

TOXICOLOGY STUDIES IN CAENORHABDITIS ELEGANS

By

Chloe Denisha Budd, BSc (Hons).

Thesis submitted to the University of Nottingham
for the degree of Master of Research

September 2008

Abstract

The nematode *Caenorhabditis elegans* was established as a model organism to study in 1965. In this study, *C.elegans* are used as a model system in which to examine the possible mechanisms of toxicity caused in humans by the anti-malarial drugs quinine and chloroquine, as well as toxicity caused in rodents by furan and investigating the pharmacology of the insecticides DDT and deltamethrin and how they affect invertebrates.

Toxicity assays were used to determine what effects these 5 toxins have on *C.elegans* at different stages of its life cycle. The effects on growth, brood size and lethality were determined using three types of assays. These assays were robust, producing reproducible results and identifying specific toxicity.

The results of these assays enabled the identification of a phenotype for each type of assay, and the inhibiting concentration (if any), for all the toxins tested.

Quinine had a dose-dependent inhibitory effect on larval growth and brood size.

Chloroquine, furan and DDT all had an inhibitory effect on growth, brood size and lethality of *C.elegans*. Chloroquine and DDT showed a greater potency on larval growth while furan showed a higher potency on brood size. Deltamethrin showed inhibitory effects on larval growth but showed no significant effect to the brood size and lethality of *C.elegans*. Deltamethrin shows stage-specific toxicity, as larval growth is significantly inhibited and effects on brood size and lethality are insignificant.

The *lat-1* (ok1465) *C.elegans* strain has a 2209bp deletion of the *lat-1* gene, and approximately 97% of these worms die before adulthood, with only 2-3 adult offspring per animal. The *lat-1* (ok1465) *C.elegans* was crossed with the CB4856 (Hawaiian) wild-type *C.elegans* strain for 6 generations to produce a strain with the ok1465 allele in a Hawaiian *C.elegans* background.

A mutagenesis could then be performed to screen and select for mutants that were resistant to the effects of the drug tested at concentrations that are toxic to wild-type animals. In the case of lat-1 (ok1465) *C.elegans*, screening would be to identify mutant individuals that no longer showed lethality; a resistant mutant would produce 300+ viable offspring. Gene mapping would then be used to identify these mutations.

The hypothesis is that for the toxins tested, the mechanism by which they exert their toxic effects in *C.elegans* would be the same as for mammals and invertebrates.

Acknowledgements

I would like to thank my supervisor, David Bell for everything he taught me and for explaining things over and over when I didn't understand without ever making me feel stupid (although he may have thought it at times), I couldn't have asked for a better supervisor.

I would like to thank my parents for their constant support not only this year, but throughout my whole life, for which I am sincerely grateful.

I would like to thank all the members of the Bell lab for all their support and advice, it was a pleasure to work with you all, and made my lab experience an enjoyable one.

A special thank you goes to Declan Brady, who taught me everything I know in the lab and without whom I would have been lost; (which I was, on days he was off.)

Finally, I'd like to thank Richard Wall, for making my year at Nottingham so much fun and for making me laugh more than I ever thought I could; and to Nabil, for his encouragement this past year and for always lifting my spirits when my experiments didn't work, (which was a lot!)

Dedication

To My Mother

Nilu

And my Father

Shyama

This thesis is dedicated in memory of my loving

grandmother

Madura Amarasekera (1931-2006)

Abbreviations:

Bp	Base pair
LB	Luria-Bertani
C. elegans	Caenorhabditis elegans
CI	Confidence interval
DDT	Dichlorodiphenyltrichloroethane.
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
L1-L4	C.elegans larval stages
NGM	Nematode growth medium
O.D	Optical density
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
TEMED	N,N,N',N' - Tetramethylethylenediamine
UHP	Ultra high purity

Contents

Abstract	1
Acknowledgements	3
Dedication	4
Abbreviations:	5
Contents	6
List of figures	8
1. Introduction	10
1.3 Chloroquine	15
1.4 Furan	17
1.5 DDT	19
1.7 The Lat-1 (ok1465) C.elegans	21
1.8 Summary / Aim of the study	22
2. Materials and Methods	24
2.1 Reagents	24
2.2 General Methods	29
2.2.1 E.coli cultures	29
2.2.2. Gel electrophoresis.	29
2.3 General methods for C.elegans	30
2.3.1 Maintenance of C.elegans	30
2.3.2 Microscopy of C.elegans	30
2.3.3 Egg Isolation	31
2.3.4 Freezing worms	31
2.4 C.elegans toxicity assays	32
2.4.1 L1 growth assay	32
2.4.2 Brood size assay	36
2.4.3 Lethality assay	38
2.5 Data presentation and statistics	40
2.6 Polymerase chain reaction (PCR)	41
2.7 Breeding	42
3. Results	47
3.1 C.elegans toxicity assays	47
3.1.1 L1 growth assay	47
3.1.2 Brood assay	47
3.1.3 Lethality assay	47
3.2 Optimisation of C.elegans toxicity assays	48
3.3 Sodium azide positive control	49
3.4 Quinine results	52
3.5 Chloroquine Results	56
3.6 Furan Results	61
3.7 DDT Results	66
3.8 Deltamethrin Results	71
3.9 Vehicle control assays	76
3.9.1 DMSO as a vehicle/solvent	76
3.9.2 Acetone as vehicle/solvent	80
3.9.3 Ethanol as a vehicle/solvent	83
3.10 PCR	87
3.10.1 PCR optimisation	87
3.11 Breeding results	88
4. Discussion	94
4.1 Reliability of methods and results	94

4.2 Vehicles	96
4.3 Quinine	98
4.4 Chloroquine	98
4.5 Furan	99
4.7 DDT	100
4.8 Deltamethrin	100
4.8 Screening and mutagenesis.	101
5. Conclusion and further studies.	103
5.1 Further Studies	103
5.2 Genetic Mapping	103
6. Appendix	108
7. References	117

List of figures

Figure 1.1.2 Anatomy of an adult hermaphrodite <i>C.elegans</i> worm.....	12
Figure 1.1.3 Life cycle of <i>C.elegans</i> at 20°C adapted from wormatlas.org.....	13
Figure 1.2 Chemical structure of quinine.....	15
Figure 1.3 Chemical structure of chloroquine.....	17
Figure 1.4 Chemical structure of furan.....	18
Figure 1.5 Chemical structure of DDT.....	19
Figure 1.6 Chemical structure of deltamethrin.....	21
Figure 1.7 A photograph showing the green fluorescent pharynx phenotype.....	22
Figure 2.4.1 Preparation of agarose pads on microscope slides.....	34
Figure 2.4.2 Worm outlined using the magnetic lasso tool.....	35
Figure 2.7.1 Punnett square showing genotypes for F1 offspring.....	43
Figure 2.7.2 Punnett square showing genotype for F2 offspring.....	44
Figure 2.7.3 Punnett square showing genotype for F3 offspring.....	45
Figure 2.7.4 Punnett square showing genotype of offspring from selfed F6 animal.....	46
Figure 3.3 Chemical structure of sodium azide.....	49
Figure 3.4.1 Effect of quinine on brood size.....	52
Figure 3.4.2 Effect of sodium azide on brood size.....	52
Figure 3.4.3 Effect of quinine on growth.....	53
Figure 3.4.4 Effect of sodium azide on growth.....	53
Figure 3.5.1 Effect of chloroquine on brood size.....	56
Figure 3.5.2 Effect of sodium azide on brood size.....	56
Figure 3.5.3 Effect of chloroquine on growth.....	57
Figure 3.5.4 Effect of sodium azide on growth.....	57
Figure 3.5.5 Effect of chloroquine on lethality.....	58
Figure 3.5.6 Effect of sodium azide on lethality.....	58
Figure 3.6.1 Effect of furan on brood size.....	61
Figure 3.6.2 Effect of sodium azide on brood size.....	61
Figure 3.6.3 Effect of furan on growth.....	62
Figure 3.6.4 Effect of sodium azide on growth.....	62
Figure 3.6.5 Effect of furan on lethality.....	63
Figure 3.6.6 Effect of sodium azide on lethality.....	63
Figure 3.7.1 Effect of DDT on brood size.....	66
Figure 3.7.2 Effect of sodium azide on brood size.....	66
Figure 3.7.3 Effect of DDT on growth.....	67
Figure 3.7.4 Effect of sodium azide on growth.....	67
Figure 3.7.5 Effect of DDT on lethality.....	68
Figure 3.7.6 Effect of sodium azide on lethality.....	68
Figure 3.8.1 Effect of deltamethrin on brood size.....	71
Figure 3.8.2 Effect of sodium azide on brood size.....	71
Figure 3.8.3 Effect of deltamethrin on growth.....	72
Figure 3.8.4 Effect of sodium azide on growth.....	72
Figure 3.8.5 Effect of deltamethrin on lethality.....	73
Figure 3.8.6 Effect of sodium azide on lethality.....	73
Figure 3.9.1 DMSO vehicle control for growth assay.....	76
Figure 3.9.2 DMSO vehicle control for brood size assay.....	77
Figure 3.9.3 DMSO vehicle control for lethality assay.....	78
Figure 3.9.4 Acetone vehicle control for growth assay.....	80
Figure 3.9.5 Acetone vehicle control for brood size assay.....	81
Figure 3.9.6 Ethanol vehicle control for growth assay.....	83
Figure 3.9.7 Ethanol vehicle control for brood size assay.....	84

Figure 3.9.8 Ethanol vehicle control for lethality assay.....	85
Figure 3.11.1 Acrylamide gel showing specificity of primers.....	88
Figure 3.11.2 Acrylamide gel showing genotype of hermaphrodites from breeding.....	90
Figure 3.11.3 Acrylamide gel showing genotype of F6 Lat C.elegans.....	92
Figure 5.2 Two point SNP mapping.....	106

1. Introduction

1.1 *Caenorhabditis elegans*

1.1.1 *Caenorhabditis elegans* as a model organism.

