppm lead acetate resulted in expression of higher concentrations of kynurenine. Indole 3-pyruvic acid showed up in higher concentrations in the lead treated middle age population and was expressed in significant concentrations ($p \leq 0.05$) in adult populations treated with lead levels of 500ppm. It appears that the exposure of *C.elegans* to lead acetate for 2.5 hours affects two branches of the tryptophan pathway. These same metabolites were perturbed as a result of lead exposure in the lead treated axenic *C.elegans*.

Some changes in metabolites of tyrosine were also observed. 3-Methoxy-4-hydroxy-mandelic acid increased ($p \leq 0.05$) dramatically with lead treatment in all age groups. Kynurenine and indole pyruvic acid were expressed in increased concentrations in 1000 ppm treated young cohort. Uric acid increased in 1000 ppm treated middle age and adult cohorts. Dose dependent increase in 3-Methoxy-4-hydroxy-mandelic acid was expressed in treated young cohort. With an exposure time of 2.5 hours, the perturbations observed were similar in axenic and monoxenic treated cohorts.

The biochemical and electrophysiological effects of lead on the nervous system have been studied in lead-exposed children. Exposure to low levels (≤15ug/dl) of lead *in utero* may affect development in children. Cord blood levels of the dopamine metabolite,

homovanillic acid, and the serotonergic metabolite, 5-hydroxy-indoleacetic acid, were compared to levels found in 9-month old infants showing that the lead exposure produced a neurotoxic effect on the serotonergic system resulting in a negative effect on their behavior. Homovanillic acid exposure affected social behavior, whereas 5-hydroxyindole-acetic acid affected coordination in 9-month old infants (Lidsky & Schneider, 2003). An inverse relationship between blood lead level and intellectual decrement was found in children exposed to lead (Lanphear et.al, 2005).

4.2.1. Age fractionated axenic *C.elegans* culture exposed to lead acetate for 1.5 days

This sub-section (section 4.2.1) describes the results for axenic age fractionated *C.elegans* treated with different concentrations of lead acetate over a 1.5 day time period. The data obtained from axenic mixed culture at time zero showed ~77.4% cohort Y, ~6% cohort M and ~16.6% cohort A. Age fractionation of this mixed culture resulted in cohort Y containing 100% Y, cohort M containing approximately 64.9% Y, 31.6% M and 3.5% A and cohort A containing approximately 13.2% Y, 4.5% M and 82.3% A. Appendix 7 presents population distribution of the axenic cultures for different treatments at the end of the treatment time point, i.e. 1.5 days. Since the exposure time was 1.5 days, the population distribution at the end of the experiment varied greatly from the time zero population as well as among respective treatments and controls. The difference in population distribution results in different metabolic fingerprints from the lead treated age fractionated *C.elegans* populations obtained from Coularray analysis.

Population data for the axenic age fractionated cohorts treated with different concentrations of lead acetate for 1.5 days are presented in Figure 20 (a to c). The percentages were calculated based on the total number of worms in each replicate. Standard error of mean ranged from 0.51 to 7.73.

Start of experiment: cohort Y ~ (100%Y)

End of experiment: cohort Y - Control ~ (78.2% Y, 14.2% M and 7.5% A)

250ppm ~ (87% Y, 8.9 % M, and 3.9%A)

500ppm ~ (85.8%Y, 11.9%M and 2.2%A)

1000ppm ~ (91.5%Y, 0%M and 8.5%A)

Start of experiment: cohort M ~ (64.9% Y, 31.6% M, 3.5%A)

End of experiment: cohort M - Control ~ (95.2% Y, 4.76% M and 0% A)

250ppm ~ (100% Y, 0 % M, and 0%A)

500ppm ~ (100%Y, 0%M and 0%A)

1000ppm ~ (100%Y, 0%M and 0%A)

Start of experiment: cohort A ~ (12.4% Y, 5.9% M and 81.6%A)

End of experiment: cohort A - Control ~ (8.6% Y, 4.5% M and 86.7% A)

250ppm ~ (20.5% Y, 8.1 % M, and 71.3%A)

500ppm ~ (24.5%Y, 6.8%M and 68.6%A)

1000ppm ~ (38.1%Y, 8.2%M and 53.6%A)

The data above shows that with increasing lead concentration, growth was affected in the treated young cohort with an exposure time of 1.5 days. This could be since there was no increase in number of middle age or adult worms in the 1000ppm

treated young cohort. In the treated middle age cohort, there was no increase in either middle -aged or adult *C.elegans* compared to the control. At the start of the experiment, cohort M had a large % of individuals in "M" range. Young, in eggs not yet laid in cohort M are likely protected from lead's effects. Therefore, the data indicates that lead inhibits growth as well as reproductive processes as only "Y" are seen after 1.5 days of lead treatment. In the treated adult cohort there was a decrease in the number of adult *C.elegans* (reduced by 40%) possibly because the senescent adult *C.elegans* lysed. There was an increase in the number of middle age *C.elegans* possibly because of the hatching eggs and the growth of some of the young *C.elegans*. Adults containing fully developed L1 or developing eggs not yet at full somatic cell state are likely protected from lead's effects.

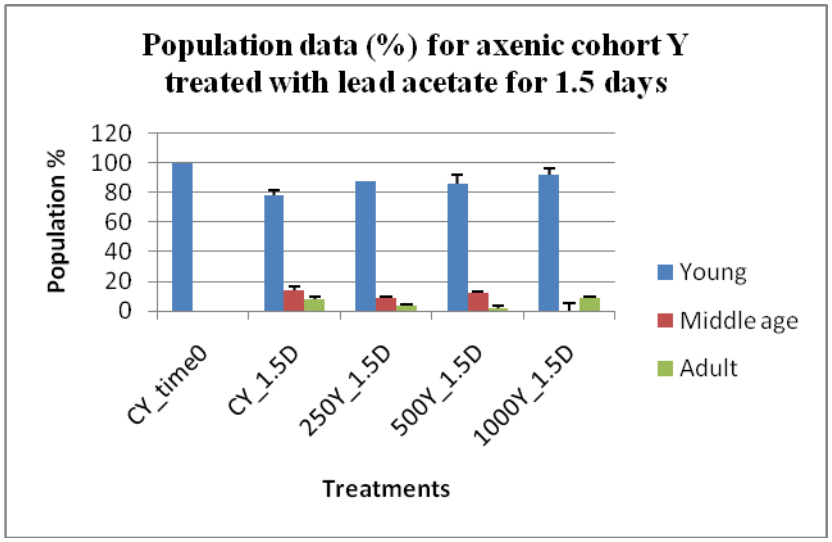


Figure 20(a): Population data for axenic age fractionated cohort Y treated with lead acetate for 1.5 days

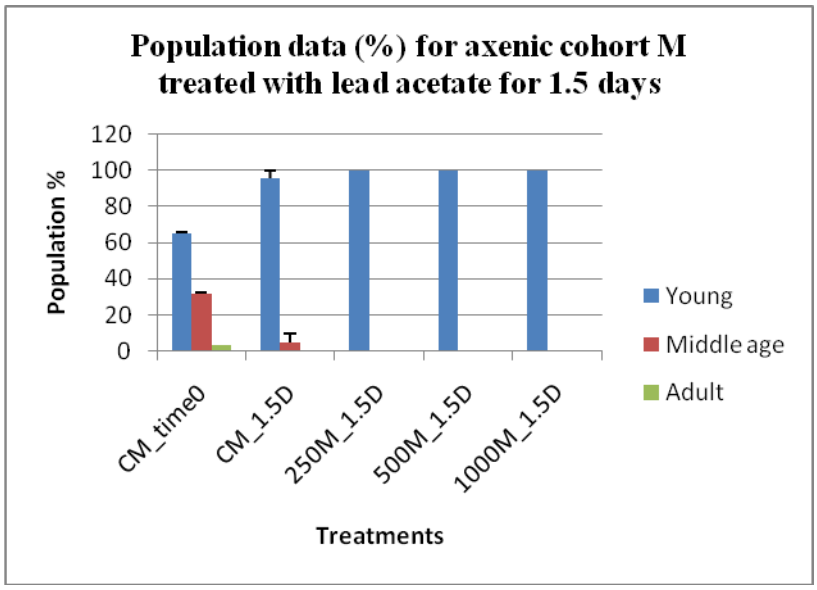


Figure 20(b): Population data for axenic age fractionated cohort M treated with lead acetate for 1.5 days

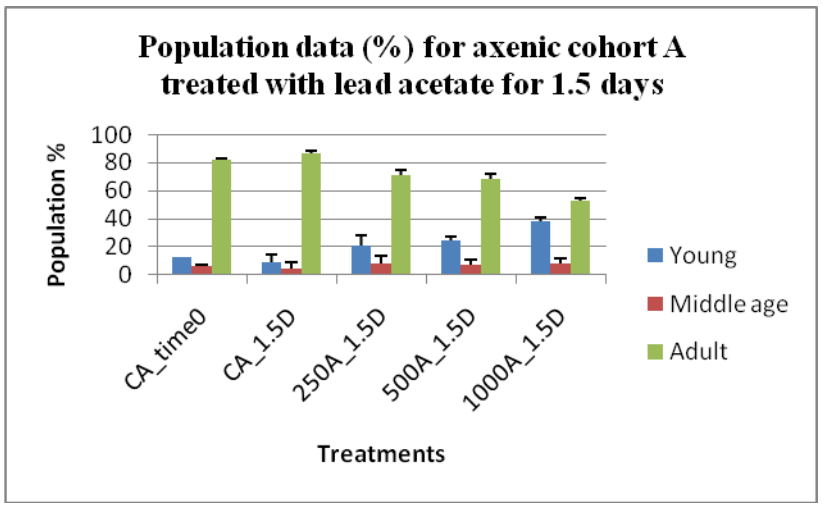


Figure 20(c): Population data for axenic age fractionated cohort A treated with lead acetate for 1.5 days

Figure 21 represents the PCA of the normalized chromatographic data for the age fractionated axenic *C.elegans* treated with 250ppm, 500ppm or 1000ppm of lead acetate for an exposure time of 2.5 hours. Figures 21 (a) and (b) represent the young (85.72% metabolic differences) and adult (78.84% metabolic differences) cohorts respectively treated with different dosages of lead acetate as indicated in the legend. PCA demonstrated that all four sub groups parsed the treated young nematodes from the controls ($p \leq 0.05$, Figure 21) separating the sub groups. This could be because the middle age fraction consists of mixture of pre-reproductive and reproductive adults providing data points difficult to separate compared to young and middle age PCA. 250 and 500ppm treated adults clustered whereas the control and 1000ppm treated were separated ($p \leq 0.05$, Figure 21 (b)). Differences in unknown as well as known compounds were confirmed in the different treatments showing age and dose dependent responses. Figure 22 represents the snapshot of complete metabolic profiles from control young and young treated with varying concentrations of lead acetate for 1.5 days. The black arrows point towards the differences in the peaks as a result of lead treatment which are dose dependent. A metafile helps to select files for simultaneous display and comparison in the same chromatogram plot. Each of these chromatograms is quite different and contains shared peaks only present on treatment with lead. Figure 23 represents the metafile to show the unique peaks present at 19.47 and 24.97 minutes in all age groups as a result of lead treatment. The peaks were present in all dosages of lead treated age groups (Appendix 10 represents the metafiles for middle age and adult cohorts). For clarity of representation of the peaks in the age groups, three replications of control and 1000ppm

treated samples have been presented at a time. The ratios calculated (Figure 24) for these two unknown-source peaks for all age groups were significant compared to their respective controls ($p \leq 0.05$) for channel 6 (360 mV). These peaks ('a' and 'b') were present in all 2.5 hour samples as well as in 1.5 days lead treated cohorts (Y, M, A). This shows that these peaks are characteristic of the lead treatment effects in all cohorts of *C.elegans*.

