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Caenorhabditis elegans stress related gene responses to selected pesticides.

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Abstract

Pesticides are used widely and more than 2 million tons are released in the environment annually (data for 2000-2001, Kiely 2004). Details of their toxicity towards non-target organisms, are not complete for many of these pesticides and serious environmental issues have previously arisen as a result (e.g. effects of DDT on reproduction of wild birds).

Unfavourable conditions, including the presence of toxicants, can induce the stress response pathways through which an organism attempts to metabolise the harmful chemicals or counter their effects. The stress response network contains a number of gene pathways controlled by transcriptional regulators which control expression of genes in one or several groups. In their attempt to counter stress, stress-response genes act in an interactive manner and should therefore be studied together as a network, rather than individually.

This study examines the effect of a number of commonly used pesticides on *C. elegans*, a non-target organism. We focus on stress-response gene expression patterns and in some cases perform assays for physiological effects. We also present supplementary qPCR experiments to confirm previous results on the effect of dichlorvos on *C. elegans* gene expression.

Our results show that some tested pesticides are not toxic to the nematode, whereas rotenone proves highly toxic and chlorpyrifos, endosulfan, DDT and carbendazim are moderately toxic with DDT showing significant inhibition of feeding as well.

Our data partly confirm the gene array results previously obtained for dichlorvos.

Our study provides information on how a number of pesticides affect stress-response gene expression. Together with previous data on the effect of heavy metals and parallel data from *Drosophila*, these findings will inform the development of a dynamic mathematical model of the stress-response network (SRN).

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Abbreviations

| | |
|-------------------|------------------------------------|
| ABC | ATP-Binding Cassette |
| ATP | Adenosine TriPhosphate |
| bp | base pairs |
| CFP | Cyan Fluorescent Protein |
| CT | Threshold Cycle |
| DC | dichlorvos |
| DDD | dichlorodiphenyldichloroethane |
| DDE | dichlorodiphenyldichloroethylene |
| DDT | dichlorodiphenyltrichloroethane |
| DEPC | diethylpyrocarbonate |
| dH ₂ O | distilled water |
| DIC | Differential Interference Contrast |
| DMF | DiMethylFormamide |
| DMSO | dimethyl sulfoxide |
| DNA | DeoxyriboNucleic Acid |
| dsRNA | double stranded RNA |
| EDTA | ethylenediaminetetraacetic acid |
| eq. | equivalent |

| | |
|------|--|
| EtBr | Ethidium Bromide |
| EtOH | ethanol |
| GABA | γ -aminobutyric acid |
| GFP | Green Fluorescent Protein |
| GO | Gene Ontology |
| hsp | heat shock protein |
| hsf | heat shock factor |
| IPCS | International Programme on Chemical Safety |
| Kb | Kilo base pairs |
| LB | Luria Bertolli |
| MeOH | methanol |
| min | minutes |
| NGM | Nematode Growth Media |
| nt | nucleotides |
| NTC | No Template Control |
| OD | Optical Density |
| PAN | Pesticide Action Network |
| PBO | Piperonyl ButOxide |
| ppm | parts per million |
| RNA | RiboNucleic Acid |

| | |
|------|----------------------------|
| RNAi | RNA interference |
| ROS | Reactive Oxygen Species |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| RT | Reverse Transcriptase |
| SDS | Sodium Dodecyl Sulfate |
| sec | seconds |
| SEM | Standard Error of the Mean |
| SOD | SuperOxide Dismutase |
| SRN | Stress Response Network |
| TAE | Tris Acetate EDTA |
| TBE | Tris Borate EDTA |
| UTR | UnTranslated Region |
| UV | Ultra Violet |
| V | Volts |
| YFP | Yellow Fluorescent Protein |

Nomenclature

| Rules followed | example |
|--|----------------|
| Letters, but not numbers within gene names are written in <i>italics</i> . | <i>daf</i> -16 |
| Protein names are written in CAPITALS. | DAF-16 |
| Cosmid names are written in CAPITALS. | Y45F10D.4 |

1 Introduction

1.1 *Caenorhabditis elegans* as a model organism.

Usually a free-living (non parasitic) soil nematode, *C. elegans* feeds on bacteria and fungi present in its environment. Use of *C. elegans* as an experimental model spread after 1974 with Sydney Brenner's extensive research in *C. elegans*' genetics (Brenner 1974). Today, the ease of culture and speed of its life cycle as well as its small size and robustness make *C. elegans* an ideal experimental model. Its simplicity makes it easy to use as a model organism for toxicology or genetic studies and its similarity to higher organisms gives research on *C. elegans* potential applications in other fields including medicine. Several properties of the organism facilitate a series of cellular and molecular techniques making this nematode a powerful tool for experimental research. The ease of manipulation of *C. elegans* and its applications have led to it becoming one of the leading model organisms in many fields of biological research and work on *C. elegans* has been awarded three Nobel prizes in recent years for Physiology and Medicine in 2002 and 2006 and Chemistry in 2006.

1.1.1 *C. elegans* in the laboratory

Because a fully grown adult nematode is only ca. 1mm long, culturing *C. elegans* does not require large spaces. In fact, *C. elegans* can easily be cultured on Petri dishes in the laboratory. Agar containing bacterial growth-promoting nutrients and salts is seeded with *E. coli* bacteria on which *C. elegans* feeds (2.2.1). A liquid culture can be used instead, where *E. coli* is resuspended in a nutrient liquid medium and *C. elegans* is allowed to grow in the suspension (2.2.2). Oxygen must be abundant and the temperature must be set within the range that *C. elegans* grow stress-free namely 15°C-25°C (Byerly et al. 1976). Under favourable conditions (presence of food and oxygen, 20°C temperature, no stress-inducing toxicants) a full life-cycle from egg to egg-laying adult lasts 3 days; thus *C. elegans* is a rapidly growing organism, ideal for developmental and genetic studies. Its ability to go into the dauer stage in the absence of nutrients or when overcrowded means that, even if left unwatched for

weeks, a culture can still be revived. Freezing of strains at -80°C is also possible, providing a laboratory with frozen stocks of previously cultured strains and avoiding genetic drift. The fact that *C. elegans* is an invertebrate means that there are essentially no ethical issues involved in its use as an experimental model.

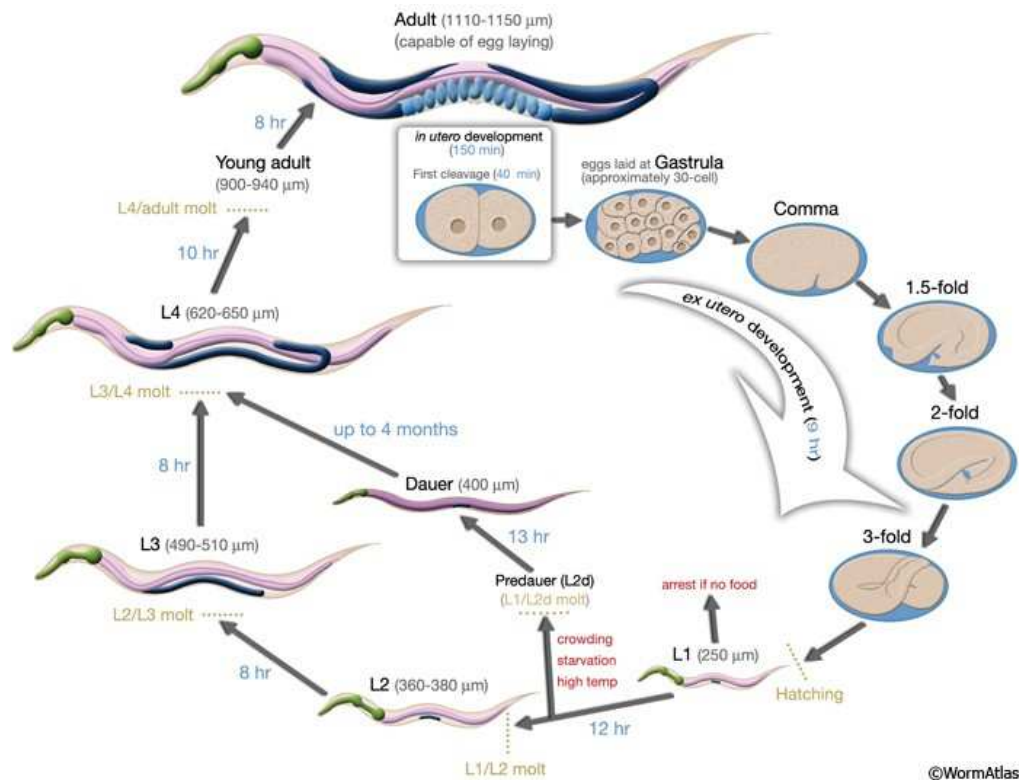


Figure 1.1 The *C. elegans* life cycle at 22°C. 0min is fertilization. At each stage, the length of the animal is marked next to the stage name. The length of time the animal spends at a certain level is indicated by the numbers in blue.

Image taken from WormAtlas © <http://www.wormatlas.org/hermaphrodite/introduction/IMAGES/introfig6leg.htm>

1.1.2 *C. elegans* anatomy

C. elegans is a transparent roundworm that reaches ca. 1mm in length. Its body comprises of the head, the main body and the tail. Food is consumed through the mouth (head region), travels through the intestine (main body) and is excreted through the anus (tail region) (fig. 1.2).

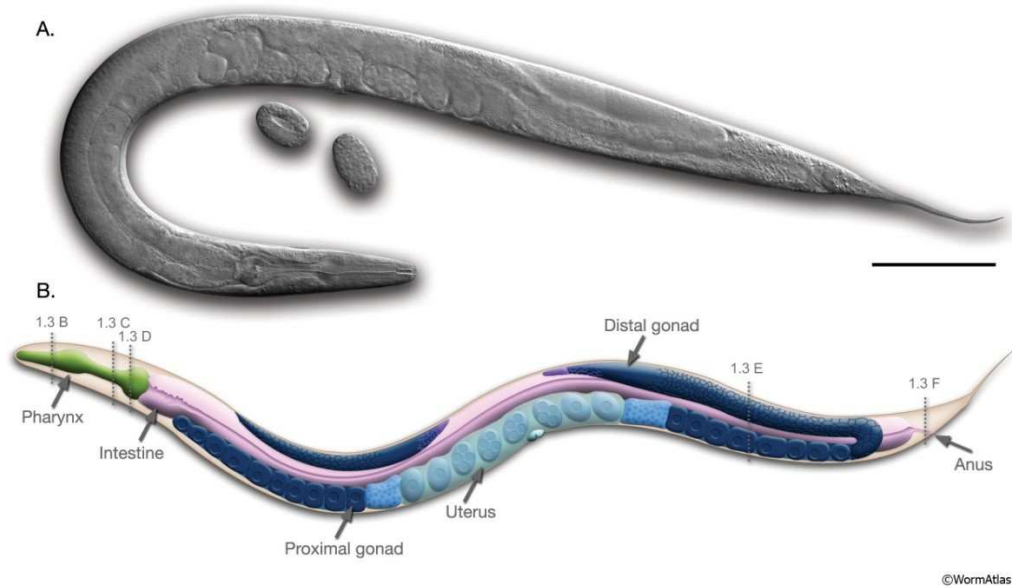


Figure 1.2 Anatomy of a hermaphrodite *C. elegans*. **A.** Differential Interference Contrast (DIC) image showing an adult hermaphrodite and laid eggs. Scale bar 0.1mm. **B.** Schematic drawing of anatomical structures. Dotted lines and labels mark position of each section shown in Fig. 1.3 B-F.

Image adapted from WormAtlas © <http://www.wormatlas.org/hermaphrodite/introduction/IMAGES/introfig1leg.htm>

The main body consists of an outer tube, an inner tube and the pseudocoelomic space, separating them. The outer tube, or body wall, is made up of cuticle, hypodermis, excretory system, neurons, and muscles. It protects the animal from the outside world and assists in locomotion. The inner tube includes the internal organs (pharynx, intestine) and its main function is through feeding. The gonad is also part of the inner tube, partly filling the pseudocoelomic space in adults. In the gonad, sperm matures first during L4 stage; when the individual has reached adulthood, the gonad switches to egg production and oocytes start to mature. Ovulation of the first oocytes results in pushing of the spermatids from the gonadal sheath (where they are generated) into the spermatheca where they mature into spermatozoa (L'Hernault 1997). In the spermatheca, spermatozoa fertilise the oocytes (Singson 2001) as they pass from the gonadal sheath toward the uterus. From the uterus, eggs are forced out of the body through the vulva. The coelomocyte system is made up of 6 scavenger cells, the

coelomocytes. Their function is to endocytose fluid and macromolecules from the body cavity, giving them an immune and hepatic role. In some larger nematode species, coelomocytes also have a phagocytic role, being able to endocytose invading organisms (Bolla et al. 1972).

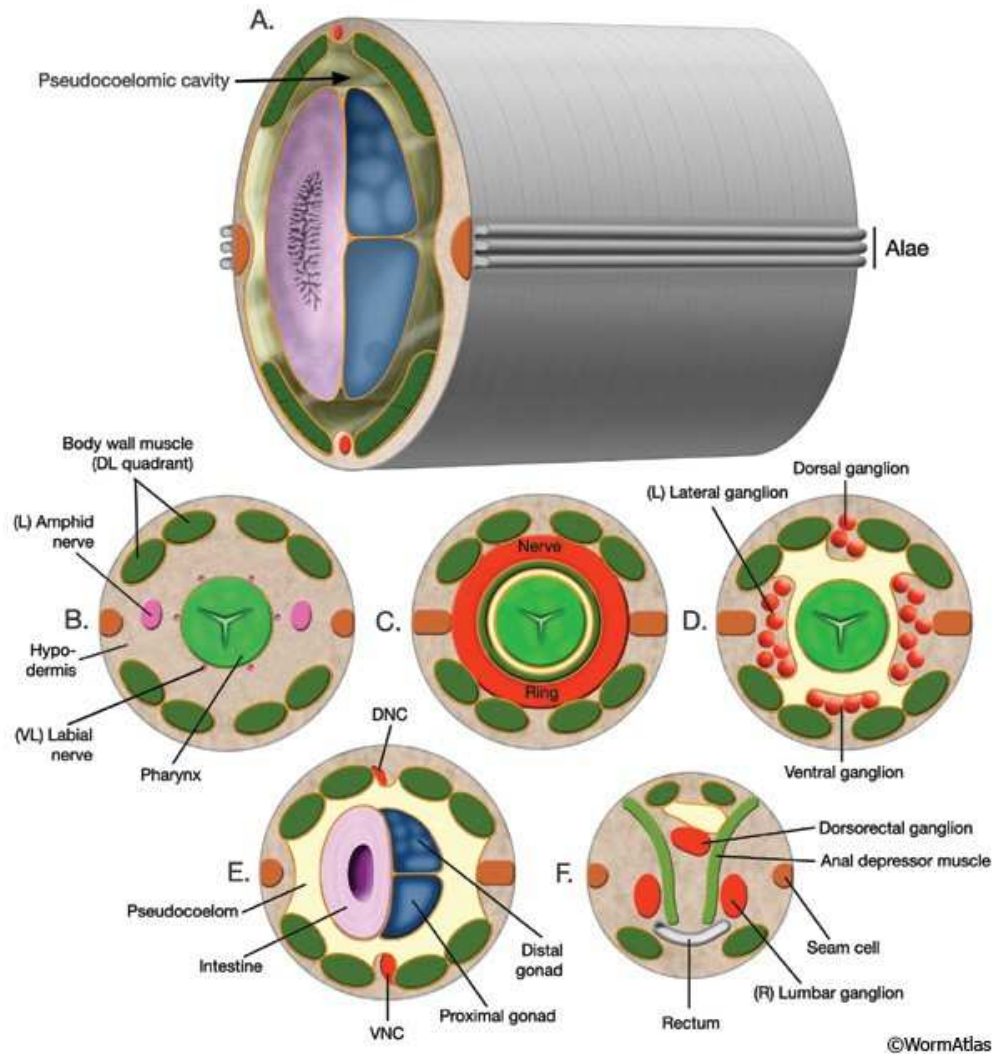


Figure 1.3 The body plan. The position of each section is labelled in Fig. 1.2 B. **A.** Posterior body region. A pseudocoelom separates the body wall from the inner tube. **B.** Section through anterior head. **C.** Section through the middle head. **D.** Section through posterior head. **E.** Section through posterior body. **F.** Section through tail. (NC) Nerve Cord. Orange lines indicate basal laminae.

Image taken from WormAtlas © <http://www.wormatlas.org/hermaphrodite/introduction/IMAGES/introfig2leg.htm>

1.1.3 *C. elegans* as a model for human diseases

Genetically, *C. elegans* has a much smaller genome than that of a human (ca. 10^7 bp compared to 3×10^9 bp) but approximately 65% of human disease genes have corresponding genes in the worm (Sonnhammer & Durbin 1997), making it a simple and powerful model to study human disease.

The structures inside a *C. elegans* nematode may be simple, but simplified versions of most animal basic organs are present. There is a nervous system with a complete set of 302 neurons (White et al. 1986) which makes *C. elegans* a very good model for the study of neural development and function. Disease models for neurodegenerative diseases have been made using the nematode, including Spinal Muscular Atrophy (Burt et al. 2006), Parkinson's disease (Nass et al. 2001; Kuwahara et al. 2006), Alzheimer's disease (Daigle & Li 1993; Ewald & Li 2010) and Huntington's disease (Parker et al. 2001; Jeong et al. 2009). A muscle system is present containing smooth muscle in the pharynx, and striated muscle in the body wall, similarly to cardiac and skeletal muscle in vertebrates (Kagawa et al. 2007). The nematode can be used for studying muscle development and mutant strains are used as disease models of a number of muscular dystrophies such as Duchenne Muscular Dystrophy (Gaud et al. 2004; Giacomotto et al. 2009) and Emery–Dreifuss muscular dystrophy (Liu et al. 2003).

1.1.4 Special properties of *C. elegans*

C. elegans was the first multicellular organism to have its genome fully sequenced and after *Saccharomyces cerevisiae* was only the second eukaryote. Naturally a lot is known about this organism's genetics and many powerful tools have been developed for its study. Most of its 22,227 (*C. elegans* Sequencing Consortium 1998; Spieth & Lawson 2006) known protein-coding genes are well characterised and many have counterparts in higher eukaryotes.

C. elegans is the sole organism for which the developmental lineage of all its somatic cells is known. Cell number between individuals is highly invariant with the final

number of nuclei in a mature hermaphrodite being 959 and that in a male being 1031 (Sulston et al. 1983). This invariability is due partly to the nature of *C. elegans* fertilisation. The vast majority of individuals are XX hermaphrodites with only 0.05% XO males. Hermaphrodites usually self-fertilise resulting in progeny identical to the parent. Self-fertilisation also gives rise to homozygosity, which is why, other than in the case of novel mutations, homozygosity is extremely common in *C. elegans*. This property is particularly useful in an experimental model, since genetic variation is a variable one has to account for when interpreting experimental results. Using genetically identical (homozygous) strains reduces their source of variability, but does not eliminate variability in response between individuals.

Another useful property of *C. elegans* is its transparency. An egg is transparent which means that visualisation of the embryo is possible in non-invasive ways. Study of *C. elegans* embryos is possible using only a microscope. The mature animal is also transparent, facilitating techniques such as anatomical imaging, cell migration studies, organ visualisation and fluorescent protein (GFP, YFP, CFP, DsRed) detection. This property also assists in the microinjection techniques used, for example, in the production of transgenic strains. By injecting the gonad with a DNA desired construct, this is taken up by maturing oocytes and inherited by some of the progeny (Mello et al. 1991; Mello & Fire 1995). Such transgenic constructs are normally inherited extra-chromosomally (transmission frequency may vary greatly), or can be integrated to the genome following γ - or x-irradiation (Mello et al. 1991; Evans 2006).

Another technique developed in *C. elegans* is the use of RNA interference (RNAi) (Fire et al. 1998). This is extremely easy to apply on the nematode, simply through feeding of bacteria containing a plasmid carrying the sequence of interest; such bacterial strains now cover most of the *C. elegans* genome (Kamath & Ahringer 2003). RNAi is a simple way to produce gene knock-downs, although the effect may vary in magnitude from a slight effect to almost complete knock-out of the gene function. Essentially, RNAi involves short sections of dsRNA which enter the cell and bind to mRNA molecules containing complementary sequences, leading to their degradation.

1.1.5 *C. elegans* in toxicology

C. elegans, being a simple organism, is easy to use in toxicology studies and findings are significant in relation to more complex organisms. It is sensitive to molecules that can penetrate its nervous system and allow study of neurotoxicity. Symptoms such as abnormal movement or lethality are easily detected under a dissecting microscope. Lethality can also be quantified using fluorescent dyes which identify dead animals by binding to DNA in compromised cells (Gill et al. 2003). Other aspects that can be studied in toxicology studies are behavioural endpoints, such as locomotion, through motility assays (Arena et al. 1995); reproduction, through a sprinting or sperm expulsion assay (Barker 1994); growth, through a count of gravid adults or a measure of body size; or feeding, through a feeding inhibition (Jones & Candido 1999). Feeding inhibitions entail worms being incubated in bacterial suspension in the presence or absence of the toxicant and measurement of the optical density to determine differences in feeding between these conditions.

The fact that *C. elegans* is transparent makes possible the use of reporter gene fusions for visualisation of protein expression patterns or cellular morphology. An example is transcriptional fusion constructs (1.5) with a reporter gene fused to the regulatory region of the gene of interest (David et al. 2003). A series of strains with such constructs for stress-response genes is available through the Caenorhabditis Genetics Centre (CGC) and the Baillie GFP genome project (Simon Fraser University, Vancouver). Such strains can be used to explore the nematode's stress response network.

1.1.6 *C. elegans* in genetics

This nematode is ideal for genetic studies. Unlike other model organisms used in genetics research, it combines a number of advantages. Unlike yeast and bacteria, it is multicellular, allowing study of the organism as a whole, or in cell culture (Christensen et al. 2002), but retains the advantage of a simple model. Unlike the fruit fly, its organs are much less complex (e.g. nervous system comprises ca. 300 cells rather than 10^5 in *Drosophila melanogaster*). Unlike the mouse, its life cycle ranges

between 2.5-6 days depending on the temperature and it can give 300-1000 progeny per hermaphrodite individual (300 in the case of self-fertilisation, 1000 following fertilisation by a male). A variety of techniques has been developed in the nematode allowing production of knock-out or knock-in strains as well as knock-down effects. Gene expression can be measured on microarrays and whole mount embryos or larvae can be stained *in situ* to localise protein distribution (using antibodies) or gene expression (using RNA). Reporter gene fusion strains can also be used to quantify differences in gene expression as well as to localise gene products.

1.1.7 *C. elegans* in developmental biology

Several properties of the nematode also make it ideal for developmental research. The fact that the embryo develops inside an egg rather than within the mother means that study of the embryo is not invasive. The transparency of the eggs makes it possible to study them under a microscope and to visualise cell lines using dyes and other markers. The nature of fertilisation in *C. elegans* is also interesting since repeated self-fertilisation results in homozygosity with little variation between individuals in the species. *C. elegans* development is strictly conserved and the lineage of every somatic cell in the adult animal is known. Male individuals are also available for out-crossing where required.

1.2 Stress response in the nematode

Stress responses are an organism's attempt to survive in an unfavourable environment. Stress can be caused by a number of factors, the main ones being changes in temperature (*C. elegans* is under stress at temperatures lower than 15°C or higher than 25°C), lack of oxygen or nutrients, presence of reactive oxygen species (ROS), infection by a pathogen, or the presence of toxic chemicals in its environment. Under stress conditions, an organism will direct energy towards cell repair in an attempt to overcome the stressor, thus limiting growth and reproduction. One way by which this is done is through the activation of stress-response gene networks.

Different types of stress are related to different networks, although there is extensive overlap. In most cases, activation of stress responses is also associated with longevity. Since energy is focused on cell maintenance and repair, growth is stunted and cellular faults, that might have otherwise been ignored to focus on reproduction, are repaired. If stressors are present in the environment at an early larval stage (L1), the animal may shift to the dauer diapause, the alternative developmental decision triggered by stress, heat, lack of nutrients or overcrowding. In this state, the animal's nose and pharynx are constricted (Riddle et al. 1981) and it possesses a specialised cuticle, thus blocking entry of chemical stressors into the organism.

Heat-induced stress is mainly regulated by the heat shock factor HSF-1 (Walker et al. 2003) which activates expression of heat shock proteins, whose main role is to act as molecular chaperones. These assist in stabilisation of proteins that are unfolded or misfolded due to heat stress.

Oxidative stress refers to the damage caused by ROS. In response to this, eukaryotes utilise a conserved detoxification system; in nematodes, the major organ involved in this response is the intestine. Reactive oxygen species are inactivated by enzymes, including superoxide dismutases (SOD), catalases and glutathione (Baumeister et al. 2006). SKN-1 appears to play an important part in the *C. elegans* oxidative stress network, it is a transcription factor involved in the regulation of many stress response genes. Other known functions of SKN-1 are in embryonic development and extended lifespan (An & Blackwell 2003).

The presence of toxic chemicals is dealt with through the xenobiotic metabolism. Organic lipophilic molecules are solubilised (Phase 1) and reactive species are inactivated (Phase 2) and possibly removed from the cell (Phase 3). The main group of genes responsible for Phase 1 is the cytochrome P450 family (Menzel et al. 2001; Schafer et al. 2009). This system operates by adding functional groups (often an epoxide group) onto the molecules. The resulting compounds may still be damaging to the organism; in which case they are inactivated by conjugation in Phase 2.

Enzymes involved in Phase 2 detoxification include glutathione-S-transferases (gst) (Lindblom & Dodd 2006).

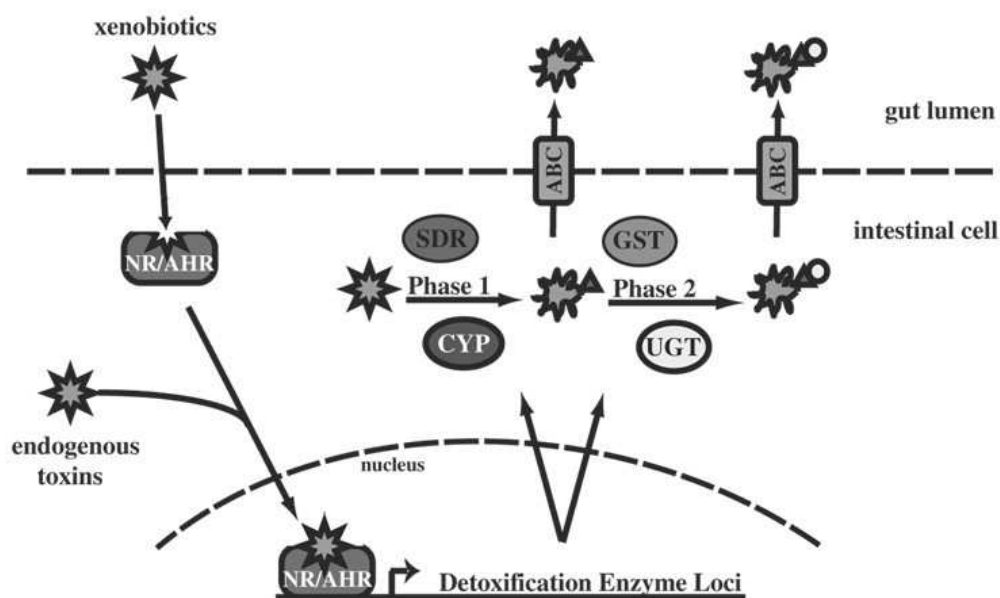


Figure 1.4 A model for the xenobiotic metabolism network. Dangerous chemical activates expression of xenobiotic stress response genes. CYPs are involved in Phase 1 and GSTs in Phase 2 detoxification. ABC transporters actively export Phase 1-2 products from the cell. *Figure taken from (Lindblom & Dodd 2006).*

Metal toxicity is dealt with in part through the metallothioneins. The main known metallothionein genes are *mtl-1* and *mtl-2*, known to be regulated by the transcription factor ELT-2, as well as DAF-16 (in the case of *mtl-1*). Metallothioneins are metal-binding proteins involved in maintenance of trace metal homeostasis and metal detoxification.

DAF-16 is a major stress response gene regulator. It has been shown to play a major part in the response to many types of stress such as heat and ultraviolet light (Henderson & Johnson 2001), hypertonicity (Lamitina & Strange 2005), heavy metal resistance (Barsyte et al. 2001), oxidative stress (Honda & Honda 1999), bacterial and

fungal infection (TeKippe & Aballay 2010), innate immunity (Shivers et al. 2008) and is also necessary for dauer morphogenesis and longevity. It is also suspected to interact with many other stress-related transcription factors, like HSF-1 (Hsu et al. 2003) and SKN-1 (Baumeister et al. 2006) and to be involved in most stress response pathways. There are however other networks that work independent of DAF-16 (Kirienko & Fay 2010).

Genotoxic stress causes DNA damage through erroneous DNA replication or due to the presence of a stressor (oxidative, irradiation, mutagens). In these cases, a cell might respond by activating pathways that direct DNA repair, cell cycle arrest or apoptosis. One gene known to be induced by this pathway is the p53 orthologue *cep-1* (Derry et al. 2001).