C. elegans is a free living nematode, present in soil and found in temperate regions of the world. The *C. elegans* was selected by Dr. Sydney Brenner in 1965, as an experimental model organism to study animal development and behaviour (Riddle 1997). In the wild, *C. elegans* feed on the micro-organisms that develop on decaying vegetable matter. In the laboratory *C. elegans* are easily cultured on agar medium plates with *Escherichia coli* OP50 as a food source. The ease of laboratory cultivation, its small size, large brood size, short development time and well studied biology make the *C. elegans* an ideal model organism for biological studies. The transparency of the *C. elegans* allows for high quality microscopic images to be taken.

1.1.2 Anatomy

C. elegans is small with the adult hermaphrodite growing to a maximum length and diameter of approximately 1mm and 80µm respectively. An adult hermaphrodite has a total of 959 somatic cells and 1031 in males, of which 302 are nerve cells. The *C. elegans* body consists of two concentric tubes which are separated by the pseudocoelom; a fluid filled space. Internal hydrostatic pressure maintains the *C. elegans* shape (Riddle 1997 & White 1988). A collagenous, extra-cellular cuticle, secreted by the underlying hypodermis covers the outer tube (Kramer 1997). Between each of the four larval molts the old cuticle is shed and a new stage specific cuticle is secreted. The body of the *C. elegans* has four strips of muscle along its length which is attached to the cuticle. Muscle contraction and relaxation causes movement of *C. elegans* in a sinusoidal movement in the dorsal-ventral plane, which can be observed on an agar plate (Mee

2002). *C.elegans* can move forward or backward in response to attractants or repellents in the environment (Riddle 1997). The water in the medium of agar plates creates surface tension which confines the *C.elegans* to the surface (Mee 2002).

The nematode *C.elegans* can be found as either hermaphrodite or male, however in wild-type N2 strain populations the majority of worms are hermaphrodite and males arise infrequently at a rate of 0.1%. The male *C.elegans* only produces sperm and must mate with a hermaphrodite to reproduce. The *C.elegans* hermaphrodite is self-fertilising, producing both sperm and oocytes. The sperm is produced in the spermatheca and the oocytes are produced in the gonads. A self-fertilised hermaphrodite produces approximately 300 hermaphrodite only offspring, however if mating with a male occurs the hermaphrodite can produce more than 1000 offspring. The offspring resulting from a mating will be 50% male and 50% hermaphrodite. Because of the very low occurrence of males in normal *C.elegans* populations, mating rarely occurs, and in the laboratory most *C.elegans* populations are generated by self-fertilisation. *C.elegans* hermaphrodites have 5 autosomes and two X chromosomes (XX), the male has one X chromosome designated as XO (Hodgkin 1979). Males occur spontaneously by means of nondisjunction in the germ line of hermaphrodites, causing the loss of an X chromosome to produce the XO male.

Figure 1.3 below shows a diagram of the anatomy of an adult *C.elegans* hermaphrodite. The *C.elegans* is an unsegmented nematode with a cylindrical shape body that is tapered at the ends (wormatlas.org). The mouth is at the very tip of the head of the worm, followed by the pharynx which connects to the intestine and runs along the length of the body ending at the rectum. The male *C.elegans* is a similar size, although the male is a little shorter and thinner. They have a similar general anatomy to the hermaphrodite, but males *C.elegans* have a clearly visible fan shaped tail which is used for clasping the hermaphrodite during mating (Mee 2002).

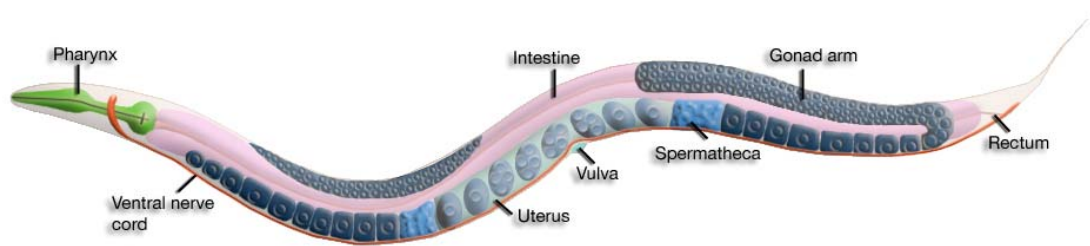


Figure 1.1.2 Diagram showing the anatomy of an adult hermaphrodite *C.elegans* worm. (Taken from www.wormatlas.org) Basic *C.elegans* anatomy showing the mouth, pharynx, gut and gonads.

1.1.3 Life cycle

The *C.elegans* has a short generation time, the entire complete life cycle from egg to egg producing hermaphrodite occurs over 3 days at 20°C under normal laboratory conditions. The length of the life cycle can vary with temperature, as *C.elegans* can grow over a range of temperatures, with the rate of growth increasing with temperature (Mee 2002) *C.elegans* populations in the laboratory are typically grown between 15°C and 25°C. The length of the life cycle can vary between 6 days at 15°C and 3 days at 25°C. Optimal growth occurs at 20°C with approximately 300 offspring produced over 4 days, and a life span of 2-3 weeks.

The *C.elegans* life cycle consists of an embryonic stage followed by four larval stages designated L1-L4 and a final adult stage. Each of the larval stages is marked with a molt where the old cuticle is shed and a new stage-specific cuticle is synthesised. (wormatlas.org) The *C.elegans* eggs are fertilised within the hermaphrodite and are laid at approximately the 30-cell stage. At 20°C embryonic development occurs over 14 hours and postembryonic development occurs over 43 hours. Larval molts occur successively at the following hours following fertilisation: L1- L2 = 29 hours, L2-L3 =

38 hours, L3-L4 = 47 hours L4 -Adult = 57 hours. The *C.elegans* life cycle is summarised below in figure 1.1.3.

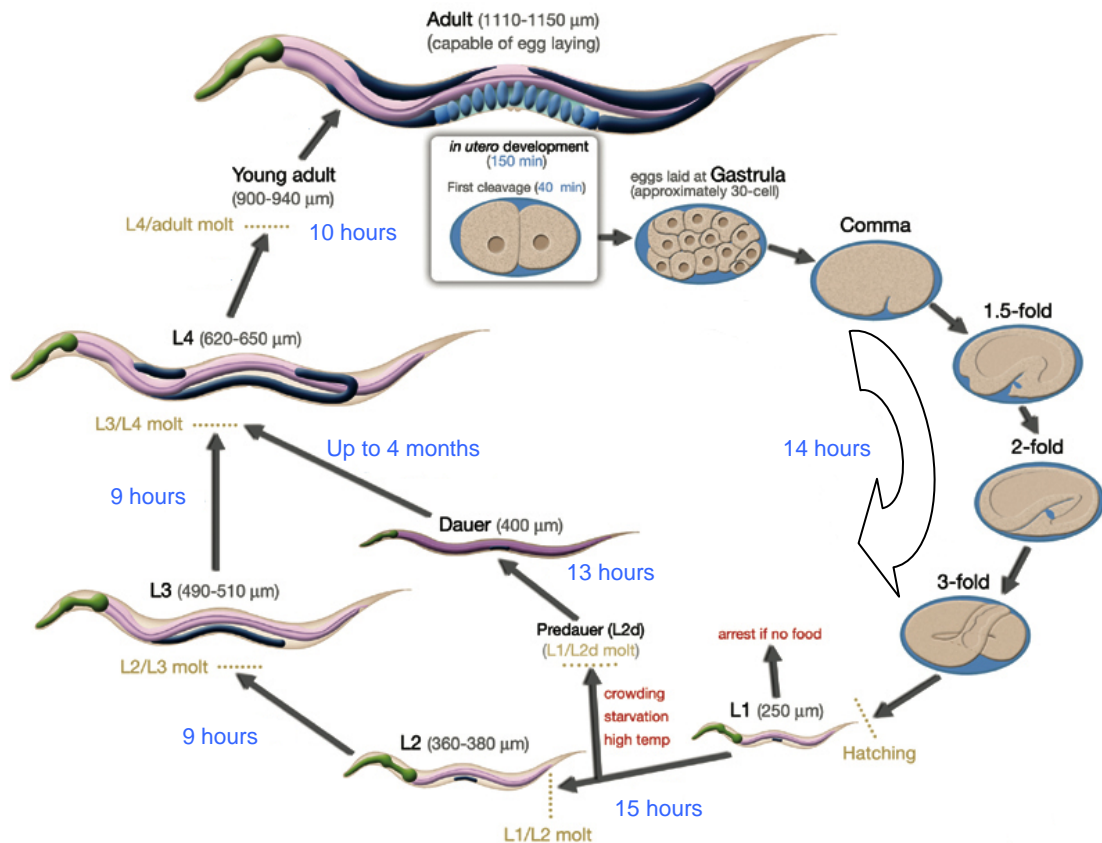


Figure 1.1.3 Life cycle of *C.elegans* at 20°C adapted from wormatlas.org.