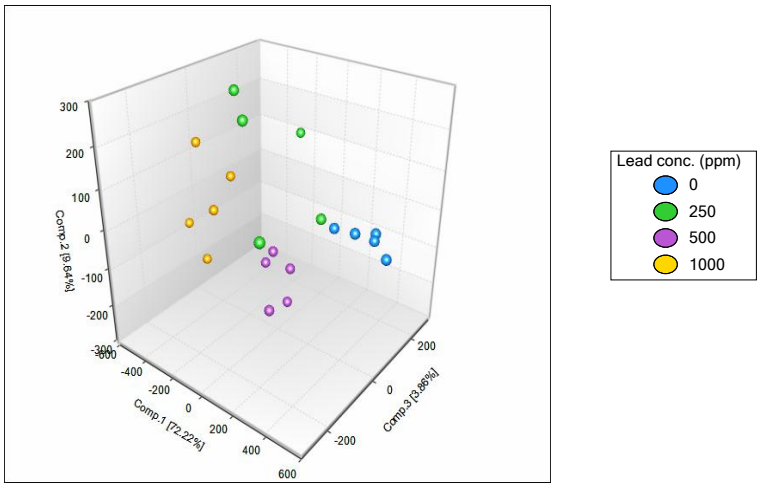


Figure 21(a): PCA for the axenic control young *C.elegans* samples and the axenic young *C.elegans* samples treated with different concentrations of lead acetate

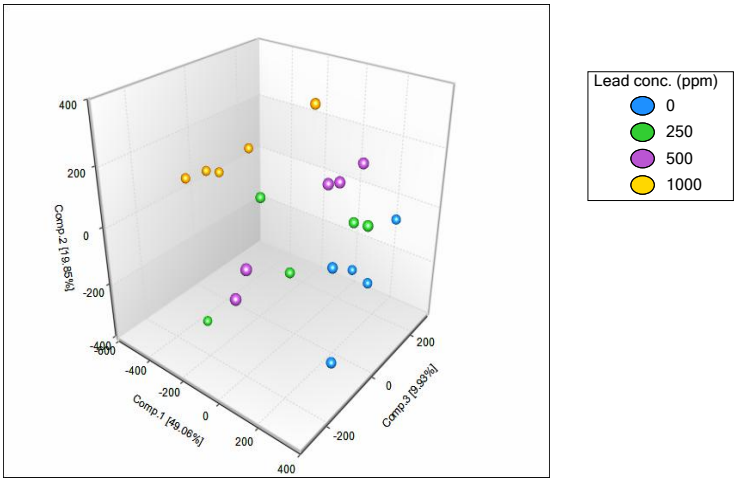


Figure 21(b): PCA for the axenic control adult *C.elegans* samples and the axenic adult *C.elegans* samples treated with different concentrations of lead acetate

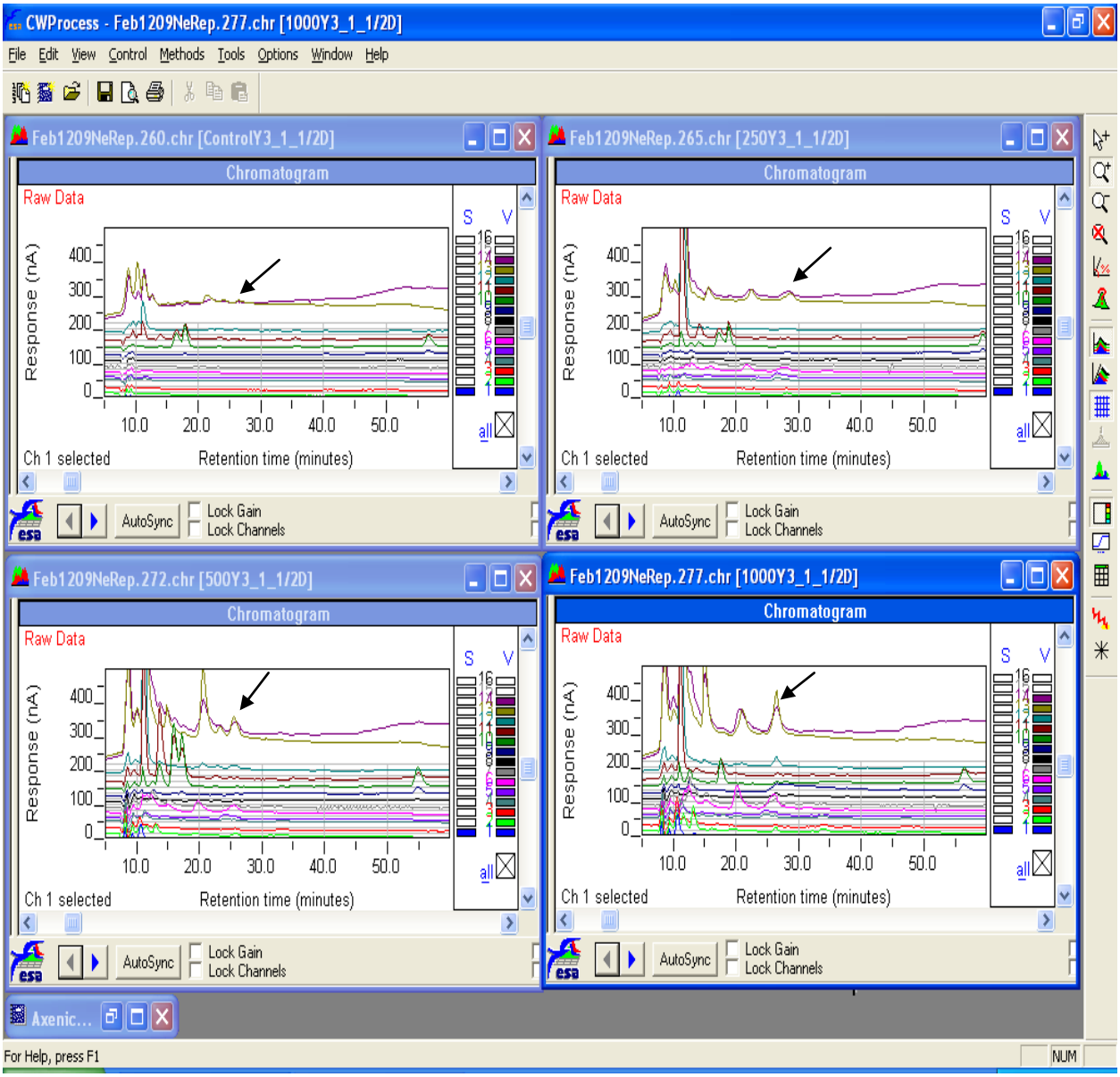


Figure 22: Snapshot of complete metabolic profiles from control young and young treated with varying concentrations of lead acetate for 1.5 days. The black arrows point towards the differences in the peaks as a result of lead treatment which are dose dependent

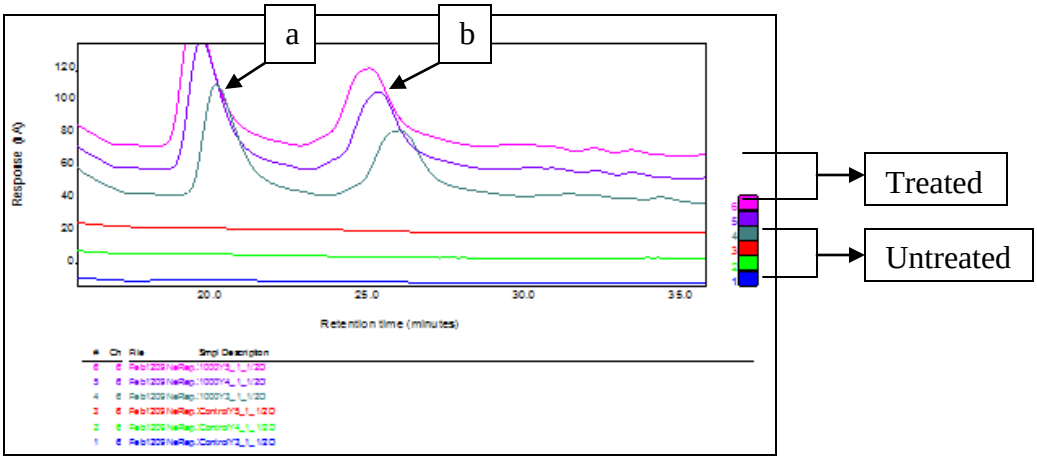


Figure 23: Metafile showing differences for channel 6 between control and 1000ppm lead treated young *C.elegans*. Blue, green and red represent three replications of untreated young; sea green, purple and pink represent three replications of 1000ppm lead treated young

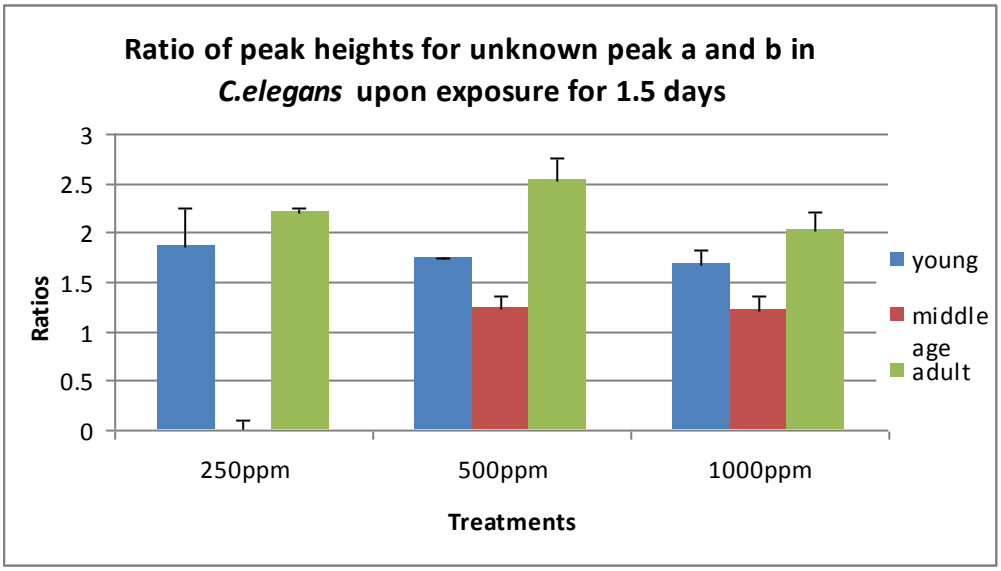


Figure 24: Ratios of two unknown peaks 'a' and 'b' in channel 6 in *C.elegans* upon exposure for 1.5 days

Figure 25 presents the metafile showing the unique peak present at 25.81 minutes in the young cohort as a result of lead treatment (Appendix 11 represents the metafiles for middle age and adult cohorts). The peaks were present in all dosages of lead treated age

groups. For clarity in representing the peak in the age groups, three replications of control and 1000ppm lead have been used. The ratios were calculated (Figure 26) with respect to guanosine for the unknown peak ‘c’ for all age groups and were significant compared to their respective controls ($p \leq 0.05$) for channel 13 (720mV). The ratios for young to middle-age to adult for the different lead dosages resulted in similar patterns. This shows that the effects of lead on these age cohorts, irrespective of lead dosage, are similar.

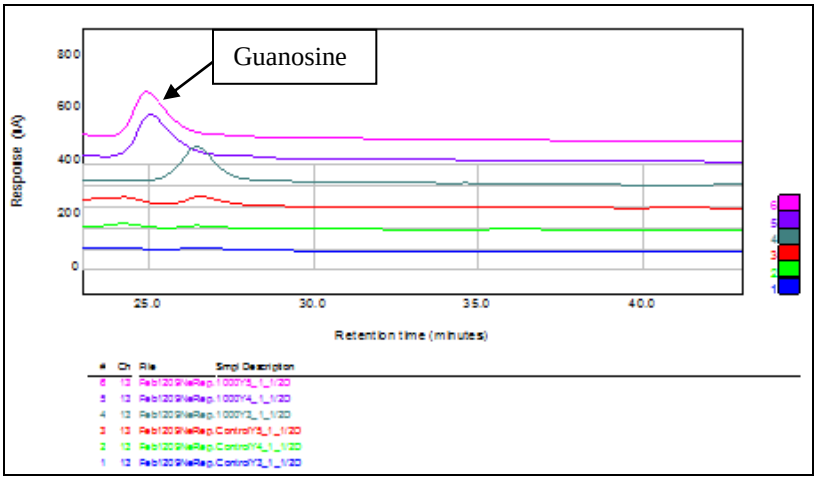


Figure 25: Metafile showing differences for channel 13 between control and 1000ppm lead treated young *C.elegans*. Blue, green and red represent three replications of untreated young; sea green, purple and pink represent three replications of 1000ppm lead treated young

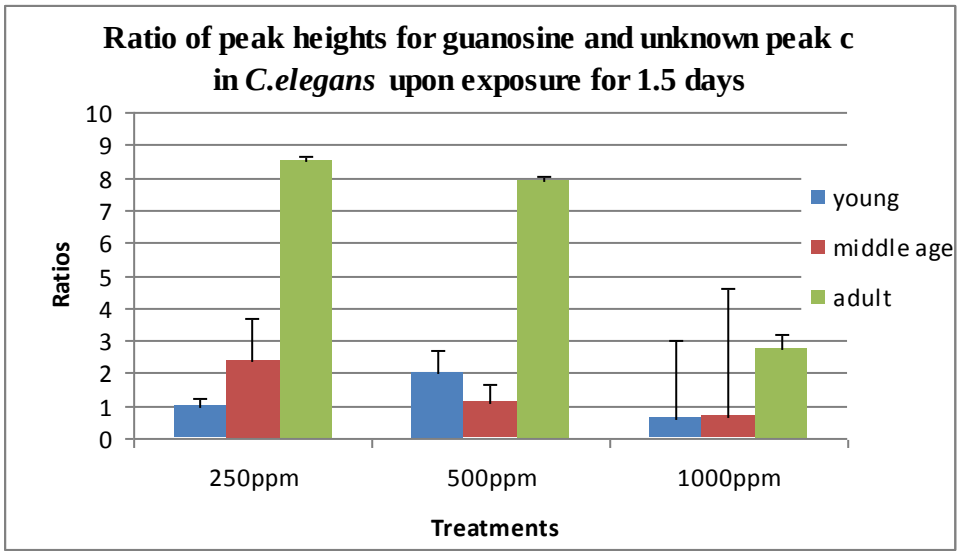


Figure 26: Ratios of guanosine to unknown peak 'c' in channel 13 in *C.elegans* upon exposure for 1.5 days

All purine analyte concentrations showed increases ($p \leq 0.05$) in the treated middle-age population ($p \leq 0.05$). Uric acid showed a concentration increase ($p \leq 0.05$) following all three treatments in the young and adult populations. The ratios (Table 9, $p \leq 0.05$) of uric acid/xanthine and guanine/guanosine increased in the treated young population. This is of importance since the uric/xanthine ratio increased for treated young in 2.5 hours lead exposure as well. Lead treated young demonstrated increases in uric acid with all dosages of lead as well as different exposure times of lead. There was no noteworthy change in the xanthine/guanine ratio in the treated young population. Xanthine/guanine and guanine/guanosine increased for the treated middle-age population whereas the uric acid/xanthine ratio decreased dramatically ($p \leq 0.05$) for the treated middle-age compared to the control. The ratios of uric acid/xanthine and

xanthine/guanine increased for the treated adult population whereas the guanine/guanosine decreased compared to the control. Figure 27 represents the purine, tyrosine and tryptophan pathways in axenic young and adult *C.elegans* treated with 1000ppm lead acetate for 1.5 days.