1.3 List of stress response genes mentioned in this project

A brief description of the genes studied in this project follows:

(Information was obtained from wormbase.org)

Genes examined by GFP fusion constructs

| Gene name | Mode of action | Localisation | References |
|-------------------------|--|---|---------------------------------------|
| <i>daf-16</i> | transcription factor – major stress response network regulator. | expressed in most cell types except in the pharynx | Murphy et al. 2003 Ogg et al. 1997 |
| <i>cep-1</i> | involved in meiotic segregation and DNA damage-induced apoptosis | expressed in the embryo, the germ line and a subset of pharyngeal cells | Derry et al. 2001 |
| Oxidative stress | | | |
| <i>skn-1</i> | transcription factor – oxidative stress response regulator | expressed in the intestine | An & Blackwell 2003 |

| | | | |
|--|---|---|---|
| <i>ctl-2</i> | catalase | found mainly in the peroxisomes of intestinal epithelial cells | Taub et al. 1999 |
| <i>sod-1</i> | copper/zinc superoxide dismutase | ubiquitously expressed in most tissues – localised in the cytosol and mitochondria | Yanase et al. 2009 |
| <i>sod-3</i> | iron/manganese superoxide dismutase | mitochondrial | Henderson et al. 2006 |
| <i>sod-4</i> | extracellular Cu ²⁺ /Zn ²⁺ superoxide dismutase | expressed in the intestine | Doonan et al. 2008 |
| T09A12.2 (designated GPA for use in this project) | glutathione peroxidase | expressed in body wall muscle cells and the nervous system | McKay et al. 2003; Hunt-Newbury et al. 2007 |
| C11E4.1 (designated GPB for use in this project) | glutathione peroxidase | expressed in the pharynx, intestine, rectal gland cells, hypodermis and head | McKay et al. 2003; Hunt-Newbury et al. 2007 |
| Metal stress | | | |
| <i>elt-2</i> | GATA-type transcription factor – metallothionein regulator | expressed in the intestine | Moilanen et al. 1999 |
| <i>mtl-1</i> | metallothionein – involved in metal detoxification, homeostasis and stress adaptation | expressed in the posterior bulb of the pharynx and, after induction, in the intestine | Moilanen et al. 1999; Li et al. 2008 |
| <i>mtl-2</i> | metallothionein – involved in metal detoxification, homeostasis and stress adaptation | only expressed upon induction in intestinal cells | Moilanen et al. 1999 |

| Heat stress | | | |
|--------------------------|--|---|---|
| <i>hsf1</i> | transcription factor – major regulator of inducible heat shock genes | inactive-cytosolic; active-nuclear | |
| <i>hsp16-1</i> | small heat shock protein – dealing with misfolded proteins | expressed mainly in muscle and hypodermis | Leroux et al. 1997 |
| <i>hsp16-2</i> | small heat shock protein – dealing with misfolded proteins | expressed mainly in the intestine and pharynx | |
| <i>hsp3</i> | molecular chaperone (HSP70 superfamily) | constitutively expressed throughout development; expression is induced under endoplasmic reticulum stress | McKay et al. 2003; Hunt-Newbury et al. 2007 |
| <i>hsp6</i> | molecular chaperone (HSP70 superfamily) | induced in response to disruptions to mitochondrial protein handling | |
| <i>hsp60</i> | mitochondrial-specific chaperone | induced in response to disruptions to mitochondrial protein handling | |
| <i>hsp70</i> | large molecular chaperone (HSP70 superfamily) | | |
| Xenobiotic stress | | | |
| <i>cyp-29A2</i> | mono-oxygenase enzymes (cytochrome P450 superfamily) | | |
| <i>cyp-35A2</i> | | | |
| <i>cyp-34A9</i> | | | |
| <i>gst-1</i> | Glutathione-S-transferases | | Hasegawa et al. 2008 |
| <i>gst-4</i> | | | |

Table 1.1 Genes tested using the GFP assay.

The GFP expression patterns of uninduced (control) and induced worms of all these transgenic strains have been tested and were found to be essentially similar to those described in the literature or on wormbase (Figure 2.2).

These 24 genes were selected to represent the main, well characterised stress response pathways. However, other pathways known to be involved in stress response have been identified (Kirienko & Fay 2010).

The selection criteria for the chosen genes involved functionality, localisation as well as practicality issues. The heat shock genes were chosen to include the main transcription factor, small as well as large heat shock proteins and molecules that localise in the cytoplasm as well as in the mitochondria. Xenobiotic stress genes were chosen from a large list of candidates (86 cyps, 44 gsts) and the main criterion was the availability of stable transgenic lines at the time that the project commenced. From the oxidative stress genes, the three out of four superoxide dismutases and two of the putative glutathione peroxidases, for which stable lines were available, were used.

By combining these sets of genes, a broad picture of cellular stress response can be generated. This thesis will focus on work done on the first 12 genes listed, however, some findings on the latter genes will be mentioned, particularly where one or more of them show a strong response.

Genes examined by qPCR

These genes were selected from genes that showed similar responses in both *C. elegans* and *Drosophila melanogaster* after 24 hours exposure to dichlorvos according to gene array data and that belong to different Gene Ontology (GO) groups.

| Gene name | Mode of action | Other characteristics | Reference |
|---------------|---|--|------------------------|
| C30F12.7 | sperm chromatin protein with evolutionarily conserved fertility factors | | Chu et al. 2006 |
| <i>aman-1</i> | predicted to be involved in glycoprotein digestion through mannose residue removal | | Paschinger et al. 2006 |
| <i>snf-1</i> | neurotransmitter transporter | | Mullen et al. 2002 |
| C10C5.3 | predicted to be involved in cellular amino acid metabolic process and proteolysis | | |
| <i>paf-2</i> | acetylhydrolase essential for embryonic morphogenesis | | Inoue et al. 2004 |
| <i>gln-1</i> | glutamine synthetase involved in the glutamine biosynthetic process and nitrogen compound metabolic process | | |
| <i>rpl-16</i> | ribosomal subunit L13 | | |
| <i>pmp-3</i> | putative ABC transporter | expression levels remain stable under most conditions | Hoogewijs et al. 2008 |
| Y45F10D.4 | thought to be involved in iron-sulphur cluster assembly | expression levels remain stable under different conditions | Hoogewijs et al. 2008 |

Table 1.2 Genes tested using qPCR.

1.4 Pesticides

In agriculture, a large variety of pesticides is used annually, often in large quantities. These chemicals can have effects on the environment other than the ones intended; a lasting example is dichlorodiphenyltrichloroethane (DDT), which was widely used from the 1940s until its ban in the 1970s as an insecticide and contact poison. Being highly hydrophobic and resistant to environmental degradation, DDT accumulates within animal tissue causing biomagnification. Even its metabolites, dichlorodiphenyldichloroethylene (DDE) and dichlorodipenyldichloroethane (DDD) show similar chemical effects and persistence. DDT acts by opening the sodium ion channels of neurons in insects but can also be toxic to other animals (especially marine animals) or cause an effect on the population of bird species through eggshell thinning.

In this project, a number of pesticides were tested for their toxicity on the nematode *C. elegans*. The pesticides tested were selected because they are either currently being used or have been used broadly in the past. Selected toxicants comprised carbendazim, chlorpyrifos, dichlorvos, diuron, DDT, endosulfan, rotenone and the pyrethroids cypermethrin and deltamethrin. A brief description of each pesticide's chemical properties and known mechanisms of action are given in Table 1.3.

| Pesticide | Use | Mode of action | Other properties | Reference |
|--------------|-------------|--|---|---|
| carbendazim | fungicide | Inhibits mitotic microtubule formation | has been banned in the European Union since January 2009 | World Health Organisation data sheet |
| chlorpyrifos | insecticide | acetylcholinesterase inhibitor | persistent chemical – also toxic to birds, fish, small mammal | Pesticide Action Network (PAN), North America |

| | | | | |
|--------------------|-----------------------------------|--|--|--|
| dichlorvos | insecticide | cholinesterase inhibitor | Veterinary, agriculture and home use | PAN, UK |
| diuron | herbicide | blocks photosynthesis | also affects general cell metabolism, growth and mitochondrial structure | Metz et al. 1986, Calvayrac et al. 1979 |
| DDT | insecticide | opens sodium channels | agricultural use banned in most developed countries | Davies et al. 2007 |
| endosulfan | insecticide | chloride channel blocker | persistent organic pollutant – banned in many countries | Bloomquist 2003, International Programme on Chemical Safety (IPCS) |
| rotenone | insecticide, piscicide, pesticide | inhibits mitochondrial electron transport – possibly also inhibits proteasome activity | moderately toxic to humans | PAN UK |
| Pyrethroids | | | | |
| cypermethrin | insecticide | stimulate nerve cells to produce repetitive discharges | | Davies et al. 2007 |
| deltamethrin | | | | |

Table 1.3 Pesticides selected for use in this project.

Pyrethroids act on the voltage-gated sodium channels of insect nerve cell membranes. *C. elegans* does not possess a classical sodium channel gene; however it is thought that pyrethroids may affect nematode potassium channels, which show biochemical similarities to mammalian sodium channels. Pyrethroids affect both the peripheral and central nervous systems. Their mode of action involves stimulation of nerve cells to

produce repetitive discharges, which results in paralysis and, finally, death. Type I pyrethroids, such as permethrin, are not as effective as type II pyrethroids, such as deltamethrin and cypermethrin, which cause an irreversible depolarisation of the neurons, due to a long-lasting effect (Davies et al. 2007). Pyrethroids are synthetic constructs, based on the chemical structure of pyrethrin I (chrysanthemic acid) and pyrethrin II (pyrethric acid); originally isolated from the flower *Chrysanthemum cinerifolius* (Davies et al. 2007).

1.5 Reporter Gene Fusions

A very powerful technique used in *C. elegans* is the application of reporter gene fusion. For this technique, initially the *lacZ* gene (Fire et al. 1990) was fused with part, or the whole of a gene and its regulatory region, expecting that the reporter would be expressed in a similar pattern as the gene of interest. Today GFP (Chalfie et al. 1994), and its variants (CFP, YFP) (Miller et al. 1999) or DsRed (Matz et al. 1999) can also be used. The advantage of GFP over *lacZ* is that it can be visualised in live animals, rather than in fixed preparations that are required for β -galactosidase staining.

Reporter gene fusions are possible in *C. elegans* due to several convenient properties of the organism. Its transparency means that *in vivo* microscopic analysis is possible without dissecting the animal. The thinness of the nematode reduces the need for high-powered confocal microscopy. The nature of this technique requires generation of transgenic animals, a process that can be performed easily and rapidly in *C. elegans* through germline transformation techniques.

There are three categories of reporter constructs. a) A *transcriptional reporter*, which is the most common kind of construct used, contains the reporter gene fused with the 5' regulatory region (promoter, enhancers) of the gene of interest. It is the easiest construct to produce, since the sequence of the gene itself is not used. This however means that intronal or 3' UTR regulatory elements will not be present, so the result may not always provide a complete representation of the gene's expression pattern. It

is however a rapid way to establish a general outline of that pattern. b) *A translational reporter* which is fused in an exonic region of the gene of interest. This construct contains the whole of the gene sequence, including *cis* regulatory elements across the gene region as well as the 5' and 3' untranslated regions (UTRs) and flanking regulatory sequences. The GFP is produced fused to the protein of interest and, where possible, the gene function is not impaired, allowing for rescue experiments where a normal copy of the protein is not present. A translational reporter gives a more faithful representation than a transcriptional reporter, but due to protein degradation, it reports a lower signal. Translational reporter constructs can also give information on the localisation of the expressed protein. c) *An smg-1-based reporter*. This construct comprises a reporter gene fused within the first exon of the gene of interest. *smg-1*-based reporter constructs include all *cis* regulatory information present in a translational reporter, but also contain the reporter gene's stop codon meaning that the protein of interest is not translated, since there is a stop codon present within the first exon. However, the presence of an early stop codon attracts nonsense-mediated mRNA decay mechanisms (Rebbapragada & Lykke-Andersen 2009); to avoid mRNA degradation, these constructs are inserted into a genetic background such as *smg-1*, which is deficient for nonsense-mediated mRNA decay (Pulak & Anderson 1993).

A series of strategies can be employed to produce these constructs, which are then inserted by microinjection in the gonad to generate a transgenic strain carrying the construct (as described in 1.1.4). The construct could be inserted into a vector using standard cloning techniques (fragment and vector amplification, restriction enzyme digestion, ligation) which gives rise to a reusable construct. PCR can be used to fuse the fragments, providing a rapid method for construct production (Hobert 2002). For large transgenes, *in vivo* recombination can be employed (Mello et al. 1991; Mello & Fire 1995). Since homologous recombination in *C. elegans* is largely uncharacterised, such constructs could be generated in yeast cells. Invitrogen have developed the Gateway cloning technology using a series of recombination events across vectors assisted by bacteriophage λ integrase proteins (Dupuy et al. 2004). For cell-type-

specific expression, in cases where a cell-type specific promoter is not available, a reconstituted reporter gene construct can be used, where the reporter is expressed as two domains, each fused with small peptides that naturally interact *in vivo* (such as leucine zippers) and transcription is controlled by different regulatory regions yielding overlapping expression patterns (Ghosh et al. 2000).

Today, several groups are generating genome-wide gene expression constructs ((Dupuy et al. 2004), British Columbia *C. elegans* Gene Expression Consortium, The Hope Laboratory Expression Pattern Database, *C. elegans* Promoter/Marker Database). Transgenic strains for a large number of genes have been generated and are available to the scientific community.

Detection of GFP fluorescence requires excitation in the 480-490nm range and measuring emission in the 525-550nm range.

1.6 Aims of this project

This project attempts to examine the effect of widely used pesticides on non-target organisms, in this case the nematode *C. elegans*. We will use transgenic strains carrying reporter constructs to test the effect of single toxicants (as well as mixtures) on the expression of genes involved in the stress responses outlined above (0). Stress-response genes usually act in an interlinked fashion and also affect other processes, such as feeding, growth and reproduction. This suggests that toxicants can have ecological effects by affecting the stress response network. Parallel studies will be conducted in India using the fruit fly *Drosophila melanogaster*. The results from both organisms will be pooled and used to inform a mathematical model of the stress response network, based on the known regulatory modules controlling each pathway. This model will provide the first theoretical framework for predicting the effects of chemical mixtures, which are more commonly found in nature than single toxicant exposures. This mixture work has been initiated for simple metal mixtures (each of

which induces multiple stress responses), but this has yet to be attempted for pesticide responses described here.

1.7 Plan of action

Transgenic strains carrying a GFP reporter gene fusion construct will be used to assay the effect on the expression of stress-response genes in the presence of a range of pesticides chosen because they have been used in the past or are currently being used widely, either in agriculture or for home use as insecticides. Worms will be exposed to different concentrations of each toxicant alongside a water control and a solvent control (in cases where the toxicant is not water soluble) and GFP expression will be measured at three timepoints, for early, intermediate and late response. Feeding inhibition assays will also be utilised in some cases, to test for physiological effects of pesticides on the nematode.

qPCR will be performed to confirm regulation of a number of genes by the pesticide dichlorvos, for which gene array assays have previously been performed in both *C. elegans* (non-target) and *Drosophila melanogaster* (target-related). The test genes will be run alongside two control genes with relatively stable expression in the nematode under different conditions.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

| Source | Compounds |
|----------------------------|---|
| Applied Biosystems, USA | RT Buffer Mix/RT Enzyme Mix, TaqMan Master Mix |
| BDH, UK | KCl, Na ₂ HPO ₄ ·7H ₂ O, NaH ₂ PO ₄ ·2H ₂ O, chloroform, isopropanol, Bromophenol Blue, |
| Courtin & Warner, UK | glycerol, |
| East Anglian Chemicals, UK | MgSO ₄ , |
| Eurofins, Germany | PCR primers, qPCR probes |
| Fisher Scientific, UK | NaCl, K ₂ HPO ₄ , C ₆ H ₅ K ₃ O ₇ , C ₆ H ₈ O ₇ , FeSO ₄ ·7H ₂ O, KOH, NaOH, C ₂ H ₃ O ₂ NH ₄ , CH ₃ COOH, H ₃ BO ₃ , K ₄ [Fe(CN) ₆]·3H ₂ O, K ₃ [Fe(CN) ₆], Kanamycin, X-gal, DMF, methanol, EDTA, ethidium bromide, xylene cyanol, |
| Fisons, UK | CaCl ₂ , KH ₂ PO ₄ , |
| Invitrogen, USA | Triazol Reagent, 1kb DNA Ladder |
| Melford, UK | agar, peptone, SDS, agarose, |
| Pestanal, USA | diuron, dichlorvos, cypermethrin deltamethrin, endosulfan, chloropyrifos, |
| Promega, USA | 100bp DNA Ladder |
| Qiagen, Germany | DNase I, Buffer RDD |

| | |
|-----------------------|--|
| Sigma, USA | MgCl ₂ , Na ₂ EDTA, MnCl ₂ .4H ₂ O, ZnSO ₄ .7H ₂ O, CuSO ₄ .5H ₂ O, NaOCl, cholesterol, DMSO, ethanol, DDT, carbendazim, rotenone, acetone, Tris, DEPC, H ₂ O ₂ , RNase ZAP, |
| Stratagene, USA | SYBR Green Master Mix, qPCR Reference Dye |
| Thermo Scientific, UK | ReddyMix PCR MasterMix |

Table 2.1 List of reagents used in this project.

2.1.2 Solutions

⁺Solution was autoclaved and stored at room temperature until used.

*Reagent was added after autoclaving.

2.1.2.1 Nematode Liquid Media

K medium⁺:

32mM KCl

53mM NaCl

M9 Buffer⁺:

22mM KH₂PO₄

42.3mM Na₂HPO₄

85.5mM NaCl

1mM MgSO₄

S Medium⁺:

100mM NaCl

5% Potassium Phosphate Buffer (K_2HPO_4/KH_2PO_4) (1M, pH6.0)

0.1% Cholesterol (diluted 5mg/ml in EtOH)*

1% Potassium Citrate Buffer ($C_6H_5K_3O_7/C_6H_8O_7$) (1M, pH6.0)*

1% Trace Metal Solution*

3mM $CaCl_2$ *

3mM $MgSO_4$ *

2.1.2.2 Growth Media**NGM⁺:**

51.3mM NaCl

17g/L Agar

2.5g/L Peptone

0.1% Cholesterol (diluted 5mg/ml in EtOH)

1mM $CaCl_2$ *

1mM $MgSO_4$ *

2.5% Potassium Phosphate Buffer (K_2HPO_4/KH_2PO_4) (1M, pH6.0)*

LB⁺:

LB was made using LB Broth-High Salts (Melford, UK). A concentration of 25g/L was used.

2.1.2.3 Agarose Gel Electrophoresis

TAE:

40mM Tris

19mM Acetic Acid

1mM EDTA

TBE:

89mM Tris

89mM Boric Acid

2mM EDTA

Loading Dye:

1x TBE

0.2% Bromophenol Blue

0.25% Xylene Cyanol

50% Glycerol

2.1.2.4 X-gal Staining Mix

0.2M Sodium Phosphate Buffer ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ / $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)

1mM MgCl_2

5mM $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$

5mM $\text{K}_3[\text{Fe}(\text{CN})_6]$

0.004% SDS

75 $\mu\text{g}/\text{ml}$ Kanamycin

The mixture was warmed up to 65°C in a water bath and then 1% of 0.4% solution X-gal substrate in dimethylformamide (DMF) was added.

2.1.2.5 Miscellaneous

Trace Metal Solution:

2.5mM FeSO₄·7H₂O

5mM Na₂EDTA

1mM MnCl₂·4H₂O

1mM ZnSO₄·7H₂O

0.1mM CuSO₄·5H₂O

The solution was autoclaved and stored at 4°C protected from the light. It was replaced once its colour had changed from ferrous green to ferric yellow.

Bleaching solution:

1% NaOCl

0.5M KOH

Due to tendency of the sodium hypochlorite to break down over time, the bleaching solution was made fresh before every egg preparation (2.2.3).

Freezing Solution:

3.26M Glycerol

0.1M NaCl

5% Potassium Phosphate Buffer (K₂HPO₄/KH₂PO₄) (1M, pH6.0)

0.3mM MgSO₄*

2.1.3 Strains

Escherichia coli strains:

| Strain name | Genotype |
|--------------|---|
| P90C | F ⁻ , <i>ara-600</i> , $\Delta(gpt-lac)5$, λ^- , <i>relA1</i> , <i>spoT1</i> , <i>thi-1</i> , |
| DH5 α | F ⁻ , $\phi 80dlacZ\Delta M15$, $\Delta(lacZYA-argF)$ U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>rk</i> ⁻ , <i>mk</i> ⁺), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> |

Table 2.2 E. coli strains used and their genotypes. Strains were provided by Prof. Andrew Chisholm of the University of California, San Diego (P90C) and Prof. Liz Socket of the medical centre, University of Nottingham.

Caenorhabditis elegans strains:

| Strain Name | Gene of Interest | Genotype | Provided by |
|----------------------|---|--------------------|--|
| Bristol N2 | | wild type | Andrew Chisholm, University of California, San Diego |
| GFP reporter strains | | | |
| CF1553 | sod-3 | <i>sod-3::gfp</i> | Cynthia Kenyon, University of California San Francisco |
| JR2474 | cep-1 | <i>cep-1::gfp</i> | Joel Rothman, University of California Santa Barbara |
| TJ356 | daf-16 | <i>daf-16::gfp</i> | Caenorhabditis Genetics Center (funded by the NIH National Center for Research Resources) |
| BC17553 | T09A12.2 glutathione peroxidase designated GPA | <i>GPA::gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20305 | C11E4.1 glutathione peroxidase designated GPB | <i>GPB::gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20309 | mtl-1 | <i>mtl-1::gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20342 | mtl-2 | <i>mtl-2::gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |

| | | | |
|-----------------------|-------|--------------------|---|
| BC20314 | elt-2 | <i>elt-2:: gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20336 | ctl-2 | <i>ctl-2:: gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20333 | sod-4 | <i>sod-4:: gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20350 | sod-1 | <i>sod-1:: gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| BC20329 | skn-1 | <i>skn-1:: gfp</i> | Baillie Genome GFP Project, Simon Fraser University, Burnaby, Vancouver, Canada |
| Georgia mtl-2::GFP | mtl-2 | <i>mtl-2:: gfp</i> | Phil Williams' lab (Ma et al, 2009, Environ Toxicol Chem 28, 1311) |
| lacZ reporter strain | | | |
| JF2.1 | mtl-2 | <i>mtl-2::lacZ</i> | Jonathan Freedman, National Institute of Environmental Health Sciences, USA |

Table 2.3 C. elegans strains used and their genotypes.

2.2 Methods

2.2.1 Pouring Plates

C. elegans was generally grown on NGM agar plates seeded with bacteria. NGM was melted (in a steamer or a microwave) until in a homogeneous liquid form and then poured into plates. The plates were spread with *E. coli* bacterial strain P90C and left overnight at 36°C.

2.2.2 Liquid culture

For mass growth of *C. elegans* a liquid culture was used. S medium was used as a base and *E. coli* bacterial strain DH5 α was diluted to an optical density (OD) of 1.5-1.7. Worms were added and the suspension was incubated for 5-6 days at 20°C on a shaker (240rpm) to ensure adequate aeration.

Bacteria were grown in liquid LB broth overnight on a shaker (37°C, 240rpm).

2.2.3 Egg Preparation

C. elegans was grown on agar plates or in liquid culture. For production of synchronised cultures the worms were bleached through a procedure that allowed survival of the eggs alone. The eggs were then allowed to hatch in the absence of food which resulted in a culture of synchronised L1s.

Worms were washed off the plate using ice-cold K medium or isolated from the liquid culture by settling on ice. The suspension was centrifuged at 1,125xg for 5min. The supernatant was discarded and the pellet resuspended in bleaching solution (2.1.2). The worms were then vortexed in the bleaching solution for ca. 7min until the bodies of adult worms started to break open, releasing the eggs. The suspension was then centrifuged at 1,125xg for 2min. The supernatant was discarded and the pellet washed in K medium and re-centrifuged at 1,125xg for 2min. At least 3 washing steps

were carried out to ensure sufficient dilution of the bleach. The pellet was then resuspended in M9 buffer allowing enough empty space in the centrifuge tube for air supply (6-11ml of liquid in a 15ml tube or 15-30ml of liquid in a 50ml tube) and the tube was left on a rotary shaker overnight.

All steps were carried out at room temperature. Wash steps after bleaching were carried out under a fume hood.

2.2.4 Quantification of GFP fluorescence

Worms were left to settle on ice and washed to discard excess bacteria. Washed worms were aliquoted into wells on a 24-well-plate and exposed to different concentrations of the toxicant. Aliquoting of worms was carried out utilizing a beaker and magnetic flea to achieve more accurate division. In each well no more than 0.3ml of total solution was added to allow air access by the worms. Worm suspension was transferred to a black 96-well-plate (Thermo Fisher Scientific, UK) before each reading and then transferred back to the 24-well-plate for the remaining incubation period. Readings were taken at 3 time points (early: 4 hours, intermediate: 16-18 hours, late: 28-30 hours). Quantification of GFP was performed using a Perkin-Elmer Victor 1420 Plate Fluorometer, excitation wavelength was set at 485nm and the emission wavelength at 535nm.

Exposures were set up using 4 biological replicates at 4 concentrations of the toxicant (plus a water control and a solvent control). All exposures were incubated at 20°C.

To avoid loss of worms due to sticking on pipette tips, an extra water control was set up for each timepoint and was used for resuspending before each transfer.

The fluorometer took 4 readings for each well, calculated the average and represented the results in the form of a colour coded table (Figure 2.1).

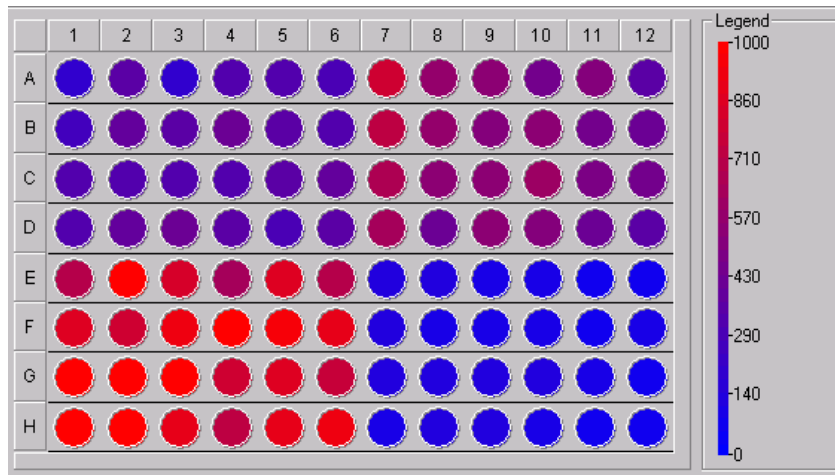


Figure 2.1 Fluorometry readout output. Circles represent the mean value of 4 fluorescence readings taken for each well. Colour scheme is shown on the left.

The strains used were checked for correct localisation of expression. In Figure 2.2 expression in uninduced animals is shown to localise in the posterior bulb of the pharynx (Figure 2.2, A), whereas induced animals appear to express GFP along the intestine (Figure 2.2, B), as would be expected for the *mtl-1* gene (Freedman et al. 1993).

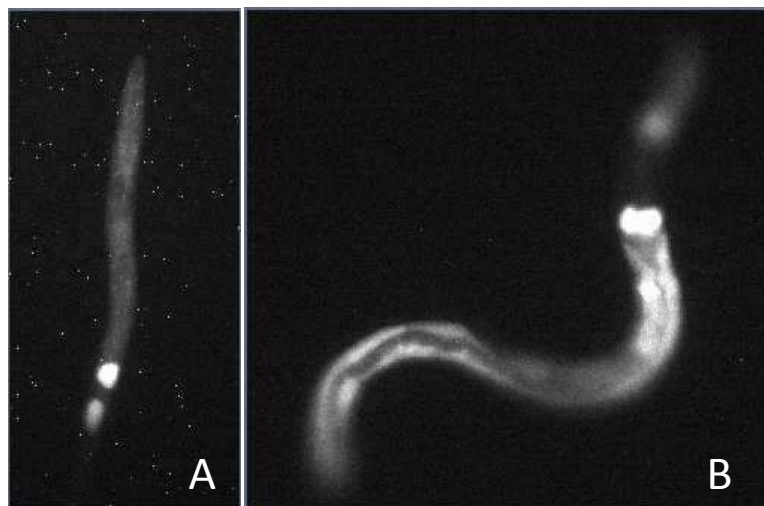


Figure 2.2 *mtl-1*::GFP expression in uninduced and induced animals. **A** Basal expression of *mtl-1*::GFP in an uninduced animal. **B** Expression of *mtl-1*::GFP after exposure to 10ppm Hg.

2.2.5 Feeding Inhibition Assay

For the feeding inhibition assay, synchronised L1 cultures of N2 wild type worms were used. Bacterial strain DH5 α was grown and diluted in K medium to a final optical density of ca. 1. This was used as a basis for all of the toxicant dilutions the worms were then exposed to.

Worms were left to settle on ice and washed to discard excess bacteria. Washed worms were aliquoted into 6-well-plates and exposed to different concentrations of the toxicant, as described above (section 2.2.4). In each well no more than 1.5ml of total solution was added to allow air access by the worms. Before each reading, worm suspension from each well was transferred into an Eppendorf tube and left to settle on ice. Once the worms had settled, the supernatant was transferred into a disposable 1ml plastic cuvette and the OD reading was taken. The solution along with the pellet was then transferred back to the 6-well-plate for the remaining incubation period. OD readings were taken using a Biochrom Libra S6 machine, with the wavelength set at $\lambda=550\text{nm}$.

Exposures were set up using 4 biological replicates, at 4 concentrations (plus a water control). Equivalent concentrations of the solvent were set up in parallel. To account for OD differences due to presence of the toxicant (dilution of the toxicant in water based solutions resulted in a cloudy solution at times), zero-bacteria controls were also performed. To account for differences in the OD due to bacterial clumping, zero-worm controls were performed. All exposures were incubated at 20°C.

2.2.6 RNA Extraction

Mass worm cultures were used. Worms were exposed to the specified concentration of the toxicant and an equivalent concentration of the solvent only as a negative control. Incubations were set up in 50ml tubes. Tubes were filled with no more than 15ml and left on a shaker (200rpm) at 20°C for the specified amount of time.