The numbers in blue show the length of time between each larval molt. The length of the *C.elegans* at each stage of development is shown in micrometers (µm) next to the stage name.

The dauer stage is an alternative larval stage which occurs in crowded populations or in the absence of food. The dauer is formed at the second molt and are non-feeding and non-developing. Whereas wild-type *C.elegans* only have a life span of 2-3 weeks, under normal laboratory conditions, dauer larvae can survive for up to 3-4 months. In order to survive dauer larvae alter their energy metabolism and accumulate fat in the hypodermal

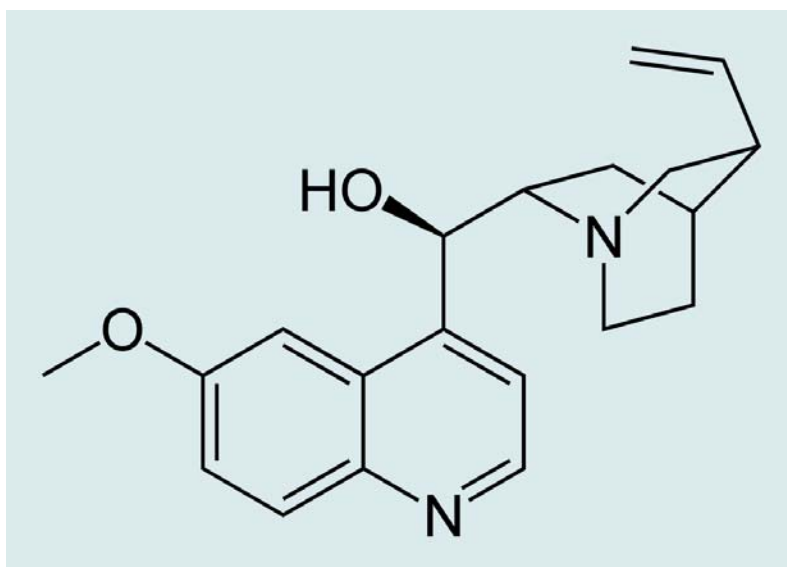
and intestinal cells. Dauer larvae are thin and have plugged mouths such that they cannot eat; they remain motionless most of the time unless touched when they can move faster than L3 larvae, which is thought to be a survival technique. Normal development is resumed when dauer larvae encounter food (Mee et al 2002).

1.2 Quinine

Quinine is a natural white crystalline alkaloid derived from the bark of the cinchona tree, native to the Andes mountain region in South America (Howard 2003). It was discovered in 1737 by Charles Marie de La Condamine and was the first effective treatment for malaria caused by *Plasmodium falciparum*. Until the 1940s quinine was the only known drug in western medicine for the treatment and prevention of malaria, after which, new more effective synthetic antimalarial drugs were developed and took over. Quinine was the first successful chemical compound ever used to treat an infectious disease, and more people have benefited from it than from any other drug in history. Today, quinine is the drug of choice for chloroquine resistant or severe *P. falciparum* malaria (Huston 2006). Antimalarial drugs can be used for prophylaxis or treatment of malaria. The clinical indication of quinine is only for treatment and has no role in prophylaxis (AlKadi 2007). As a monotherapy quinine is given for 7 days to achieve cure, however due to its unpleasant adverse effects patient adherence is poor (Taylor W 2004). Treatment with quinine is often associated with cinchonism, hypoglycaemia and hypotension, which are dose-related toxicities. Cinchonism usually occurs after several days of therapeutic use or an overdose of quinine. It consists of visual and hearing disturbances, abdominal pain, diarrhoea, vomiting, mental status changes, fever, flushed skin and vertigo. Cinchonism is a reversible syndrome (Huston 2006). Other common adverse effects of quinine are nausea, diarrhoea, headache tinnitus, dysphoria and blurred vision. Quinine causes toxicity in humans at approximately $<2\text{mM}$, causing

severe adverse affects such as retinopathy, hypoglycaemia, ototoxicity, hearing loss and thrombocytopenia Severe hypotension frequently occurs if the quinine is injected too rapidly (AlKadi 2007).

The mechanism by which quinine causes this toxicity in humans is not understood, and identifying this is important as then related compounds that do not have such an effect could be selected.

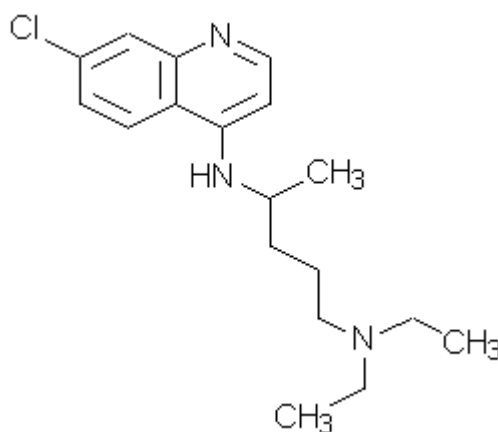


1.2 Chemical structure of Quinine – taken from www.wikipedia.org

1.3 Chloroquine

Like quinine, chloroquine is also an anti-malarial drug. It is effective against *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and drug sensitive *Plasmodium falciparum* (AlKadi 2007). Chloroquine is used as prophylaxis or treatment. Quinine is used for treatment of strains of the malarial parasite *Plasmodium falciparum* that have developed resistance to chloroquine. The intraerythrocytic stages of *P.falciparum* are responsible for the clinical manifestation of malaria and are the target of chloroquine. These stages feed on the haemoglobin in erythrocytes, which is digested

by lysosomes, producing the toxic by product, ferriprotoporphyrin (FP) or heme, which is detoxified by dimerization to into non-toxic β -hematin (Fitch 2006) (Sanchez CP 1997). β -hematin (or hemozoin) collects in the lysosome as insoluble crystals. This detoxification of FP is inhibited by chloroquine in the lysosomes. β -hematin molecules are capped by chloroquine to prevent further detoxification of FP, causing it to accumulate to lethal levels. In vivo experiments have demonstrated that significantly less chloroquine was accumulated in chloroquine resistant parasites than those that were sensitive to chloroquine. It has been proposed that this reduced accumulation of chloroquine is thought to provide resistance suggesting that resistant parasites are able to lower the concentration of chloroquine in the lysosomes to below than that which is required for heme polymerisation (Sanchez 1997). Membrane function is disrupted when chloroquine binds to FP forming a highly toxic complex called the FP-chloroquine. Action of this toxic complex and FP causes cell lysis and ultimately results in parasite cell auto-digestion. Due to its low cost and tolerability, chloroquine continues to be used despite its decreasing efficiency against *P.falciparum*. Chloroquine resistant *P.vivax* is also emerging (Taylor 2004). Unlike quinine, chloroquine is normally well tolerated. Mild side effects of include headache, mild gastrointestinal upset, malaise and nausea. However, long term chloroquine use can lead to severe adverse effects such as retinopathy and neuromyopathy. For treatment of malaria, chloroquine is administered once daily. Weekly chloroquine is used for prophylaxis alone or in combination with proguanil. Chloroquine causes acute toxicity in humans at $<3\text{mM}$, causing vomiting, convulsions, cardio-respiratory arrest, drowsiness and shock. This toxicity is most frequently encountered when therapeutic or high doses are administered too rapidly by parenteral routes (AlKadi 2007). The mechanism, by which chloroquine causes this toxicity in humans is not understood, and identifying this is important as then related compounds that do not have such an effect could be selected.