Table 9: Effect of lead acetate for 1.5 day on purine ratios ($p \leq 0.05$) of axenic age fractionated cohorts

Metabolites	250 ppm			500 ppm			1000 ppm		
	Y	M	A	Y	M	A	Y	M	A
Uric/xan	↑	↓	↑	↑	↓	↑	↑	↓	↑
Xan/guan	-	↑	↑	-	↑	↑	-	↑	↑
Guan/guanosine	↑	↑	↓	↑	↑	↓	↑	↑	↓

Table 10: Effect of lead acetate for 1.5 day on tyrosine ratios ($p \leq 0.05$) of axenic age fractionated cohorts

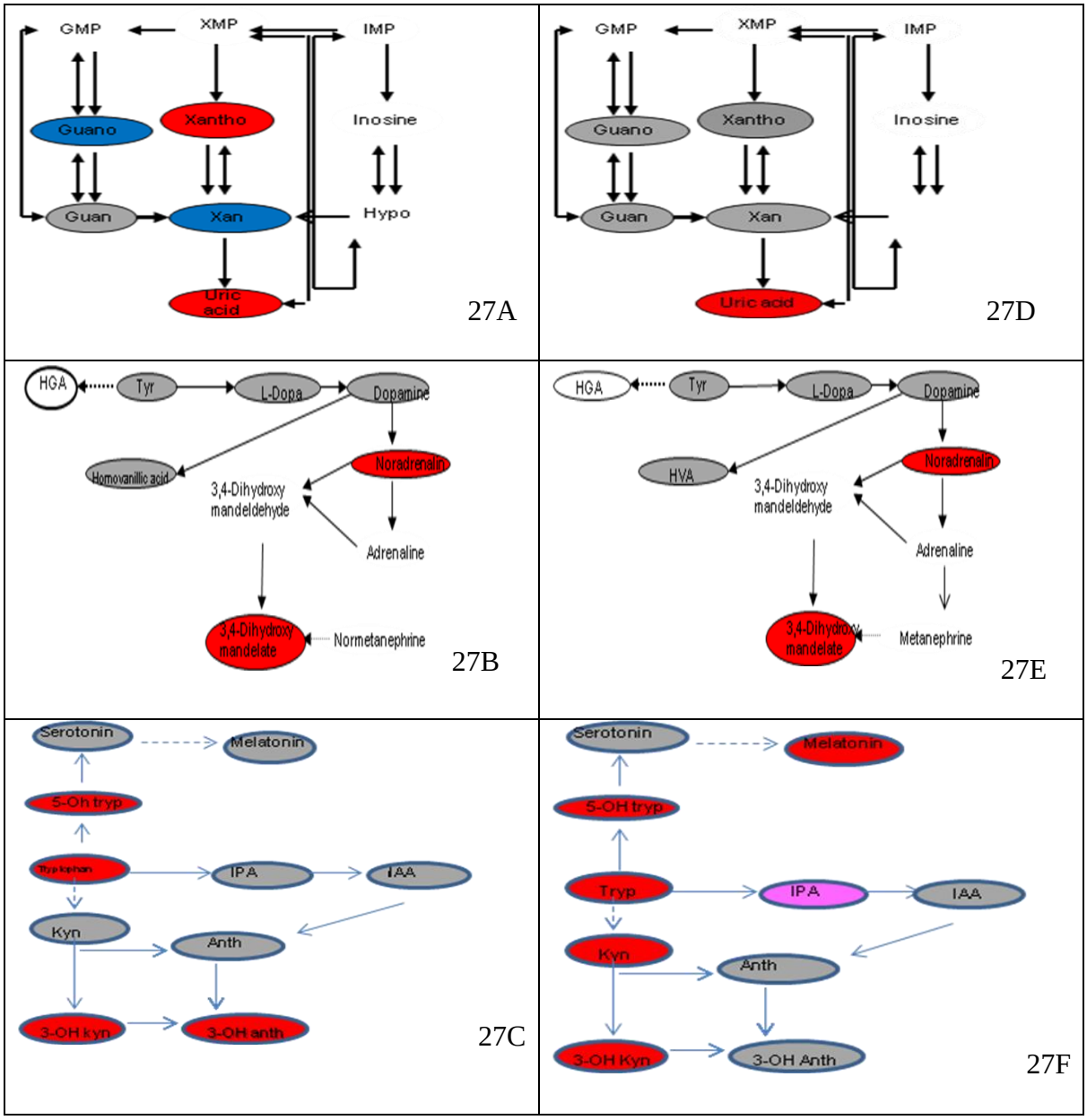
Metabolites	250 ppm			500 ppm			1000 ppm		
	Y	M	A	Y	M	A	Y	M	A
MA/tyr	↓	-	↓	↓	-	↓	↓	↑	↓
Norepi/tyr	↓	↑	↓	↓	↑	↓	↓	↑	↓
Dopamine/tyr	-	-	↓	-	-	↓	-	-	↓
L-dopa/tyr	↓	-	↓	↓	-	↓	↓	-	↓
HVA/tyr	↓	-	↓	↓	-	↓	↓	↑	↓
HGA/tyr	↓	-	↓	↓	-	↓	↓	↑	↓
p-OH-paa/tyr	↓	-	↓	↓	-	↓	↓	↑	↓

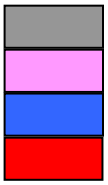
Table 11: Effect of lead acetate for 1.5 day on tryptophan ratios ($p \leq 0.05$) of axenic age fractionated cohorts

Metabolites	250 ppm			500 ppm			1000 ppm		
	Y	M	A	Y	M	A	Y	M	A
Mel/tryp	-	-	↑	-	-	↑	-	-	↑
Ser/tryp	-	↓	-	-	↓	-	-	↓	-
5-OH-tryp/tryp	-	-	-	-	-	-	↑	-	-
IAA/tryp	-	-	-	-	-	-	-	-	-
IPA/tryp	-	-	↑	-	-	↑	-	-	↑
3-OH-anth/tryp	-	-	-	↑	-	-	↑	-	-
Anth/tryp	↓	-	↑	↓	-	↑	↓	↑	↑
3-OH-kyn/tryp	-	↓	-	↑	↓	-	↑	↓	-
Kyn/tryp	-	↓	↑	-	↓	↑	-	↓	↑

Tryptophan concentrations values per mg soluble protein dramatically increased ($p \leq 0.001$) in the 1000 ppm treated young population whereas it also increased and for all three treatments in the middle-age population. In the treated young and adult populations, tryptophan showed a dose dependent increase in response to different doses of lead acetate treatment. In the middle-age population tryptophan was sharply reduced following the 1000 ppm treatment. 3-hydroxy anthranilic acid was detected in increased amounts following 500 and 1000 ppm ($p \leq 0.001$) treatment of the young population. There was a significant increase in anthranilic acid following 500 ppm treatment of the middle-age population ($p \leq 0.05$). 3-hydroxy kynurenine and 5-hydroxy tryptophan were expressed in greater concentrations ($p \leq 0.05$) in the 1000 ppm treated adult population whereas kynurenine, indole-3 pyruvic acid and melatonin were expressed in elevated ($p \leq 0.05$) amounts in all three of the treated adult populations. 5-OH tryptophan was not

detected in any of 2.5 hour treated axenic cohorts. Melatonin has been reported to provide protection to biological membranes from oxidative damage, e.g. several tryptophan metabolites provide protection against iron chloride and ascorbic acid to synaptosomes of rat brain (Milan-Plano, 2010). It could be speculated that increase in melatonin provides protection to nematode cell membranes during lead toxicity.





Compound was measured and no change was found (p>0.1)

Compound was measured and was increased in *C.elegans* (p<0.1)

Compound was measured and was decreased in *C.elegans* (p<0.05)

Compound was measured and was increased in *C.elegans* (p<0.05)
No box represented compound was not measured

Figure27: Changes in purine (27A for young and 27D for adult), tyrosine (27B for

young and 27E for adult) and tryptophan (27C for young and 27F for adult) in 1000ppm lead acetate treated *C.elegans* for 1.5 days

Comparing the ratios (Table 11, $p \leq 0.05$) of the metabolites from the tryptophan pathway to tryptophan, metabolites leading to anthranilate (anthranilate, 3-hydroxy anthranilate, kynurenine and 3-hydroxy kynurenine) were perturbed in treated young, whereas metabolites leading to melatonin (5-hydroxy tryptophan, melatonin) were perturbed in treated adults. Figure 28(a) represents the ratios of tryptophan to different metabolites and end-products in the same pathway. More ratios were perturbed for the treated young compared to middle- age and adult nematodes. This implies that lead exposure has deeper impact on the metabolic pathways of the treated young. Tracking these ratios is useful in attempting to develop a better understanding of leads' impact on these particular biochemical pathways and end products. Anthranilate was present in all age groups that were exposed to soluble lead and can serve as an indicator or potential marker for lead exposure in this organism. The relative levels of anthranilate observed on an individual basis are more pronounced in young as compared to the adult (Troast, 2006).

Norepinephrine shows an increasing dose response to exposure of lead compared to the control (Figure 28b). Table 10 ($p \leq 0.05$) represents the tyrosine ratios for 1.5 days lead treated age fractionated cohorts. All the branches in the pathways produce components with a range of known physiologic functions in other systems, e.g. mammalian. 3-methoxy-4-hydroxy-mandelic acid was expressed in increased concentrations ($p \leq 0.05$) in the treated young population following all the treatments.

Tyrosine increased ($p \leq 0.05$) concentration in all the treated middle- age populations. 3-Methoxy-4-hydroxy-mandelic acid increased ($p \leq 0.05$) with the treatment when compared to the controls in the adult population.