Worms were pelleted (1,200xg for 10min. Then the supernatant was removed and the pellet was dropped in liquid nitrogen (ca. 5ml) in a sterile mortar. Triazol reagent (1ml) was added and the worms were ground thoroughly with a sterile pestle. The resulting mixture was transferred to an Eppendorf tube and centrifuged (12,000xg for 10min at 4°C). Clear supernatant was transferred to a fresh tube and allowed to stand at room temperature for 5min. Chloroform was added (0.2ml) and the solution was shaken vigorously for 15sec before allowing to stand at room temperature for 10min. The mixture was centrifuged (12,000xg for 15min at 4°C) to separate into 3 phases: a settled red organic phase (protein), an interphase (DNA) and a clear aqueous upper phase (RNA). The RNA phase was transferred into a fresh tube and 0.5ml of isopropanol was added. The solution was allowed to stand at room temperature for 5-10min before centrifuging (12,000xg for 10min at 4°C) to form an RNA pellet. The supernatant was removed and the pellet washed with 75% ethanol (EtOH) and centrifuged (7,500xg for 5min at 4°C). The supernatant was removed again and the tube was left open under a fume hood to allow drying of EtOH. RNA was reconstituted in 50µl DEPC-treated water. RNA was quantified using a nanodrop machine (Thermo Scientific Nanodrop 1000 Spectrophotometer) and its quality was checked by running on a 1xTAE/1% SDS/1% agarose gel (2.2.11).

Precautions:

Appropriate training was undertaken before using liquid nitrogen.

All water used was treated with diethylpyrocarbonate (DEPC) to inactivate RNase and other robust enzymes. DEPC was diluted 1:10 in EtOH. This solution was used in a 1% dilution to treat distilled water. Water was left under a fume hood for ca. 1 hour and then autoclaved.

Pipettes were sterilised using hydrogen peroxide (H₂O₂). Pipette ends were removed and left in a 3% H₂O₂ solution under a fume hood for ca. 1 hour. Pipette ends were then rinsed using DEPC-treated water and left to dry overnight.

The mortar and pestle used were sprayed with RNase ZAP, rinsed with DEPC-treated water and sterilised before each use.

All eppendorf tubes used were sterile. All pipette tips used were sterile barrier tips.

All working surfaces were sprayed with RNase ZAP before each experiment.

Gloves were worn at all times and changed frequently to avoid contamination.

2.2.7 DNase digestion of DNA-contaminated RNA

Digestions were set up as follows:

| Component | Volume |
|--|---------------------------------------|
| RNase-free water | to give a final volume of 100 μ l |
| RNA solution | \leq 87.5 μ l |
| DNase I stock (2.7 Kunitz units per μ l) | 2.5 μ l |
| Buffer RDD | 10 μ l |

Table 2.4 DNase digestion components.

Digestions were incubated at room temperature for 10min. EDTA was added to a final concentration of 20mM and DNase was inactivated by incubating at 75°C for 10min.

2.2.8 RNA Precipitation

After DNase digestion, EDTA is present in the RNA solution. EDTA protects RNA from degradation during the DNase I inactivation step, but can inhibit further reactions (e.g. PCR) by chelating metal ions in the buffer. Thus, after DNase digestion, RNA was precipitated before proceeding to further experiments.

Ammonium acetate was added to a final volume of 2.5M. EtOH was added to a final volume of 60%. Solution was incubated at -20°C for 1 hour or overnight, then was centrifuged at 13,000xg for 10min at 4°C and supernatant was removed. The pellet was washed with ice-cold 70% EtOH and then centrifuged at 13,000xg for 1min at 4°C. The supernatant was removed and the tube was re-centrifuged (13,000xg, 1min, 4°C) to remove dregs. The final pellet was resuspended in RNase-free water.

2.2.9 Conversion of RNA to cDNA

Before running a Polymerase Chain Reaction (PCR) RNA needs to be converted to DNA to be used as a template. A Reverse Transcriptase (RT) reaction was run using the RT set (Applied Biosystems) in order to produce cDNA from the RNA template.

In each reaction the following were added:

| Component | Volume |
|---------------|--------|
| RT Buffer Mix | 10µl |
| RT Enzyme Mix | 1µl |
| RNA solution | 9µl |

Table 2.5 RNA to cDNA reaction components.

The reaction was run in a BioRad MJR Block 96V PCR machine in the following conditions:

| | Temperature | Time |
|--------|-------------|-------|
| STEP 1 | 37°C | 60min |
| STEP 2 | 94°C | 5min |
| STEP 3 | 4°C | hold |

Table 2.6 RNA to cDNA reaction conditions.

cDNA was quantified using a nanodrop machine.

2.2.10 DNA Amplification by Polymerase Chain Reaction (PCR)

2.2.10.1 Primer design

Since cDNA was used as the template, one primer per set was designed to span exon boundaries in order to avoid amplification of any genomic DNA. All primers were designed to have a similar melting temperature (ca. 56°C) in such a way as to minimise chances of self annealing and primer dimer formation.

| Oligo name | Gene amplified | Primer sequence | Tm (°C) | expected amplified fragment size |
|----------------------|------------------|--------------------------------------|--------------|----------------------------------|
| w03g9-1for | snf-1 | TGGCATATTTGTTTCGCAATG (20) | 53.2 | 211bp |
| w03g9-1rev | snf-1 | ACTCCGATTCTCTCGGAAGAC (20) | 59.4 | |
| c10c5-3for | C10C5-3 | ATGGAGAAACTCGAGCGGTA (20) | 57.3 | 195bp |
| c10c5-3rev | C10C5-3 | GGACGGCGTCAATCTTATGT (20) | 57.3 | |
| c30f12-7for | C30F12-7 | CCAGGTCACAGACTCCCACT (20) | 61.4 | 201bp |
| c30f12-7rev | C30F12-7 | TTCAATTGCCAGCATAGCAG (20) | 55.3 | |
| c45b2-5for | gln-1 | GGGAGATCAACTGTGGGTGT (20) | 59.4 | 183bp |
| c45b2-5rev | gln-1 | TCGATAGCTTCCACCCTGT (20) | 57.3 | |
| c52b9-7for | paf-2 | AACGGACTTCCAAAAGCAA (20) | 53.2 | 233bp |
| c52b9-7rev | paf-2 | ACGATGGGAAATGAATGGA (20) | 53.2 | |
| f55d10-1for | aman-1 | CAGGGATGCACACAAAATG (20) | 55.3 | 234bp |
| f55d10-1rev | aman-1 | CCTGCGATGAGGTATTCGTT (20) | 57.3 | |
| rpl-16 left | rpl-16 | GGAGTCCAGCCAAATACCA (20) | 59.93 | 174bp |
| rpl-16 right | rpl-16 | GGCTCCCTTCACCTTCTCT (20) | 59.82 | |
| pmp-3 FOR | pmp-3 | GTTCCCGTGTTCATCACTCAT (21) | 57.9 | 115bp |
| pmp-3 REV | pmp-3 | ACACCGTCGAGAAGCTGTAGA (21) | 59.8 | |
| Y45F10D.4 FOR | Y45F10D.4 | GTCGCTTCAAATCAGTTCAGC (21) | 57.9 | 157bp |
| Y45F10D.4 REV | Y45F10D.4 | GTTCTTGTC AAGT GATCCGACA (22) | 58.4 | |

Table 2.7 Primers used to amplify test and reference genes from *C. elegans* cDNA. All primer sequences are written in the 5' to 3' direction. The number of nucleotides in the primer sequence is displayed in brackets “()”. Genes shown in bold were used as reference genes.

2.2.10.2 Setting up a PCR

In each reaction tube the following were added:

| Component | Volume/final concentration |
|-------------------|----------------------------|
| dH ₂ O | to a final volume of 25µl |
| PCR ReddyMix | 22µl |
| Forward primer | 0.5pmol/µl |
| Reverse primer | 0.5pmol/µl |
| Template cDNA | 40ng/µl |

Table 2.8 PCR components.

Negative controls were run alongside each reaction. In the negative controls, DNA was replaced by dH₂O. Reaction was run in a BioRad MJR Block 96V PCR machine in the following conditions:

| | Temperature | Time | |
|-----------|-------------|------|-------------|
| STEP 1 | 94°C | 2min | |
| STEP 2 .1 | 94°C | 20s | X 31 cycles |
| .2 | 56°C | 30s | |
| .3 | 72°C | 1min | |
| STEP3 | 4°C | hold | |

Table 2.9 PCR conditions.

All steps were performed under a fume hood and all components were kept on ice prior to the reaction.

2.2.11 Quantification of mRNA using a Quantitative Polymerase Chain Reaction (qPCR)

To quantify differences in mRNA levels for test genes caused by the presence of the toxicant, a qPCR was performed. Toxicant-treated and solvent control exposures were set up in 4 biological replicates (2.2.6). RNA was extracted (2.2.6), cleaned of DNA contamination (2.2.7), precipitated (2.2.8) and used to produce cDNA (2.2.9). PCR was used to optimise primer concentration and reaction conditions (2.2.10). Test reactions were run to confirm that reference gene expression remained virtually unchanged in different conditions. Initial reactions were run to test primer pair and probe sets singly and together.

Each test gene was run alongside one reference gene using probes to detect amplification. The second reference gene was run in a separate reaction in parallel and SYBR Green was used to detect amplification, according to the manufacturer's instructions.

Probes were designed for each test gene and one of the two reference genes.

| Oligo name | Gene amplified | Probe sequence | Tm (°C) | Fluorochrome |
|------------|----------------|-------------------------------------|-------------|--------------|
| w03g9-1 | snf-1 | AATTGGACAAGTGACCGGAC (20) | 57.3 | FAM |
| c10c5-3 | | CGTGATGAGCAAAAGGCTCT (20) | 57.3 | FAM |
| c30f12-7 | | CCGAGATGATTGCCCATATC (20) | 57.3 | FAM |
| c45b2-5 | gln-1 | TTTGGACCCTAAACCAGTGC (20) | 57.3 | FAM |
| c52b9-7 | paf-2 | TTGAATGTTGGAGATTGGCA (20) | 53.2 | FAM |
| f55d10-1 | aman-1 | CGAGAAAGGAAGCAAACCTG (20) | 57.3 | FAM |
| | pmp-3 | CGTTTCACCTGCAGAATTGA (20) | 55.3 | CY5 |

Table 2.10 Probes used to detect rate of amplification of test and reference genes using a Quantitative PCR. Probe sequence is written in the 5' to 3' direction. The number of nucleotides in the probe sequence is displayed in brackets “()”. The gene in bold was used as a reference gene.

Each reaction was prepared in an Eppendorf tube to a total volume of 50µl, 20µl were loaded into each of two wells per reaction on a qPCR plate which was sealed and kept cool until loaded into the machine. For each condition [toxicant-treated, solvent control and no template control (NTC)] 4 biological replicates were run. Each reaction was set up as follows:

| Component | TaqMan Probes qPCR | | SYBR Green qPCR | |
|--------------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| | test reaction | NTC | test reaction | NTC |
| dH ₂ O | to give a final volume of 50µl | to give a final volume of 50µl | to give a final volume of 50µl | to give a final volume of 50µl |
| TaqMan MasterMix | 25µl | 25µl | - | - |
| SYBR Green MasterMix | - | - | 25µl | 25µl |
| reference dye | - | - | 300nM | 300nM |
| test gene forward primer | 0.5pmol/µl | 0.5pmol/µl | - | - |
| test gene reverse primer | 0.5pmol/µl | 0.5pmol/µl | - | - |
| reference gene forward primer | 0.5pmol/µl | 0.5pmol/µl | 0.5pmol/µl | 0.5pmol/µl |
| reference gene reverse primer | 0.5pmol/µl | 0.5pmol/µl | 0.5pmol/µl | 0.5pmol/µl |
| test gene probe | 100nM | 100nM | - | - |
| reference gene probe | 100nM | 100nM | - | - |
| cDNA | ca. 40ng | - | ca. 40ng | - |

Table 2.11 qPCR components. NTC: no template control.

All steps were performed under a fume hood and all components were kept on ice prior to the reaction.

2.2.12 Agarose Gel Electrophoresis

To separate nucleic acid samples according to size, a 1xTAE/1% agarose gel was used. RNA samples were loaded onto a 1xTAE/1% agarose/1% SDS gel alongside a 1kb plus DNA Ladder. DNA samples were loaded onto a 1xTAE/1% agarose gel and run alongside a 100bp DNA Ladder. The gel was run at 107V for ca. 45min. After running, the gel was stained with 0.0025% ethidium bromide (EtBr) for 30min. The gel was destained in distilled water for 30min (changing the water every 10min) and photographed using a BioRad Gel Imaging System with a ChemiDoc XRS camera.

DNA ladders were used even in the case of RNA samples because RNA ladders are not robust and prove hard to keep. For comparison DNA samples are equivalent to RNA of half the size of base pairs (bp) in nucleotides (nt).

Since EtBr is a mutagen, care was taken to avoid contact with the skin and contamination. Gloves were worn at all times when handling EtBr and were disposed of immediately after use. EtBr-stained gels were disposed of as contaminated waste. EtBr-containing reagents were run through an EtBr-binding filter before disposal. Equipment that may have been in contact with EtBr was cleaned thoroughly.

2.2.13 Staining and Quantification of Worms Expressing the β -galactosidase Gene

β -galactosidase needs to be stained before visualisation. X-gal staining was carried out for worms expressing the β -galactosidase gene after exposure to toxicants.

Worms were left to settle on ice and washed to discard excess bacteria. Washed worms were aliquoted into 15ml tubes and exposed to different concentrations of the toxicant, as well as a negative and a positive control. Samples from each condition were removed at each timepoint, washed 2-4 times with ice-cold K medium (2.1.2) and placed in Eppendorf tubes. Each sample was centrifuged (425xg, 1min), the supernatant was removed and 100 μ l of acetone was added. The samples were left to stand at room temperature for 5min before centrifuging (425xg, 1min). The

supernatant was removed and the samples left under a fume hood, with the caps open, until the acetone had evaporated, before storing at 4°C. When samples for all timepoints had been collected, 50-100µl of X-gal staining mix (425xg, 1min) was added to each worm pellet. Samples were incubated at 37°C until the positive control, but not the negative control had turned blue. Samples were centrifuged (425xg, 1min), the supernatant was removed and the pellet washed with ice-cold K medium. Approximately 10µl of worm suspension (10-15 worms) for each replicate of a condition was dropped on a slide. Slides were left to dry and then 7µl of 10% glycerol was dropped onto the dried worms. A cover slip was placed and the edges were sealed with nail varnish.

Exposures were set up using 3 biological replicates at 4 concentrations of the toxicant. Three controls were set up in parallel to the toxicant concentrations. In these the toxicant was substituted with a) distilled water and b) the solvent concentration present at the highest toxicant concentration, to be used as negative controls and c) 8ppm Zn to be used as a positive control.

A scoring system was used to measure the approximate strength of staining, where fully or strongly stained worms were given a score of 2, weakly or partly stained worms were given a score of 1 and unstained worms were given a score of 0. Scores for all the worms in each condition were added, divided by the number of worms and multiplied by 50, giving a final score with a maximum of 100 and a minimum of 0.

Examples of strongly stained, weakly stained and non-stained worms are shown in Figure 2.3.

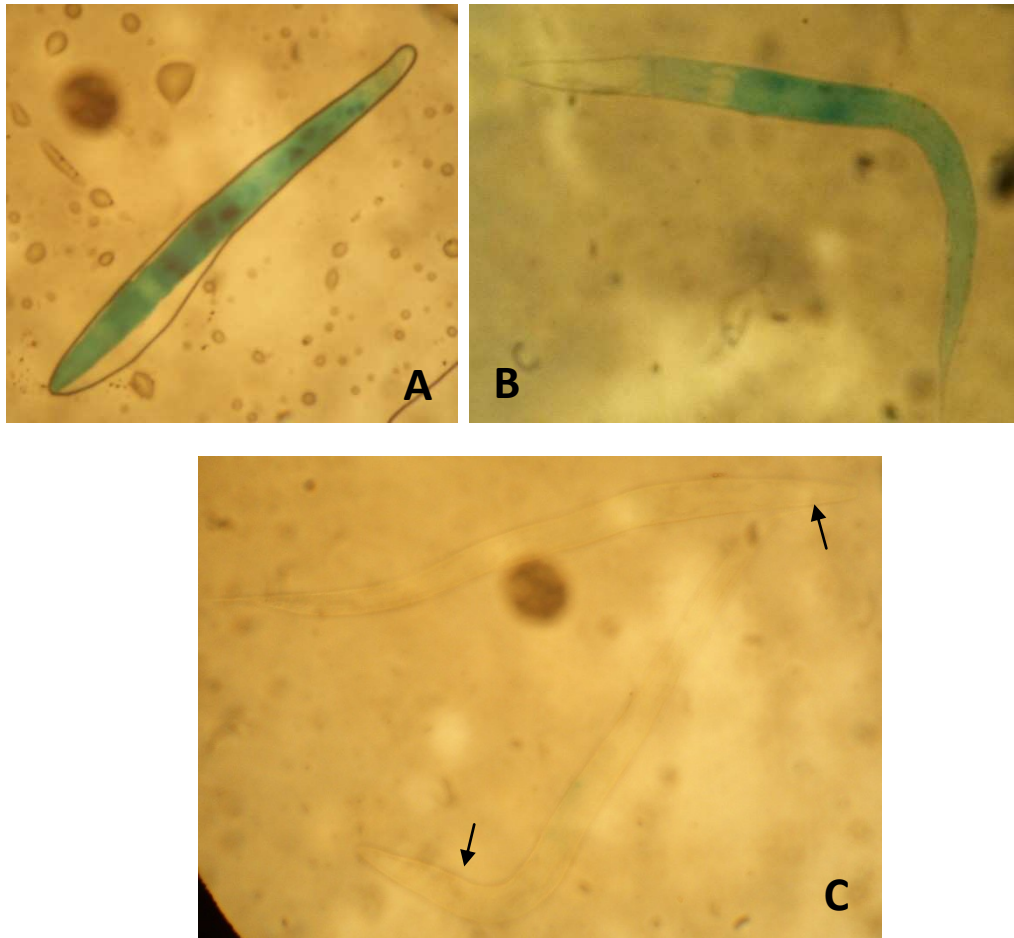


Figure 2.3 *mtl-2::lacZ* expressing worms after staining. **A** Example of strongly stained worm. **B** Example of weakly stained worm. **C** Two examples of not-stained worms. Arrows point to head region of the worms.

3 Results

3.1 Responses of transgenic GFP strains exposed to toxicants

Twenty-four genes were chosen to represent the known groups of stress-response genes (heat shock, oxidative stress, xenobiotic stress, metal stress), including the 4 main transcription regulators, ELT-2, DAF-16, SKN-1 and HSF-1. Transgenic strains, each expressing one of the chosen genes in a transcriptional fusion with GFP, were obtained and used to assay gene expression after exposure to toxicants. Here, we will focus on 12 of those genes, the oxidative stress-responsive genes *sod-1*, *sod-3*, *sod-4*, *ctl-2*, GPA (T09A12.2), GPB (C11E4.1) and transcription factor *skn-1*; the metal stress-responsive genes *mtl-1*, *mtl-2* and the transcription factor *elt-2*; the major stress-response transcription factor *daf-16* and *cep-1*, which is involved in DNA damage-induced apoptosis.

These transgenic strains were exposed to a number of selected pesticides and GFP fluorescence was measured at different timepoints. The pesticides used were rotenone, carbendazim, chlorpyrifos, endosulfan, cypermethrin, deltamethrin, diuron and DDT. Pesticides were insoluble in water so each was diluted in a solvent. Solvents used comprised ethanol (EtOH), methanol (MeOH) and dimethyl sulfoxide (DMSO). Since the solvents themselves could cause stress on the animals, the solvent concentration was kept at no higher than 0.2%, limiting the highest concentration of toxicant achievable. The highest concentration used was chosen as the highest concentration achievable where the solvent did not appear to have a major effect on gene expression (0.1-0.2%) and the toxicant did not have a lethal effect on the worms. The rest of the concentrations were chosen as a serial dilution reaching a lowest concentration of below 1 ppm.

Alongside a water (dH₂O) control, the solvent, at the concentration it was present in the highest concentration of the pesticide, was also used as a second control. All exposures were set up in 4 replicates and readings were taken at three timepoints to represent early (4 hours), intermediate (16-18 hours) and late (28-30 hours) response.

The data was plotted, showing the readings for all replicates at different concentrations at each timepoint (Figure 3.1) and as a mean \pm SEM (Figure 3.2). A Dunnett's multiple comparison test was performed using the solvent control for comparison on the software Prism (GraphPad) (Figure 3.3). All the data for each pesticide was summarised in a PowerPoint file with each slide contained the data for one gene (Figure 3.4).

It should be noted that the worms were washed clean of bacteria before each experiment and therefore were under starving conditions during the exposures. This was done to reduce background fluorescence from bacteria but may cause a difference in expression for some genes in the later timepoints due to response to starvation. Such effects should be normalised through comparison to controls.

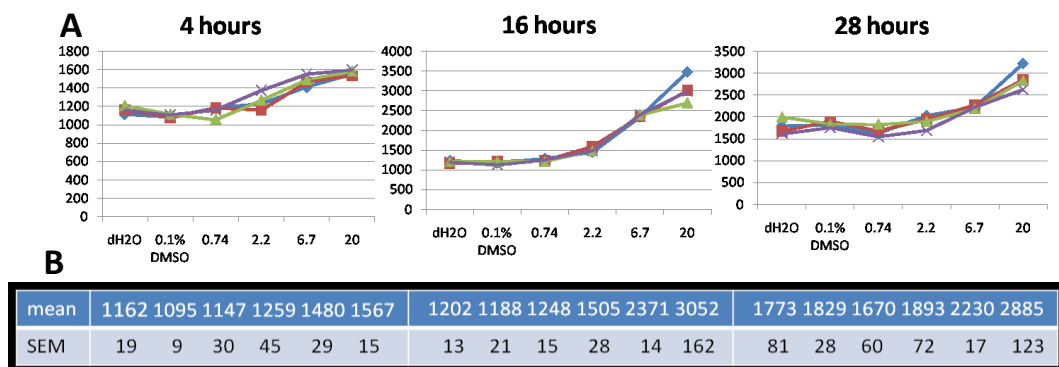


Figure 3.1 Readings for *skn-1::GFP* at all concentrations for all three timepoints.

A Graphs showing all replicates for each condition. **B** Mean and SEM for each condition.

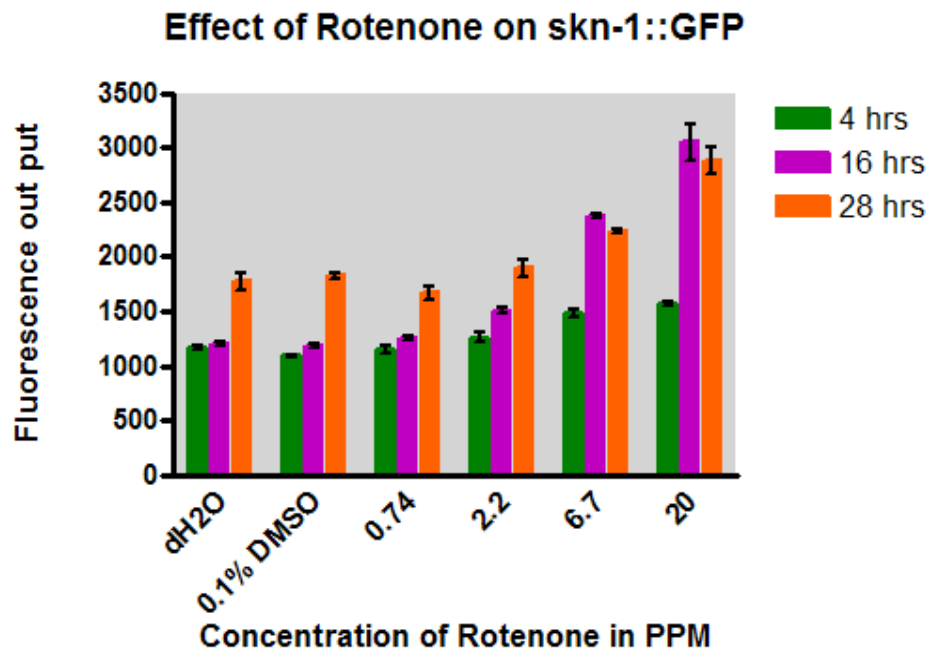


Figure 3.2 Mean measurement for *skn-1::GFP* at all concentrations for all three timepoints. Error bars represent SEM.

Dunnett's Multiple Comparison Test for *skn-1*

| Groups | P value 4h | P value 16h | P value 28h |
|-------------------|------------|-------------|-------------|
| 0.1% DMSO vs dH2O | P > 0.05 | P > 0.05 | P > 0.05 |
| 0.1% DMSO vs 0.74 | P > 0.05 | P > 0.05 | P > 0.05 |
| 0.1% DMSO vs 2.2 | P < 0.01 | P < 0.05 | P > 0.05 |
| 0.1% DMSO vs 6.7 | P < 0.01 | P < 0.01 | P < 0.01 |
| 0.1% DMSO vs 20 | P < 0.01 | P < 0.01 | P < 0.01 |

Figure 3.3 Dunnett's multiple comparison test for *skn-1::GFP*. Significant values are highlighted, light purple representing significance (P<0.05) and dark purple representing high significance (P<0.01).

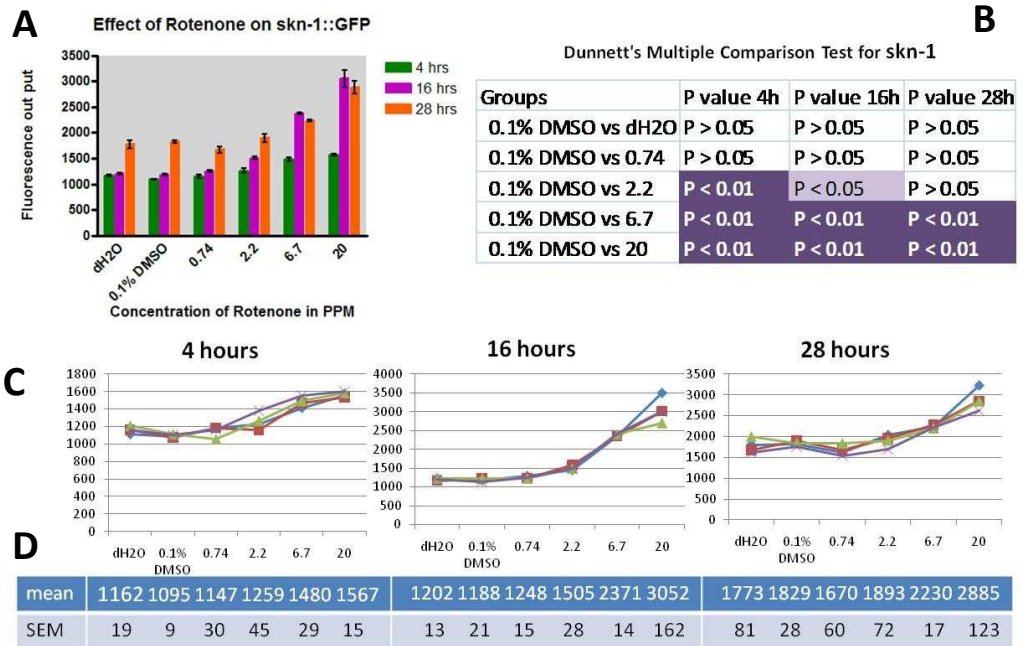


Figure 3.4 Effect of rotenone on *skn-1* expression. An example of a slide summarising the effect of a pesticide (rotenone) on a specific gene (here, *skn-1*). **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

The mean fluorescence output for each condition was divided by the solvent control of the same set to give an expression ratio. These were then represented in a table, summarising the results for one pesticide. A colour coding system was used to identify significant expression changes involving up- or down-regulation of the GFP transgene (Figure 3.5).

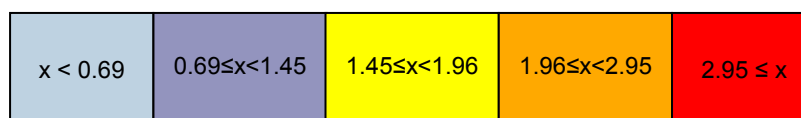


Figure 3.5 Colour coding system used for GFP fluorescence readout tables to depict high and low expression changes. “x” represents the GFP fluorescence readout ratio: mean value for given condition, divided by the mean value for the solvent control in the same set.