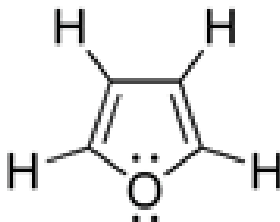


1.3 Chemical structure of Chloroquine – taken from www.wikipedia.org

1.4 Furan

Furan is a heterocyclic organic compound typically derived by the thermal decomposition of pentose containing materials. It is a toxic, highly volatile, flammable, colourless liquid and has a boiling point close to room temperature. Furan is insoluble in water but soluble in alcohol and organic solvents. Since the 1970's the occurrence of furan in some food items has been known; since then, a survey conducted by the US Food and Drug Administration (FDA) found furan to occur in a variety of foods, particularly canned and jarred products that undergo heat treatment. The mechanism of furan formation in foods is still unknown, although it has been proposed that thermal degradation of carbohydrates is a possible route. The presence of furan in so many food items has made it more important to clarify its toxicological properties (Durling 2007). Furan is carcinogenic in rats and mice, and has been classified as a possible human carcinogen (Peterson 2006). Studies have suggested both non-genotoxic and genotoxic pathways as possible mechanisms behind the carcinogenic effect of furan (Durling 2007). Furan and furan compounds are cytotoxic, most frequently targeting the liver, lungs and kidneys causing necrosis. Organ specificities have been observed in some

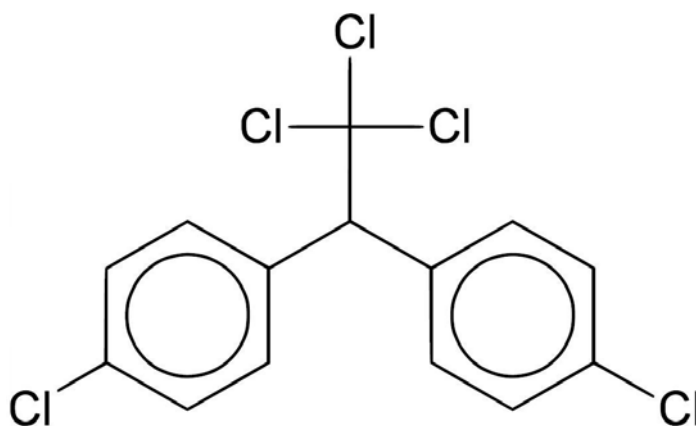
furan compounds. Based on necrosis development, furan was found to be equally toxic to the liver and kidney in acute short term studies where 100 to 300 mg/kg of furan was intraperitoneally administered to mice over a 48 hour period. In the same experimental system 2-ethyl furan was found to be more toxic to the kidney than the liver whereas furosemide was found to be more toxic to the liver than to the kidney (NTP 1993). Studies have shown a dose-dependent increase in hepatocellular adenomas and carcinomas at 30mg/kg – 60mg/kg (Heppner 2007). The mechanism of tumour induction is unknown, however furan hepatotoxicity is initiated following bio-activation by cytochrome P450 2E1-catalyzed oxidation to cis-2-butene-1,4-dial (Kedderis 1996). Cis-2-butene-1, 4-dial is a cytotoxic metabolite which binds to nucleosides and proteins. Metabolism studies have shown that this reactive metabolite is formed in vivo and is also an intermediate that leads to other metabolites whose role in furan derived toxicities is yet to be discovered (Peterson 2006). The data available shows that there is a relatively small difference between the doses required to produce carcinogenic effects in experimental animals and possible exposure to humans (Heppner 2007). Due to the high occurrence of this compound in foods it is important to identify its toxicological effects in humans. Studies that have been carried out in rodents have not been able to successfully determine the mechanism of furan toxicity.



1.4 Chemical structure of Furan – taken from www.wikipedia.org

1.5 DDT

DDT is a colourless crystalline solid, best known for its insecticidal properties. It is an organochlorine insecticide first synthesised by Othmar Zeidler, in 1874, however it was only identified as an insecticide in 1939 by Paul Hermann Müller. DDT is a highly effective insecticide which affects the neurons. It acts on the voltage gated sodium channel proteins in nerve cell membranes. These proteins are integral membrane proteins and are responsible for the conduction of sodium ions. During an action potential the sodium channel undergoes transitions between open and closed in response to changes in the membrane potential. DDT binds to these sodium channels in the open state which inhibits its transition to the non-conducting deactivated state. Thus the channel is kept open and there is a prolonged inward conductance of sodium causing repetitive nerve firing which leads to paralysis and death of the insect. Resistance to DDT is seen in insects with particular point mutations in the sodium ion channel gene. In some insect species, the up-regulation of genes expressing cytochrome P450 also results in DDT resistance. DDT was the insecticide widely used for malaria vector control programmes; however its use has gradually declined due to concerns for the environment, and development of vector resistance. There are no sodium channels recognised in *C.elegans* and the mechanism by which DDT causes toxicity is not understood as the channel that mediates sodium transport is unknown.



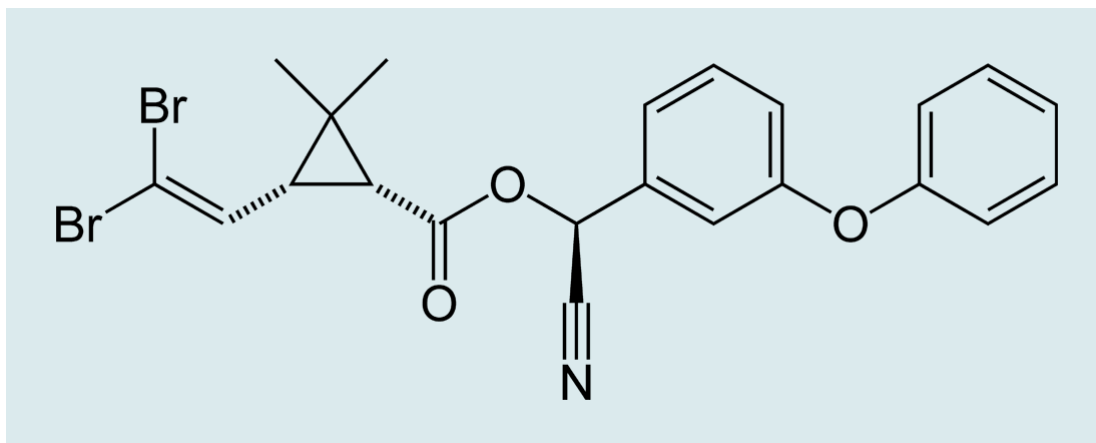
1.5 Chemical structure of DDT – taken from www.wikipedia.org

1.6 Deltamethrin

Deltamethrin is a widely used pyrethroid ester insecticide. It is a synthetic pyrethroid, and considered to be one of the safest classes of pesticides.

Like DDT, the target for deltamethrin is voltage-gated sodium channels; disturbing the ionic exchanges during nerve impulses. In insects pyrethroids cause severe paralysis (knockdown) (Daaboub 2008). At operational doses, deltamethrin has relatively low toxicity in mammals, with exposure to mammals classified as safe. Deltamethrin has quick knockdown effects and high insecticidal potency; it is highly toxic to aquatic life, particularly fish. Non-bioaccumulation and efficiency against arthropods with resistance to cyclodienes, carbamates and organophosphate insecticides make deltamethrin and pyrethroids suitable for veterinary and public health purposes (Daaboub 2008). Widespread use of pyrethroids has seen an increase in pyrethroid resistance in many insect species. Target site insensitivity (or resistance) to deltamethrin is acquired by a point mutation in the insects voltage-gated sodium channel proteins (Perera 2008). Over 20 unique polymorphisms in the amino acid sequence of sodium ion channels have been associated with resistance to pyrethroids. (Hemingway 2004). The most common mutation identified is a substitution at position 1014 of leucine to phenylalanine in the S6 segment of domain II of the sodium channel gene (Daaboub 2008). This mutation has been identified in mosquitoes and other insect species resistant to pyrethroids. Another mechanism conferring pyrethroid resistance is increased activity of detoxification enzymes such as carboxylesterases; however this often has effects on fitness. Deltamethrin has played a key role in controlling malaria vectors and preventing the spread of diseases carried by rodents and prairie dogs infested by ticks as well as other burrowing animals. It is also used in the manufacture of mosquito nets, where the net is saturated with deltamethrin. Studies have shown that mosquito resistance to deltamethrin is correlated with resistance to DDT, suggesting there may be cross resistance between

the two insecticides. There are no sodium channels recognised in *C.elegans* and the mechanism by which deltamethrin causes toxicity is not understood, as the channel that mediates sodium transport in *C.elegans* is unknown.



1.6 Chemical Structure of Deltamethrin – taken from www.wikipedia.org

1.7 The Lat-1 (ok1465) *C.elegans*

The latrophilin protein, encoded by the lat-1 gene is a member of the secretin/calcitonin family of G-protein coupled receptors. It was first isolated from bovine membranes and is mostly found in neuronal or neuroendocrine tissues (Adenle 2008). Latrophilin is the major protein to bind α -latrotoxin. Ligand binding to the latrophilin receptor triggers an intracellular signalling cascade.

The lat-1 (ok1465) has a 2209bp deletion of the lat-1 gene that removes exons 3, 4 and 5 and inserts an AT encoding a truncated protein and lacks the first transmembrane domain (Guest 2007). This deletion renders latrophilin dysfunctional. Approximately 97% of these worms die before adulthood, with only 2-3 adult offspring per animal. Studies have shown that the lat-1 gene is essential for embryonic and larval development and this lethality is due to the deletion in the lat-1 gene. Adenle, (2008) found that 33% of lat-1 (ok1465) offspring had embryonic lethality and 65% larval lethality, whereas in wild-type offspring 100% survived to adulthood. The phenotype of lat-1(ok1465) animal offspring is characterised by the failure of gut attachment to pharynx in larvae and

failure of epithelial migration. Due to the lethality of the *lat-1* (*ok1465*) allele, it is balanced with a *mIn* gene, where it is maintained as a stable heterozygote *mIn/lat-1*, which has a wild-type phenotype. The *mIn* gene is tagged with dumpy (*dpy*) and green fluorescent protein (*gfp*). A selfed *mIn/lat-1* worm will produce offspring of *mIn/mIn*, *mIn/lat-1* and *lat-1/lat-1* genotypes. *C.elegans* carrying the *mIn* gene have a fluorescent pharynx; but homozygous *mIn/mIn* worms also express a recessive dumpy (short and fat) phenotype. Homozygote *lat-1* worms are of a normal size but are not fluorescent. These differences can be identified under a microscope using a *gfp* filter.