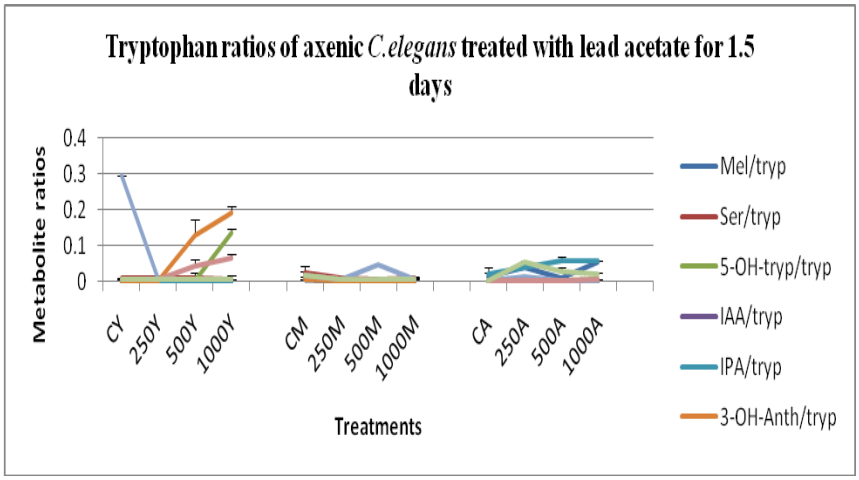


Figure 28(a): Metabolite to tryptophan ratios of *C.elegans* (young, middle and adult) treated with varying concentrations of lead acetate for 1.5 days

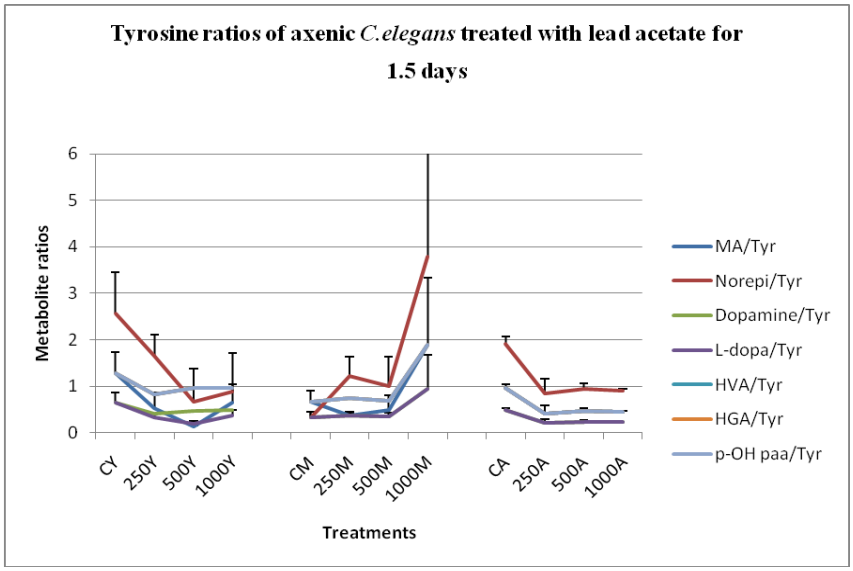


Figure 28(b): Metabolite to tyrosine ratios of *C.elegans* (young, middle and adult) treated with varying concentrations of lead acetate for 1.5 days

4.2.2. Age fractionated monoxenic *C.elegans* culture exposed to lead acetate for 1.5 days

This sub-section (section 4.2.2) describes the results of age fractionated monoxenic culture treated with lead acetate for 1.5 days time period. Population data details for the monoxenic age fractionated cohorts treated with different concentrations of lead acetate for 1.5 days are presented below. The percentages were calculated based on the total number of worms in each replicate. Reproduction is affected in cohort Y since, increasing concentrations of lead reduced the number of young. Middle-age and adult worm numbers increased therefore, growth is not affected in treated cohort Y (Figure 29 (a to c)). In the treated adult cohort, lead affected the reproduction since there was no increase in number of young. Standard error of mean ranged from 0.17 to 10.83.

Start of experiment: Cohort Y ~ (100%Y)

End of experiment: Cohort Y- Control ~ (98.5% Y, 1.4% M and 0% A)

250ppm ~ (76.3% Y, 9.6 % M, and 14%A)

500ppm ~ (77.3%Y, 18.3%M and 4.4%A)

1000ppm ~ (83.3%Y, 11.8%M and 4.9%A)

Start of experiment: Cohort M ~ (64.9% Y, 31.6% M, 3.5%A)

End of experiment: Cohort M- Control ~ (84.4% Y, 8.6% M and 10% A)

250ppm ~ (74.5% Y, 10.5 % M, and 14.8%A)

500ppm ~ (67.6%Y, 6.1%M and 26.3%A)

1000ppm ~ (59.3%Y, 12%M and 28.5%A)

Start of experiment: Cohort A ~ (24.8% Y, 1.6% M and 73.5%A)

End of experiment: Cohort A- Control ~ (49.7% Y, 8.6% M and 41.5% A)

250ppm ~ (35.6% Y, 10.4 % M, and 53.8%A)

500ppm ~ (32.4%Y, 17.2%M and 50.3%A)

1000ppm ~ (48.3%Y, 17.8%M and 33.7%A)

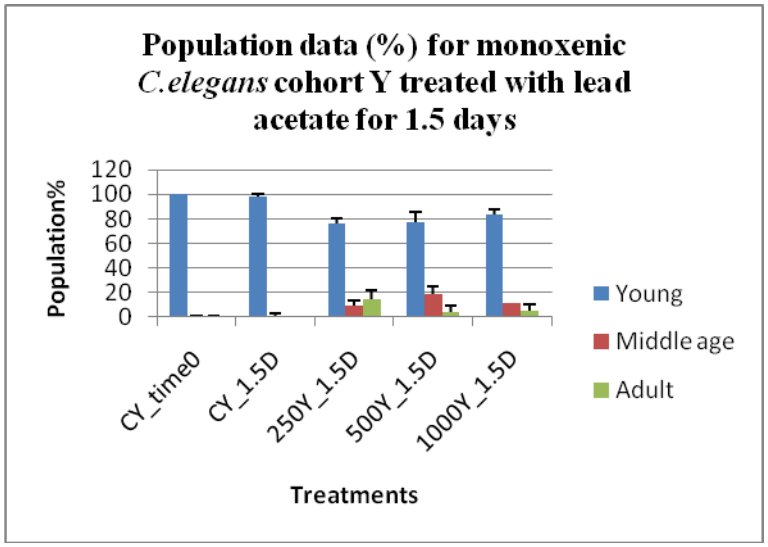


Figure 29(a): Population data for monoxenic age fractionated cohort Y treated with lead acetate for 1.5 days

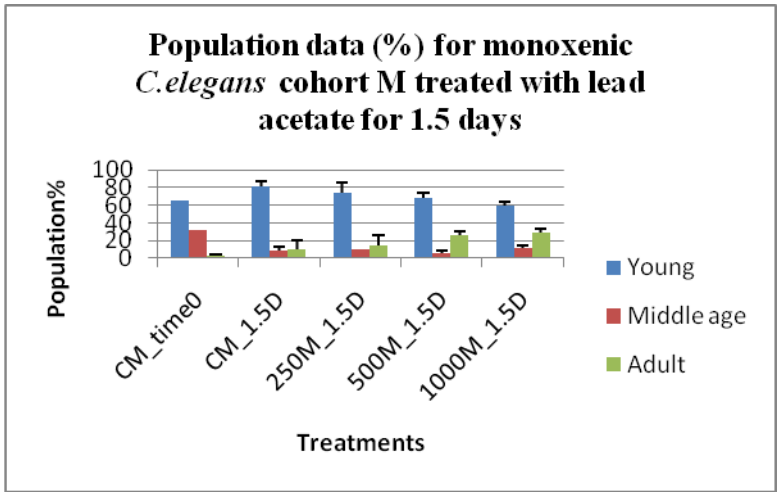


Figure 29(b): Population data for monoxenic age fractionated cohort M treated with lead acetate for 1.5 days

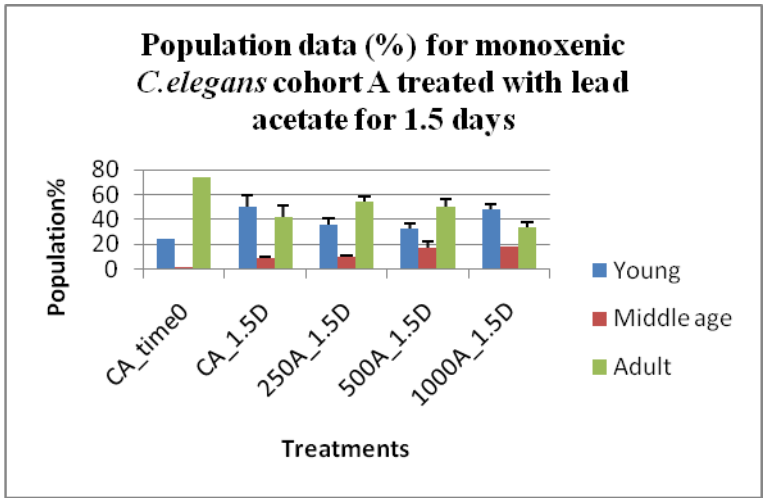


Figure 29(c): Population data for monoxenic age fractionated cohort A treated with lead acetate for 1.5 days

Figure 30 (a) and (b) represent the monoxenic middle age (capturing 82.98% metabolic differences) and adult (capturing 73.64% metabolic differences) (capturing 82.98% metabolic differences) cohorts treated with different dosages of lead acetate respectively. The data was analyzed through PCA to determine if it was possible to distinguish different subgroups (including controls and the different treatments) using ~ 2000 detectable analytes through Coularray. PCA was able to separate the four treatment groups in middle age nematodes ($p \leq 0.05$, Figure 30 (a)). PCA was performed for the young population which did not result in separating the treatment groups: the treated fractions grouped together. In the treated adult nematodes all four treatment groups parsed clustered based on dosage of lead (Figure 30(b)).

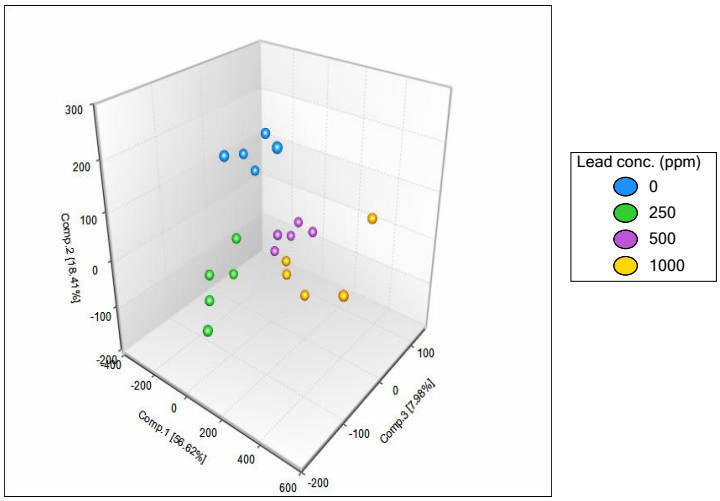


Figure 30 (a): PCA for the monoxenic control middle age *C.elegans* samples (dodge blue) and the middle age *C.elegans* samples treated with different concentrations of lead acetate

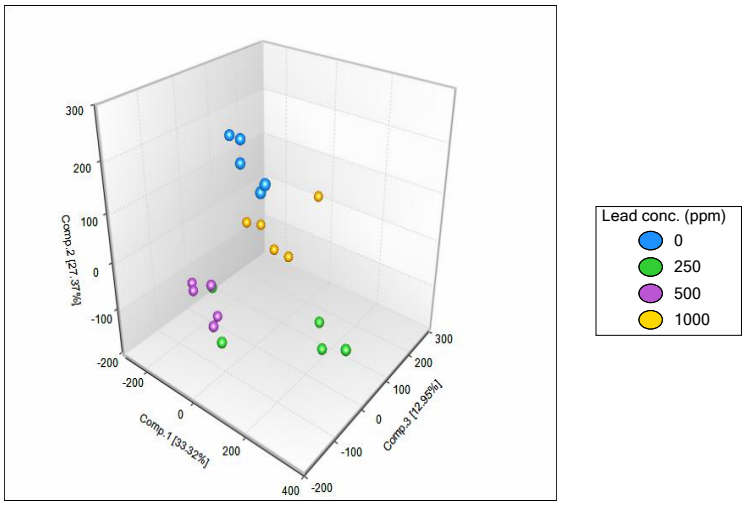


Figure 30 (b): PCA for the monoxenic control adult *C.elegans* samples and the adult *C.elegans* samples treated with different concentrations of lead acetate

Uric/xanthine and xanthine/guanine ratios increased ($p \leq 0.05$) in treated young populations compared to the control indicating an increase of uric acid. There was a dose

dependent increase in uric/xanthine and guanine/guanosine ratios in treated middle-age populations compared to the control. Uric acid and 8-hydroxy guanine levels were elevated ($p \leq 0.05$) in the 500ppm treated middle-age population. 8-hydroxy guanine is considered as one of the major products of oxidative DNA damage (Chang *et.al*, 1992; Arai *et.al*, 2002). The ratio of xanthine/guanine decreased significantly ($p \leq 0.05$) in the treated adult population compared to the control. Xanthosine showed a decreasing dose-dependent response with the increasing lead acetate concentrations, only in the monoxenic adult cohort. Xanthosine was only detected in the axenic adult cohort with lead levels of 1000 ppm. Uric acid increased in all axenic and monoxenic cohorts. The trend of perturbation in purine analytes in treated monoxenic cohorts was the opposite of the trend in treated axenic cohorts. All purine analytes in treated monoxenic cohorts were reduced, whereas there was an in increase in the purine analytes in treated axenic cohorts.

Tryptophan, indole 3-pyruvic acid, kynurenine and melatonin concentrations were reduced ($p \leq 0.05$) as a result of 1000ppm treatment in adult population compared to the control. Responses of cohorts Y, M and A samples differ with respect to the dose of lead received with respect to values observed in controls. All three treated adult populations expressed p-OH phenyl acetic acid in larger concentrations ($p \leq 0.05$). Tyrosine concentrations decreased in all the treated middle age populations resulting in greater concentrations ($p \leq 0.05$) of 3-Methoxy-4-hydroxy-mandelic acid, L-dopa and norepinephrine in treated middle-age populations compared to their controls.

4. CONCLUSIONS

The hypothesis for this study is based on the assumption there will be alterations in metabolic profiles, especially those involving changes in the metabolites of tryptophan, tyrosine and purine pathways in *C.elegans* as a result of stress. Common metabolite changes in *C.elegans* will occur irrespective of the stressors used for exposure. Unique metabolic alterations to tryptophan, tyrosine and purine pathways will occur following exposure of *C.elegans* to specific stress.