3.1.1 Rotenone

The natural pesticide rotenone was dissolved in DMSO, achieving a stock concentration of 20,000ppm. The highest concentration used in exposures was 20ppm with 0.1% DMSO present. A 3-fold dilution series was used for the toxicant concentrations and a solution containing 0.1% DMSO was used as a solvent control.

| Rotenone | | EARLY (4 hours) | | | | | | INTERMEDIATE (16-18hours) | | | | | | LATE (28-30 hours) | | | | | | |
|-----------------------|---------------------------|-----------------|------|------|------|------|------|---------------------------|--------------|------|------|------|------|--------------------|-------------------|--------------|------|------|-----|----|
| Gene | Dose dH ₂ O | 0.1% DMSO | ppm | | | | ppm | dH ₂ O | 0.1% DMSO | ppm | | | | ppm | dH ₂ O | 0.1% DMSO | ppm | | | |
| | | | 0.74 | 2.2 | 6.7 | 20 | | | | 0.74 | 2.2 | 6.7 | 20 | | | | 0.74 | 2.2 | 6.7 | 20 |
| sod-1 | 0.97 | 1.00 | 1.04 | 1.06 | 1.19 | 1.18 | 1.07 | 1.00 | 1.01 | 1.15 | 1.97 | 2.44 | 0.99 | 1.00 | 1.01 | 1.05 | 1.38 | 1.57 | | |
| sod-3 | 0.96 | 1.00 | 0.84 | 0.86 | 1.03 | 0.99 | 1.02 | 1.00 | 0.90 | 0.91 | 1.10 | 1.27 | 1.20 | 1.00 | 0.91 | 0.76 | 0.81 | 0.96 | | |
| sod-4 | 0.99 | 1.00 | 0.83 | 0.82 | 0.83 | 0.81 | 0.98 | 1.00 | 0.98 | 1.00 | 1.17 | 1.17 | 1.01 | 1.00 | 1.00 | 0.96 | 1.03 | 1.11 | | |
| ctl-2 | 1.11 | 1.00 | 1.16 | 1.18 | 1.19 | 1.25 | 0.99 | 1.00 | 1.01 | 1.02 | 1.20 | 1.42 | 0.98 | 1.00 | 0.93 | 0.92 | 1.04 | 1.28 | | |
| gpA (T09A 12.2) | 0.99 | 1.00 | 0.93 | 0.84 | 0.81 | 0.87 | 1.02 | 1.00 | 0.99 | 1.03 | 1.28 | 1.43 | 1.04 | 1.00 | 1.02 | 1.04 | 1.23 | 1.48 | | |
| gpB (C11E 4.1) | 1.00 | 1.00 | 1.05 | 1.09 | 1.02 | 0.98 | 1.02 | 1.00 | 0.99 | 1.02 | 1.19 | 1.23 | 1.07 | 1.00 | 0.98 | 0.97 | 0.94 | 1.03 | | |
| skn-1 | 1.06 | 1.00 | 1.05 | 1.15 | 1.35 | 1.43 | 1.01 | 1.00 | 1.05 | 1.27 | 2.00 | 2.57 | 0.97 | 1.00 | 0.91 | 1.03 | 1.22 | 1.58 | | |
| cep-1 | 1.01 | 1.00 | 0.88 | 0.90 | 0.93 | 0.88 | 1.03 | 1.00 | 0.97 | 0.96 | 1.09 | 1.10 | 1.03 | 1.00 | 0.95 | 0.91 | 1.05 | 1.10 | | |
| daf-16 | 1.05 | 1.00 | 0.76 | 0.73 | 0.72 | 0.75 | 1.09 | 1.00 | 1.02 | 1.01 | 1.19 | 1.24 | 1.05 | 1.00 | 1.04 | 1.00 | 1.18 | 1.29 | | |
| mtl-1 | 1.01 | 1.00 | 1.03 | 1.08 | 1.25 | 1.27 | 0.97 | 1.00 | 1.02 | 1.09 | 1.30 | 1.51 | 0.99 | 1.00 | 1.04 | 0.94 | 1.03 | 1.22 | | |
| mtl-2 | 1.06 | 1.00 | 1.03 | 1.06 | 1.25 | 1.25 | 1.08 | 1.00 | 1.00 | 1.12 | 1.82 | 2.10 | 1.03 | 1.00 | 0.99 | 0.98 | 1.14 | 1.38 | | |
| elt-2 | 1.07 | 1.00 | 1.02 | 1.00 | 1.14 | 1.14 | 1.09 | 1.00 | 1.03 | 1.09 | 1.59 | 1.54 | 1.04 | 1.00 | 1.02 | 0.98 | 1.15 | 1.24 | | |

Table 3.1 GFP fluorescence ratios for transgenic strains after exposure to rotenone. Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

Rotenone seems to induce expression of about half of the genes tested, at intermediate and/or late timepoints (Table 3.1). *sod-1* and *skn-1* among the oxidative stress genes and all three metal stress genes show up-regulation at the intermediate timepoint which then fades by the late timepoint (Figure 3.4, Figures 3.6-3.9)

Among the oxidative stress genes, GPA shows up-regulation at the late timepoint (Figure 3.10).

From the full list of genes tested, the heat shock genes showed no effect whereas most of the xenobiotic genes tested showed up-regulation. Among the glutathione-S-transferases, *gst-1* showed a 1.5-1.8-fold up-regulation at the highest concentration for the intermediate and late timepoints respectively but *gst-4* showed no effect. Among the cytochrome P450 genes *cyp-29A2* showed a time-dependent induction for the highest concentration, reaching 2-fold up-regulation; *cyp-34A9* showed a 1.5-1.8-fold induction for the highest concentration at the intermediate timepoint; and *cyp-35A2* showed a 2-fold up-regulation for the highest concentrations at the early and intermediate timepoints (information provided by Charumathi Anbalagan, data not shown).

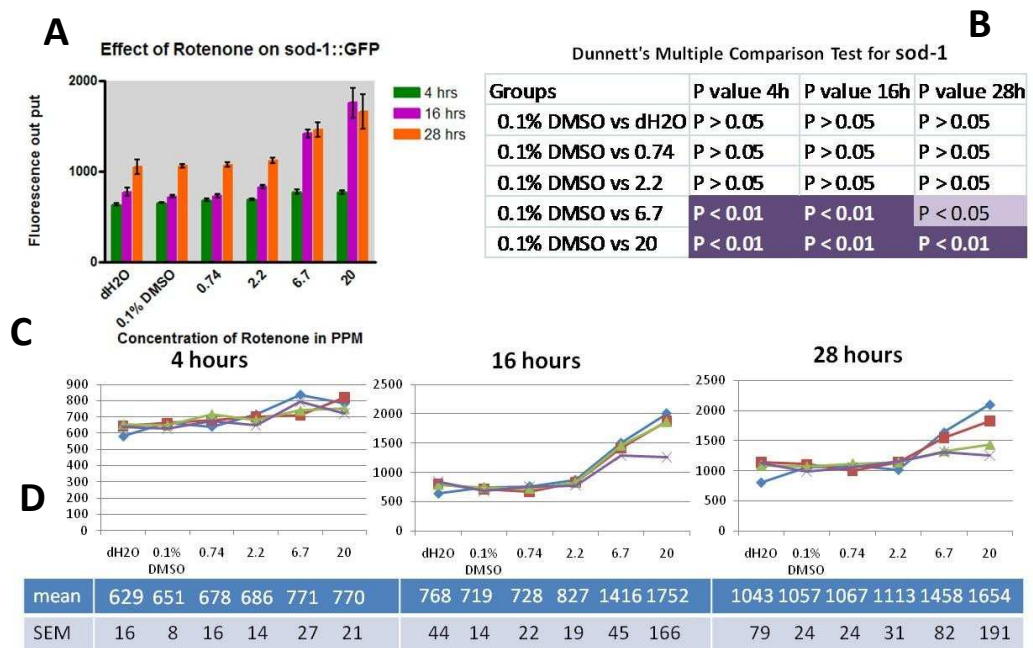


Figure 3.6 Summary of the effect of rotenone on *sod-1*. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

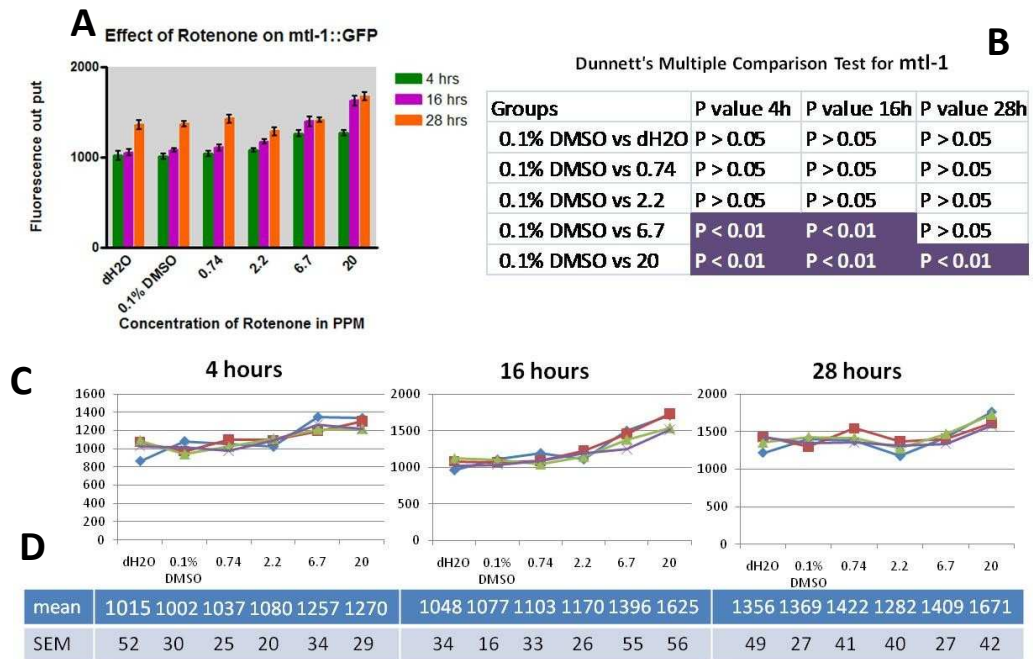


Figure 3.7 Summary of the effect of rotenone on *mtl-1*. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

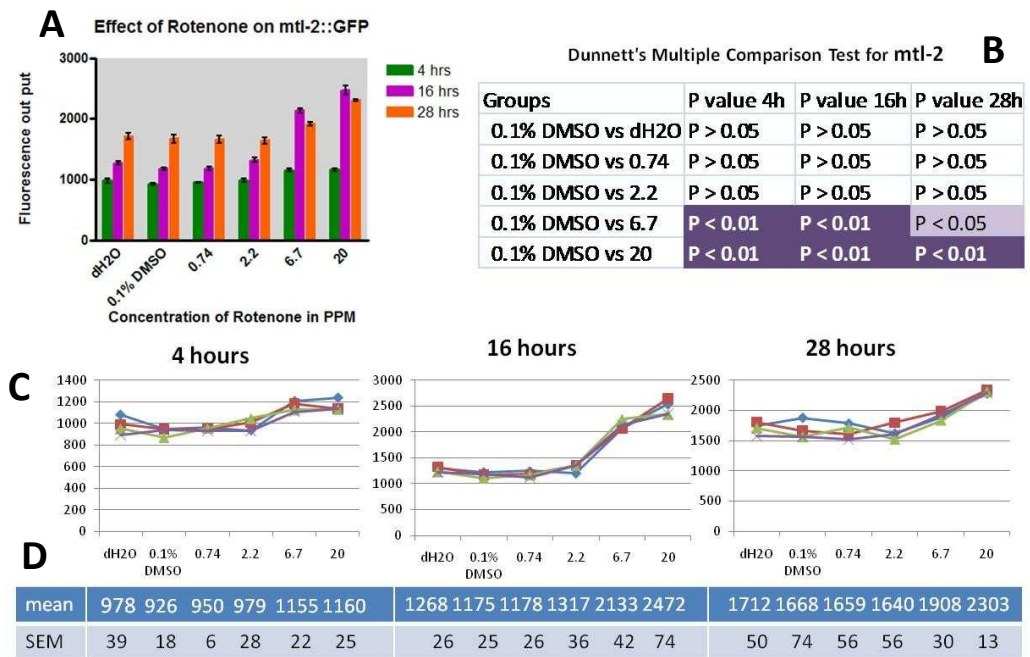


Figure 3.8 Summary of the effect of rotenone on *mtl-2*. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

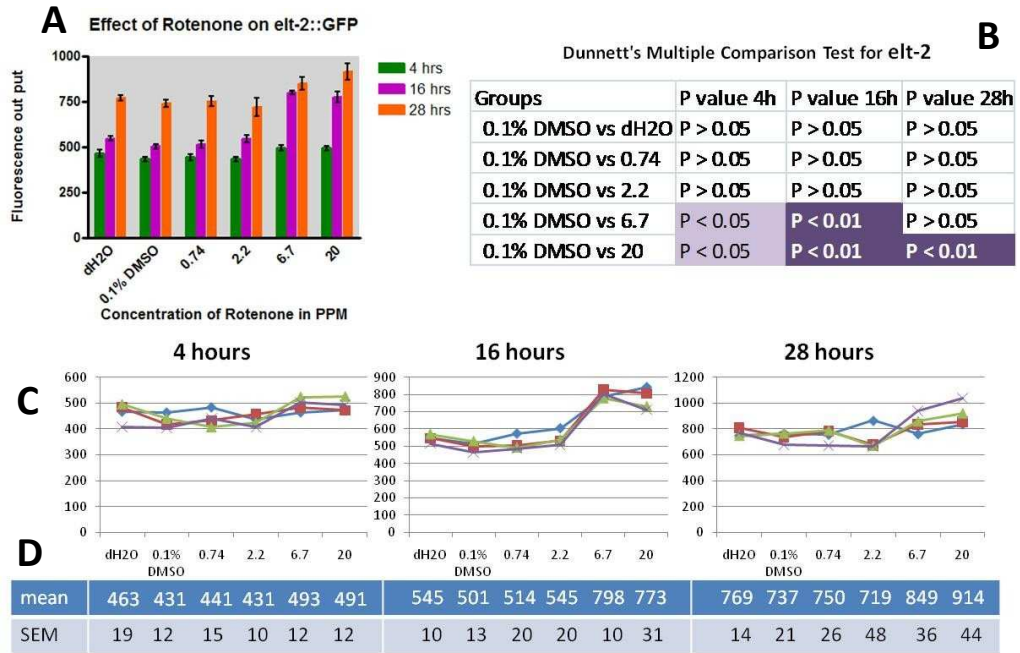


Figure 3.9 Summary of effect of rotenone on *elt-2*. **A** Chart summarising the data for all the concentrations at all timepoints. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test; showing significance of results. If $0.01 < P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

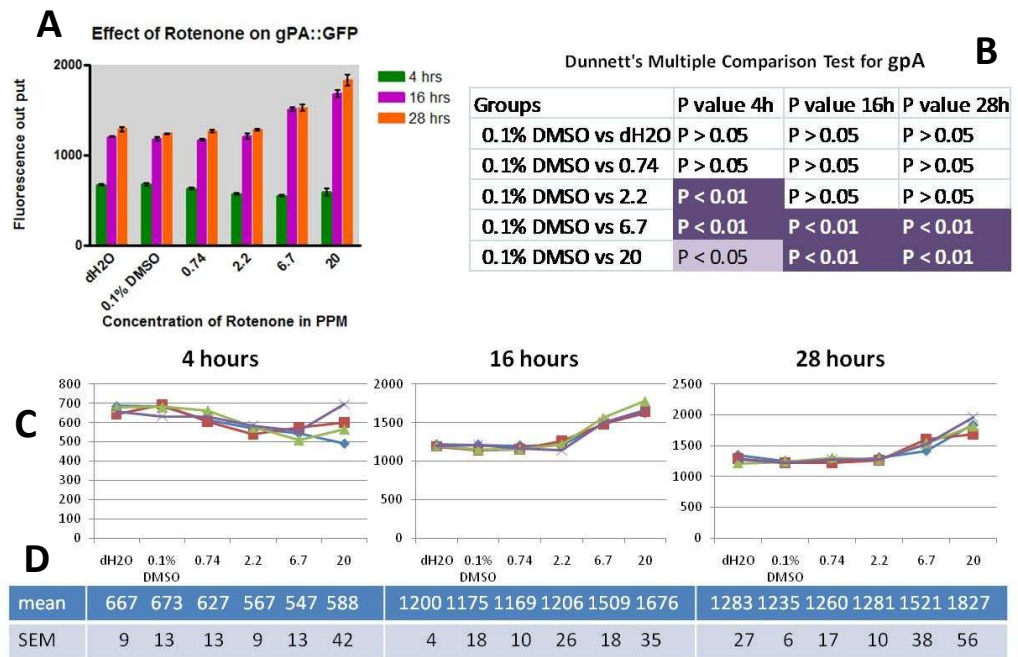


Figure 3.10 Summary of the effect of rotenone on GPA. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

3.1.2 Carbendazim

Carbendazim was dissolved freshly in EtOH before setting up each exposure to avoid precipitation. A maximum stock concentration of 50,000ppm was achieved, giving a top concentration of 100ppm (with 0.2%EtOH present) in exposures. A series of 5-fold dilutions was utilised for the toxicant concentrations and a 0.2% EtOH solution was used as the solvent control.

| Carbendazim | | EARLY (4 hours) | | | | | | INTERMEDIATE (16-18hours) | | | | | | LATE (28-30 hours) | | | | | | |
|-----------------|-------------------|-----------------|------|------|------|------|------|---------------------------|-----------|------|------|------|------|--------------------|-------------------|-----------|------|------|----|-----|
| Dose Gene | Dose | | ppm | | | | ppm | Dose | | ppm | | | | ppm | Dose | | ppm | | | |
| | dH ₂ O | 0.2% EtOH | 0.8 | 4 | 20 | 100 | | dH ₂ O | 0.2% EtOH | 0.8 | 4 | 20 | 100 | | dH ₂ O | 0.2% EtOH | 0.8 | 4 | 20 | 100 |
| sod-1 | 1.04 | 1.00 | 1.04 | 0.99 | 1.01 | 0.99 | 1.01 | 1.00 | 1.01 | 0.96 | 0.94 | 0.92 | 1.09 | 1.00 | 1.17 | 0.94 | 1.00 | 0.93 | | |
| sod-3 | 1.15 | 1.00 | 0.91 | 0.93 | 1.04 | 0.89 | 1.01 | 1.00 | 0.93 | 1.14 | 1.33 | 1.13 | 1.01 | 1.00 | 1.12 | 2.07 | 2.13 | 1.71 | | |
| sod-4 | 1.01 | 1.00 | 0.92 | 0.94 | 0.91 | 0.95 | 0.96 | 1.00 | 0.85 | 0.76 | 0.75 | 0.88 | 1.06 | 1.00 | 0.99 | 0.94 | 0.92 | 0.97 | | |
| ctl-2 | 1.05 | 1.00 | 1.33 | 1.33 | 1.27 | 1.19 | 1.06 | 1.00 | 1.13 | 1.09 | 1.09 | 1.10 | 1.07 | 1.00 | 1.07 | 1.13 | 1.10 | 1.15 | | |
| gpA (T09A 12.2) | 1.12 | 1.00 | 0.99 | 1.22 | 1.24 | 1.23 | 1.10 | 1.00 | 1.07 | 1.12 | 1.13 | 1.17 | 1.12 | 1.00 | 1.09 | 1.05 | 1.09 | 0.99 | | |
| gpB (C11E4 .1) | 1.14 | 1.00 | 1.02 | 1.05 | 1.08 | 1.16 | 1.17 | 1.00 | 1.07 | 1.00 | 0.98 | 1.15 | 1.12 | 1.00 | 1.05 | 1.02 | 1.09 | 1.15 | | |
| skn-1 | 1.09 | 1.00 | 1.17 | 1.18 | 1.32 | 1.13 | 1.04 | 1.00 | 1.04 | 0.97 | 1.10 | 1.02 | 1.03 | 1.00 | 0.91 | 0.94 | 1.05 | 0.95 | | |
| cep-1 | 1.07 | 1.00 | 0.91 | 1.03 | 0.93 | 0.99 | 1.10 | 1.00 | 0.96 | 0.93 | 0.91 | 0.97 | 1.12 | 1.00 | 0.92 | 0.88 | 0.84 | 0.89 | | |
| daf-16 | 0.88 | 1.00 | 0.93 | 0.94 | 0.93 | 0.93 | 0.95 | 1.00 | 1.01 | 0.97 | 0.97 | 0.97 | 1.01 | 1.00 | 0.94 | 0.89 | 0.93 | 0.91 | | |
| mtl-1 | 1.02 | 1.00 | 0.91 | 1.08 | 1.07 | 1.10 | 1.05 | 1.00 | 1.05 | 1.09 | 1.14 | 1.17 | 1.07 | 1.00 | 1.10 | 1.06 | 1.14 | 1.11 | | |
| mtl-2 | 1.06 | 1.00 | 0.88 | 0.87 | 0.87 | 0.88 | 1.08 | 1.00 | 1.06 | 1.06 | 0.97 | 1.14 | 1.06 | 1.00 | 0.94 | 0.96 | 0.95 | 0.91 | | |
| elt-2 | 1.06 | 1.00 | 1.03 | 1.10 | 1.04 | 1.02 | 1.10 | 1.00 | 0.93 | 0.96 | 0.97 | 1.01 | 1.02 | 1.00 | 1.05 | 1.00 | 1.01 | 1.00 | | |

Table 3.2 GFP fluorescence ratios for transgenic strains after exposure to carbendazim. Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

Carbendazim shows a significant up-regulation of the mitochondrial superoxide dismutase *sod-3*, involved in the response to oxidative stress (Figure 3.11). It does not appear to have much effect on the expression of other stress-response genes.

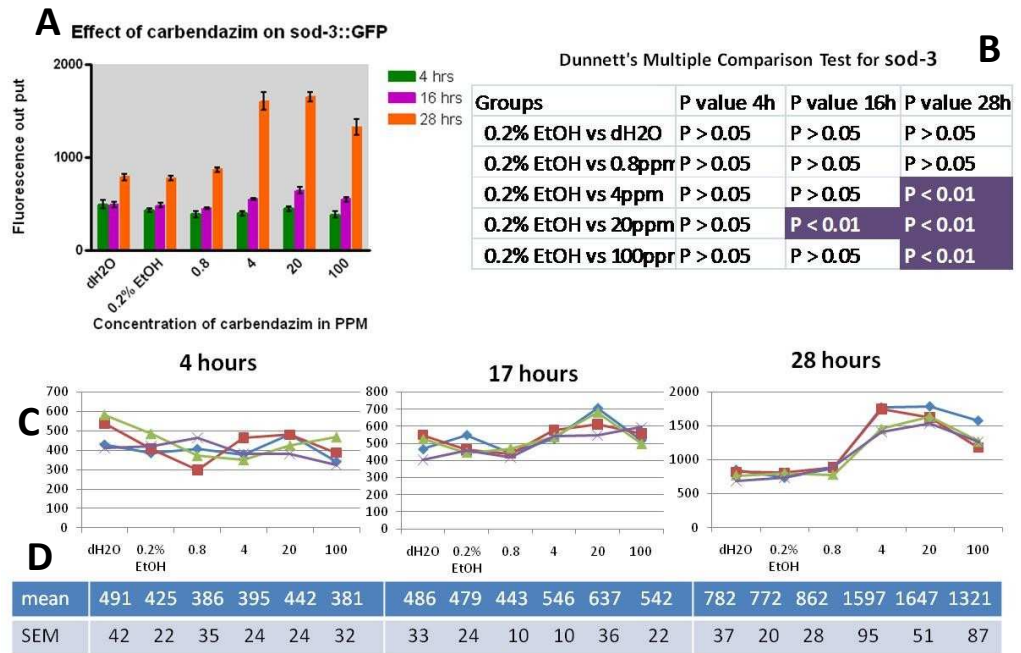


Figure 3.11 Summary of effect of carbendazim on *sod-3*. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

3.1.3 Chlorpyrifos

Chlorpyrifos was dissolved in MeOH and a 150,000ppm maximum stock concentration was achieved. A top concentration of 300ppm was used where MeOH was present at 0.2%. A 10-fold dilution series was used for the toxicant concentrations and a solution of 0.2% MeOH was used as the solvent control.

| Chlorpyrifos | | EARLY (4 hours) | | | | | | INTERMEDIATE (16-18hours) | | | | | | LATE (28-30 hours) | | | | | | |
|-----------------|-------------------|-----------------|------|------|------|------|-----|---------------------------|-----------|------|------|------|------|--------------------|-------------------|-----------|------|------|------|------|
| Dose Gene | dH ₂ O | 0.2% MeOH | 0.3 | 3 | 30 | 300 | ppm | dH ₂ O | 0.2% MeOH | 0.3 | 3 | 30 | 300 | ppm | dH ₂ O | 0.2% MeOH | 0.3 | 3 | 30 | 300 |
| sod-1 | 1.04 | 1.00 | 0.92 | 0.90 | 0.94 | 0.98 | | 1.05 | 1.00 | 0.97 | 0.92 | 1.01 | 1.15 | | 1.03 | 1.00 | 0.91 | 0.87 | 0.86 | 1.03 |
| sod-3 | 0.99 | 1.00 | 0.90 | 0.87 | 0.98 | 1.19 | | 0.98 | 1.00 | 0.91 | 0.86 | 1.06 | 1.12 | | 1.02 | 1.00 | 0.91 | 0.84 | 0.84 | 0.89 |
| sod-4 | 0.96 | 1.00 | 0.88 | 0.88 | 0.89 | 0.83 | | 1.06 | 1.00 | 0.92 | 0.96 | 0.98 | 1.26 | | 1.16 | 1.00 | 0.94 | 0.94 | 0.77 | 0.93 |
| ctl-2 | 1.12 | 1.00 | 1.01 | 0.91 | 0.98 | 1.10 | | 1.15 | 1.00 | 1.04 | 0.94 | 0.93 | 1.16 | | 1.06 | 1.00 | 1.03 | 0.92 | 0.86 | 1.06 |
| gpA (T09A 12.2) | 1.04 | 1.00 | 0.99 | 0.87 | 0.87 | 0.98 | | 1.10 | 1.00 | 0.96 | 0.86 | 0.93 | 1.14 | | 1.04 | 1.00 | 0.93 | 0.87 | 0.70 | 0.98 |
| gpB (C11E 4.1) | 1.08 | 1.00 | 1.00 | 0.96 | 0.89 | 0.96 | | 1.04 | 1.00 | 0.97 | 0.96 | 0.88 | 0.83 | | 1.05 | 1.00 | 0.99 | 0.97 | 0.79 | 0.77 |
| skn-1 | 1.03 | 1.00 | 0.96 | 0.90 | 0.96 | 0.81 | | 0.91 | 1.00 | 0.93 | 0.89 | 0.89 | 0.85 | | 0.90 | 1.00 | 0.98 | 0.86 | 0.85 | 0.94 |
| cep-1 | 1.01 | 1.00 | 0.89 | 0.93 | 0.82 | 0.91 | | 0.96 | 1.00 | 0.93 | 0.92 | 0.82 | 1.11 | | 1.05 | 1.00 | 0.91 | 0.96 | 0.78 | 1.14 |
| daf-16 | 0.92 | 1.00 | 0.87 | 0.84 | 0.83 | 0.86 | | 1.11 | 1.00 | 0.98 | 0.95 | 0.90 | 1.03 | | 0.99 | 1.00 | 0.90 | 0.87 | 0.91 | 0.94 |
| mtl-1 | 1.29 | 1.00 | 0.92 | 0.97 | 0.95 | 1.17 | | 1.08 | 1.00 | 0.98 | 0.89 | 0.88 | 0.97 | | 1.09 | 1.00 | 1.05 | 0.88 | 0.79 | 0.82 |
| mtl-2 | 0.96 | 1.00 | 0.86 | 0.84 | 0.81 | 0.82 | | 1.08 | 1.00 | 0.99 | 0.95 | 1.13 | 1.03 | | 0.95 | 1.00 | 0.86 | 0.84 | 0.93 | 0.99 |
| elt-2 | 0.98 | 1.00 | 0.98 | 0.91 | 0.90 | 0.89 | | 0.99 | 1.00 | 0.97 | 0.89 | 0.89 | 1.09 | | 1.04 | 1.00 | 0.98 | 0.93 | 0.78 | 1.04 |

Table 3.3 GFP fluorescence ratios for transgenic strains after exposure to chlorpyrifos. Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

Chlorpyrifos shows little effect on the expression of these stress-response genes in the nematode. However, looking at the full data, among the xenobiotic stress genes, *cyp-34A9* shows a high induction at the highest concentration only, of the scale of 4-6-fold. Induction is apparent at the early timepoint (ratio: 6.07) and shows signs of fading, reaching a ratio of 3.96 by the late timepoint (Figure 3.12). Note that the lower concentrations gave ratio values of 0.85-0.95, showing no signs of up-regulation.

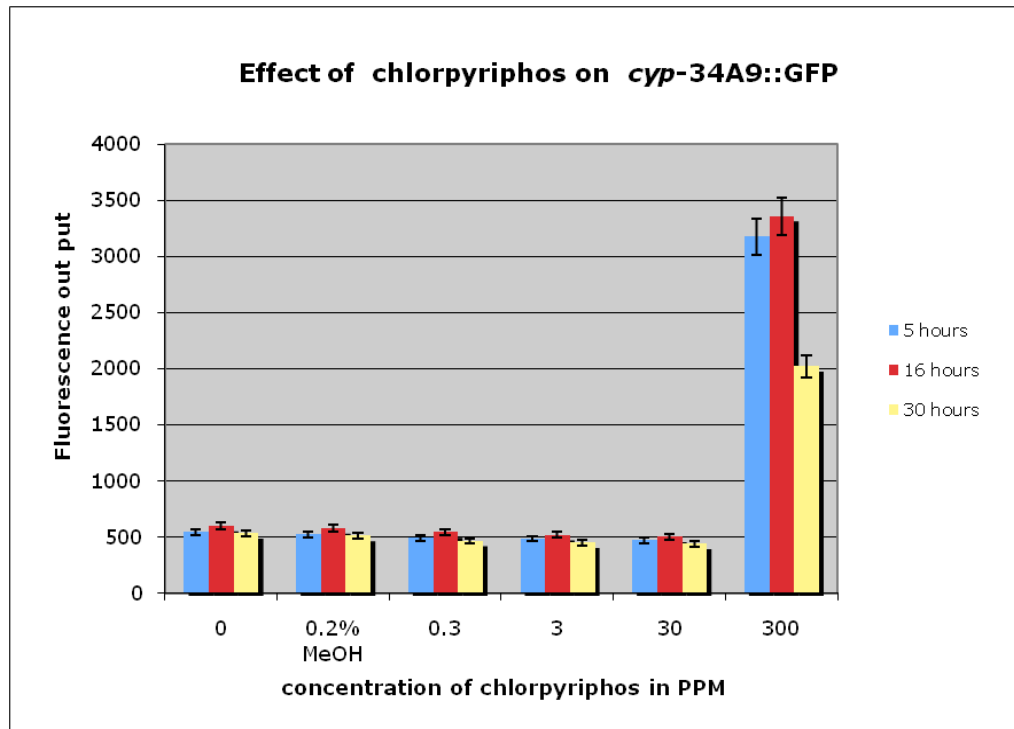


Figure 3.12 Effect of chlorpyrifos on *cyp-34A9::GFP*. Error bars represent SEM.

Figure kindly provided by Charumathi Anbalagan.