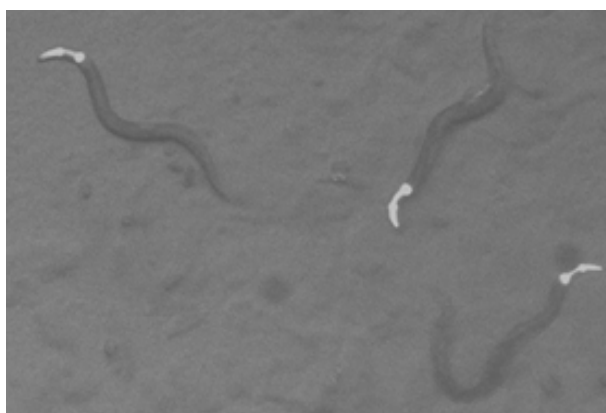


Figure 1.7 A photograph taken under a microscope using the *gfp* filter, showing the green fluorescent pharynx phenotype seen in *C.elegans* carrying the *mIn* gene.

1.8 Summary / Aim of the study.

Chloroquine and quinine are both antimalarial drugs, still widely used today, for the treatment and prophylaxis of malaria. The mechanism of action of the drugs on the malaria causing strains of *Plasmodium* has been demonstrated in experiments *in vivo*; however the pathway of toxicity in humans is still unclear and *C.elegans* are used in this study to help determine the genes that may be involved in this pathway.

Furan is a highly toxic, volatile organic compound which is carcinogenic in rats and mice and is thought to be a possible human carcinogen. Furan is present in a variety of foods, with the highest levels found in jarred and canned products, and is formed during

the heat-treatment of food. Due to the high occurrence of this compound in foods it is important to identify its toxicological effects in humans. Studies that have been carried out in rodents have not been able to successfully determine the mechanism of furan toxicity. In this study *C.elegans* are used as a model for rodent toxicity.

DDT and Deltamethrin are potent synthetic insecticides widely used in agriculture today. These insecticides have had a significant impact in the control of malaria and other insect borne diseases. DDT and Deltamethrin act on the sodium ion channels in neurons by opening them, which therefore cause them to fire spontaneously which lead to spasms and eventually death. Little is known about the pharmacology of these insecticides and how they work in *C.elegans*. There are no sodium channels recognised in *C.elegans* and the channels that mediate sodium transport is unknown, and in this study the *C.elegans* is used to try identify this.

Toxicity assays will be used in this study to test how the above compounds effect the growth, brood size and lethality of *C.elegans* by exposing them to various concentrations of each compound. Once a phenotype is established for how the *C.elegans* are affected by each compound, a mutagenesis can be performed to screen and select for mutants that are a resistant to the effects of the compound, and gene mapping would be used to identify these mutations. The hypothesis is that the mechanism by which these toxins exert their toxic effects on *C.elegans* would be the same as for mammals and invertebrates.

The lat-1 (ok1465) *C.elegans* have a deletion in the lat-1 gene and ~ 97% homozygous worms die before reaching adulthood. In this study the lat-1(ok1465) *C.elegans* will be crossed with the Hawaiian (CB4856) *C.elegans* for 6 generations to produce a strain with the ok1465 allele in a Hawaiian *C.elegans* background. A mutagenesis could then be performed to screen and select for mutants that did not have 97% lethality. Gene mapping would then be used to identify these mutations.

2. Materials and Methods

LB Agar, LB Broth and Agar were obtained from Melford laboratory Ltd. Peptone, Gelatine, Tween 20, TEMED, Ethanol, Quinine, DDT, Sodium azide, Deltamethrin, Furan, Chloroquine, DMSO and Acetone were obtained from Sigma. Primers were from Eurofins. 100bp ladder was from New England Biolabs. The PCR master mix was obtained from Thermo Scientific. All other chemicals or compounds were from Fisher Scientific unless otherwise stated.

2.1 Reagents

All solutions were made up to 1 litre with UHP water and sterilized by autoclaving. Storage was at room temperature unless otherwise stated.

Nematode Growth Medium (NGM) agar.

NaCl – 3.0g

Agar – 17.0g

Peptone – 2.5g

Cholesterol (5mg/ml ethanol) – 1ml

S- Basal

NaCl – 5.84g

Potassium Phosphate Buffer – 50ml

Potassium Phosphate Buffer (pH 6.0)

1M KH_2PO_4 – 434ml

1M K_2HPO_4 – 66ml

The above volumes mix to make the buffer pH6.

LB Broth – made up using 25g of a ready mix.

Tryptone – 10g

NaCl – 10g

Yeast extract – 5g

LB Agar – made up using 35g of a ready mix.

Tryptone – 10g

NaCl – 10g

Yeast extract – 5g

Micro Agar – 10g

Sodium azide – dissolved in H₂O, made up to 1ml each at the following concentrations by serial dilution.

1M, 0.1M, 0.01M, 0.001M, 0.0001M.

Chloroquine – dissolved in H₂O; 150mM stock solution made up to 10ml, this was diluted to make 1ml each of the following concentrations.

125mM, 100mM, 30mM, 10mM

Chloroquine was stored at -20°C.

Furan – 150mM stock solution made up to 10ml in 100% ethanol, this was diluted to make 1ml each of the following concentrations.

125mM, 100mM, 30mM, 10mM.

Furan was stored at 4°C.

Quinine – 250mM stock solution made up to 2ml in 100% ethanol, this was diluted to make 1ml each of the following concentrations.

250mM, 100mM, 30mM, 10mM

Quinine was stored at -20°C.

DDT – stock solution made up to 1mg/ml in 100% DMSO this was serially diluted to make up 1ml each of the following concentrations.

0.1mg/ml, 0.01mg/ml, 0.001mg/ml, 0.0001mg/ml.

DDT was stored at -20°C.

Deltamethrin – stock solution made up to 1mg/ml in 100% DMSO this was serially diluted to make 1ml each of the following concentrations.

0.1mg/ml, 0.01mg/ml, 0.001mg/ml, 0.0001mg/ml

Lysis Buffer – made up to 100ml as described.

Tris-HCL (pH 8.0) – 1ml

KCL – 0.37275g

MgCl₂ – 0.0508275g

IGEPAL (Nonidet P40) - 450μl

Tween 20 - 450μl

Gelatin - 10μg

Proteinase-K (Stock 10mg/ml) - 500μl (to give 50μg/ml)

Proteinase-K must be kept frozen so once added, 10μl of the solution were aliquot into 500 μl

PCR tubes and stored at -20°C.

12% TBE-PAGE Gel – made up to 100ml as described.

Polyacrylamide (30%) - 40ml

H₂O – 50ml

10 x TBE – 10ml

TEMED - 100μl

This solution was kept at 4°C, and the following was added when assembling a gel.

Ammonium phosphate (Stock 0.1g/ml) - 10μl per ml of 12% TBE page gel.

Ammonium phosphate (APS) stock was aliquot to fill 1.5ml centrifuge tubes and stored at -20°C.

TBE – this was made up as a 10X concentrate. 100ml of the concentrate was then made up to 1 litre.

Tris – 108g

Bomic Acid – 55g

0.5M EDTA (pH 8.0) – 40ml

Freezing Solution

NaCl – 5.85g

KH₂PO₄ – 6.8g

Glycerol – 300g

1M NaOH – 5.6ml

0.1M MgSO₄ – 3ml (this was only added after autoclaving).

PCR Master Mix – Ready to use, each vial contains 1.8ml at a 1.1x working concentration.

Thermoprime plus DNA polymerase – 1.25 units

Tris-HCL (pH8.0) – 75mM

(NH₄)₂SO₄ – 20mM

MgCl₂ – 1.5mM

Tween 20 – 0.01% (v/v)

dATP, dCTP, dGTP and dTTP each at – 20mM

The master mix was stored at 4°C.

Loading Buffer – made up to 10ml

Glycerol – 5ml

Bromophenol blue – 25μl

Xylene cyanol FF – 25μl

10 X TBE – 1ml

Primers – made as per company instructions to 100pm.

Lat1 ok1465 null 5'

5'- AGC TTG GTC AGG GTA GTA CT – 3'

Lat1 ok1465 null 3'

5' GGT TTT TGG TCC AAA TTT CGA CC – 3'

Lat1 ok1465 wild-type 5'

5' – TTC ACA TTC ATT GTG ATG GA – 3'

Lat1 ok1465 wild-type 3'

5' – TCC GGT AAT CCA GAG ACA TC – 3'

To avoid repeat freeze-thawing, primers were aliquot (15µl) into PCR tubes and stored at -20°C.