In tracking the metabolic profiles following stress exposure, majority of metabolites detected by Coularray were not changed. Our data shows that a relationship exists between the initial stress response and subsequent changes in the metabolic constituents of purine, tyrosine and tryptophan pathways of *C.elegans* to the specific stress factor used. No single metabolite changed in common across all of the treatments, but there were some peaks shared across the life stages and lead treatment levels. Spore and toxin treatments produced unique peaks not found following treatment with the rest of the stress factors.

Although most of the research with *C.elegans* has used monoxenic cultures, our research explored the effects of the same stress factors on axenic and monoxenic cultures (nutritional differences). The metabolic profiles of the two control culture types, axenic and monoxenic, exhibited alterations which were confirmed using PCA to cluster the data

depending on life stage and type of culture analyzed (Willett et al., 2010). One of the differences is shown in Figure 31 representing the snapshot of metabolic profiles of axenic and monoxenic young. The black arrow shows one of the differences between the two profiles as a result of the nutritional differences. This peak was only present in the axenic cultures. Other changes from exposure to the different stressors will be discussed below. Axenic cultures demonstrated metabolic differences as a result of exposure to *B.anthracis* spores and three toxin combination. Monoxenic cultures did not exhibit any observable metabolic changes with exposure to any biological stressors. With lead exposure axenic culture analysis showed alterations to many more metabolites in the three pathways studied than from the monoxenic culture analysis.

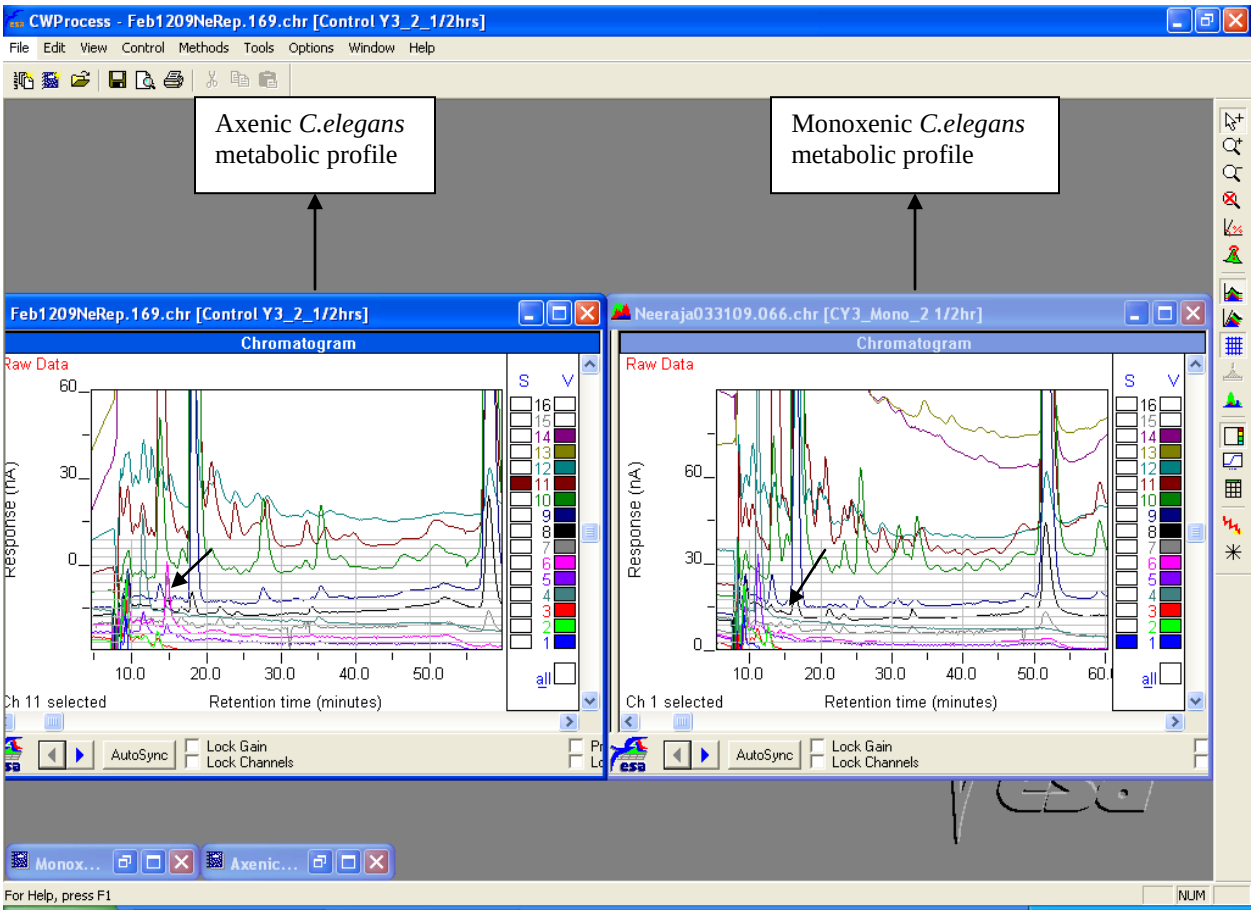


Figure 31: Snapshot of complete metabolic profiles from control axenic and monoxenic young

The biological stress factors derived from *B.anthracis* used in this research (vegetative cells and culture supernatant) led to no detectable stress effects or changes in metabolites of either axenic or monoxenic mixed cultures.

The *B.anthracis* spores and the three combined *B.anthracis* toxins perturbed axenic *C.elegans*' purine metabolite levels and their relative ratios, changing the overall metabolic fingerprint. Uric acid concentrations increased as a result of spore treatment in axenic *C.elegans* after an exposure time of 2.5 hours, whereas the monoxenic *C.elegans*

remained unchanged. Unique peaks were found as a result of spore treatment and following toxins (PAEFLF) treatment when data were subjected to a subtraction algorithm (Sudama, 2008) and represent early state changes in the metabolic profiles of axenic *C.elegans*.

Mixed populations (including all life stages) of axenic, but not monoxenic, *C.elegans* responded only to the combined exotoxins (PAEFLF). Perturbations were found in purine, tyrosine and tryptophan pathways following 2.5 hours exposure, as demonstrated by PCA. Guanosine was detected in elevated concentrations in axenic *C.elegans* samples treated with the *B.anthracis* three-toxin combination (PA+EF+LF) compared to the control axenic *C.elegans* samples, possibly providing protection against the toxicity of *B.anthracis* exotoxins. In the tryptophan pathway, the ratios demonstrate that metabolic flux is perturbed in branches leading to melatonin, indole acetate and anthranilate with PAEFLF when compared to control *C.elegans*. PAEFLF treatment in *C.elegans* also resulted in the flux change in products from the tyrosine pathway including norepinephrine, homogenistic acid and homovanillic acid.

Lead is a known chemical toxicant that causes harmful effects in humans by affecting development (e.g. learning, memory and behavior in children) (Anibal et al., 2006). Our research demonstrated that lead results in dose-dependent behavior in *C.elegans.* In terms of metabolic changes, 2.5 hour lead exposure did not affect monoxenic young and middle age *C.elegans*, whereas the purine, tyrosine and tryptophan metabolites perturbed in young and middle age of axenic *C.elegans* were the same as the metabolites perturbed in human subjects exposed to lead for longer periods of time (Blum

& Manzo, 1985). Increasing concentrations of lead at 1.5 days affected the following: growth in axenic young, reproductive processes in axenic middle-age, and led to lysis of mature adult *C.elegans*. In comparison, in 1.5 days lead treated monoxenic cohorts, reproduction is affected in cohort Y since, increasing concentrations of lead reduced the number of young. Middle-age and adult worm numbers increased therefore, growth is not affected in treated cohort Y. In the treated adult cohort, lead affected the reproduction since there was no increase in number of young. In the purine pathway, uric/xanthine and xanthine/guanine ratios increased, resulting in lead dose-dependent elevated concentrations of uric acid in all cohorts exposed to lead. Precursor-to-product ratios demonstrated that norepinephrine and 3-methoxy-4-hydroxy-mandelic acid, components of the tyrosine pathway, were increased only in axenic young and adult cohorts. In the tryptophan pathway, elevated concentrations of 3-hydroxy-kynurenine and 3-hydroxy anthranilic acid were detected in all treated young cohorts. Melatonin, also from the tryptophan pathway, was elevated only in treated adult cohorts.

Some of the unknown compounds perturbed by lead exposure were found to be dose-dependent across life stages. A selected set of three unique peaks (labeled ‘a’,’b’ and ‘c’ herein) appeared following two lead exposure times (2.5 hours and 1.5 days) in all age cohorts of *C.elegans*. The ratios of ‘a/b’ and ‘guanosine/c’ (guanosine was the nearest known compound in the metabolic profile) yielded similar values and changes across the age cohorts (Y, M and A) and at varying dosages, indicating that modulation of these analytes is stable with respect to final lead concentration. Thus, once identified, it may be a stable marker for 2.5 hours and 1.5 days of lead exposure. Because there were

age differences for some factors, we examined the axenic population profiles over time in detail, as a way to monitor for the sensitivity of different developmental stages to lead toxicity and to determine if these compounds (‘a’,’b’ and ‘c’) may be key markers of lead as a toxicant. The ratios for young to middle-age to adult for the different lead dosages resulted in similar patterns, showing that the effects of lead on all cohorts (Y, M and A) was independent of lead dosage.

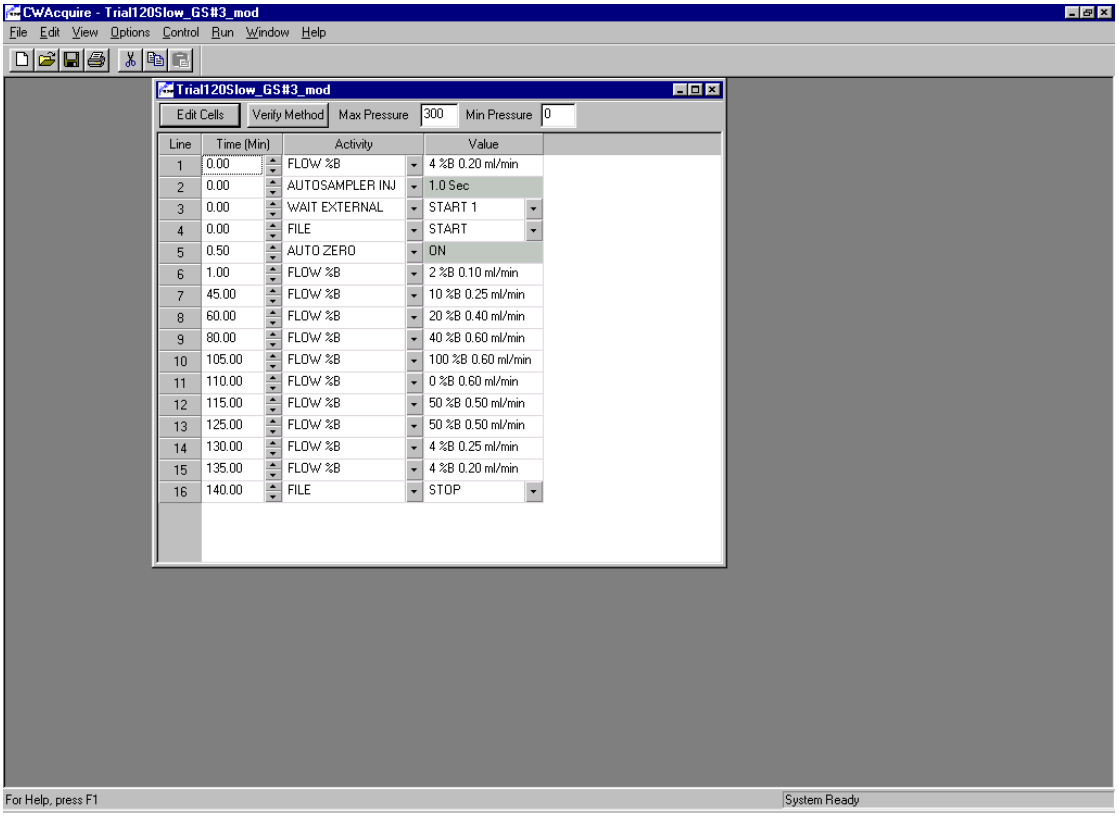
This research has demonstrated that the perturbation of specific metabolites in the purine, tyrosine and tryptophan pathways, as well as a number of unique but currently unidentified analytes, resulted in reproducible signature patterns in the nematode. The patterns emerged after introduction of a stress factor, and were characteristic of type, dosage, life stage of the nematode and the nutritional source of the *C.elegans* culture (axenic, monoxenic) tested.