3.1.4 Endosulfan

Endosulfan was dissolved in EtOH, reaching a maximum stock concentration of 100,000ppm. A top concentration of 200ppm was used where EtOH was present at 0.2%. A 10-fold dilution series was used for the toxicant concentrations and the solvent control used contained 0.2% EtOH.

| Endosulfan | | EARLY (4 hours) | | | | | | INTERMEDIATE (16-18hours) | | | | | | LATE (28-30 hours) | | | | | | |
|-----------------|-------------------|-----------------|------|------|------|------|-------|---------------------------|-----------|------|------|------|------|--------------------|-------------------|-----------|------|------|------|------|
| Dose Gene | dH ₂ O | 0.2% EtOH | 0.2 | 2 | 20 | 200 | p p m | dH ₂ O | 0.2% EtOH | 0.2 | 2 | 20 | 200 | p p m | dH ₂ O | 0.2% EtOH | 0.2 | 2 | 20 | 200 |
| sod-1 | 0.97 | 1.00 | 0.96 | 0.94 | 0.99 | 1.01 | | 1.04 | 1.00 | 0.97 | 0.88 | 1.06 | 1.05 | | 1.25 | 1.00 | 1.06 | 0.99 | 1.01 | 1.10 |
| sod-3 | 1.09 | 1.00 | 0.99 | 1.03 | 1.04 | 1.01 | | 1.07 | 1.00 | 0.98 | 1.00 | 0.89 | 0.83 | | 1.10 | 1.00 | 0.96 | 0.97 | 0.72 | 0.70 |
| sod-4 | 1.05 | 1.00 | 0.95 | 0.99 | 1.05 | 1.05 | | 1.06 | 1.00 | 0.97 | 0.94 | 1.00 | 1.03 | | 1.08 | 1.00 | 0.95 | 0.94 | 0.97 | 0.97 |
| ctl-2 | 0.88 | 1.00 | 0.80 | 0.80 | 0.81 | 0.79 | | 1.04 | 1.00 | 0.98 | 0.96 | 0.93 | 0.91 | | 1.04 | 1.00 | 0.97 | 0.94 | 0.92 | 0.88 |
| gpA (T09A 12.2) | 0.94 | 1.00 | 0.91 | 0.97 | 0.92 | 0.91 | | 1.01 | 1.00 | 0.95 | 0.97 | 0.86 | 0.91 | | 0.97 | 1.00 | 0.93 | 0.91 | 0.80 | 0.88 |
| gpB (C11E 4.1) | 1.00 | 1.00 | 0.99 | 1.00 | 1.07 | 1.28 | | 1.09 | 1.00 | 1.03 | 1.02 | 1.21 | 1.28 | | 1.06 | 1.00 | 1.00 | 0.97 | 0.84 | 0.97 |
| skn-1 | 0.99 | 1.00 | 0.94 | 0.96 | 0.90 | 0.89 | | 1.02 | 1.00 | 0.96 | 0.91 | 0.90 | 1.01 | | 1.17 | 1.00 | 1.02 | 1.01 | 0.92 | 1.05 |
| cep-1 | 0.98 | 1.00 | 0.97 | 0.99 | 0.99 | 1.04 | | 0.98 | 1.00 | 1.00 | 1.03 | 1.01 | 0.96 | | 1.03 | 1.00 | 0.99 | 1.05 | 1.03 | 1.02 |
| daf-16 | 1.03 | 1.00 | 0.98 | 0.91 | 0.98 | 0.99 | | 1.07 | 1.00 | 1.02 | 0.97 | 0.91 | 0.92 | | 1.06 | 1.00 | 0.96 | 0.91 | 0.92 | 0.90 |
| mtl-1 | 1.01 | 1.00 | 0.90 | 0.94 | 0.96 | 0.97 | | 1.02 | 1.00 | 0.89 | 0.90 | 0.87 | 0.94 | | 1.04 | 1.00 | 0.92 | 0.92 | 0.89 | 0.93 |
| mtl-2 | 1.02 | 1.00 | 1.00 | 0.97 | 1.17 | 1.29 | | 1.04 | 1.00 | 0.99 | 0.99 | 1.12 | 1.23 | | 1.11 | 1.00 | 0.98 | 0.86 | 1.10 | 1.07 |
| elt-2 | 1.05 | 1.00 | 1.04 | 1.01 | 1.09 | 1.12 | | 1.03 | 1.00 | 0.98 | 0.91 | 1.01 | 1.02 | | 1.17 | 1.00 | 1.02 | 1.02 | 1.00 | 1.05 |

Table 3.4 GFP fluorescence ratios for transgenic strains after exposure to endosulfan.

Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

Endosulfan does not have much apparent effect on gene expression of these stress-response genes. However, the xenobiotic stress gene *cyp-34A9*, again shows significant up-regulation, with the ratios for all concentrations reaching 4-fold for the early timepoint and rising to around 7-fold for the intermediate and around 9-fold for the late timepoint (Figure 3.13).

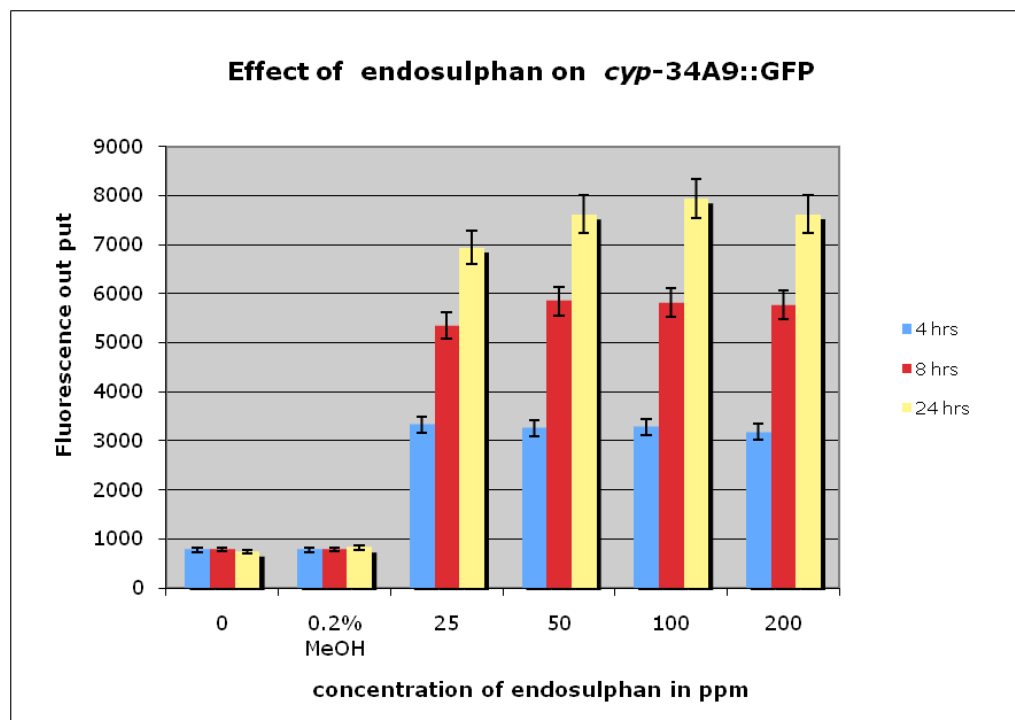


Figure 3.13 Effect of endosulfan on *cyp-34A9*. Error bars represent SEM. Figure kindly provided by Charumathi Anbalagan.

3.1.5 Cypermethrin

The pyrethroid cypermethrin was dissolved in EtOH, achieving a maximum stock concentration of 100,000ppm. The highest concentration used was 100ppm, which contained 0.1% EtOH. A series of 10-fold dilutions was used for the toxicant concentrations and a solution of 0.1% EtOH was used as a solvent control.

| Cypermethrin | | EARLY (4 hours) | | | | | | INTERMEDIATE (16-18hours) | | | | | | LATE (28-30 hours) | | | | | | | |
|-----------------|------|-------------------|-----------|------|------|------|------|---------------------------|-------------------|-----------|------|------|------|--------------------|-------|-------------------|-----------|------|------|------|------|
| Gene | Dose | dH ₂ O | 0.1% EtOH | 0.1 | 1 | 10 | 100 | p p m | dH ₂ O | 0.1% EtOH | 0.1 | 1 | 10 | 100 | p p m | dH ₂ O | 0.1% EtOH | 0.1 | 1 | 10 | 100 |
| | | sod-1 | 1.02 | 1.00 | 0.93 | 0.99 | 0.89 | | 0.79 | | 0.96 | 1.00 | 0.87 | 0.80 | | 1.00 | 0.95 | | 1.05 | 1.00 | 0.90 |
| sod-3 | 1.15 | 1.00 | 1.12 | 0.91 | 0.89 | 0.69 | | 1.08 | 1.00 | 0.95 | 0.80 | 0.78 | 0.82 | | 1.22 | 1.00 | 0.98 | 0.78 | 0.79 | 0.78 | |
| sod-4 | 1.06 | 1.00 | 0.96 | 0.99 | 0.97 | 0.83 | | 1.18 | 1.00 | 0.94 | 0.93 | 0.91 | 0.94 | | 1.24 | 1.00 | 0.93 | 0.91 | 0.90 | 1.12 | |
| ctl-2 | 1.16 | 1.00 | 1.09 | 1.05 | 1.10 | 1.06 | | 1.16 | 1.00 | 1.01 | 1.02 | 1.04 | 1.14 | | 1.32 | 1.00 | 0.98 | 1.03 | 0.96 | 1.06 | |
| gpA (T09A 12.2) | 0.99 | 1.00 | 0.96 | 1.01 | 0.92 | 0.91 | | 1.01 | 1.00 | 0.90 | 0.90 | 0.95 | 1.15 | | 1.25 | 1.00 | 0.98 | 1.01 | 0.98 | 1.06 | |
| gpB (C11E 4.1) | 0.96 | 1.00 | 0.91 | 0.81 | 0.79 | 0.71 | | 1.06 | 1.00 | 1.02 | 0.89 | 0.79 | 0.76 | | 0.96 | 1.00 | 1.03 | 0.88 | 0.89 | 0.83 | |
| skn-1 | 1.04 | 1.00 | 1.01 | 1.13 | 0.94 | 0.93 | | 1.03 | 1.00 | 1.00 | 0.97 | 0.84 | 0.86 | | 1.09 | 1.00 | 1.00 | 0.99 | 0.96 | 1.07 | |
| cep-1 | 1.10 | 1.00 | 0.99 | 0.99 | 0.97 | 0.90 | | 1.11 | 1.00 | 0.93 | 0.94 | 1.02 | 0.93 | | 1.26 | 1.00 | 1.02 | 0.87 | 0.95 | 0.94 | |
| daf-16 | 1.02 | 1.00 | 1.01 | 0.95 | 0.94 | 0.88 | | 0.92 | 1.00 | 0.98 | 0.91 | 0.81 | 0.81 | | 0.88 | 1.00 | 0.90 | 0.79 | 0.75 | 0.78 | |
| mtl-1 | 1.11 | 1.00 | 1.00 | 1.02 | 1.04 | 1.11 | | 1.15 | 1.00 | 1.01 | 1.00 | 1.01 | 1.21 | | 1.08 | 1.00 | 0.97 | 0.94 | 0.97 | 1.00 | |
| mtl-2 | 0.98 | 1.00 | 0.99 | 0.95 | 0.89 | 0.88 | | 1.02 | 1.00 | 1.05 | 1.00 | 0.96 | 0.95 | | 0.96 | 1.00 | 1.02 | 0.90 | 0.87 | 0.92 | |
| elt-2 | 1.19 | 1.00 | 1.14 | 1.11 | 1.26 | 1.05 | | 1.12 | 1.00 | 1.05 | 1.00 | 1.06 | 1.08 | | 1.14 | 1.00 | 0.94 | 0.95 | 1.06 | 1.13 | |

Table 3.5 GFP fluorescence ratios for transgenic strains after exposure to cypermethrin. Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

It appears from Table 3.5 that cypermethrin does not have a significant effect on the expression of stress-response genes in the nematode at any timepoint tested.

3.1.6 Deltamethrin

Deltamethrin, like cypermethrin, is a pyrethroid insecticide. This toxicant was dissolved in DMSO, achieving a maximum stock concentration of 50,000ppm; the highest test concentration was 100ppm, containing 0.2% DMSO. A series of 10-fold dilutions was used for the toxicant concentrations and a solution of 0.2% DMSO was used as the solvent control.

| Deltamethrin | | EARLY (4 hours) | | | | | INTERMEDIATE (16-18hours) | | | | | LATE (28-30 hours) | | | | | | | | |
|-----------------------|---------------------------|-----------------|------|------|------|------|---------------------------|-------------------|--------------|------|------|--------------------|------|------|-------------------|--------------|------|------|----|-----|
| Gene | Dose dH ₂ O | 0.2% DMSO | ppm | | | | ppm | dH ₂ O | 0.2% DMSO | ppm | | | | ppm | dH ₂ O | 0.2% DMSO | ppm | | | |
| | | | 1 | 5 | 25 | 100 | | | | 1 | 5 | 25 | 100 | | | | 1 | 5 | 25 | 100 |
| sod-1 | 1.01 | 1.00 | 0.94 | 0.94 | 0.93 | 0.96 | 1.18 | 1.00 | 1.12 | 0.96 | 1.01 | 1.06 | 1.23 | 1.00 | 1.05 | 0.87 | 0.87 | 0.92 | | |
| sod-3 | 1.10 | 1.00 | 1.09 | 0.97 | 0.94 | 0.83 | 1.09 | 1.00 | 1.00 | 0.93 | 0.92 | 0.93 | 1.15 | 1.00 | 1.00 | 0.93 | 0.92 | 0.81 | | |
| sod-4 | 1.08 | 1.00 | 1.16 | 0.97 | 0.93 | 0.97 | 0.96 | 1.00 | 0.97 | 0.96 | 0.85 | 0.91 | 0.92 | 1.00 | 0.98 | 0.91 | 0.81 | 0.81 | | |
| ctl-2 | 1.09 | 1.00 | 1.03 | 1.07 | 0.97 | 0.84 | 0.97 | 1.00 | 0.98 | 0.96 | 0.90 | 0.90 | 1.14 | 1.00 | 0.98 | 0.98 | 0.93 | 0.98 | | |
| gpA (T09A 12.2) | 1.07 | 1.00 | 0.96 | 0.90 | 0.86 | 0.79 | 1.03 | 1.00 | 0.95 | 0.95 | 0.89 | 0.93 | 1.12 | 1.00 | 1.05 | 1.02 | 0.99 | 0.95 | | |
| gpB (C11E 4.1) | 1.06 | 1.00 | 0.96 | 0.94 | 0.87 | 0.78 | 1.05 | 1.00 | 0.90 | 0.87 | 0.89 | 0.94 | 1.11 | 1.00 | 0.89 | 0.87 | 0.85 | 0.87 | | |
| skn-1 | 0.98 | 1.00 | 0.76 | 0.80 | 0.76 | 0.66 | 0.85 | 1.00 | 0.69 | 0.74 | 0.77 | 0.70 | 0.80 | 1.00 | 0.72 | 0.69 | 0.70 | 0.67 | | |
| cep-1 | 1.05 | 1.00 | 0.91 | 0.93 | 0.87 | 0.75 | 1.06 | 1.00 | 0.98 | 0.99 | 0.96 | 0.90 | 1.09 | 1.00 | 1.03 | 1.10 | 1.00 | 0.81 | | |
| daf-16 | 1.00 | 1.00 | 0.97 | 0.97 | 0.97 | 0.99 | 1.04 | 1.00 | 0.98 | 0.96 | 0.98 | 1.04 | 1.09 | 1.00 | 1.00 | 0.94 | 0.96 | 1.07 | | |
| mtl-1 | 0.92 | 1.00 | 0.87 | 0.85 | 0.87 | 0.77 | 0.95 | 1.00 | 0.80 | 0.77 | 0.89 | 0.76 | 0.96 | 1.00 | 0.83 | 0.82 | 0.81 | 0.75 | | |
| mtl-2 | 1.05 | 1.00 | 0.93 | 0.93 | 0.89 | 0.72 | 0.94 | 1.00 | 0.90 | 0.86 | 0.85 | 0.77 | 0.97 | 1.00 | 0.99 | 0.91 | 0.90 | 0.72 | | |
| elt-2 | 0.99 | 1.00 | 0.92 | 0.92 | 0.95 | 0.79 | 0.96 | 1.00 | 0.85 | 0.88 | 0.90 | 0.83 | 1.01 | 1.00 | 0.94 | 0.89 | 1.02 | 0.85 | | |

Table 3.6 GFP fluorescence ratios for transgenic strains after exposure to deltamethrin. Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

Other than a down-regulation of *skn-1* (Figure 3.14), deltamethrin does not appear to have much effect on stress-response gene expression in the nematode (Table 3.6).

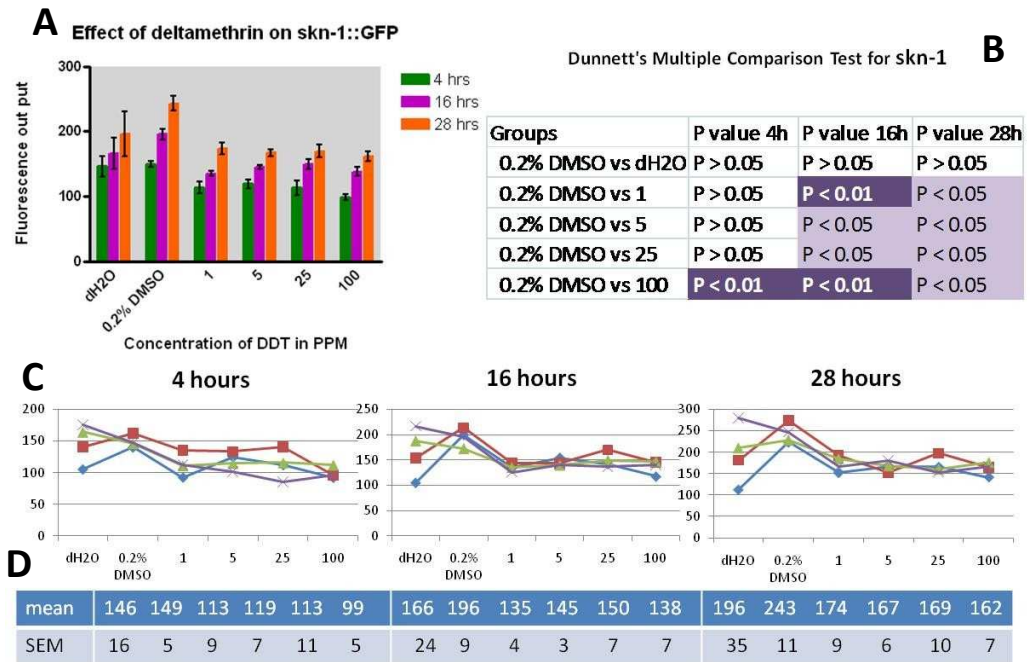


Figure 3.14 of Summary of effect of deltamethrin on *skn-1*. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

On the basis of our GFP quantification assays, pyrethroids appear to have little effect on nematode stress-response gene expression. Further assays were therefore carried out to test for possible physiological effects of pyrethroids on the nematode and these are described later in section 3.2.

3.1.7 Diuron

Diuron is a herbicide acting on the photosynthetic pathway. It is very insoluble in water and a solution of 50% EtOH/50% DMSO was used to dissolve it. In this solution, diuron was dissolved to reach a stock concentration of 33,333 parts per million (ppm). The highest achievable test concentration was 66.7ppm, in which the two solvents were present at 0.1% each. A series of 10-fold dilutions was used with 4 concentrations of the toxicant; a water control and a solvent control containing 0.1%EtOH/0.1%DMSO were run parallel to the toxicant exposures.

Since diuron was not expected to act specifically on *C. elegans* and the highest concentration achieved was relatively low, only 2 readings were taken, for the early and late timepoints.

| Diuron | | EARLY (4 hours) | | | | | | LATE (28-30 hours) | | | | | |
|-----------------|-------------------|---------------------|--------|-------|------|------|-----|--------------------|---------------------|--------|-------|------|------|
| Dose Gene | dH ₂ O | 0.1% DMSO/0.1% EtOH | 0.0667 | 0.667 | 6.67 | 66.7 | ppm | dH ₂ O | 0.1% DMSO/0.1% EtOH | 0.0667 | 0.667 | 6.67 | 66.7 |
| sod-1 | 1.02 | 1.00 | 1.02 | 0.96 | 0.96 | 0.98 | | 0.99 | 1.00 | 0.88 | 0.89 | 0.91 | 0.77 |
| sod-3 | 1.18 | 1.00 | 1.08 | 1.07 | 0.95 | 0.77 | | 0.94 | 1.00 | 0.91 | 0.85 | 0.88 | 0.74 |
| sod-4 | 1.06 | 1.00 | 1.04 | 1.04 | 1.04 | 1.05 | | 1.02 | 1.00 | 0.98 | 1.00 | 0.95 | 0.79 |
| ctl-2 | 1.03 | 1.00 | 1.07 | 1.09 | 1.02 | 0.99 | | 0.87 | 1.00 | 0.98 | 0.94 | 0.90 | 0.89 |
| gpA (T09A 12.2) | 1.09 | 1.00 | 1.07 | 1.11 | 1.02 | 1.11 | | 1.10 | 1.00 | 0.99 | 0.97 | 0.94 | 1.00 |
| gpB (C11E4 .1) | 0.95 | 1.00 | 0.90 | 0.87 | 0.85 | 0.87 | | 1.00 | 1.00 | 0.96 | 0.87 | 0.80 | 0.81 |
| skn-1 | 0.96 | 1.00 | 0.91 | 0.90 | 0.92 | 0.91 | | 0.95 | 1.00 | 0.89 | 0.81 | 0.78 | 0.84 |
| cep-1 | 0.90 | 1.00 | 0.87 | 0.89 | 0.87 | 0.84 | | 0.83 | 1.00 | 0.86 | 0.97 | 1.05 | 1.08 |
| daf-16 | 1.05 | 1.00 | 1.00 | 0.97 | 0.98 | 0.88 | | 1.06 | 1.00 | 0.96 | 0.95 | 0.91 | 0.79 |
| mtl-1 | 0.95 | 1.00 | 1.00 | 1.14 | 0.99 | 0.95 | | 0.84 | 1.00 | 1.02 | 1.23 | 1.11 | 1.04 |
| mtl-2 | 0.96 | 1.00 | 0.96 | 1.00 | 0.96 | 1.00 | | 0.95 | 1.00 | 0.90 | 0.94 | 0.87 | 0.94 |
| elt-2 | 0.91 | 1.00 | 1.04 | 1.00 | 0.98 | 0.82 | | 0.88 | 1.00 | 0.99 | 0.96 | 0.88 | 0.77 |

Table 3.7 GFP fluorescence ratios for transgenic strains after exposure to diuron.

Mean readout for each condition was divided by the solvent control of the same set to give the expression ratio shown. Cells were shaded according to the colour coding system shown in Figure 3.5.

It appears from Table 3.7 that diuron has little effect on the expression of stress-response genes in the nematode, with most genes showing light but not significant down-regulation at the late timepoint. Figure 3.15 shows an example of a non-responsive gene in the presence of diuron.

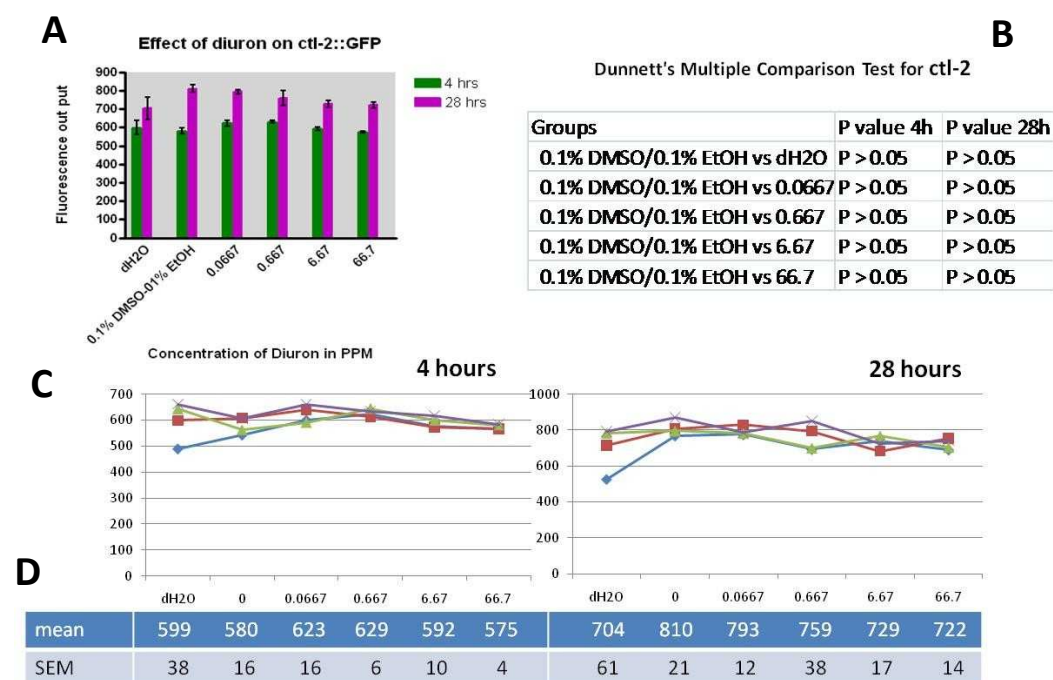


Figure 3.15 Summary of effect of diuron on *ctl-2*. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

Further analysis into the effect of diuron on the nematode was performed as presented in section 3.2.

3.1.8 DDT

DDT was dissolved in DMSO giving a stock concentration of 50,000ppm. A maximum test concentration of 100ppm was used, where DMSO was present in 0.2%. A 10-fold dilution series was used for the toxicant concentrations and a solution of 0.2% DMSO was used as the solvent control.

| DDT Gene | EARLY (4 hours) | | | | | | | INTERMEDIATE (16-18hours) | | | | | | | LATE (28-30 hours) | | | | | | |
|-----------------------|-------------------|--------------|------|------|------|------|-----|---------------------------|--------------|------|------|------|------|-----|--------------------|--------------|------|------|------|------|--|
| | dH ₂ O | 0.2% DMSO | 0.1 | 1 | 10 | 100 | ppm | dH ₂ O | 0.2% DMSO | 0.1 | 1 | 10 | 100 | ppm | dH ₂ O | 0.2% DMSO | 0.1 | 1 | 10 | 100 | |
| sod-1 | 1.00 | 1.00 | 0.98 | 0.94 | 0.88 | 0.88 | | 1.04 | 1.00 | 0.91 | 0.90 | 0.78 | 0.87 | | 1.11 | 1.00 | 0.92 | 0.92 | 0.85 | 0.88 | |
| sod-3 | 1.10 | 1.00 | 1.00 | 0.99 | 1.01 | 0.89 | | 1.04 | 1.00 | 0.93 | 0.93 | 0.93 | 0.94 | | 1.14 | 1.00 | 0.89 | 0.90 | 0.93 | 1.07 | |
| sod-4 | 1.04 | 1.00 | 0.99 | 1.02 | 1.04 | 0.93 | | 1.06 | 1.00 | 0.92 | 0.96 | 0.96 | 0.97 | | 1.09 | 1.00 | 0.94 | 0.93 | 0.92 | 0.95 | |
| ctl-2 | 1.12 | 1.00 | 1.03 | 0.99 | 1.07 | 1.02 | | 1.05 | 1.00 | 0.93 | 0.87 | 0.88 | 0.91 | | 1.05 | 1.00 | 0.93 | 0.80 | 0.84 | 0.77 | |
| gpA (T09A 12.2) | 1.02 | 1.00 | 0.93 | 0.92 | 0.94 | 0.80 | | 1.04 | 1.00 | 0.93 | 0.89 | 0.92 | 0.90 | | 1.08 | 1.00 | 0.95 | 0.93 | 0.93 | 0.98 | |
| gpB (C11E 4.1) | 0.91 | 1.00 | 0.81 | 0.76 | 0.69 | 0.63 | | 1.00 | 1.00 | 0.83 | 0.80 | 0.70 | 0.67 | | 1.08 | 1.00 | 0.85 | 0.85 | 0.74 | 0.70 | |
| skn-1 | 1.03 | 1.00 | 0.99 | 1.01 | 0.99 | 0.88 | | 1.11 | 1.00 | 0.98 | 0.91 | 0.96 | 0.94 | | 1.05 | 1.00 | 0.93 | 0.88 | 0.87 | 0.85 | |
| cep-1 | 1.00 | 1.00 | 0.91 | 0.95 | 0.97 | 0.95 | | 1.06 | 1.00 | 1.02 | 0.93 | 0.93 | 0.93 | | 1.08 | 1.00 | 0.96 | 0.88 | 0.82 | 0.86 | |
| daf-16 | 1.13 | 1.00 | 0.95 | 0.93 | 0.91 | 0.86 | | 1.06 | 1.00 | 0.92 | 0.87 | 0.84 | 0.85 | | 1.07 | 1.00 | 0.95 | 0.95 | 0.90 | 0.89 | |
| mtl-1 | 1.10 | 1.00 | 1.00 | 0.91 | 0.95 | 0.97 | | 1.15 | 1.00 | 0.96 | 0.88 | 0.91 | 1.12 | | 1.07 | 1.00 | 0.90 | 0.91 | 0.82 | 0.87 | |
| mtl-2 | 0.96 | 1.00 | 0.94 | 0.99 | 0.94 | 0.87 | | 0.98 | 1.00 | 0.98 | 0.88 | 0.90 | 1.07 | | 1.01 | 1.00 | 0.99 | 0.85 | 0.90 | 1.15 | |
| elt-2 | 1.09 | 1.00 | 1.00 | 0.97 | 0.95 | 0.89 | | 1.15 | 1.00 | 1.00 | 0.95 | 0.91 | 0.93 | | 1.11 | 1.00 | 1.03 | 0.96 | 0.84 | 0.85 | |

Table 3.8 GFP fluorescence ratios for transgenic strains after exposure to DDT. Mean readout for each condition was divided by the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

Other than a down-regulation of GPB (Figure 3.16), DDT does not appear to have much effect on the gene expression of these stress-response genes (Table 3.8). Looking at the full list, among the xenobiotic stress genes, *cyp-35A2* shows an up-regulation for the highest concentration compared to the solvent control. The ratio value reaches 1.77 for the early timepoint and fades to 1.61 for the intermediate and close to 1 for the late timepoint (Figure 3.17). Further assays were carried out to test for physiological effects of DDT on the nematode as described in section 3.2.