Egg Isolation Bleach Solution – made up to 24 ml as described.

NaOCl (stock at 13%) – 3ml

NaOH (1M) – 6ml

H₂O – 15ml

2.2 General Methods

2.2.1 E.coli cultures.

E.coli OP50 cells were grown up in LB broth medium. LB agar (section 2.1) was microwaved for 10-15 minutes until molten and poured under sterile conditions in a class II hood to make LB agar plates. These plates were then streaked with a single colony of OP50 E.coli and incubated at 37°C overnight. (Only one plate was streaked at a time, the remaining LB agar plates were sealed and stored at 4°C) A single colony from this plate was used to inoculate 5ml of LB broth (section 2.1) in a universal (20ml) tube under sterile conditions in a class II hood. This was grown overnight at 37°C and 240rpm. OP50 E.coli plates and liquid media were stored at 4°C. A new LB agar plate was streaked with OP50 E.coli every 4 weeks.

2.2.2. Gel electrophoresis.

TBE-PAGE gel was made up as described in section 2.1. 5.5ml of this solution was poured into a gel casting mould and allowed to set with a comb of 10 wells. When set, the comb was removed and the gel was placed in an electrophoresis tank filled with 1x TBE buffer. 3µl of loading buffer (section 2.1) was added to each of the DNA samples and gently mixed. The DNA samples were carefully loaded into the wells of the gel

alongside a 100bp DNA ladder as a marker. The gels were run using the Bio-Rad gel system for 1.5 hours at 100V. The gel was then removed from the mould and placed in a solution of 0.0001% ethidium bromide in H₂O for 30 minutes and washed with H₂O for a further 10 minutes. The gel was visualised under UV trans-illuminator, and photographed using Bio Rad Gel-Doc system and the Quantity One software.

2.3 General methods for *C.elegans*

2.3.1 Maintenance of *C.elegans*

C.elegans were grown and maintained on NGM agar plates seeded with an OP50 *E.coli* lawn at 15°C throughout the study. NGM was prepared as described in section 2.1. After autoclaving the solution was microwaved until molten and allowed to cool to 65°C. The NGM plates were poured into 3cm or 9cm under sterile conditions in a class II hood, until ½ full. Once the agar was set, a drop of OP50 *E.coli* was placed in the centre of each of the plates. A flamed glass spreader was used to evenly spread the *E.coli* on 9cm plates. The plates were left to air-dry under sterile conditions and incubated overnight at 37°C. Plates were stored at room temperature. 3cm plates were used for breeding, and 9cm plates were used for keeping *C.elegans* stocks. These plates were chunked when plates were overcrowded or worms became starved. Chunking involves using a sterile heat flamed stainless steel scalpel, to cut out and transfer a chunk of an old starved NGM plate of *C.elegans* to a new plate. *C.elegans* were handled using a worm pick; which is a platinum wire heat sealed to the end of glass pipette.

2.3.2 Microscopy of *C.elegans*

C.elegans were viewed using a Wild M3 stereo microscope, at 6.4X, 16X and 40X magnification. Fluorescent worms were viewed using the Olympus SZX12 stereo

microscope (using a gfp filter) and worms from the L1 growth assay, were viewed on microscope slides using the Axiovert 135TV inverted microscope.

2.3.3 Egg Isolation

1. A plate of gravid hermaphrodite *C.elegans* was washed with 2-3mls of S-basal and 0.5ml was transferred using a pipette to a 1.5ml centrifuge tube.
2. 0.5ml of the egg isolation bleach solution was added to the centrifuge tube, and a timer was started.
3. The centrifuge tube was immediately closed and mixed by vortexing at the maximum speed for 3 minutes.
4. It was then spun in a centrifuge held at 4°C for 1 minute at 500g. A 1.5ml centrifuge tube filled with an equal volume of H₂O was used as a balance.
5. After spinning, the supernatant was carefully discarded ensuring not to disturb the pellet and fresh S-basal was added to fill the centrifuge tube. A 1.5ml centrifuge tube filled with an equal volume of H₂O was used as a balance.
6. Steps 4 and 5 were repeated for three further spins.
7. After the final spin the supernatant was discarded and the pellet re-suspended in s-basal a final time.
8. The solution is then transferred to a 6-well plate using a sterile Pasteur pipette.
9. All steps were then repeated until all the wells in the 6-well plate are full.
10. The 6-well plate is sealed with laboratory film and incubated at 15°C overnight for the eggs to hatch into larvae.

2.3.4 Freezing worms

C.elegans worms can be frozen and stored at -80°C for an indefinite period of time. Starved L1 animals are most likely to survive this freezing; whereas adult and well fed

animals do not survive.(Dawe 2004) In order to obtain starved animals worm populations to be frozen, are either bleached to obtain starved L1 larvae or allowed to grow on plates until the OP50 E.coli was exhausted. Plates with starved C.elegans worms were washed with 2-3ml of S-basal using a Pasteur pipette, into a 5ml tube. An equal volume of freezing solution (section 2.1) was added and mixed by inverting several times. 1ml aliquots were dispensed into 1.5ml cryotubes. The tubes were closed, labelled and frozen at -80°C.

2.4 C.elegans toxicity assays.

These methods are based on Bischof et al (2006) taken from C.elegans: Methods and applications; Methods in Molecular Biology.

2.4.1 L1 growth assay

This assay is to measure the effect of toxins on growth and development of C.elegans. The assay is set up and run over 4 days.

1. On day 1 5ml of LB broth was inoculated with a single colony of E.coli OP50, and incubated overnight at 37°C and 240rpm. A plate of gravid hermaphrodite worms were bleached as described in section 2.3.3.
2. On day 2, 1ml of the OP50 bacterial culture was spun for 5 minutes at 2000g. The supernatant was discarded and the pellet was re-suspended in S-basal medium.
3. L1 worms were spun for 1 minute at 500g. The supernatant was discarded and the pellet was re-suspended in s-basal with a volume sufficient to give 6-8 worms/μl. The number of worms in a 5μl sample was counted. If the worms were too concentrated

s-basal was added until there were 30-40 worms per 5 μ l. If the worms were too diluted, they were re-spun and the appropriate amount of supernatant was removed.

4. The absorbance of the bacterial culture was measured at 600nm. The absorbance was adjusted to be 0.70, by adding S-basal. The spectrophotometer was zeroed using S-basal.

5. The assay was set up in a 24-well plate, with 3 wells for each concentration. Each well contained 5 μ l of L1 worms in s-basal (30-40 worms in total), 40 μ l of E.coli OP50 bacterial culture in s-basal, the toxin and s-basal to make a total volume of 400 μ l.

6. The toxin was made up to give a range of doses in order to obtain a response curve. The toxin was serially diluted to prepare 100X stocks of each concentration to be tested.

7. A master cocktail was made up for each concentration, sufficient for 4 wells, combining the bacterial culture, s-basal medium and L1 worms. (See appendix for volumes added.) This was then repeated for the rest of the concentrations and the control.

8. Once the cocktail was aliquot into the wells, the toxin was added. (See appendix for volumes added.) S-basal was added to control wells in place of the toxin.

9. The 24-well plate was then sealed with laboratory film (to prevent evaporation), wrapped in a damp paper towel (to provide humidity) and placed in an air tight plastic container. The assay was incubated at 15°C for 3 days.

10. After 3 days, 4µl of 1M Sodium azide was added to each well in order to immobilise the worms.

11. The contents of each well was transferred into 1.5ml centrifuge tubes and spun for 1 minute at 500g. The supernatant was removed leaving approximately 10-20µl. the pellet was re-suspended by briefly vortexing

11. Agarose pads were prepared on microscope slides using a 2% (w/v) agarose solution. A microscope slide was aligned in between two microscope slides with tape adhered to the surface. (See figure 2.4.1 below) Using a Pasteur pipette, 2 drops of agarose was placed on the middle slide and another slide was quickly placed on top of the agarose drops in a perpendicular orientation, as shown below. Once the agarose had set (after approximately 1 minute) the top slide was removed. This was then repeated to make the rest of the agarose pad microscope slides. Each slide was labelled with a concentration.