Over the last few decades much has been learned about pathway contributions to homeostasis of metabolism in different living systems. The acute responses detected in axenic but not in monoxenic *C.elegans* suggest that axenic *C.elegans* is likely a more sensitive model system for detecting metabolic perturbations resulting from stress. The virulence of Anthrax in humans corresponds to the presence of the three exotoxins: protective antigen, edema toxin and the lethal toxin. Axenic *C.elegans* showed similar stress responses when exposed to the three exotoxins. This is the first report of the effect of *B.anthracis* toxins on *C.elegans,* demonstrating that only *C.elegans* exposed to the tri-part combination produces a response; single and bi-part mixtures led to no detectable responses.

Following short- term exposure to lead, minimal changes were observed in the metabolic profiles of *C.elegans*, but after longer exposure metabolic differences accumulated. This is analogous to the observed effects of lead in young humans, with slowly accumulating neurological deficits. Another similarity is that it took the same three- toxin combination required for the virulence of *B.anthracis* in humans to elicit a response in the nematode. It appears that using small molecule studies to monitor *C.elegans* responses to stress factors is a productive way to identify core pathways leading to physiologic state changes in both organisms. While the more complex system undoubtedly has its own elaborations, the response of this model system likely reflects some of the actions of stressors in mammalian systems as well. Understanding the underlying mechanisms to such responses in an organism with a much shorter life cycle and with few regulatory limits on its use as a test animal may lead to its deployment for early detection of toxicity. In terms of more basic research, the somewhat simpler metabolic profiles may allow more rapid identification of molecules responsible for state changes resulting in altered physiology.

Appendix

The HPLC method developed and used in this research to analyze samples

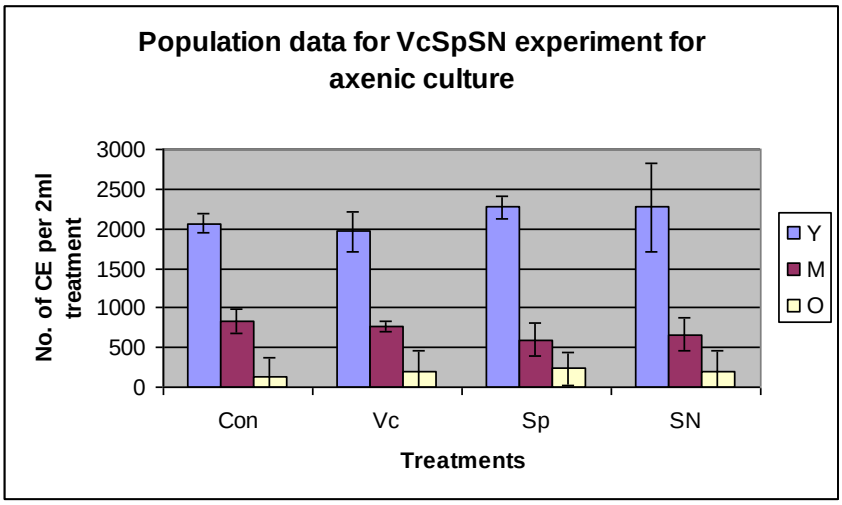


Line	Time (Min)	Activity	Value
1	0.00	FLOW %B	4 %B 0.20 ml/min
2	0.00	AUTOSAMPLER INJ	1.0 Sec
3	0.00	WAIT EXTERNAL	START 1
4	0.00	FILE	START
5	0.50	AUTO ZERO	ON
6	1.00	FLOW %B	2 %B 0.10 ml/min
7	45.00	FLOW %B	10 %B 0.25 ml/min
8	60.00	FLOW %B	20 %B 0.40 ml/min
9	80.00	FLOW %B	40 %B 0.60 ml/min
10	105.00	FLOW %B	100 %B 0.60 ml/min
11	110.00	FLOW %B	0 %B 0.60 ml/min
12	115.00	FLOW %B	50 %B 0.50 ml/min
13	125.00	FLOW %B	50 %B 0.50 ml/min
14	130.00	FLOW %B	4 %B 0.25 ml/min
15	135.00	FLOW %B	4 %B 0.20 ml/min
16	140.00	FILE	STOP

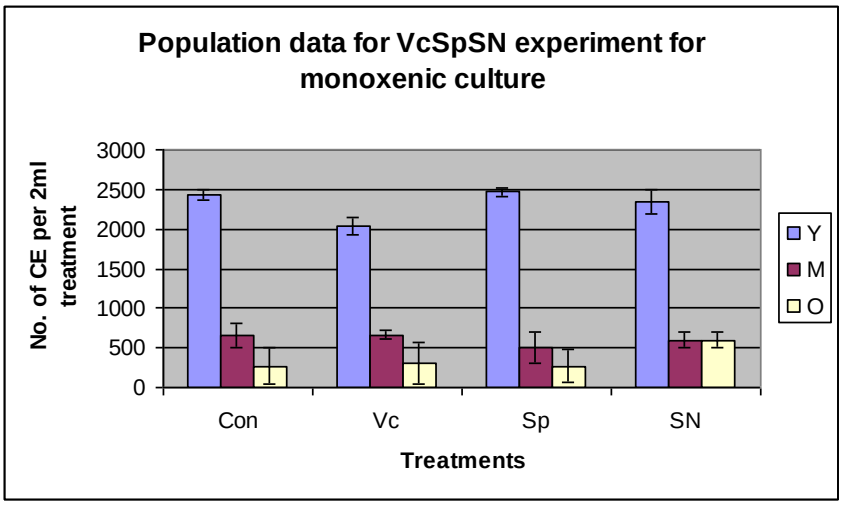
Appendix 2

Raw data (as counts) for the population distribution of vegetative cells, spores and culture supernatant experiment for axenic and monoxenic *C.elegans* cultures at the end of the time point (2.5hours)

Axenic data	Y	M	A	Total	Mono data	Y	M	A	Total
Con_Ax1	2000	500	300	2800	Con_Mono1	2500	1000	800	4300
Con_Ax2	1800	1000	200	3000	Con_Mono2	2100	800	600	3500
Con_Ax3	2000	1000	0	3000	Con_Mono3	2400	700	400	3500
Con_Ax4	2000	700	400	3100	Con_Mono4	2400	800	400	3600
Con_Ax5	2200	800	0	3000	Con_Mono5	2500	500	0	3000
Vc_Ax1	1700	800	300	2800	Vc_Mono1	2900	500	0	3400
Vc_Ax2	2300	800	200	3300	Vc_Mono2	2300	400	400	3100
Vc_Ax3	1700	700	500	2900	Vc_Mono3	2100	700	500	3300
Vc_Ax4	2000	800	0	2800	Vc_Mono4	1900	600	0	2500
Vc_Ax5	2200	800	100	3100	Vc_Mono5	2100	700	400	3200
Sp_Ax1	1700	700	500	2900	Sp_Mono1	2700	400	0	3100
Sp_Ax2	2000	700	300	3000	Sp_Mono2	2300	600	400	3300
Sp_Ax3	2300	600	300	3200	Sp_Mono3	2500	500	500	3500
Sp_Ax4	2100	800	400	3300	Sp_Mono4	2500	700	200	3400
Sp_Ax5	2400	400	0	2800	Sp_Mono5	2400	300	100	2800
SN_Ax1	2400	700	0	3100	SN_Mono1	2300	600	600	3500
SN_Ax2	2000	700	400	3100	SN_Mono2	2100	700	700	3500
SN_Ax3	2100	900	100	3100	SN_Mono3	2500	600	600	3700
SN_Ax4	1800	600	500	2900	SN_Mono4	2200	700	700	3600
SN_Ax5	2900	500	0	3400	SN_Mono5	2300	500	500	3300



Population data for Young (Y), middle-age (M) and adult (A) for VcSpSN experiment for axenic *C.elegans* culture.



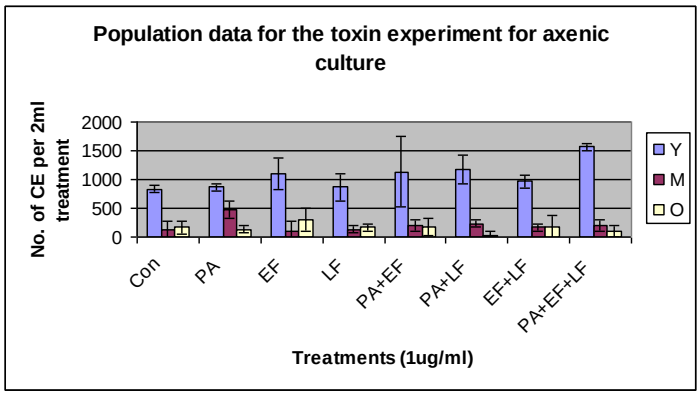
Population data for Young (Y), middle-age (M) and adult (A) for VcSpSN experiment for monoxenic *C.elegans* culture.

Appendix 3

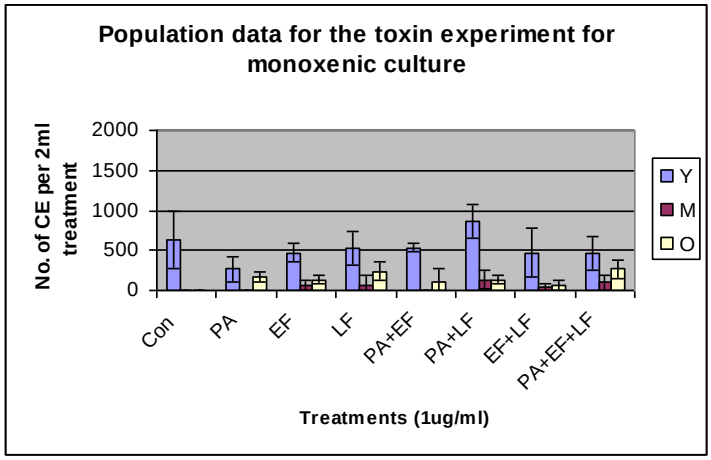
Raw data (as counts) for the population distribution for the toxin experiment for axenic and monoxenic *C.elegans* cultures at the end of the time point (2.5 hours)

Axenic	Y	M	A	Total	**Monoxenic**	Y	M	A	Total
Con1	1100	200	0	1300	Con1	500	0	200	700
Con2	800	0	200	1000	Con2	600	0	100	700
Con3	800	300	300	1400	Con3	300	0	0	300
Con4	800	0	100	900	Con4	600	0	0	600
Con5	900	100	100	1100	Con5	1000	0	0	1000
PA1	1300	100	200	1600	PA1	700	0	200	900
PA2	1700	400	0	2100	PA2	300	0	0	300
PA3	900	600	100	1600	PA3	400	0	100	500
PA4	900	300	100	1300	PA4	100	0	200	300
PA5	800	500	200	1500	PA5	300	0	200	500
EF1	1500	100	0	1600	EF1	200	0	100	300
EF2	800	0	200	1000	EF2	400	0	0	400
EF3	900	300	300	1500	EF3	400	0	100	500
EF4	1400	0	500	1900	EF4	400	100	200	700
EF5	1000	0	100	1100	EF5	600	100	100	800
LF1	1200	400	100	1700	LF1	1200	200	100	1500
LF2	900	300	0	1200	LF2	300	0	0	300
LF3	1000	100	200	1300	LF3	700	0	100	800
LF4	600	100	100	800	LF4	600	200	300	1100
LF5	1000	200	200	1400	LF5	300	0	300	600
PA+EF1	1600	200	200	2000	PA+EF1	500	0	0	500
PA+EF2	700	100	100	900	PA+EF2	400	0	100	500
PA+EF3	600	300	200	1100	PA+EF3	500	0	300	800
PA+EF4	1800	200	300	2300	PA+EF4	500	0	0	500
PA+EF5	1000	100	0	1100	PA+EF5	600	0	0	600
PA+LF1	500	100	100	700	PA+LF1	400	100	100	600
PA+LF2	700	100	100	900	PA+LF2	100	0	200	300
PA+LF3	900	200	0	1100	PA+LF3	800	200	100	1100
PA+LF4	1400	300	0	1700	PA+LF4	1100	0	200	1300
PA+LF5	1200	200	100	1500	PA+LF5	700	200	100	1000
LF+EF1	1300	200	100	1600	LF+EF1	300	0	0	300
LF+EF2	1400	200	100	1700	LF+EF2	700	100	300	1100
LF+EF3	900	200	100	1200	LF+EF3	200	0	100	300
LF+EF4	1100	100	400	1600	LF+EF4	400	0	0	400
LF+EF5	900	200	0	1100	LF+EF5	800	100	100	1000

PA+EF+LF 1	1200	200	300	1700	PA+EF+LF 1	600	0	100	700
PA+EF+LF 2	1500	300	0	1800	PA+EF+LF 2	1300	100	200	1600
PA+EF+LF 3	1600	200	0	1800	PA+EF+LF 3	700	0	200	900
PA+EF+LF 4	1600	100	200	1900	PA+EF+LF 4	300	100	200	600
PA+EF+LF 5	1500	300	100	1900	PA+EF+LF 5	400	200	400	1000



Population data for Young (Y), middle-age (M) and adult (A) for toxin experiment for 14 days old axenic *C.elegans* culture



Population data for Young (Y), middle-age (M) and adult (A) for toxin experiment for 14 days old monoxenic *C.elegans* culture

Appendix 4

Population data (as counts) for LPS experiment for axenic culture at the end of 2.5 hours.