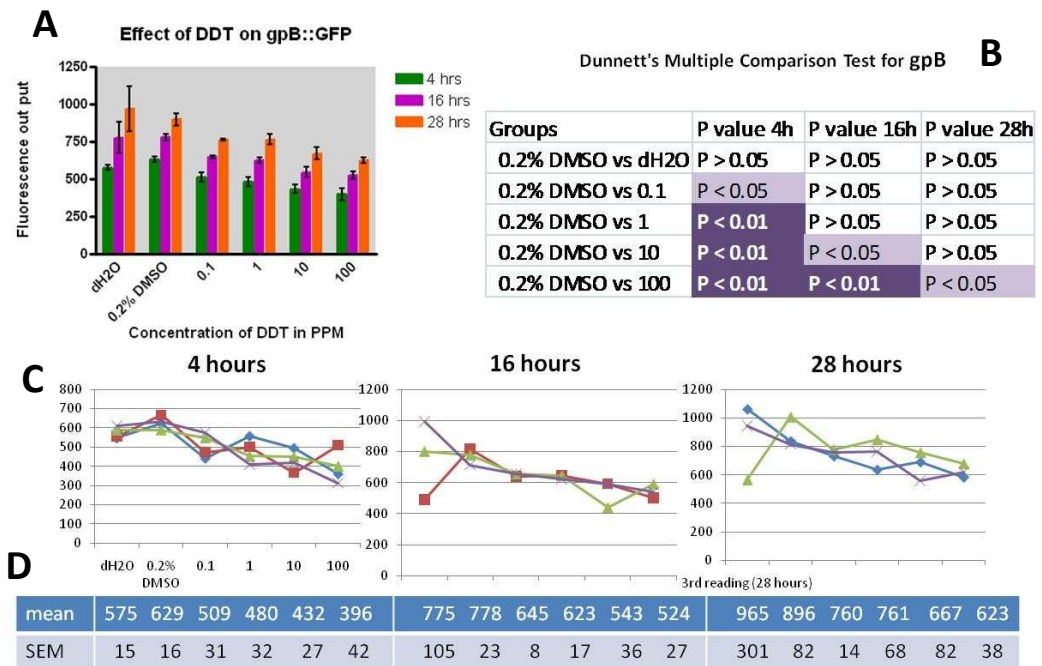


Figure 3.16 Summary of effect of DDT on GPB. **A** Chart summarising the data for all the concentrations at all the time points. Each column shows the average fluorescence output for all the replicates at one test condition. Error bars represent the standard error of the mean. **B** Table showing the results of a Dunnett's multiple comparison test, showing significance of the results. If $0.01 \leq P < 0.05$ the cell is shown with a light purple background, if $P < 0.01$ the cell is shown with a dark purple background. **C** Graphs showing results for all the replicates in all the concentrations at each timepoint. **D** Table showing the results depicted in each graph above.

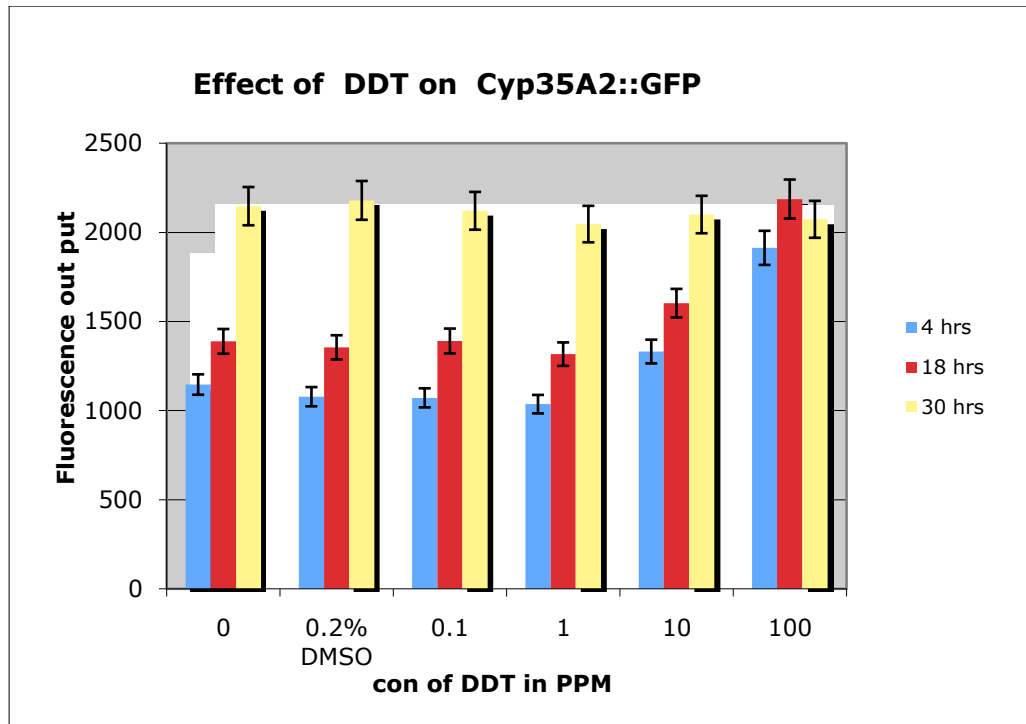


Figure 3.17 Effect of DDT on *cyp-35A2*. Error bars represent SEM. Figure kindly provided by Charumathi Anbalagan.

3.2 Feeding inhibition assay

In some cases where it was determined that further experiments were needed, a feeding inhibition assay was used. This entailed exposing the worms to different concentrations of the toxicant while being suspended in a bacterial solution of given optical density and then measuring the optical density with a spectrophotometer. Readings were taken at different timepoints and test samples were compared to control samples. Where worms fed normally, bacterial solution optical density was reduced; where worms detected a threat in the environment and restricted food intake, bacterial solution optical density would show a slower reduction.

Diuron showed no significant up- or down-regulation on any of the genes tested. Acknowledging that this may have been due to the low concentrations of the toxicant achieved, we performed a feeding inhibition assay which shows lower sensitivity to the presence of solvent compared to the GFP assay; this allowed us to use a higher top concentration of the toxicant. The exposures were performed in parallel to equivalent concentrations of the solvent for each point, which reached a top concentration of 0.2%DMSO/0.2%EtOH (Figure 3.188).

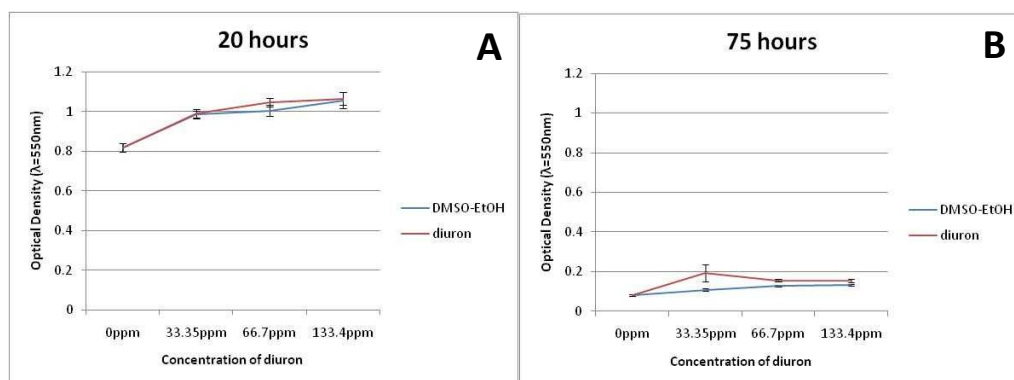


Figure 3.18 Comparison of feeding inhibition in the presence of diuron and equivalent concentrations of the solvent. A Effect on feeding of diuron compared to the solvent alone after 20 hours exposure. **B** Effect on feeding of diuron compared to the solvent alone after 75 hours exposure. Error bars represent standard error of the mean.

It is apparent from Figure 3.188 that diuron does not significantly inhibit feeding compared to the solvent alone, even at a concentration double that tested in the GFP assays.

To explore physiological effects of DDT and deltamethrin on the nematode, feeding inhibition assays were performed. Both these toxicants gave a clear solution when dissolved in DMSO, but this became opaque when further diluted in water; this will clearly affect the optical density (OD). To account for this change, two controls were set up for each toxicant concentration, one with zero-worms plus zero-bacteria (zw/zb) and the other with zero-worms in the presence of bacteria (zw+b). These controls were incubated alongside the tests as the lowest possible and highest possible OD values for each condition. The results are plotted below, showing the two controls along with the feeding inhibition test values (Figures 3.19, 3.20).

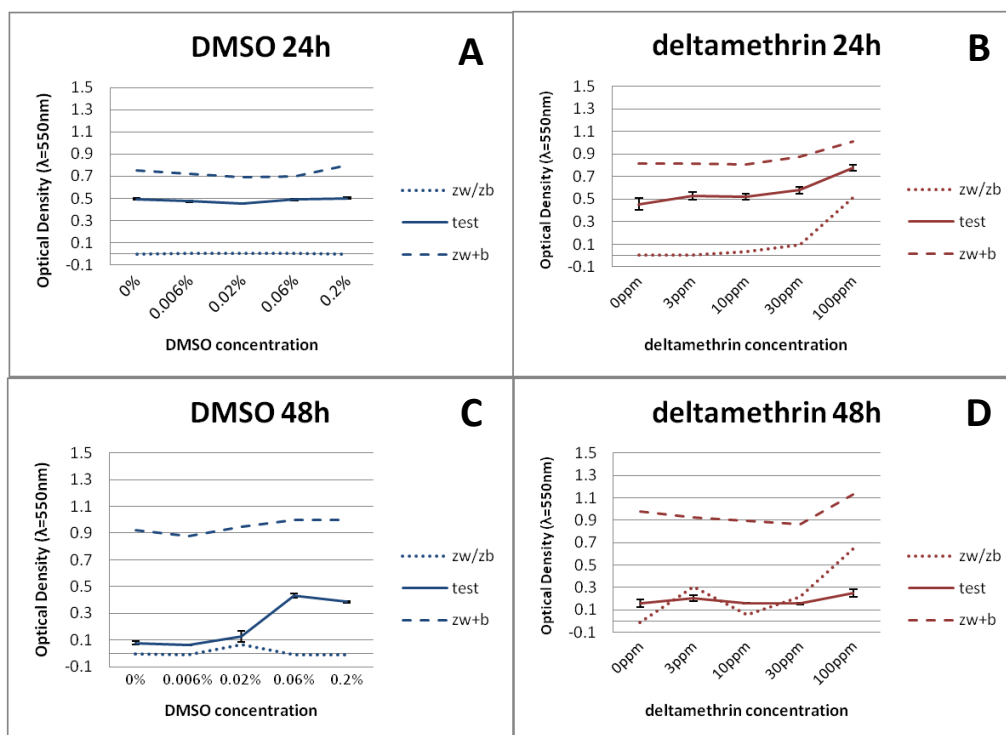


Figure 3.19 Comparison of feeding inhibition in the presence of deltamethrin and equivalent concentrations of the solvent DMSO. A Effect on feeding of DMSO after 24 hours exposure. **B** Effect on feeding of deltamethrin after 24 hours exposure. **C** Effect on feeding of DMSO after 48 hours exposure. **D** Effect on feeding of deltamethrin after 48 hours exposure. Results are plotted alongside the zero-worms/zero-bacteria (zw/zb) and the zero-worms/+bacteria (zw+b) controls. Error bars for the test series represent standard error of the mean.

Point 2 in Figure 3.19, D shows a higher value than in the previous timepoint. Since neither bacteria nor worms were added to this series, there should be no great change to the value between timepoints, as is seen for the remaining points on the series. This change to one sample can be contributed to contamination and, probably, bacterial or yeast growth.

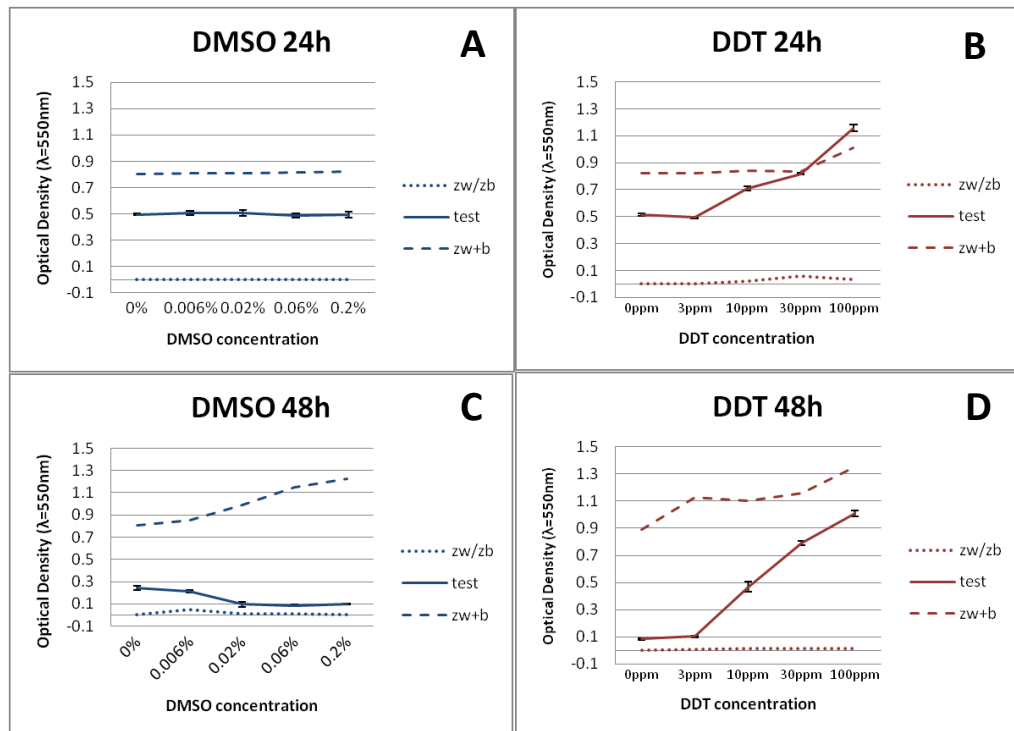


Figure 3.20 Comparison of feeding inhibition in the presence of DTT and equivalent concentrations of the solvent DMSO. A Effect on feeding of DMSO after 24 hours exposure. **B** Effect on feeding of DDT after 24 hours exposure. **C** Effect on feeding of DMSO after 48 hours exposure. **D** Effect on feeding of DDT after 48 hours exposure. Results are plotted alongside the zero-worms/zero-bacteria (zw/zb) and the zero-worms/+bacteria (zw+b) controls. Error bars for the test series represent standard error of the mean.

Whereas deltamethrin does not appear to have an effect on feeding (Figure 3.19), DDT shows a clear inhibitory effect (Figure 3.20). We therefore conclude that pyrethroids are not obviously toxic to the nematode *C. elegans*. However, DDT may cause feeding inhibition in this organism.

3.3 Assays for confirmation of previous dichlorvos results

3.3.1 Previous GFP data on dichlorvos

One of the pesticides tested by previous students as part of the same project was dichlorvos (DC). The effect of dichlorvos on stress-response gene expression in the nematode was the largest recorded in the project with almost all genes showing an effect at some level (Table 3.9). The highest concentration of dichlorvos contained a concentration of 0.1% DMSO, however, a solvent control was not run in parallel with the exposures, a water control alone was used instead.

| Dc | Early | | | | | P P m | Intermediate | | | | | P P m | Late | | | | |
|-----------------|-----------|------|------|------|------|-------|--------------|------|------|------|------|-------|------|------|------|------|------|
| | Gene Dose | 0.04 | 0.4 | 4 | 40 | | 400 | 0.04 | 0.4 | 4 | 40 | | 400 | 0.04 | 0.4 | 4 | 40 |
| sod-1 | 1.18 | 1.30 | 1.02 | 1.12 | 0.97 | | 1.27 | 1.63 | 1.25 | 1.36 | 1.26 | | 1.24 | 1.22 | 1.03 | 1.04 | 1.18 |
| sod-3 | 1.09 | 1.29 | 1.01 | 0.55 | 0.52 | | 1.04 | 1.04 | 0.92 | 0.86 | 0.72 | | 1.23 | 1.04 | 0.87 | 0.90 | 0.94 |
| sod-4 | 1.26 | 1.29 | 0.96 | 0.90 | 0.86 | | 1.38 | 1.49 | 1.32 | 1.31 | 1.22 | | 1.28 | 1.33 | 1.51 | 1.34 | 1.18 |
| ctl-2 | 1.07 | 1.03 | 0.63 | 0.72 | 0.62 | | 1.16 | 1.01 | 0.98 | 0.86 | 0.85 | | 0.92 | 0.77 | 0.81 | 0.75 | 0.76 |
| GPA (T09A1 2.2) | 1.09 | 1.16 | 1.02 | 0.93 | 0.97 | | 1.10 | 1.15 | 1.26 | 1.15 | 1.26 | | 1.28 | 1.55 | 1.61 | 1.49 | 1.76 |
| GPB (C11E4.1) | 1.15 | 1.27 | 1.27 | 1.12 | 1.17 | | 1.31 | 1.16 | 1.38 | 1.31 | 1.63 | | 1.48 | 1.49 | 2.03 | 2.00 | 2.74 |
| skn-1 | 1.09 | 1.17 | 1.13 | 1.08 | 1.11 | | 1.10 | 1.16 | 1.19 | 1.19 | 1.40 | | 1.27 | 1.46 | 1.55 | 1.32 | 1.75 |
| gst-1 | 1.06 | 1.24 | 1.22 | 1.02 | 0.97 | | 1.20 | 1.46 | 1.45 | 1.44 | 1.53 | | 1.27 | 1.48 | 1.47 | 1.51 | 1.71 |
| gst-4 | 1.03 | 0.95 | 1.18 | 1.16 | 1.05 | | 1.06 | 1.08 | 1.12 | 1.14 | 1.27 | | 1.08 | 1.08 | 1.33 | 1.42 | 1.72 |
| cyp29A 2 | 1.07 | 1.11 | 0.95 | 0.83 | 0.84 | | 1.08 | 1.18 | 1.15 | 1.14 | 1.24 | | 1.08 | 1.35 | 1.40 | 1.36 | 1.51 |
| cyp34A 9 | 1.10 | 1.06 | 1.06 | 1.06 | 2.16 | | 1.26 | 1.42 | 1.59 | 1.74 | 2.94 | | 1.33 | 1.50 | 1.78 | 2.00 | 3.47 |
| cyp35A 2 | 1.07 | 0.97 | 0.79 | 0.68 | 0.98 | | 1.14 | 1.29 | 1.23 | 1.42 | 1.94 | | 1.10 | 1.25 | 1.23 | 1.43 | 2.05 |
| mtl-1 | 1.03 | 1.25 | 1.15 | 0.93 | 0.88 | | 1.06 | 0.93 | 0.90 | 0.88 | 1.05 | | 1.11 | 1.06 | 1.08 | 1.05 | 1.34 |
| mtl-2 | 1.08 | 1.12 | 1.31 | 1.79 | 1.66 | | 1.12 | 1.19 | 1.29 | 1.74 | 1.87 | | 1.22 | 1.48 | 1.69 | 2.17 | 2.31 |
| elt-2 | 1.03 | 1.17 | 1.04 | 0.86 | 0.69 | | 1.15 | 1.22 | 1.17 | 1.16 | 1.17 | | 1.01 | 1.06 | 1.14 | 1.13 | 1.17 |
| hsp-16.1 | 1.12 | 1.01 | 0.98 | 1.07 | 0.84 | | 1.14 | 1.19 | 1.22 | 1.31 | 1.79 | | 1.28 | 1.44 | 1.64 | 1.86 | 3.05 |
| hsp-16.2 | 1.06 | 1.04 | 1.16 | 1.50 | 2.05 | | 1.37 | 1.50 | 1.49 | 1.82 | 2.22 | | 1.52 | 1.57 | 1.78 | 2.47 | 3.63 |
| hsp-3 | 1.09 | 1.41 | 1.61 | 1.48 | 1.46 | | 1.30 | 1.66 | 1.70 | 1.55 | 1.91 | | 1.29 | 1.81 | 1.94 | 1.78 | 2.33 |
| hsp-6 | 1.16 | 1.26 | 1.24 | 1.34 | 1.19 | | 1.26 | 1.24 | 1.18 | 1.56 | 2.01 | | 1.42 | 1.60 | 1.50 | 2.31 | 3.30 |
| hsp-60 | 1.06 | 1.01 | 0.92 | 0.80 | 0.80 | | 1.17 | 1.30 | 1.48 | 1.57 | 1.98 | | 1.18 | 1.47 | 1.64 | 1.81 | 2.12 |
| hsp-70 | 1.10 | 1.13 | 1.31 | 1.17 | 1.47 | | 1.18 | 1.33 | 1.63 | 1.39 | 1.76 | | 1.26 | 1.63 | 1.97 | 1.73 | 2.11 |
| hsf-1 | 1.11 | 1.42 | 1.37 | 1.00 | 0.75 | | 1.08 | 1.13 | 1.10 | 1.00 | 1.06 | | 1.09 | 1.23 | 1.16 | 1.05 | 1.24 |
| cep-1 | 1.01 | 1.05 | 0.73 | 0.76 | 0.75 | | 1.10 | 0.92 | 1.05 | 0.94 | 1.03 | | 1.10 | 1.10 | 1.51 | 1.20 | 1.36 |
| daf-16 | 0.96 | 1.47 | 1.45 | 1.24 | 1.17 | | 1.28 | 1.71 | 1.81 | 1.77 | 1.67 | | 1.35 | 2.32 | 2.24 | 2.14 | 2.11 |

Table 3.9 GFP fluorescence ratios for all 24 transgenic strains after exposure to dichlorvos. Mean readout for each condition was divided by the water control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5. Information kindly provided by Charumathi Anbalagan and Ivan Lafayette.

3.3.2 Use of further transgenic strains to determine effect of dichlorvos on *mtl-2* expression

It was considered somewhat anomalous that dichlorvos should have an effect on *mtl-2* but not on *mtl-1*. For this reason, repeat experiments were performed on strains carrying a construct with various reporter genes fused to *mtl-2* regulatory sequences. These included the Georgia strain carrying an *mtl-2::GFP* fusion with a smaller promoter region (compared to the Vancouver strain) and JF2.1 carrying an *mtl-2::lacZ* fusion. The Georgia (G-*mtl-2*) strain results are shown in Table 3.10. For comparison, ratios were calculated against the water control, as was done in Table 3.9.

| Dichlorvos | | | EARLY (4 hours) | | | | | INTERMEDIATE (16-18hours) | | | | | LATE (28-30 hours) | | | | | | | |
|-----------------|-------------------|------------|-----------------|------|------|------|-------|---------------------------|------------|------|------|------|--------------------|-------|-------------------|------------|------|------|------|------|
| Dose | dH ₂ O | 0.11% DMSO | 16.6 | 50 | 150 | 450 | p p m | dH ₂ O | 0.11% DMSO | 16.6 | 50 | 150 | 450 | p p m | dH ₂ O | 0.11% DMSO | 16.6 | 50 | 150 | 450 |
| G- <i>mtl-2</i> | 1.00 | 1.44 | 0.82 | 0.78 | 0.67 | 0.68 | | 1.00 | 1.34 | 1.10 | 1.22 | 1.18 | 1.20 | | 1.00 | 1.17 | 1.19 | 1.21 | 1.35 | 1.94 |

Table 3.10 GFP fluorescence ratios for G-*mtl-2* transgenic strain after exposure to dichlorvos. Mean readout for each condition was divided by the water control of the same set to give the ratio. Cells are shaded according to the colour coding system shown in Figure 3.5.

The JF2.1 strain expressed the β -galactosidase *lacZ* reporter gene, which requires staining and is then visible under a light microscope. Worms were given a score of 0-100 according to strength of staining as described in 2.2.12. A water control, a solvent control (0.11% DMSO) and a positive control (8ppm Zn) were run alongside the test concentrations. The final score for each condition was divided by the score of the solvent control for that set, giving a ratio. The ratios for the controls and test concentrations are shown in Table 3.11. Note that timepoints differ to GFP data.

| Dichlorvos | | EARLY (8 hours) | | | | | | LATE (20 hours) | | | | | | |
|------------|--|-------------------|------|------|------|------|------|-----------------|-------------------|------|------|------|------|------|
| Dose | | dH ₂ O | 1.5 | 15 | 150 | 400 | Zn 8 | p p m | dH ₂ O | 1.5 | 15 | 150 | 400 | Zn 8 |
| Gene | | | | | | | | | | | | | | |
| JF2.1 | | 0.75 | 0.79 | 0.80 | 0.60 | 0.71 | 1.36 | | 1.60 | 1.24 | 1.11 | 1.72 | 1.34 | 2.49 |

Table 3.11 β -galactosidase staining strength after exposure to dichlorvos. Staining was measured using a scoring system described in 2.2.12. Scores for each condition were divided by the score of the solvent control of the same set to give the ratio. Cells were shaded according to the colour coding system shown in Figure 3.5.

The Georgia *mtl-2::GFP* strain showed some up-regulation after exposure to dichlorvos (Table 3.10), although the effect was not as strong as that recorded previously (Table 3.9). The JF2.1 *lacZ* strain showed signs of up-regulation at high concentrations (Table 3.11), although, like the Georgia strain, it did not reach the levels observed by previous experiments (Table 3.9). Note that whereas the GFP results are presented as ratios compared with the water control, the *lacZ* data are shown as ratios compared with the solvent control.

3.3.3 Gene array data

Since dichlorvos appeared to have a broad effect on *C. elegans* gene expression, a series of gene arrays were carried out to test for other genes being affected. The gene arrays picked up a variety of genes showing up- or down-regulation after exposure to dichlorvos. Some of the principal genes affected, along with a brief summary of each gene's function, are listed in Table 3.12.

| Gene/group (by name) | Fold expression change at 1.5 ppm DC | Fold expression change at 150 ppm DC | Gene function / gene ontology term |
|--|--------------------------------------|--------------------------------------|---|
| Stress genes | | | |
| hsp-12.6 | 8.6 down | 12.9 down | Small heat-shock protein expressed in L1s/dauers |
| hsp-16.1 | - | 2.2 up | Stress-responsive small heat-shock protein |
| hsp-16.48 | 3.0 up | - | Stress-responsive small heat-shock protein |
| hsp-16.2/-16.41 | - | 2.7 / 2.6 up | Stress-responsive small heat-shock proteins |
| gst-4 / gst-7 | - | 5.3 / 2.3 up | Phase II glutathione S-transferases |
| gst-26 / gst-27 | - | 2.3 / 2.4 down | Phase II glutathione S-transferases |
| gst-5 / gst-42 | 2.2 / 2.1 down | - | Phase II glutathione S-transferases |
| mtl-1 / mtl-2 | 2.6 / 2.7 down | 3.2 / 2.6 down | Metal-binding metallothioneins |
| cyp-37B1 | 3.8 down | 3.1 down | Cytochrome P450 mono-oxygenase |
| cyp-34A10 | - | 5.5 up | Cytochrome P450 mono-oxygenase |
| cyp-34A9 | 2.1 down | - | Cytochrome P450 mono-oxygenase |
| cyp-35A2/-35C1 | - | 2.1 / 2.3 up | Cytochrome P450 mono-oxygenases |
| cyp-13A5/-14A3 | - | 2.1 / 6.7 up | Cytochrome P450 mono-oxygenases |
| ugt-46 | 5.2 down | 5.4 down | Phase II UDP gluc(uron)osyl transferase |
| ugt-17 | 4.8 up | 6.2 up | Phase II UDP gluc(uron)osyl transferase |
| ugt-9/-22/-53 | - | 2.8 / 2.4 / 2.0 up | Phase II UDP gluc(uron)osyl transferases |
| aip-1 | - | 2.1 up | Arsenite-inducible protein-1 |
| cdr-2 | - | 3.0 down | Cadmium-response protein-2 |
| Neuropeptides and insulin-like signalling peptides | | | |
| nlp-26/-28/-29/-30 | 6.5/2.8/2.8/2.7 up | 6.3 / 2.5/2.9 / ? up | Neuropeptide-like proteins |
| nlp-35 | 2.0 down | 4.1 down | Neuropeptide-like protein |
| nlp-17 | - | 2.1 down | Neuropeptide-like protein |
| ins-11 | 5.3 up | 2.3 up | Insulin-like peptide |
| ins-35 | 4.3 down | 6.5 down | Insulin-like peptide |
| ins-7 | - | 4.4 up | Insulin-like peptide |
| C-type lectins, thaumatins, saposins and lysozymes. | | | |
| clec-76/-13 | 8.8/3.6 down | - | C-type lectin |
| clec-47 | 3.3 up | - | C-type lectin |
| clec-61/-82 | - | 9.9/2.3 down | C-type lectin |
| clec-41/-5/-47/-43/-7 | - | 7.6/2.9/2.4/2.3/2.3 up | C-type lectin |
| ssp-11/-6 | 5.6/2.7 down | 8.8 / 3.5 down | Saposin-like defensive protein |
| ssp-15/-18/-1 | - | 4.5/2.3/2.1 down | Saposin-like defensive protein |
| lys-3/-1/-8 | 2.9 / 2.5 / 2.2 up | - | Lysozyme-like defensive protein |
| lys-10 | - | 5.4 up | Lysozyme-like defensive protein |
| thn-1 | 3.1 up | 7.5 up | Thaumatins: pathogenesis-related proteins group 5 |
| lec-4 / lec-9 | 2.4 up / - | - / 2.3 down | Galectins |
| Metabolic enzymes | | | |
| cht-1 | 6.2 up | 6.0 up | Chitinase |
| cpr-2 | 21.6 down | 33.4 down | Cysteine protease 2 |
| T19B4.1 | 4.6 up | 2.8 up | Peptidyl mono-oxygenase |
| fat-5/-6 | 3.7 / 2.6 up | - / - | Fatty acid desaturase-5/-6 |
| sulp-8/-2 | 3.7 / 3.1 up | 2.8 / 2.9 up | Sulphate permease-8/-2 |
| odc-1 | 3.2 up | 2.3 up | Ornithine decarboxylase |
| sodh-2 | 2.5 down | 2.5 up | Sorbitol dehydrogenase-2 |
| alh-12 | 2.3 down | - | Aldehyde dehydrogenase-12 |
| alh-5 | - | 2.7 up | Aldehyde dehydrogenase-5 |
| sdha-1 | 2.1 up | - | Succinate dehydrogenase subunit A |
| cts-1 | 2.0 up | - | Citrate synthase-1 |
| plc-1 | 2.4 down | - | Phospholipase C-1 |
| ech-9 | - | 3.5 down | Enoyl coenzyme A hydratase |
| nnt-1 | - | 3.0 down | Nicotinamide nucleotide transhydrogenase |
| ppt-1 | - | 2.6 down | Palmitoyl protein thioesterase |
| ckb-2 | - | 2.3 up | Choline kinase B-2 |
| pef-1 | - | 2.3 down | PPEF-related serine/threonine phosphatase |
| pssy-1 | - | 2.1 up | Phosphatidylserine synthase 1 |
| asp-4 | - | 2.1 down | Aspartyl protease 4 |
| Assorted other | | | |
| ttni-1 | 3.5 up | 2.3 up | Troponin 1 |
| nhr-99 | 2.7 down | - | Nuclear hormone receptor-like-99 |
| nhr-3 / -61 | 2.8 / 2.0 up | 2.7 / 2.9 up | Nuclear hormone receptor-like-3/-61 |
| nhr-167/-58 | - | 2.2 / 2.1 up | Nuclear hormone receptor-like-167/-58 |
| nhr-144 | - | 2.0 down | Nuclear hormone receptor-like-144 |
| vit-3/-4 / -6 | - | 2.7 / 2.1 / 2.0 up | Vitellogenin yolk proteins expressed in adult gut |
| C35D5.8a/F10D7.3 | 3.2 up / - | 3.7 / 4.5 up | Thioredoxin / Glutaredoxin |
| jnk-1 | - | 2.7 down | Jun-related kinase 1 |

Table 3.12 Gene array results for some of the genes showing up- or down-regulation after exposure to 1.5ppm or 150ppm dichlorvos. Table kindly provided by David de Pomerai, based on unpublished data from Neil Graham, Ram Prakash Gupta, Ivan Lafayette and Pradip Sinha.