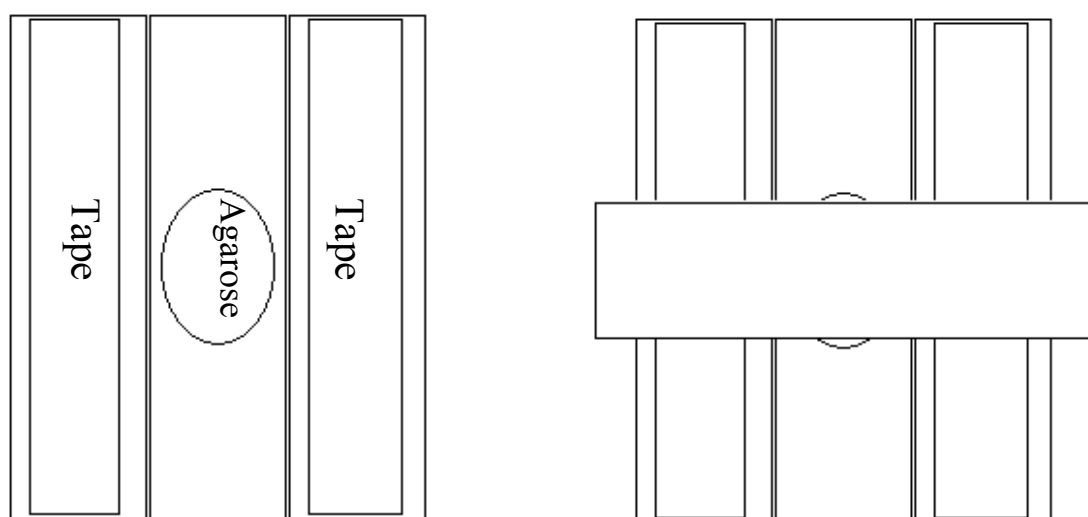


Figure 2.4.1 Preparation of agarose pads on microscope slides

12. The worms were gently mixed by hand and then 5-7 μ l was carefully pipetted onto the agarose pad, making sure it was for the correct concentration. A coverslip was then gently placed on top.

13. The worms were then imaged with a 10X objective using an inverted microscope and photographed using a Scion 1310M CCD camera and the Visicapture software. The worm images were saved as .tiff files and a minimum of 30 worms were photographed per concentration.

14. The worms' sizes were then measured in pixels using Adobe Photoshop CS3 and the magnetic lasso tool.



Figure 2.4.2 An example of a worm that has been photographed and outlined using the magnetic lasso tool in Adobe Photoshop CS3. The outlining is as close as possible to the edge of the worm's body.

15. The measurements recorded in pixels were converted to mm^2 by photographing a haemocytometer (of known area) in the same manner. The photographed area was then also measured in pixels. The measurement in pixels was divided by the measurement in mm^2 to obtain the number of pixels per mm^2 . The number of pixels per mm^2 is 1,709,750. All the worm measurements were converted to mm^2 in this way.

16. An identical assay was set up and run alongside, with Sodium azide (see appendix) as a positive control.

2.4.2 Brood size assay.

This assay is to measure the effect of toxins on brood size. The assay is set up and run over 8 days.

1. On day 1 a plate of gravid adult worms were bleached as described in section 2. 3.3
2. On day 2 L1 worms were plated on an NGM plate seeded with E.coli OP50 and incubated at 15°C for 3 days. The worms will be at the L4 stage of growth.
3. On day 4, 5ml of LB broth was inoculated with a single colony of E.coli OP50, and incubated overnight at 37°C and 240rpm.
4. On day 5, 1ml of the OP50 bacterial culture was spun for 5 minutes at 2000g. The supernatant was discarded and the pellet was re-suspended in S-basal medium.
5. The absorbance of the bacterial culture was measured at 600nm. The absorbance was adjusted to be 0.70, by adding S-basal. The spectrophotometer was zeroed using s-basal.

6. The assay was set up in a 48-well plate, with 3 wells for each concentration. Each well contained a single L4 worm, 40µl of bacterial culture, the toxin and s-basal to make a total volume of 400µl.
7. A master cocktail was made up for each concentration, sufficient for 4 wells, combining the bacterial culture and S-basal medium. (See appendix for volumes added.) This was then repeated for the rest of the concentrations and the control.
8. Using the worm pick, a single L4 worm was picked from the NGM plate and placed in each well. The worm pick was sterilised in a flame after each worm was placed.
9. Once all the worms had been transferred, the toxin was added. See appendix for volume of toxin added to each well for each concentration.
10. The 48-well plate was then sealed with laboratory film (to prevent evaporation), wrapped in a damp paper towel (to provide humidity) and placed in an air tight plastic container. The assay was incubated at 15°C for 3 days.
11. After 3 days the number of progeny in each well was counted under a stereo microscope.
12. An identical assay was set up and run alongside, with Sodium azide (see appendix) as a positive control.

Due to the short generation time of *C.elegans* only the partial brood size was measured after 3 days. After 3 days the early progeny would start having progeny of their own which would make scoring difficult. In order to measure the full brood size over 5-6 days the worms would have to be transferred to new wells after the third day. It was therefore more feasible to perform this assay over three days to avoid the need of transferring the parental worm.

2.4.3 Lethality assay.

This assay is to measure the effect of toxins on lethality. This assay is set up and run over 8 days.

1. On day 1 a plate of gravid hermaphrodite worms were bleached as described in section 2.3.3
2. On day 2 L1 worms were plated on an NGM plate seeded with *E.coli* OP50 and incubated at 15°C for 3 days. The worms will be at the L4 stage of growth.
3. On day 4, 5ml of LB broth was inoculated with a single colony of *E.coli* OP50, and incubated overnight at 37°C and 240rpm.
4. On day 5, 1ml of the OP50 bacterial culture was spun for 5 minutes at 2000g. The supernatant was discarded and the pellet was re-suspended in S-basal medium.
5. The absorbance of the bacterial culture was measured at 600nm. The absorbance was adjusted to be 0.70, by adding S-basal. The spectrophotometer was zeroed using S-basal.

6. L4 worms were collected in a 1.5ml centrifuge tube by washing the NGM plate with 1-2ml of s-basal and spun for 1 minute at 500g. The supernatant was discarded and the pellet was re-suspended in s-basal with a volume sufficient to give approximately 10 worms/ μ l. The number of worms in a 5 μ l sample was counted. If the worms were too concentrated s-basal was added until there were 50 worms per 5 μ l. If the worms were too diluted, they were re-spun and the appropriate amount of supernatant was removed.

7. The assay was set up in a 24-well plate, with 3 wells for each concentration. Each well contained 5 μ l of L4 worms in s-basal (20 worms in total), 40 μ l of E.coli OP50 bacterial culture in s-basal, the toxin and s-basal to make a total volume of 400 μ l.

8. A master cocktail was made up for each concentration, sufficient for 4 wells, combining the bacterial culture, S-basal medium and L4 worms. (See appendix for volumes added.) This was then repeated for the rest of the concentrations and the control.

9. Once the cocktail was aliquot into the wells, the toxin was added. (See appendix for volumes added.) S-basal was added to control wells in place of the toxin.

10. The 24-well plate was then sealed with laboratory film (to prevent evaporation), wrapped in a damp paper towel (to provide humidity) and placed in an air tight plastic container. The assay was incubated at 15°C for 3 days.

11. After 3 days the percent alive was determined by counting the number of viable worms in each well based on movement.

12. An identical assay was set up and run alongside, with Sodium azide (see appendix) as a positive control.

2.4.4 Vehicle control assays.

Vehicle control assays were set up alongside the above three assays for each toxin tested. These assays were set up and analysed as described above for each type of assay. The vehicle was only tested if it was not H₂O. The sodium azide positive control assay set up along side the each toxicity assay was also used as the positive control for the vehicle control assay.

2.5 Data presentation and statistics.

Microsoft Excel was used to calculate the mean and standard deviation of the data and the log₁₀ concentration. These values were analysed by the GraphPad Prism 5 software which was used to generate all graphs. The data was fit to a linear non regression curve with the settings log [agonist] vs. response. The top and bottom value constraints were set as the start size and maximum measurements for the growth curves. The bottom constraint value was set as the maximum brood size for the toxin tested and the top constraint value was set as 0 for all brood size curves. The constraint values for the dose-response curves were set as 100 for the bottom and 0 for the top for all toxins. The IC₅₀ and 95% confidence intervals for all the toxicity assay results were calculated by the Graphpad Prism software.

Statistical analysis was carried out to determine if the difference between the treated and the control was statistically significant. A t-test was done on results that did not show an obvious difference from the control value. If the t value was below the threshold of 0.05 (5%) the null hypothesis was rejected. The null hypothesis was that there is no difference

between the two values. Therefore if the null hypothesis was rejected then the difference between the two values is statistically significant. However, if the t value was above the 0.05 (5%) threshold, the null hypothesis was accepted, and the difference between the two values is not statistically significant.

2.6 Polymerase chain reaction (PCR)

1. A single worm was picked from a plate using a flamed titanium worm-pick and transferred into a 500µl PCR tube with 10µl of lysis buffer.

2. In order to lyse the worm and isolate the genomic DNA, it was then frozen for 1 hour at -80°C and then heated for 6 hours at 65°C and 1 hour at 95°C. The proteinase-k is active at 65°C but is de-activated at 95°C. This heat-deactivation is crucial as the proteinase-k can cleave the polymerase during the PCR reaction.

3. Each PCR reaction contained 2µl of the DNA solution, 1µl of each 5' and 3' 100pmole primers (see section 2.1 for sequence) and 26µl of PCR master mix. These were set up in a PCR hood using filter-tips to prevent cross contamination.

4. The PCR reaction was carried out in a Progene thermal cycler and optimised for the following conditions.

94°C for 30 seconds - Denaturing

54°C for 30 Seconds - Annealing

72°C for 1 minute – Elongation

This was done for 35 cycles.