Axenic data	Y	M	A	Total
Con_Ax1	3000	500	0	3500
Con_Ax2	2400	800	400	3600
Con_Ax3	900	300	300	1500
Con_Ax4	1400	300	400	2100
Con_Ax5	1300	0	400	1700
0.2_Ax1	400	300	300	1000
0.2_Ax2	1100	300	100	1500
0.2_Ax3	1800	200	200	2200
0.2_Ax4	800	600	100	1500
0.2_Ax5	800	200	300	1300
1_Ax1	300	300	200	800
1_Ax2	1500	300	300	2100
1_Ax3	1200	700	200	2100
1_Ax4	2000	500	300	2800
1_Ax5	1000	400	300	1700
5_Ax1	500	100	100	700
5_Ax2	1600	200	500	2300
5_Ax3	600	500	300	1400
5_Ax4	1300	400	100	1800
5_Ax5	1500	700	100	2300

Appendix 5

Raw data (as counts) for the population distribution for axenic *C.elegans* cultures treated with lead acetate at the end of the time point (2.5 hours)

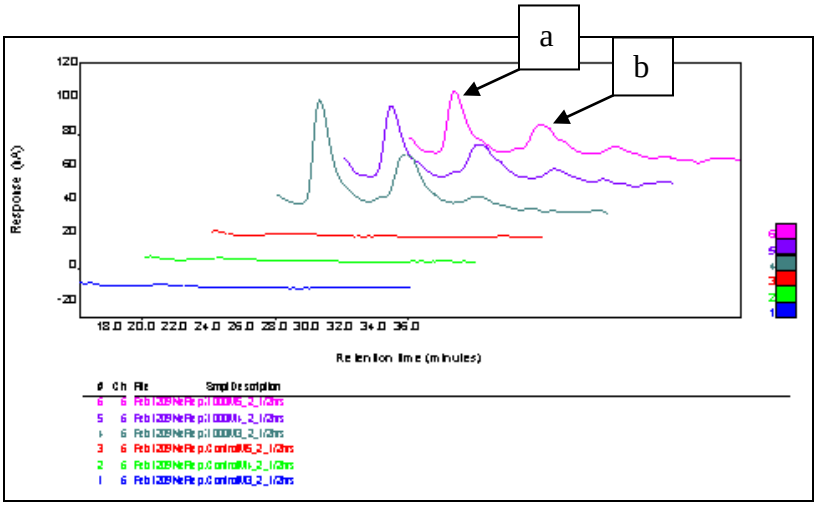
Axenic	Y	M	A	Axenic	Y	M	A	Axenic	Y	M	A
CY1	5400	0	0	CM1	2025	0	0	CA1	3450	2000	300
CY2	5625	375	75	CM2	2325	100	0	CA2	2325	1100	300
CY3	5550	0	0	CM3	3150	0	0	CA3	2550	1300	675
CY4	5025	200	0	CM4	4275	300	0	CA4	2550	1500	900
CY5	5325	0	0	CM5	2400	300	0	CA5	3525	2700	825
250Y1	7125	0	75	250M1	2325	100	75	250A1	3600	1500	225
250Y2	8175	200	0	250M2	4125	0	0	250A2	4050	2300	1125
250Y3	7800	200	0	250M3	3525	0	0	250A3	2700	1700	750
250Y4	6675	200	0	250M4	3750	0	0	250A4	3375	1800	825
250Y5	6375	100	0	250M5	4050	0	0	250A5	3525	1800	675
500Y1	6225	100	0	500M1	3750	400	0	500A1	3225	1400	375
500Y2	6750	0	0	500M2	2625	200	0	500A2	3300	1900	525
500Y3	5625	100	0	500M3	1875	300	0	500A3	2850	1900	150
500Y4	6375	100	0	500M4	2625	100	0	500A4	2850	1800	600
500Y5	8100	100	75	500M5	3000	100	0	500A5	3900	1900	675
1000Y1	7125	0	0	1000M1	1725	0	0	1000A1	3600	1300	375
1000Y2	7500	200	75	1000M2	1575	0	0	1000A2	2625	2700	975
1000Y3	7275	200	75	1000M3	3225	200	0	1000A3	6525	3300	1275
1000Y4	6375	0	0	1000M4	1875	100	0	1000A4	7800	6000	2325
1000Y5	4725	0	0	1000M5	1725	0	0	1000a5	7650	4800	2100

Appendix 6

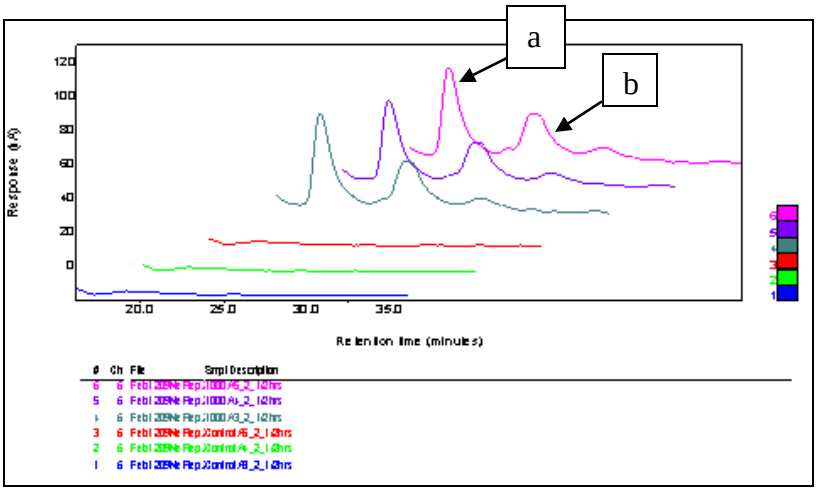
Raw data (as counts) for the population distribution for monoxenic *C.elegans* cultures treated with lead acetate at the end of the time point (2.5 hours)

Axenic	Y	M	A	Axenic	Y	M	A	Axenic	Y	M	A
CY1	22500	16875	0	CM1	600	1275	150	CA1	450	525	1050
CY2	50625	5625	0	CM2	675	600	150	CA2	750	1425	1050
CY3	28125	5625	0	CM3	675	300	150	CA3	300	375	675
CY4	22500	11250	0	CM4	750	1125	225	CA4	375	225	525
CY5	56250	16875	0	CM5	900	825	225	CA5	525	375	375
250Y1	73125	16875	0	250M1	525	900	75	250A1	450	600	825
250Y2	78750	16875	0	250M2	975	225	75	250A2	1125	225	525
250Y3	73125	5625	0	250M3	1200	975	825	250A3	675	300	825
250Y4	84375	16875	0	250M4	900	600	225	250A4	900	450	825
250Y5	84375	22500	0	250M5	975	300	225	250A5	600	225	675
500Y1	50625	22500	0	500M1	525	675	75	500A1	450	225	975
500Y2	39375	78750	0	500M2	450	375	75	500A2	450	225	675
500Y3	106875	78750	0	500M3	675	300	150	500A3	750	525	600
500Y4	28125	50625	0	500M4	300	750	225	500A4	450	750	300
500Y5	16875	39375	0	500M5	525	975	225	500A5	1350	300	1200
1000Y1	28125	28125	0	1000M1	225	1050	225	1000A1	225	1275	1050
1000Y2	45000	61875	0	1000M2	450	975	75	1000A2	675	900	975
1000Y3	16875	39375	0	1000M3	1200	1125	225	1000A3	450	450	675
1000Y4	22500	61875	0	1000M4	900	1125	300	1000A4	750	525	750
1000Y5	33750	61875	0	1000M5	750	675	300	1000A5	300	975	675

Appendix 7

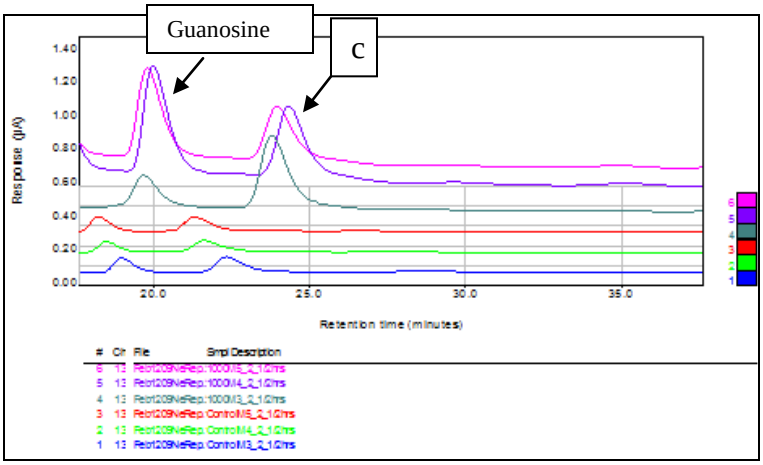


Metafile showing differences in a selected region of channel 6 for 3 replicates each of control and 1000ppm lead treated middle- age *C.elegans*. Blue, green and red represent replications of untreated middle- age; sea green, purple and pink represent replications of 1000ppm lead treated middle- age.

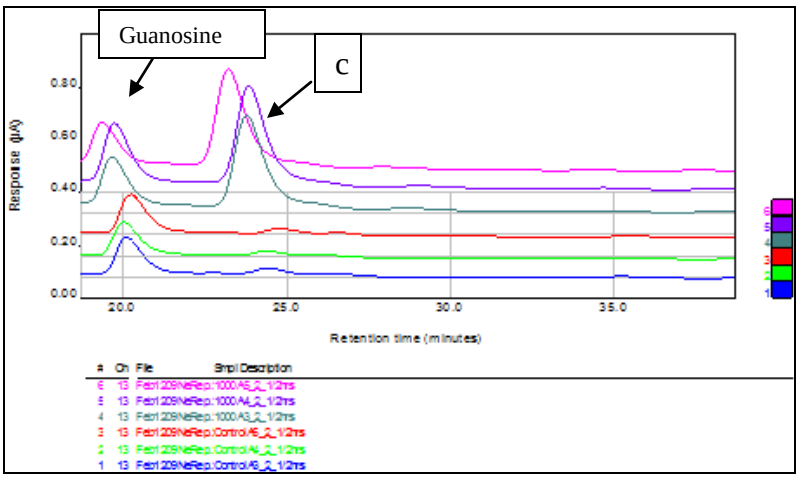


Metafile showing differences in a selected region of channel 6 for 3 replicates each of control and 1000ppm lead treated adult *C.elegans*. Blue, green and red represent replications of untreated adult; sea green, purple and pink represent replications of 1000ppm lead treated adult

Appendix 8



Metafile showing differences for channel 13 between control and 1000ppm lead treated middle -age *C.elegans*. Blue, green and red represent three replications of untreated middle- age; sea green, purple and pink represent three replications of 1000ppm lead treated middle- age



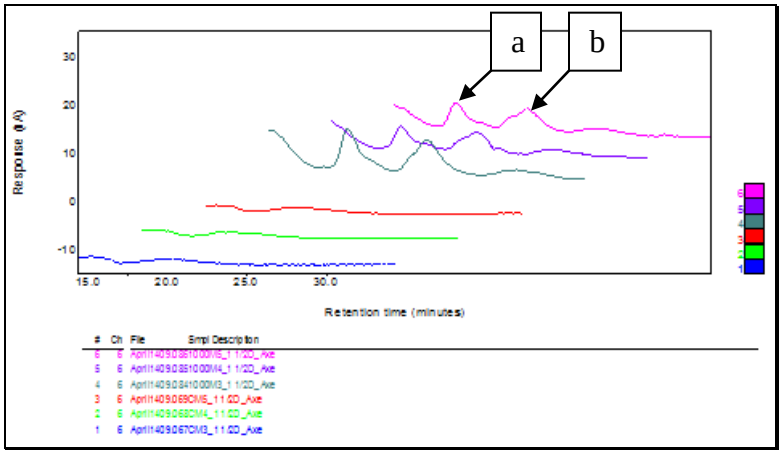
Metafile showing differences for channel 13 between control and 1000ppm lead treated adult *C.elegans*. Blue, green and red represent three replications of untreated adult; sea green, purple and pink represent three replications of 1000ppm lead treated adult

Appendix 9

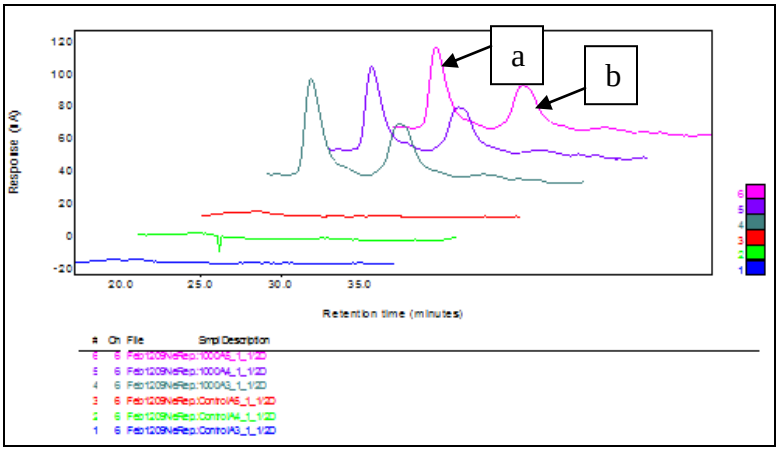
Raw data (as counts) for the population distribution for axenic *C.elegans* cultures treated with lead acetate at the end of the time point (1.5 days)