Parallel gene arrays were performed for both *C. elegans* and *Drosophila melanogaster*. Since dichlorvos is a potent insecticide, *Drosophila melanogaster* were exposed to a maximum of 15 parts per billion (ppb) dichlorvos, a concentration 10,000-fold smaller than the maximum dose used on *C. elegans*. From the results, 6 genes were chosen that showed similar patterns of regulation for both organisms (Appendix I). These genes were: *snf-1*, C10C5.3, C30F12.7, *gln-1*, *paf-2* and *aman-1*. A qPCR was performed for these genes to confirm gene array data.

3.3.4 qPCR

3.3.4.1 RNA extraction

Worms were exposed to 150ppm dichlorvos, for 24 hours at 20°C and the equivalent concentration of the solvent (0.1% DMSO) was used as the negative control. Exposures were set up in 4 biological replicates. RNA was extracted using the triazol method (2.2.6) and then run on a 1xTAE/1%SDS/1%agarose gel to check its quality (Figure 3.21). Both 18S and 28S rRNA subunits should be visible on the gel.

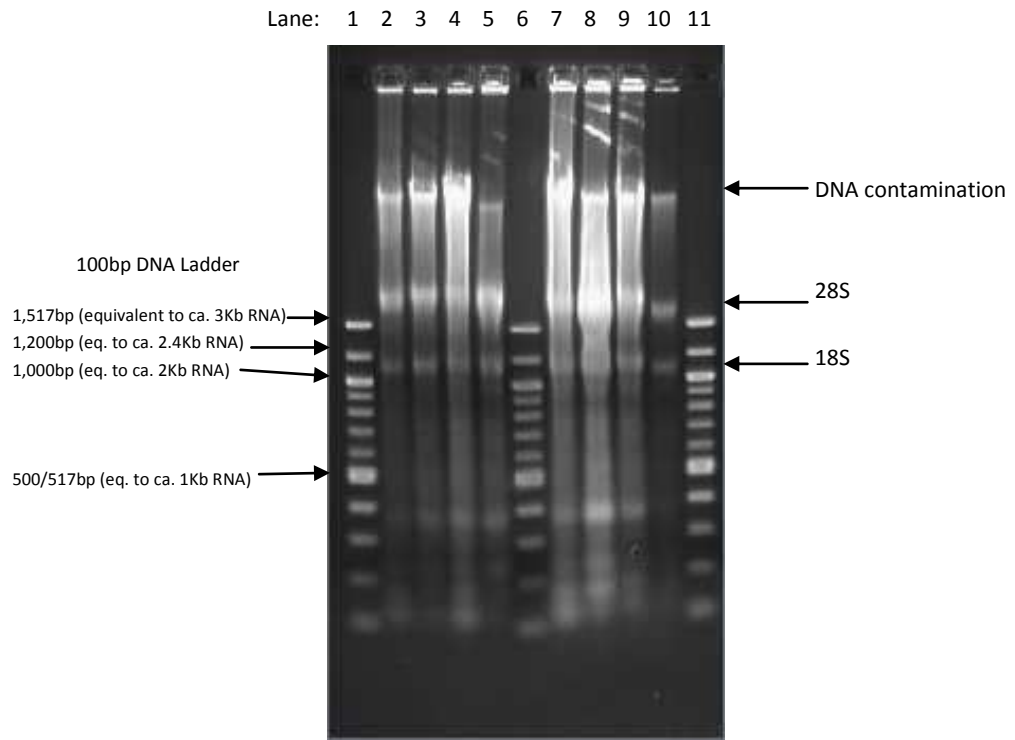


Figure 3.21 Extracted RNA. A small portion of each RNA sample (10 μ l) was mixed with 1 μ l loading dye (2.1.2) and loaded on a 1xTAE/1%SDS/1%agarose gel. The gel was run at 107V for ca. 45min, stained with EtBr and a picture was taken under UV light (2.2.11). Lanes 1,6,11: 100bp DNA Ladder; lanes 2-5: DMSO samples 1-4 respectively; lanes 7-10: DC samples 1-4 respectively.

From Figure 3.21, DNA contamination is detectable in the RNA preparation. Extracted RNA was quantified using a Thermo Scientific Nanodrop 1000 Spectrophotometer (Table 3.13) and then cleaned of DNA contamination by DNase I digestion (2.2.7). The cleaned RNA quality was then checked (Figure 3.22). Note that the method used for RNA extraction is crude and RNA quantity from different samples may vary.

| Sample | measurement (ng/ μ l) |
|--------|---------------------------|
| DMSO 1 | 118.1 |
| DMSO 2 | 110.9 |
| DMSO 3 | 249.5 |
| DMSO 4 | 197.3 |
| DC 1 | 454.8 |
| DC 2 | 612.6 |
| DC 3 | 387.5 |
| DC 4 | 130.4 |

Table 3.13 Concentration of extracted RNA samples.

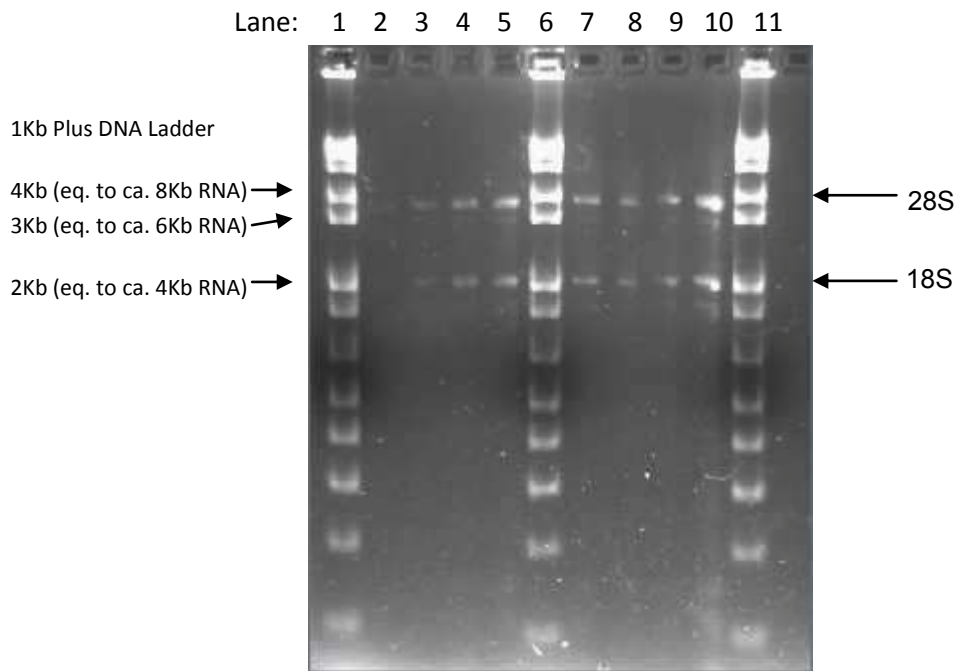


Figure 3.22 DNase I digested RNA. A small portion of each RNA sample (10 μ l) was mixed with 1 μ l loading dye (2.1.2) and loaded on a 1xTAE/1%SDS/1% agarose gel. The gel was run at 107V for ca. 45min, stained with EtBr and a picture was taken under UV light (2.2.11). **Lanes 1,6,11:** 1Kb Plus DNA Ladder; **lanes 2-5:** DMSO samples 1-4 respectively; **lanes 7-10:** DC samples 1-4 respectively.

In Figure 3.22, 2 RNA bands were visible for all 8 lanes. It is clear that DNA contamination had been successfully removed since only the expected bands are visible.

3.3.4.2 cDNA synthesis

Uncontaminated RNA was used as a template to synthesise cDNA (2.2.9). The cDNA was quantified using the nanodrop machine and all samples were found to contain approximately the same concentration of cDNA (Table 3.14).

| Sample | measurement (ng/μl) |
|--------|---------------------|
| DMSO 1 | 2085.3 |
| DMSO 2 | 2196.6 |
| DMSO 3 | 2114.5 |
| DMSO 4 | 2103.7 |
| DC 1 | 2092.9 |
| DC 2 | 2096.7 |
| DC 3 | 2111.7 |
| DC 4 | 2285.2 |

Table 3.14 Concentration of synthesised cDNA.

To check quality of the cDNA a PCR was run using the *snf-1* set of primers that were known to work (Figure 3.23). A negative control not containing a DNA template, and a positive control containing quality checked DNA, were run alongside the reaction (Figure 3.23, lanes 2, 11). A fragment of 211bp is expected to be amplified in this reaction.

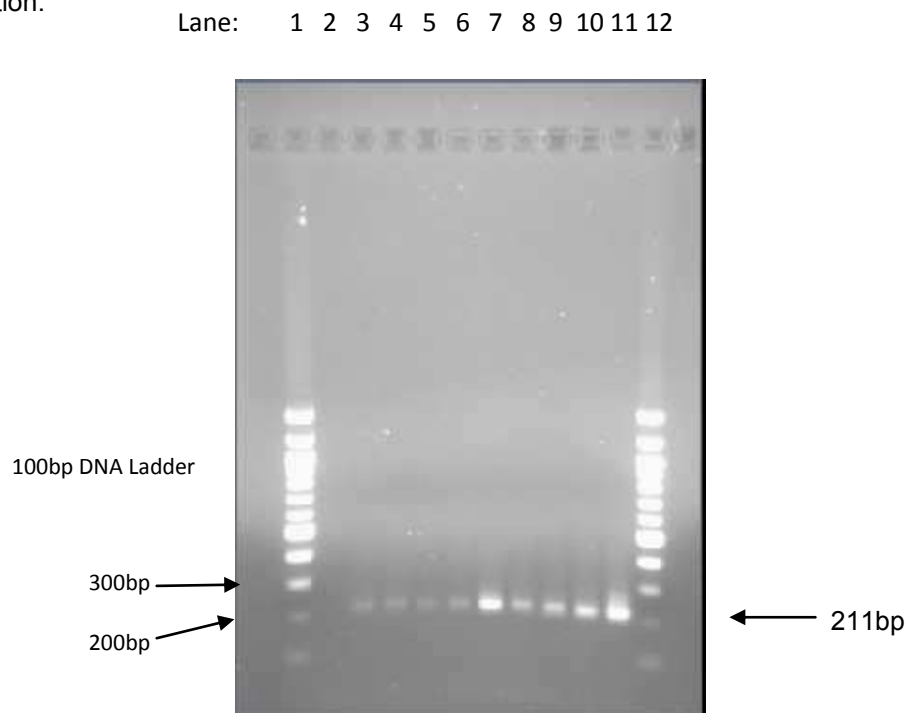


Figure 3.23 cDNA quality check by PCR. A small portion of each PCR sample (10μl) containing loading dye was loaded on a 1xTAE/1%agarose gel. The gel was run at 107V for ca. 45min, stained with EtBr and a picture was taken under UV light (2.2.11). **Lanes 1, 12:** 100bp DNA Ladder; **lane 2:** negative control; **lanes 3-6:** reactions containing DMSO samples 1-4 as template respectively; **lanes 7-10:** reactions containing DC samples 1-4 as template respectively; **lane 11:** positive control.

The expected fragment was amplified in all lanes (Figure 3.23).

3.3.4.3 PCR optimisation

The synthesised cDNA was used as a template for PCR and qPCR reactions. Primers for the 6 chosen genes (3.3.2) plus 3 control genes (*rpl-16*, *pmp-3* and Y45F10D.4), thought to have relatively stable expression under different conditions, were ordered and quality checked by PCR. Reaction conditions were optimised using a gradient PCR and primer concentrations were found optimum at 0.5pmol/ μ l (Figures 3.24, 3.25).

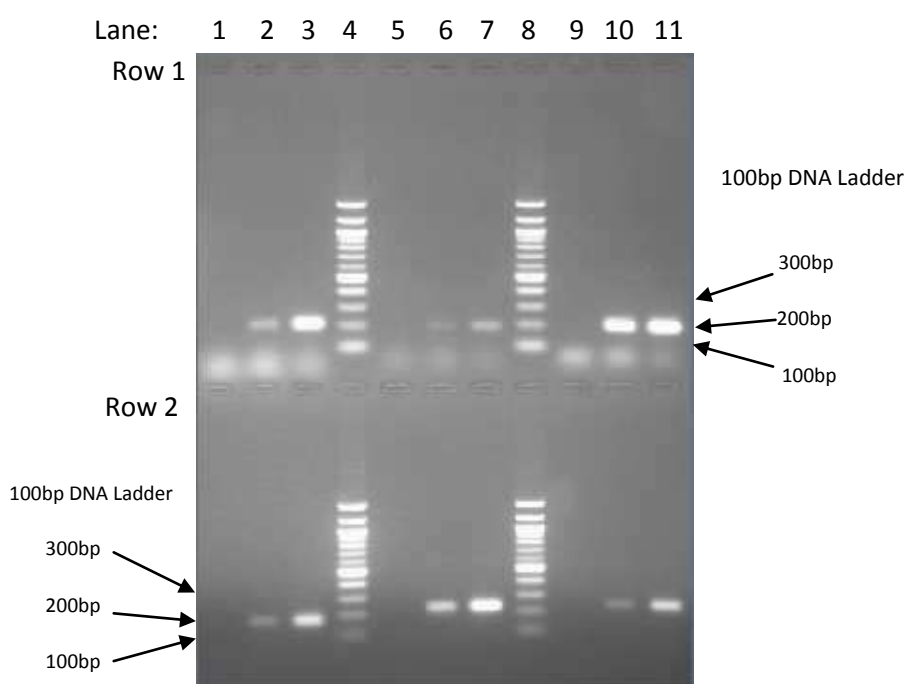


Figure 3.24 PCR optimisation using test genes' primer sets at 1pmol/ μ l and 0.5pmol/ μ l.

A small portion of each PCR sample (10 μ l) containing loading dye was loaded on a 1xTAE/1%agarose gel. The gel was run at 107V for ca. 45min, stained with EtBr and a picture was taken under UV light (2.2.11). **Rows 1, 2: lanes 4, 8:** 100bp DNA Ladder; **lanes 1, 5, 9:** negative control (not containing DNA template); **lanes 2, 6, 10:** reactions run with 1pmol/ μ l primer concentration; **lanes 3, 7, 11:** reactions run with 0.5pmol/ μ l primer concentration. **Row 1: lanes 1-3:** reactions run using primer set for *snf-1*; lanes 5-7: reactions run using primer set for C10C5.3; **lanes 9-11:** reactions run using primer set for C30F12.7. **Row 2: lanes 1-3:** reactions run using primer set for *gln-1*; **lanes 5-7:** reactions run using primer set for *paf-2*; **lanes 9-11:** reactions run using primer set for *aman-1*.

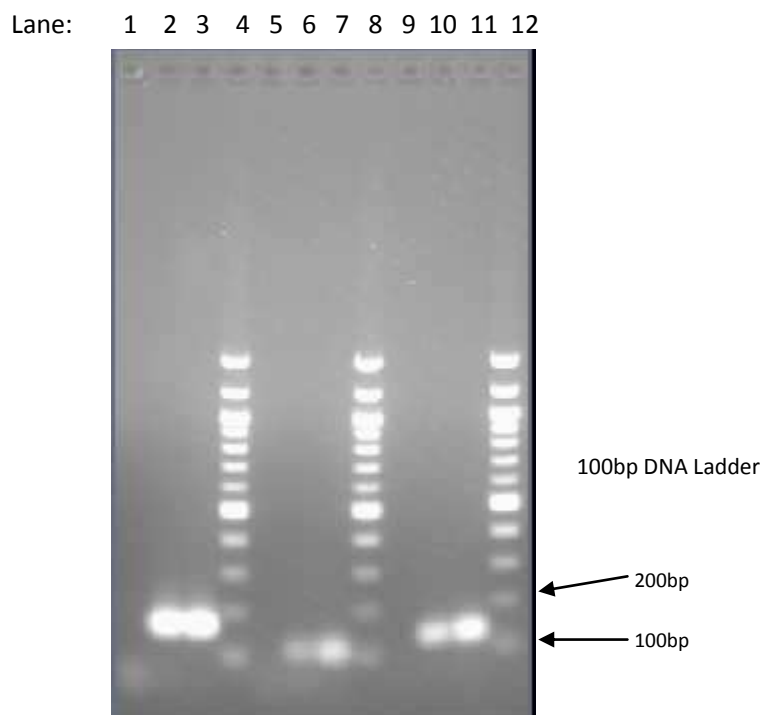


Figure 3.25 PCR optimisation using reference genes' primer sets at 1pmol/ μ l and 0.5pmol/ μ l. A small portion of each PCR sample (10 μ l) containing loading dye was loaded on a 1xTAE/1%agarose gel. The gel was run at 107V for ca. 45min, stained with EtBr and a picture was taken under UV light (2.2.11). **Lanes 4, 8, 12:** 100bp DNA Ladder; **lanes 1, 5, 9:** negative control (not containing DNA template); **lanes 2, 6, 10:** reactions run with 1pmol/ μ l primer concentration; **lanes 3, 7, 11:** reactions run with 0.5pmol/ μ l primer concentration. **Lanes 1-3:** reactions run with primer set for *rpl-16*; **lanes 5-7:** reactions run using primer set for *pmp-3*; **lanes 9-11:** reactions run using primer set for Y45F10D.4.

Bands on these gels appear to be approximately the size expected for each set of primers according to the fragment sizes listed in Table 2.6. Lanes containing reactions run with 0.5pmol/ μ l primer concentration show a lower level of primer dimer formation and more strongly fluorescing bands than the reactions run with 1pmol/ μ l primer concentration. Reactions were also run using 0.25pmol/ μ l primer concentration, but the resulting bands were not as strong as those run with 0.5pmol/ μ l primer concentration (results not shown). Therefore, the primer concentration chosen as optimum was 0.5pmol/ μ l.

3.3.4.4 qPCR

Probes were ordered for the 6 test genes and 2 of the reference genes (*rpl-16* and *pmp-3*). Amount of cDNA present is measured by the number of cycles needed for the probe to reach a certain threshold (CT), after which amplification is exponential. Before proceeding with the pPCR reaction, test reactions were run. Firstly, a reaction was run to optimise probe concentration. Reactions were run in parallel for all 8 genes and for low (30nM), medium (100nM) and high (300nM) probe concentrations. An example of the resulting graph is shown in Figure 3.26 for gene *paf-2*. The “medium” concentration was chosen for further reactions.

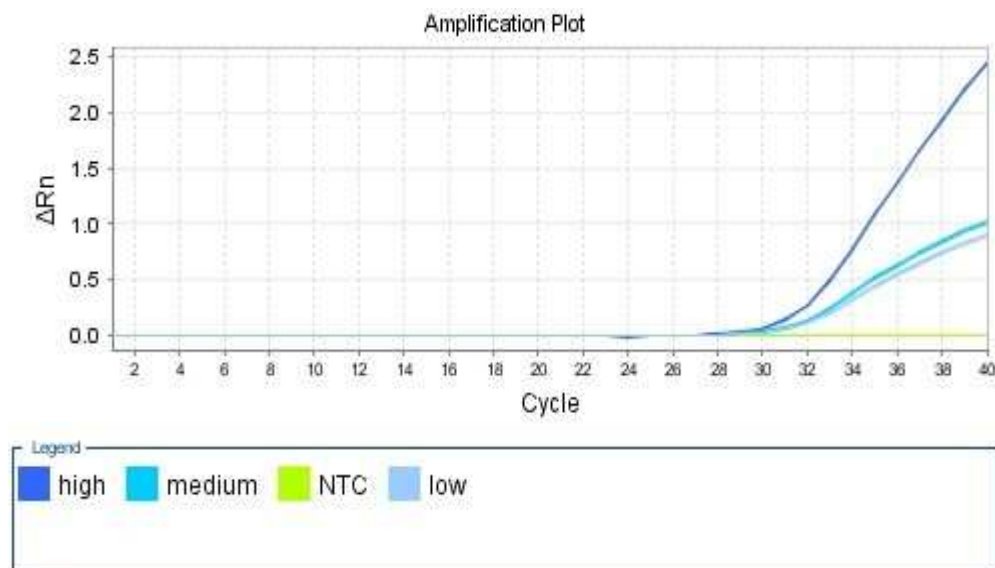


Figure 3.26 Probe concentration test qPCR for *paf-2*. Three probe concentrations were run in parallel, high concentration was 300nM, medium concentration was 100nM and low concentration was 30nM. NTC: no template control.

Reactions were run to check quality of the reference genes. DNA from solvent-treated and dichlorvos-treated worms was used as a template in reactions run in parallel to check for consistency (Figures 3.27, 3.28).

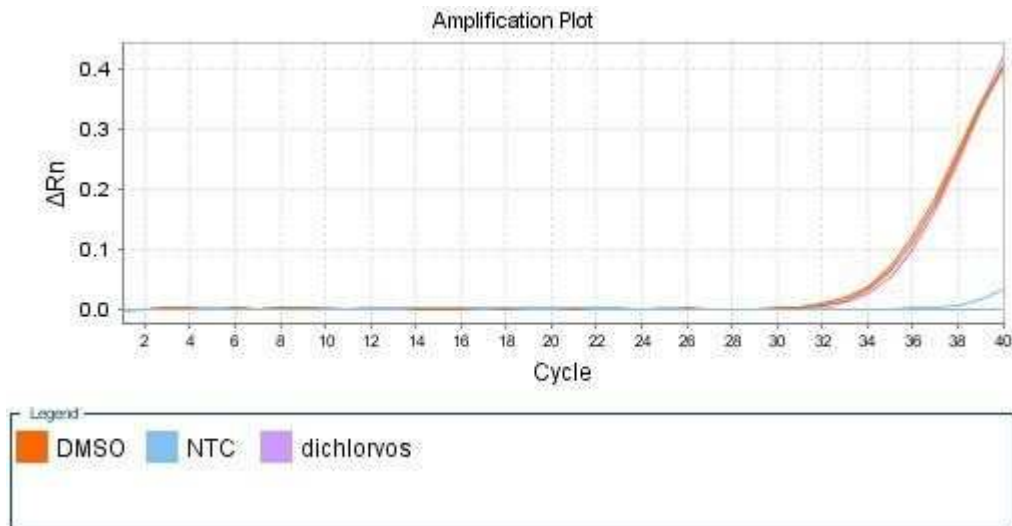


Figure 3.27 Reference gene quality check for *pmp-3*. Reactions using cDNA from solvent-treated (DMSO) and dichlorvos-treated (dichlorvos) worms were run in parallel. NTC: no template control.

It is clear from Figure 3.27 that *pmp-3* is a good reference gene since the 2 reactions do not show substantial difference in CT.

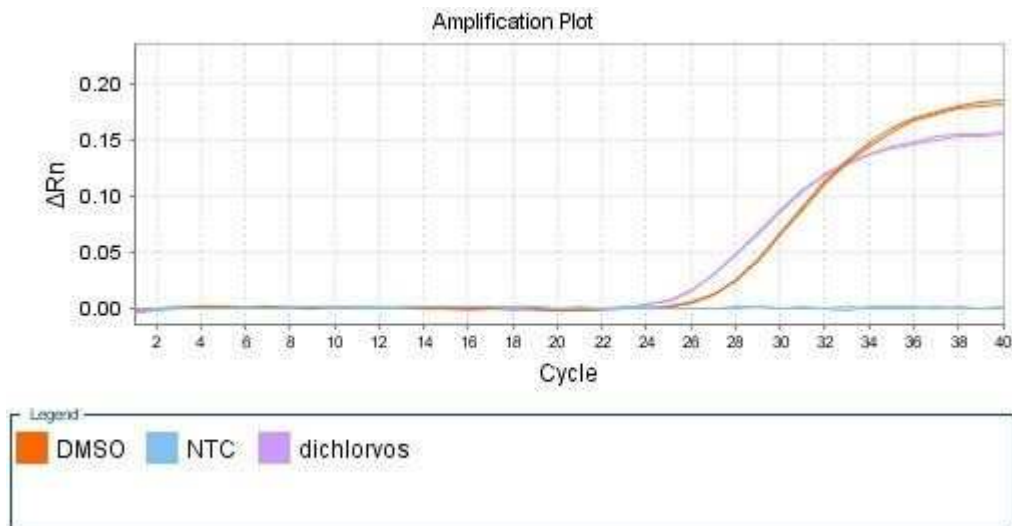


Figure 3.28 Reference gene quality check for *rpl-16*. Reactions using cDNA from solvent-treated (DMSO) and dichlorvos-treated (dichlorvos) worms were run in parallel. NTC: no template control.

From Figure 3.28 it appears that there is a slight difference in CT for *rpl-16* between reactions using cDNA from solvent-treated and dichlorvos-treated worms. This suggests that *rpl-16* is not a reliable reference gene. Therefore, the third potential reference gene, Y45F10D.4, was tested. Since a probe had not been obtained for this gene, the SYBR Green method was used instead. Y45F10D.4 was determined to be a reliable second reference gene, since there was very little difference in CT between reactions using samples from solvent-treated and dichlorvos-treated worms (Figure 3.29).

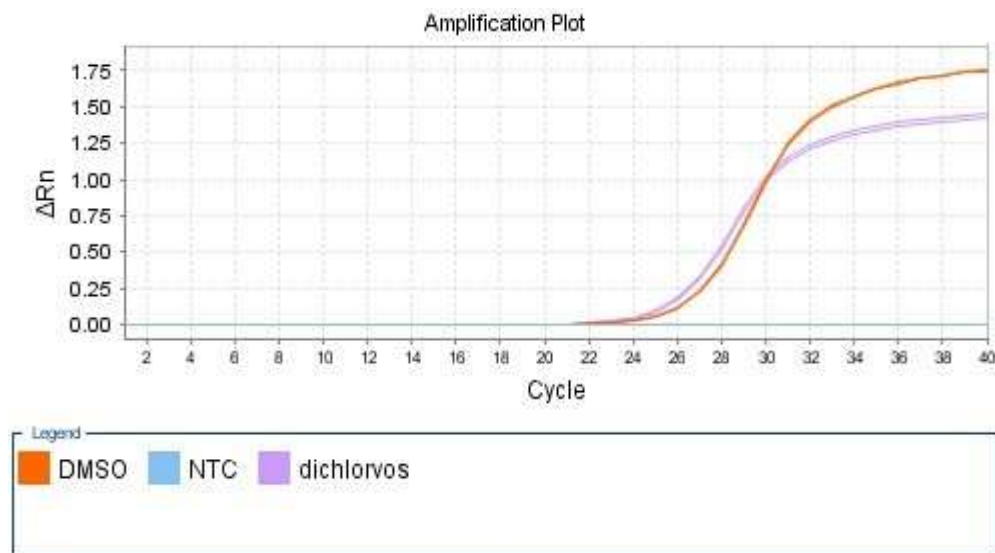


Figure 3.29 Reference gene quality check for Y45F10D.4. Reactions using cDNA from solvent-treated (DMSO) and dichlorvos-treated (dichlorvos) worms were run in parallel. NTC: no template control.

To check that running a reaction with multiple primer and probe sets does not affect the reaction results, reference gene *pmp-3* was run singly and together with the test gene C30F12.7 (Figure 3.30).

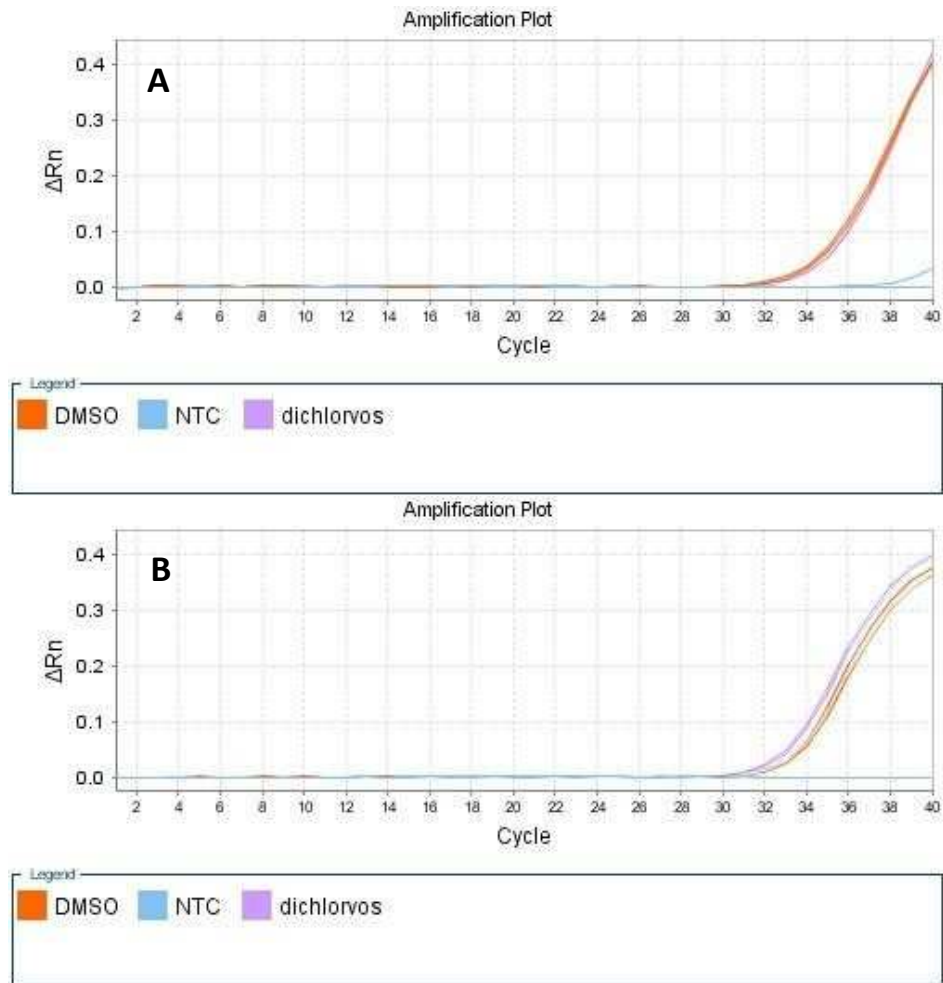


Figure 3.30 *pmp-3* run singly and together with C30F12.7. The reference gene *pmp-3* was run in 2 reactions, one with a single primer-probe set (**A**) and one with multiple primer-probe sets (**B**). NTC: no template control.

It was determined that there is no substantial difference in the reaction parameters between reactions run with single and multiple primer/probe sets. Note that in Figure 3.30 B it appears as though there is a difference in amplification between the two templates used (solvent versus dichlorvos). However, looking at the CT, there is no substantial difference.

Test reactions were set up in 2 replicates with one test gene and the reference gene *pmp-3* run in the same reaction and the second test gene Y45F10D.4 run in parallel but in a separate reaction. Results were normalised against the reference genes using the software qBasePlus (biogazelle). The mean CT and standard error (SEM) was calculated for the four biological replicates run for each reaction. The expression ratios were calculated by dividing the mean CT of each condition for each gene by that of the solvent-treated condition. The expression ratios were plotted on a graph alongside the equivalent gene array data for each gene for comparison (Figure 3.31). An unpaired *t* test was performed to determine significance (Figure 3.31, Table 3.15).

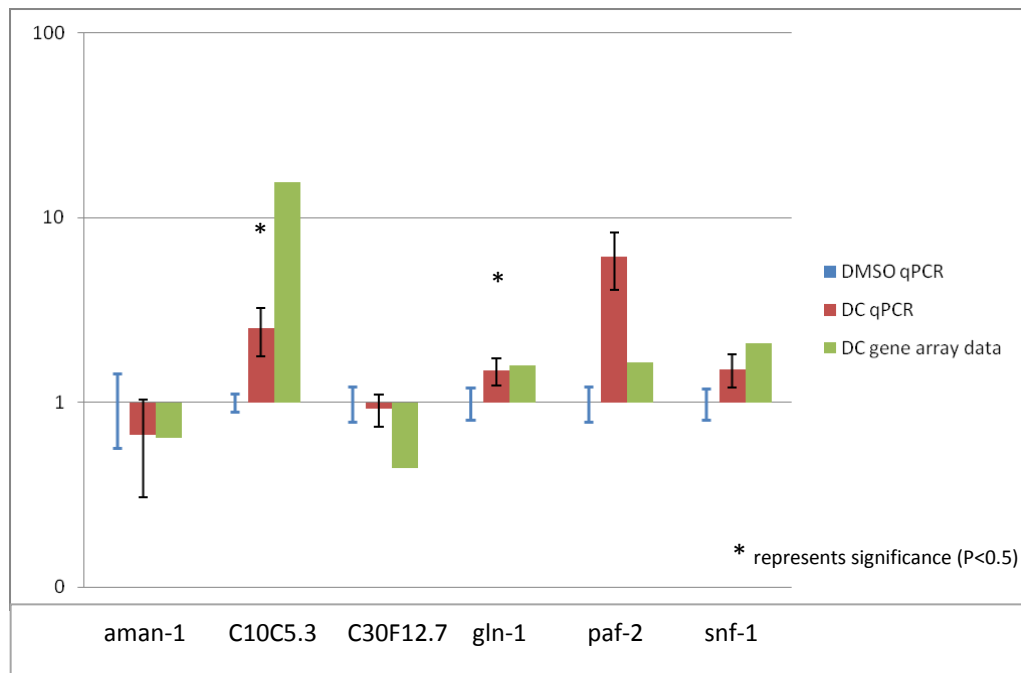


Figure 3.31 qPCR and gene array data for the 6 test genes. Data are shown as expression ratios, showing the level of up- or down-regulation caused by treatment of the toxicant dichlorvos (DC). Error bars on qPCR data represent SEM; SEM for gene array data was not available.

| Gene | P value |
|---------------|---------|
| <i>aman-1</i> | 0.1794 |
| C10C5.3 | 0.0373 |
| C30F12.7 | 0.5752 |
| <i>gln-1</i> | 0.043 |
| <i>paf-2</i> | 0.5937 |
| <i>snf-1</i> | 0.0596 |

Table 3.15 Unpaired t test for test genes. P value represents significance, where if $P < 0.05$ the result is significant and if $P < 0.01$ the result is highly significant. Significant results are highlighted.

Figure 3.31 shows that the qPCRs have confirmed the gene array data. Although there is a difference in the level of up- or down-regulation, the direction is the same in both sets of results.

4 Discussion

4.1 Effect of tested pesticides on stress response gene expression and feeding in the nematode *C. elegans*

In this study a variety of pesticides was used for exposure of transgenic nematodes. Of these pesticides, none have a known function as nematocides. The aim of this study was to look at non-target effects of widely used chemicals. From our findings, toxicants that did not affect expression of any of the genes tested, could be considered safe to the animal; those that showed significant changes for 1 or 2 genes were considered to have a limited response, but not necessarily to be safe; whereas those that affected the expression of a large number of genes were considered highly toxic. It should be noted however that the concentrations used here are much higher than those likely to be found in the environment. In cases where the toxicant was suspected to have a physiological effect on the nematode, a feeding inhibition assay was performed for further analysis.

According to our data it appears that many pesticides have rather selective effects on non-target organisms. The herbicide **diuron** showed no signs of significant up- or down-regulation of stress-response genes of the nematode *C. elegans* (Figure 3.6). It should however be noted that due to solubility issues for this chemical, the highest concentration reached was relatively low; suggesting that some effect could occur at higher concentrations. For this reason, a feeding inhibition was performed, reaching a concentration 2-fold higher than that reached in the GFP assays. No physiological effect of diuron on the nematode was detected from this assay (Figure 3.188). It is therefore concluded that diuron does not show toxicity towards the nematode *C. elegans*.

The insecticide pyrethroid **cypermethrin** also shows no detectable effects on nematode stress-response gene expression (Table 3.5). Cypermethrin acts by activating neuronal voltage-gated sodium channels. *C. elegans* does not have sodium

channels, but has potassium channels which chemically resemble insect sodium channels, so it was thought that these might be affected by pyrethroids.

The second pyrethroid tested, **deltamethrin**, shows down-regulation of *sod-3*, a gut-related gene (Table 3.6). Although pyrethroids are known to act on sodium channels of the nervous cell membrane, none of the neuronal genes tested showed any change in gene expression. To further assess the effect of deltamethrin on the nematode, a feeding inhibition assay was performed. From Figure 3.19 it is apparent that deltamethrin has no net effect on feeding, and from our data we can conclude that deltamethrin has no significant or detectable effect on *C. elegans*.

The insecticides chlorpyrifos and endosulfan show no effect on oxidative, metal and heat stress-response genes, but they both show high up-regulation for the cytochrome P450 gene *cyp-34A9*. **Chlorpyrifos** is an acetylcholinesterase inhibitor, which means it acts on neuronal cells, preventing cholinergic neurons from recovering after activation. *C. elegans* possess a nervous system, but a relatively simple one; however, the *C. elegans* acetylcholinesterase system shows great similarities with that of vertebrates and invertebrates (Erickson et al. 1994; Combes et al. 1999). It is important to note that *C. elegans* possess multiple acetylcholinesterase genes whereas insects usually possess one (Combes et al. 2001). This suggests the possibility that chlorpyrifos may display limited toxicity to *C. elegans* due to the presence of multiple targets with possibly different active sites. **Endosulfan** is known to act by blocking chloride channels. *C. elegans* possess chloride channels (Schriever et al. 1999; Ringstad et al. 2009), although they are not yet fully characterised. Our findings suggest that both chlorpyrifos and endosulfan show limited toxicity on *C. elegans* and are metabolised through the xenobiotic pathway.

Dichlorodiphenyltrichloroethane (DDT) showed a down-regulation of GPB, a glutathione peroxidase gene expressed in the gut, and an up-regulation of *cyp-35A2*, a gene involved in xenobiotic stress-response. DDT acts on neuronal sodium channels, but both genes affected by the presence of DDT are gut-expressed genes (Menzel et al. 2001; McKay et al. 2003; Hunt-Newbury et al. 2007). To explore the

possible effect of DDT on the nematode's intestine, a feeding inhibition assay was performed. It is clear from Figure 3.20 that DDT inhibits feeding at both 24 and 48 hour exposures. This may be related to the fact that DDT bioaccumulates in lipid-rich tissues; since lipids are generally broken down and stored in the gut, it is possible that the DDT concentration builds up in the nematode intestine and causes the observed inhibitory effect on feeding and expression of the gut genes GPB and *cyp-35A2*. It can therefore be concluded that DDT has a physiological effect on *C. elegans*, and is likely metabolised through the xenobiotic pathway.

The fungicide **carbendazim** acts by inhibiting microtubule formation. From our data it unexpectedly appears to induce *sod-3::GFP* expression. SOD-3 is the main mitochondrial superoxide dismutase in the nematode, so carbendazim's effect on *sod-3* expression suggests it may also affect oxidative stress in mitochondria.

Rotenone is a mitochondrial poison and, according to our data (Table 3.1), is the most toxic of the pesticides tested on the nematode. Around half of the genes tested showed an up-regulation in expression at the intermediate or late timepoints. Of the oxidative stress genes, *sod-1* and *skn-1* showed up-regulation of around 2-fold at the 2 highest concentrations (6.7ppm, 20ppm) for the intermediate timepoint, which faded out at the late timepoint; by contrast, GPA (T09A12.2) showed up-regulation of about 1.5-fold at the highest concentration for the late timepoint. Of the metal stress genes, the two metallothioneins (*mtl-1*, *mtl-2*) and their transcriptional regulator (*elt-2*) showed up-regulation at the higher concentrations for the intermediate timepoint, suggesting possible effect on metal homeostasis. Among the xenobiotic stress genes, *gst-1*, but not *gst-4* showed around 1.6-fold up-regulation for the highest concentration at the intermediate and late timepoints and all cytochrome P450 genes tested showed around 2-fold up-regulation at the highest concentrations for at least the intermediate timepoint. The heat stress genes, as well as the major transcription factor *daf-16*, and the p53 orthologue *cep-1* did not show any changes in their expression patterns.

Some limitations of the assays used should be noted:

(i) *The worms used were mixed stage cultures*, meaning that if a toxicant only affected worms of a particular stage, this would not have shown up consistently. However, using mixed cultures was overall the best approach since maintaining synchronised cultures of multiple strains for each exposure would be very laborious and multiple exposures would be necessary for each strain to include all stages. Using mixed cultures ensures detection of a significant effect on the organism as a whole, but not necessarily for specific stages. Post-exposure examination of worms under a fluorescence microscope could be used to determine whether GFP expression is confined to particular developmental stages.

(ii) *Studies were only run for 28 hours*. This limit was chosen because of the basal fluorescence of bacteria. The worm suspension was washed free of bacteria to ensure that only fluorescence in the worms would be taken into account. This however meant that worms would be under starvation conditions, resulting in up-regulation of stress-response genes irrespective of the presence of a toxicant; thus exposures were not carried out for longer than 28 hours. It is possible that some toxicants may affect animals only in the longer-term or cause developmental arrest, an effect that would not have been detected.

(iii) *Different cultures were used for each exposure assay*. Although homozygous *C. elegans* strains give genetically very similar individuals, there is some phenotypic variability which could cause differences in experimental outcomes between different cultures. This might be particularly problematic for cultures that had become starved or contaminated. However, repeat runs on the same strain from different cultures generally yield similar patterns of stress-gene response.

(iv) *GFP stability obstructs break-down in the cells*. Whereas up-regulation can be detected by increased protein production, down-regulation requires both a decrease in production and degradation of pre-existing protein. GFP is a rather stable protein,

meaning that break-down in the cells takes a long time. During that time, GFP levels will appear to be stable, even though gene expression may actually be decreased.

(v) *Regulatory elements outside the upstream region were not accounted for.* The constructs used were transcriptional reporters containing about 3Kb of upstream sequence, meaning that gene regulation was only dependent on this regulatory region. Intronal or downstream regulatory elements were not present, and therefore, GFP expression may not exactly match that of the gene of interest. However, this 3Kb region is standard for such transcriptional GFP fusions (Hunt-Newbury et al. 2007), and is much larger than the 500-1000bp of upstream region used previously for, e.g., *mtl-2::GFP* transcriptional fusions (Swain et al. 2004; Ma et al. 2009).

(vi) *Background fluorescence makes small differences hard to detect.* A curious artifact in *C. elegans* is the fact that gut granules and nucleoli of hypodermal cells exhibit autofluorescence. This background means that a relatively high GFP response is required in order to be clearly detectable above background.

(vii) *Solvents used to dissolve the toxicant may have an effect on gene expression.* Due to pesticide insolubility in water, solvents (EtOH, MeOH or DMSO) were used to dissolve the pesticide prior to further dilution in water. However, the solvents used may have toxic effects on the organism themselves. To account for this, the solvent concentration did not exceed 0.2% in exposures. A solvent control containing the same concentration as that present in the highest toxicant concentration was set up in parallel to the toxicant exposures and then used to compare against the results. However, only one concentration of the solvent was used as a control and all other toxicant concentrations were compared against this, even though lower concentrations of the solvent were present at the other doses. Distilled water controls were also run, and any signs of response at low test doses could be compared against these instead.

(viii) *Loss of worms during transfers may influence outcome.* Worms sticking to pipette tips during transfers will inevitably affect the number of worms present in the well. Especially after multiple readings, this could seriously influence the results –

leading to progressive decreases in signal over time. To account for this, extra water controls were set up and incubated in parallel with the toxicant exposures. Before each transfer, an extra water control was used to resuspend worms in the pipette tip. The same tip was used for transfer of all conditions of each strain, taking care to start from the controls and move upwards through the toxicant concentration series. This precaution minimised variability between replicates for the remaining test conditions, since the problem of worms sticking is most acute with fresh tips. Silicone tips were also tested, but did not greatly ameliorate this problem.

4.2 Confirmation of previous results for dichlorvos

According to previous experiments (Table 3.9), dichlorvos shows up-regulation for more than half the genes tested. Among the oxidative stress genes, the mitochondrial superoxide dismutase *sod-3* and catalase *ctl-2* show down-regulation at the early timepoint, whereas the superoxide dismutases *sod-1* and *sod-4* show around 1.5-fold up-regulation at the lower concentrations for the intermediate (both) and late (*sod-4* only) timepoints; the glutathione peroxidases GPA and GPB and the transcription factor *skn-1* also show 1.5- to 2-fold up-regulation at the late timepoint. Among the xenobiotic stress genes, the glutathione-S-transferases tested (*gst-1*, *gst-4*) showed around 1.5-fold up-regulation, with *gst-1* showing sensitivity to all but the lowest concentration at intermediate and late timepoints, and *gst-4* only responding to the highest concentration at the late timepoint. Cytochrome P450 genes showed varying responses, with *cyp-29A2* only showing up-regulation at the highest concentration at the late timepoint, *cyp-35A2* showing down-regulation at the early timepoint and time-dependent up-regulation at the highest concentration, and *cyp-34A9* showing dose- and time-dependent up-regulation reaching 3.5-fold for the highest concentration at the late timepoint. Of the metal response genes, only *mtl-2* showed any response with dose- and time-dependent up-regulation. The gene controlling DNA-damage-induced apoptosis *cep-1* only showed 1.5-fold up-regulation at an intermediate concentration at the late timepoint. The major transcription regulator *daf-16* showed time-dependent

up-regulation for all but the lowest concentration tested. Among the heat stress genes, all the heat shock proteins showed dose- and time-dependent up-regulation to varying levels, but they all exceeded 2-fold up-regulation for at least the highest concentration at the late timepoint; *hsp-16.1*, *hsp-16.2* and *hsp-6* all show up to 3-fold up-regulation. However, their transcription factor, *hsf-1*, did not show any change in gene expression. This could imply that the induction of heat shock protein expression was controlled by other transcription factors, such as DAF-16; or that the function of HSF-1 was enhanced without affecting transcription (e.g. through increased phosphorylation or decreased degradation).

It is uncommon for only one of the metallothioneins to show a response alone since they are usually regulated together. For this reason, other strains expressing the *mtl-2* gene in a reporter fusion construct were used to confirm effect of dichlorvos on *mtl-2* expression. Our results confirmed up-regulation of *mtl-2* in the Georgia *mtl-2::GFP* strain, but the effect was only significant for the highest concentration tested at the late timepoint (Table 3.10). The JF2.1 *mtl-2::lacZ* strain also showed up-regulation at a high concentration after 20 hours exposure (Table 3.11), but to a lower level than that observed in previous results.

Since dichlorvos was the only pesticide to show a wide effect on most of the stress response genes tested, a series of gene arrays had been performed on the non-target organism *C. elegans* and the target-related organism *Drosophila melanogaster* to identify common response patterns. It should be noted however that although insects respond to extremely low concentration of dichlorvos (a maximum of 15 parts per **billion**), *C. elegans* needs to be exposed to much higher concentrations to show a response (1.5-400 parts per **million**).

The gene array data showed significant effects of dichlorvos on a wide variety of genes (Table 3.12); over 1000 genes show significant ($P < 0.05$) up- or down-regulation after applying the Benjamini-Hochberg correction to remove false positives (Neil Graham, unpublished data). Many genes showed up- or down-regulation at either or both of the concentrations tested. To confirm the significance of these results, 6

genes were chosen, orthologs of which showed a similar response to dichlorvos in both organisms, and qPCR was performed to confirm the findings in *C. elegans*.

Our results confirmed the direction of response for all of the tested genes, but showed some variability in the level of response (Figure 3.31).

5 Conclusions and Further Work

This study shows evidence that many commonly used pesticides do not show extensive non-target toxicity toward the nematode *C. elegans*. Among the pesticides tested in this report, diuron and the pyrethroids cypermethrin and deltamethrin displayed no change in the expression of the representative stress-response genes used in this study. The insecticides chlorpyrifos, endosulfan and DDT and the fungicide carbendazim showed a limited effect on stress-response gene expression of this model organism. DDT also displayed a clear inhibitory effect on feeding. The mitochondrial poison rotenone however, showed a highly toxic effect on many genes.

The insecticide dichlorvos, previously shown to have a broad effect on *C. elegans* gene expression, was studied further and some of the previous data was confirmed.

Although our results are replicable, there are several limitations arising from the nature of the experimental procedures used. Further assays could be performed to explore other aspects of toxicity of the agents tested here. Similar experiments could be set up using synchronised cultures to look at the effect of toxicants on different developmental stages of the organism. Animals could be exposed in the presence of food for a whole life cycle and effects on the progeny could be observed. To overcome the issue of the solvent effect, multiple solvent controls could be used so that each toxicant concentration can be compared to a matched control containing the equivalent concentration of the solvent. To account for regulatory elements outside the promoter region, translational reporter fusions could be used. This however would introduce new issues; for example, translational reporter strains are much harder to produce and the reporter products prove harder to detect due to instability of the fused protein.

One way to improve results of feeding inhibitions would be to use multiple replicates for all controls, which in some cases exhibited a strange pattern (Figure 3.19, D). For DDT, for which *C. elegans* showed a physiological but not a genetic response, further

assays could be performed to explore the functions through which this effect takes place. For example, nose constriction could be observed in the presence of DDT and compared to the solvent control to determine if feeding is inhibited by pharynx constriction, similar to the mechanism activated in the dauer stage.

An important limitation we faced was the low solubility of most pesticides in water. If solvents are found in which a pesticide can be dissolved to a higher concentration, or which exhibits no toxicity to the nematode, then higher test concentrations could be achieved. It should be noted however, that the maximal doses tested on *C. elegans* were in most cases much higher than those used in the field.

Pyrethroids showed no effect on *C. elegans*. The use of piperonyl butoxide (PBO) to enhance pyrethroid activity has been examined previously (Svendsen et al. 1986; Brun-Barale et al. 2010; Matowo et al. 2010). Further assays could be performed to investigate the effect of pyrethroids on *C. elegans* simultaneously exposed to the synergistic action of PBO.

Our study could obviously be expanded by the study of further pesticides and more genes. Gene array studies could be performed for a global overview of gene expression changes. Such studies however are extremely expensive and impractical for examination of different timepoints and concentrations.

The gene Y40B10A.6 showed the largest extent of up-regulation in response to dichlorvos, according to our gene array data. After exposure to 1.5ppm of dichlorvos for 24 hours Y40B10A.6 showed a 30-fold up-regulation and after exposure to 150ppm dichlorvos for 24 hours it showed a 40-fold up-regulation. Further exploration of the function and sites of action of this gene could also prove useful to determine the mechanism through which dichlorvos acts on *C. elegans*. This gene encodes a putative O-methyltransferase, but no functional data is available for the translated protein. Other O-methyltransferases also showed high up-regulation to the scale of 10-fold suggesting a role of this group of enzymes in the metabolism of dichlorvos. A paper on the gene array is in preparation.

Our results have shown evidence on the effects of a number of pesticides on the nematode *C. elegans*, with a focus on stress-response gene expression. Our data will be further used for the development of a mathematical model of the core stress response network which will offer predictions of the effect of simple chemical mixtures on the expression of stress response genes.

6 Appendix

Common genes

| GO Class | <i>D. melanogaster</i> gene | Description | <i>C. elegans</i> gene | Regulation | Reference |
|--------------------------------|-----------------------------|---|--|--|--|
| Lipid metabolic process | FBgn0025809 (CG8962) | Molecular function 1-alkyl-2-acetyl-glycerophosphocholine esterase activity | WBgene00003907 (C52B9.7) | <i>D.mel</i> Up (1.81) <i>C.ele</i> UP (1.64) | Flybase Sheffield et al., 2000, Proteins Struct., Function Genet. 39(1): 1--8 Homologs of the α - and β -subunits of mammalian brain platelet-activating factor acetylhydrolase Ib in the <i>Drosophila melanogaster</i> genome. [FBrf0128643] Wormbase Paper evidenceInoue T et al., 2004. PAF acetylhydrolase activity is present at near wild-type levels in paf-1 mutant animals, suggesting that PAF-2 is the main source of PAF acetylhydrolase activity in <i>C. elegans</i> . Supported by: Inoue T et al., 2004 |
| | | | Primer sequence Upper: 5' gctatatctcgtcgccacaagt 3' Lower: 5' ccgtcggaaaataaagtctcat 3' | | |

| | | | | | |
|---------------------------------------|---------------------------------|--|---|---|---|
| <p>Oxidoreductase activity</p> | <p>FBgn0038922 (CG6439)</p> | <p>Molecular function Isocitrate dehydrogenase (NAD+) activity</p> <p>Biological process Tricarboxylic acid cycle, oxidation-reduction, magnesium ion binding</p> <p>Cellular Component Mitochondrion</p> | <p>WBGene00016266 (C30F12.7)</p> | <p><i>D.mel</i> Down (2.48)</p> <p><i>C.ele</i> Down (2.28)</p> | <p>Flybase 1. Zhou, D., Xue, J., Lai, J.C., Schork, N.J., White, K.P., Haddad, G.G. (2008). Mechanisms underlying hypoxia tolerance in Drosophila melanogaster: hairy as a metabolic switch. PLoS Genet. 4(10): e1000221. 2. Predicted Annotations: based on sequence similarity</p> <p>Wormbase 1. WBPaper00028451] Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. (01 JAN 2006 00:00:00) 2. Predicted annotations: Biol. Funct.:Interpro to GO mapping Mol.funct.:Interpro to GO mapping Cellular comp: Interpro to GO mapping</p> |
| | | | <p>Primer sequence Upper: 5'gaattgccccgagatgat3' Lower: 5'tccaaaagcgacgatgagact3'</p> | | |

| | | | | | |
|----------------------------------|---------------------------------|--|--|--|--|
| <p>Catalytic activity</p> | <p>FBgn0001142 (CG2718)</p> | <p>Molecular function glutamate-ammonia ligase activity</p> <p>Biological process glutamine biosynthetic process</p> | <p>WBGene00001602 (C45B2.5) (gln-1) (GLutamiNe synthetase (glutamate-ammonia ligase)</p> | <p><i>D.mel</i> Up (3.68) <i>C.ele</i> Up (1.59)</p> | <p>Flybase Caggese, Caizzi, et al.,1992, Dev. Genet. (13): 359--366 Mutations in the glutamine synthetase I (Gsl) gene produce embryo-lethal female sterility in Drosophila melanogaster.</p> <p>Wormbase Predicted annotations Biol. Funct.-Interpro to GO mapping Mol.funct.- Interpro to GO mapping</p> |
| | | | <p>Primer sequence Upper: 5' atgcgaagatggaacagg 3' Lower: 5' aagtcggcgctcattatc 3'</p> | | |

| | | | | | |
|----------------------------------|---------------------------------|--|--|---|--|
| <p>Hydrolase activity</p> | <p>FBgn0032066 (CG9463)</p> | <p>Molecular function alpha-mannosidase activity</p> <p>Biological process mannose metabolic process</p> | <p>WBGene00018877 (F55D10.1)</p> | <p><i>D.mel</i> Down (1.85)</p> <p><i>C.ele</i> Down (1.56)</p> | <p>Flybase 1.Dickson et al., 2007.7.18, RNAi construct and insertion data submitted by the Vienna Drosophila RNAi Center RNAi construct and insertion data submitted by the Vienna Drosophila RNAi Center [FBrf0200327] 2. FlyBase Curators et al., 2004-, Gene Ontology annotation in FlyBase through association of InterPro records with GO terms. Gene Ontology annotation in FlyBase through association of InterPro records with GO terms</p> <p>Wormbase [WBPaper00027768] A deletion in the golgi alpha-mannosidase II gene of Caenorhabditis elegans results in unexpected non-wild type N-glycan structures. (01 JAN 2006 00:00:00) [WBPaper00029024] Carbohydrates and glycosylation (01 JAN 2006 00:00:00)</p> |
| | | | <p>Primer sequence Upper: 5' tcgccattgacaacttccatc 3' Lower: 5' tgcatttctcgagggttagg 3'</p> | | |

| | | | | | |
|---|----------------------------------|---|--|--|--|
| <p>Metallopeptidase activity</p> | <p>FBgn0039050 (CG17110)</p> | <p>Molecular function aminoacylase activity metallopeptidase activity</p> <p>Biological process cellular amino acid metabolic process</p> <p>proteolysis</p> <p>Cellular Component cytoplasm</p> | <p>WBGene00007507 (C10C5.3)</p> | <p><i>D.mel</i> Up (1.78)</p> <p><i>C.ele</i> Up (15.48)</p> | <p>Flybase Predicted</p> <p>wormbase Predicted</p> |
| | | | <p>Primer sequence Upper: 5' aacattccgtgagcattgg 3' Lower: 5' gcctgattccctgagca 3'</p> | | |

| | | | | | |
|------------------------------------|----------------------------------|--|---|---|---|
| <p>Transporter activity</p> | <p>Fbgn0031939 (CG13796)</p> | <p>Molecular function neurotransmitter:sodium symporter activity</p> <p>Biological process neurotransmitter transport</p> <p>Cellular Component integral to plasma membrane</p> | <p>WBGene00004900 (W03G9.1)</p> | <p><i>D.mel</i> Up (8.12)</p> <p><i>C.ele</i> Up (2.08)</p> | <p>Flybase Miller, M.M., Popova, L.B., Meleshkevitch, E.A., Tran, P.V., Boudko, D.Y. (2008). The invertebrate B(0) system transporter, D. melanogaster NAT1, has unique d-amino acid affinity and mediates gut and brain functions. Insect Biochem. Mol. Biol. 38(10): 923--931.</p> <p>Wormbase [WBPaper00015781] Sodium-dependent Neurotransmitter Transporter (snf) Genes in C. elegans. (01 JAN 2002 00:00:00) [WBPaper00024940] A Na⁺/Cl⁻ coupled GABA transporter, GAT-1, from Caenorhabditis elegans. (01 JAN 2005 00:00:00)</p> |
| | | | <p>Primer sequence Upper: 5' gaatcttcggcttactga 3' Lower: 5' atatgccattgctgacac 3'</p> | | |

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PAN UK, Rotenone - <http://www.pan-uk.org/pestnews/Actives/rotenone.htm>

The Hope Laboratory Expression Pattern Database -

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WHO/FAO DATA SHEETS ON PESTICIDES No. 89 CARBENDAZIM -

http://www.inchem.org/documents/pds/pds/pest89_e.htm

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