Axenic	Y	M	A	Axenic	Y	M	A	Axenic	Y	M	A
CY1	19000	2000	2000	CM1	3000	0	0	CA1	4000	10500	4500
CY2	17500	4000	2500	CM2	3000	500	500	CA2	24500	2500	1000
CY3	23000	5000	1000	CM3	2000	0	0	CA3	59500	9000	5000
CY4	16000	4000	0	CM4	0	7000	1000	CA4	59500	500	500
CY5	0	0	0	CM5	1000	0	0	CA5	66000	10500	5000
250Y1	20500	2000	1000	250M1	500	0	0	250A1	13000	1500	500
250Y2	9000	0	500	250M2	1500	0	0	250A2	10500	4000	2500
250Y3	11000	1000	0	250M3	1000	0	0	250A3	6500	1500	1000
250Y4	6500	1500	500	250M4	1500	0	0	250A4	10500	4500	1000
250Y5	12500	1500	500	250M5	0	1500	0	250A5	32500	2500	3000
500Y1	7500	500	0	500M1	1000	0	0	500A1	6000	1000	1000
500Y2	7500	0	1000	500M2	2500	1500	500	500A2	13500	6000	1000
500Y3	9500	500	0	500M3	1000	0	0	500A3	5000	5000	1000
500Y4	9500	3000	0	500M4	1500	0	0	500A4	20500	7500	3000
500Y5	6500	500	500	500M5	1000	500	0	500A5	18500	5000	1500
1000Y1	4000	0	500	1000M1	3500	1000	0	1000A1	19000	5000	1000
1000Y2	5500	0	0	1000M2	1500	0	0	1000A2	19000	4500	1000
1000Y3	6000	0	1000	1000M3	500	500	0	1000A3	6000	5500	1000
1000Y4	15000	500	500	1000M4	1000	0	0	1000A4	8000	4500	1500
1000Y5	6000	500	0	1000M5	1500	0	0	1000A5	9500	6500	1000

Appendix 10

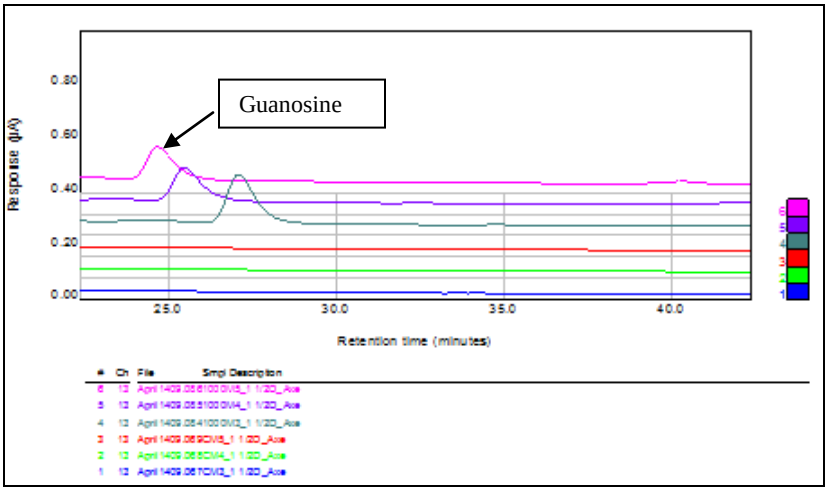


Metafile showing differences for channel 6 between control and 1000ppm lead treated middle- age *C.elegans*. Blue, green and red represent three replications of untreated middle -age; sea green, purple and pink represent three replications of 1000ppm lead treated middle- age

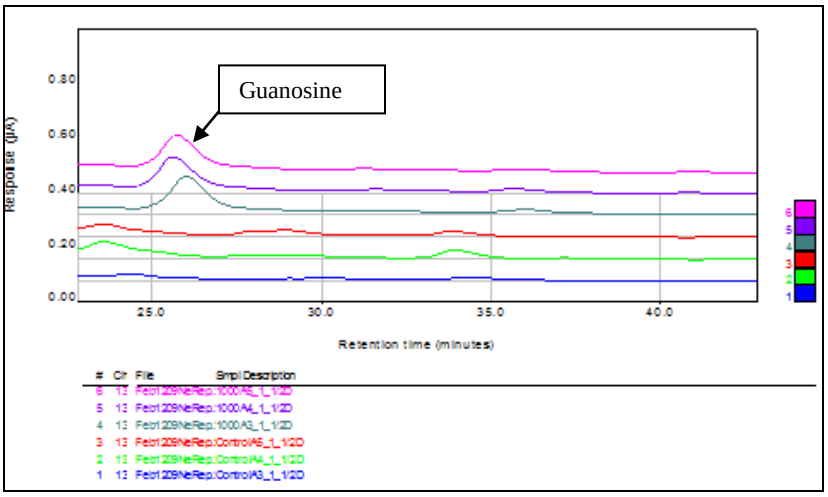


Metafile showing differences for channel 6 between control and 1000ppm lead treated adult *C.elegans*. Blue, green and red represent three replications of untreated adult; sea green, purple and pink represent three replications of 1000ppm lead treated adult

Appendix 11



Metafile showing differences for channel 13 between control and 1000ppm lead treated middle -age *C.elegans*. Blue, green and red represent three replications of untreated middle- age; sea green, purple and pink represent three replications of 1000ppm lead treated middle- age



Metafile showing differences for channel 13 between control and 1000ppm lead treated adult *C.elegans*. Blue, green and red represent three replications of untreated adult; sea green, purple and pink represent three replications of 1000ppm lead treated adult

Appendix 12

Raw data (as counts) for the population distribution for monoxenic *C.elegans* cultures treated with lead acetate at the end of the time point (1.5 days).

Axenic	Y	M	A	Axenic	Y	M	A	Axenic	Y	M	A
CY1	0	300000	0	CM1	3000	600	0	CA1	4000	400	1500
CY2	300000	0	0	CM2	2000	200	0	CA2	4000	800	3500
CY3	300000	100000	0	CM3	500	0	500	CA3	3500	1000	6000
CY4	300000	100000	0	CM4	3500	0	1500	CA4	3000	2200	9500
CY5	200000	0	0	CM5	1000	600	0	CA5	6000	2600	5000
250Y1	300000	200000	0	250M1	1000	600	500	250A1	4500	1800	8500
250Y2	300000	400000	0	250M2	4500	600	500	250A2	17000	3400	10000
250Y3	0	200000	0	250M3	3500	400	0	250A3	14000	1400	7000
250Y4	200000	300000	0	250M4	5000	600	1000	250A4	7500	1600	7500
250Y5	200000	200000	0	250M5	3000	600	2000	250A5	4000	1200	7500
500Y1	200000	300000	0	500M1	3500	0	1000	500A1	3500	800	7000
500Y2	200000	100000	0	500M2	2500	400	1500	500A2	4000	1800	5500
500Y3	500000	300000	0	500M3	3000	400	1000	500A3	6500	1400	5500
500Y4	200000	300000	0	500M4	1000	400	1500	500A4	3500	1800	3500
500Y5	700000	300000	0	500M5	1000	400	500	500A5	3500	3200	6500
1000Y1	300000	300000	0	1000M1	4500	1000	1500	1000A1	4500	1600	3000
1000Y2	300000	100000	0	1000M2	3500	400	1500	1000A2	5500	2400	5500
1000Y3	200000	300000	0	1000M3	1000	1400	1500	1000A3	3000	1000	1500
1000Y4	400000	700000	0	1000M4	2000	600	1500	1000A4	1000	1000	2000
1000Y5	500000	1200000	0	1000M5	1500	600	500	1000A5	1500	1400	2000

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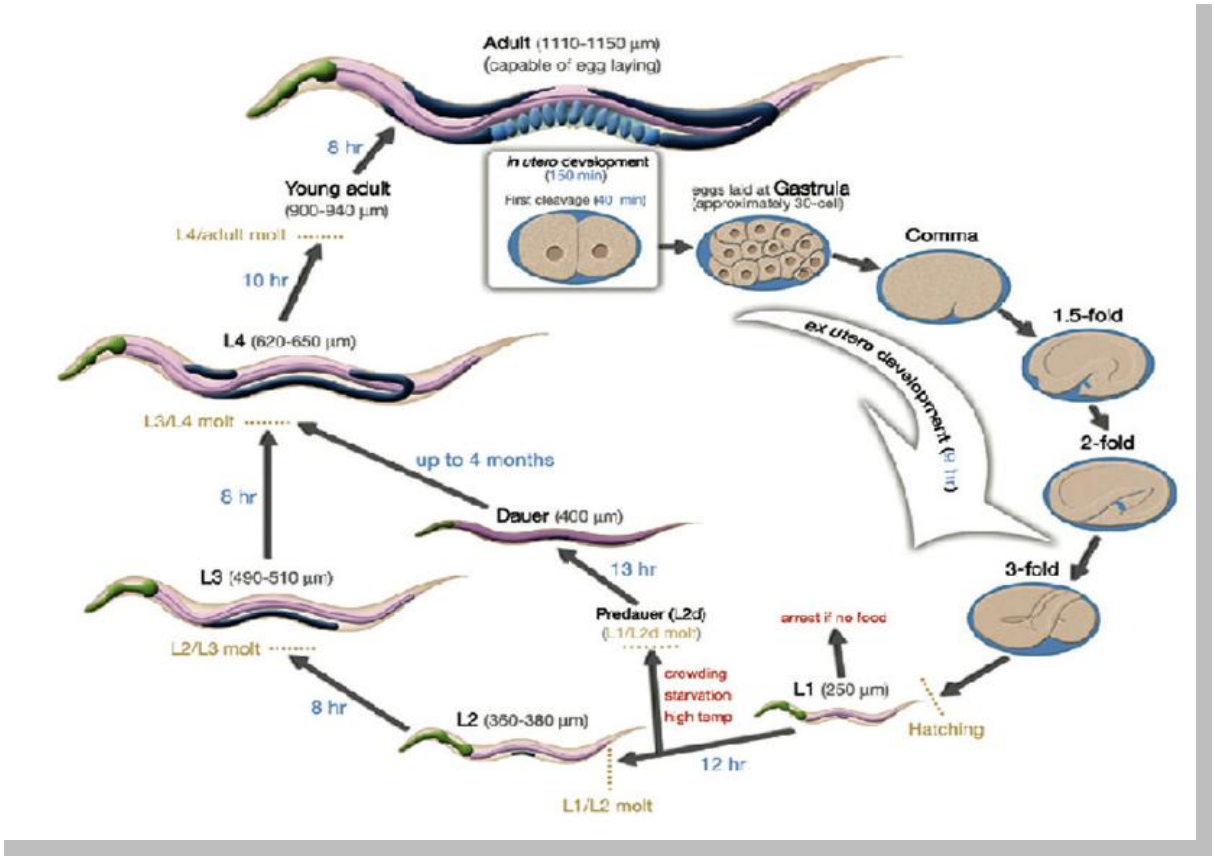
SUPPLEMENTARY MATERIAL

Tyrosine derivatives	**Observed lowest detection limits**
Norepinephrine	20 pg
DL-hydroxy mandelic acid	10pg
L-Dopa	5pg
Dopamine	5pg
Tyrosine	5pg
p-hydroxy phenyl acetic acid	10ng
Homovanillic acid	10ng

Tryptophan	**Observed lowest detection limits**
3-hydroxy kynureine	20pg
3-hydroxy anthranilate	5pg
5-hydroxy tryptophan	10pg
Kynurenine	20pg
Anthranilate	20pg
5-hydroxy Indole acetic acid	25pg
Serotonin	30pg
Tryptophan	20pg
n-acetyl serotonin	30pg
Tryptamine	30pg
Indole-3 pyruvic acid	5pg
Melatonin	5pg
5-methoxy tryptamine	30pg



Life cycle of *C.elegans* hermaphrodite at 22°C (www.wormbook.org)

CURRICULUM VITAE

Neeraja Podugu was born and raised in India. She completed her schooling from Kendriya Vidyalaya in different states of India. She graduated from Gujarat Agricultural University with Bachelor of Science in Agriculture in 2000. She completed her Master of Science degree in Plant Pathology from the same university in 2003. Her thesis was titled "Detection, transmission and serology of Soybean Mosaic Virus (SMV)". For the past four years, she has been working on her PhD with a research assistantship in Biosciences at Molecular and Microbiology Department, College of Science, George Mason University, USA.