Prior to cycling the PCR reaction was heated for 2 minutes at 94°C, this is an initialisation step and is required for the heat activation of DNA polymerase.

To confirm the amplification of the PCR product the PCR reaction was run on 12% polyacrylamide gel electrophoresis (PAGE) alongside a negative control and a DNA ladder as a marker.

2.7 Breeding

Breeding was carried out between the Lat-1 ok1465 *C.elegans* and the Hawaiian CB4856 *C.elegans*.

1. The Lat-1 ok1465 *C.elegans* were isolated by allowing the mIn/lat-1 strain of *C.elegans* to self-fertilise which would produce homozygous lat-1 worms at a rate of approximately 1/100. The mIn/lat-1 *C.elegans* population was viewed under a microscope using a gfp filter. Homozygous lat-1 worms were identified from the mIn/lat-1 population as described in section 1.7.

2. A few homozygous Lat-1 *C.elegans* were placed on a 10cm NGM agar plate seeded with OP50 and allowed to self-fertilise to produce a Lat-1 stock plate.

3. CB4856 hermaphrodites were allowed to self-fertilise, which produced males at a frequency of 1/100. 6 males were picked using a flamed titanium worm pick and transferred onto a 3cm NGM agar plate seeded with OP50 *E.coli*, along with a single CB4856 L4 hermaphrodite. The plate was then incubated at 15°C overnight.

4. The following day the 6 males and hermaphrodite were transferred to another 3cm NGM plate seeded with OP50 *E.coli*. This was repeated for the following 2 days by

which time the progeny from the first day will be L4. If the breed was successful then approximately 50% of the progeny will be male. In order to produce more males, step 3 was repeated using 6 males and 1 L4 hermaphrodite from this plate. Step 3 was repeated throughout the breed to produce males.

5. Using a flamed titanium worm pick, 6 CB4856 males were placed on a 3cm NGM agar plate seeded with OP50 E.coli, along with a single L4 Lat-1 hermaphrodite picked from the Lat-1 stock plate using a flamed titanium worm pick. This plate was then sealed using laboratory film and incubated at 15°C overnight.

6. The following day the hermaphrodite and 6 males were transferred to another 3cm NGM agar plate seeded with OP50 E.coli, using a flamed titanium worm pick. This was repeated daily until the progeny from the first day had grown to L4.

7. If the breed was successful there will be more viable offspring and 50% of the offspring will be male. The genotype of all the F1 offspring will be Lat-1 +/-.

Lat-1(-/-) ♀ X CB4856 ♂ (+/+)

		CB4856	
		+	+
Lat-1	F1	-/+	-/+
	-	-/+	-/+

Figure 2.7.1 Punnett square showing genotypes for F1 offspring from a cross between a homozygous Lat-1 hermaphrodite and homozygous CB4856 male

8. An L4 hermaphrodite was picked from this plate and crossed with 6 CB4856 males as described in step 5.

9. A successful breed was successful 50% of the offspring would be male and the F2 offspring would be 50% genotype +/- and 50% genotype +/+.

		CB4856	
		+	+
F1	F2		
	—	-/+	-/+
	+	+/+	+/+

Figure 2.7.2 Punnett square showing genotype of F2 offspring from a cross between heterozygous F1 hermaphrodite and homozygous CB4856 male.

10. From this F2 generation, 8 L4 hermaphrodites were selected at random and each was crossed with 6 CB4856 males as described in step 4. Each of the plates was labelled, the worms were transferred daily to new plates as described in step 5, and each plate was labelled. On the third day each of the 8 hermaphrodites were genotyped by PCR as described in section 2.6. The PCR products were run on 12% polyacrylamide gel electrophoresis along side a DNA marker and negative control to confirm amplification the PCR fragment, as described in section 2.2.2. The PCR was run with ok1465 null primers so the gel would only show a band to confirm the presence of a Lat-1 PCR fragment in a heterozygous +/- hermaphrodite and no band would be seen for homozygous +/+ hermaphrodites. If the breed was successful 50% of the offspring would be male. A heterozygote hermaphrodite would produce offspring with 50% genotype +/- and 50% genotype +/+. A homozygote hermaphrodite would produce only wild-type offspring of +/+ genotype.

		CB4856	
	F3	+	+
	—	-/+	-/+
F2	+	+/+	+/+

		CB4856	
	F3	+	+
	+	+/+	+/+
F2	+	+/+	+/+

Figure 2.7.3 Punnett squares showing genotype of F3 offspring from a cross between F2 heterozygous and homozygous hermaphrodite and a homozygous CB4856 male.

Any plates with offspring from a homozygote (+/+) hermaphrodite, were discarded.

11. Step 10 was repeated, with L4 hermaphrodites from plates with F3 offspring from a heterozygote hermaphrodite. Step 10 was repeated until the F6 generation.

12. F6 offspring from a heterozygote hermaphrodite were grown to L4. 15 of these L4 hermaphrodites were each placed on a 3cm NGM plate seeded with OP50 E.coli and allowed to self for 3 days at 15°C. Each day the C.elegans were transferred to a fresh plate. On the third day, these 15 F6 adult hermaphrodites were genotyped as described in step 10. A self-fertilised heterozygous (+/-) hermaphrodite would produce offspring of 50% genotype (+/-), 25% genotype (+/+) and 25% genotype (-/-). A self-fertilised homozygous hermaphrodite would produce offspring of only wild-type genotype (+/+).

	+	-
+	+/+	+/-
-	+/-	-/-

	+	+
+	+/+	+/+
+	+/+	+/+

Figure 2.7.4 Punnett squares showing the genotype of offspring from a self-fertilized F6 heterozygous hermaphrodite and a homozygous hermaphrodite.

Plates with offspring from a homozygous hermaphrodite were discarded.

13. All the offspring from heterozygous hermaphrodites were individually plated on to 3cm NGM plates seeded with OP50 E.coli and allowed to self-fertilise for one week at 15°C.

14. Although 25% of the offspring should have been of lat-1 (-/-) genotype but, due to its characteristic 97% lethality during embryogenesis and larval development only 1-2 of the lat-1 (-/-) embryos from the heterozygous hermaphrodite would survive to adulthood. After three days all the plates were examined. A plate with a lat-1 (-/-) animal can be easily recognised as it will have many embryos but only 2-3 adult C.elegans. The majority of plates had many animals and were therefore either of heterozygous (+/-) genotype or homozygous (+/+) genotype and were discarded.

15. When a plate with a lat-1 (-/-) animal was identified, it was left to grow at 15°C for 5 weeks to allow the population to grow to a sufficient number of worms. . This phenotype was confirmed by genotyping animals from this population as described step 10. This population was then frozen and stored at -80°C as described in section 2.3.4

3. Results

3.1 C.elegans toxicity assays

Three types of toxicity assays were used in this study and performed in liquid media. These include quantitative growth, brood size and lethal dose assays. The assays provide useful toxicological information about how/what effect toxins have on C.elegans at different stages in its life cycle.

3.1.1 L1 growth assay

L1 worms were exposed to various concentrations of a toxin for 3 days. The worms were photographed and measured to determine size and hence the concentration of compound which inhibited growth (if any). This assay measures the parameters of development as well as growth, and potentially allows for worms to progress through all four larval stages, which is unique to this assay. This assay can also detect if a toxin was more efficacious at a particular larval stage (Bischof 2006)

3.1.2 Brood assay

Individual L4 C.elegans were exposed to various concentrations of a toxin for 3 days. After 3 days, the brood size was counted to determine how the brood size is affected and at the concentration of compound which inhibited brood size (if any). This assay measures the ability of a toxin to prevent production of the next generation of animals.

3.1.3 Lethality assay

L4 C.elegans were exposed to various concentrations of a toxin over 3 days. After 3 days the worms were scored as dead or alive based on movement to determine the percent viability and at what concentration the toxin caused lethality. This is a standard toxicological assay which allows for determining the concentration at which 50% of the

animals are killed (LC_{50}). Due to ease of scoring worms as dead or alive, a large sample can be analyzed.

3.2 Optimisation of *C.elegans* toxicity assays

For all three assays, initial results were highly variable, and the results were not reliable. Hence it was necessary to optimise a variety of parameters to ensure reproducible results. Thus, the vehicle medium, volume of bacteria, OD of bacterial culture, and total well volume was varied as per below with all other parameters as described in methods section 2.4.1 – 2.4.3, but in the absence of a toxin.

S-basal, M9 Buffer, K-media and S-media were tested. There was no difference observed between them in any of the assays.

The volume of OP50 *Escherichia coli* was tested between 10 μ l and 60 μ l with increments of 10 μ l. There was no difference between each volume tested.

The OD of the OP50 *Escherichia coli* bacterial culture at 600nm was varied between 0.2 and 0.8 increasing with increments of 0.1. Below 0.3 all three assays were affected. The brood size was significantly reduced and absent in some wells; a small percentage of lethality was observed in wells of the growth and lethality assays as well as a lack of growth seen in the L1 worms of the growth assay. At 0.4 and 0.5 only the growth and lethality assays were affected with no affect observed in the brood size assay. Above 0.5 the assays were not affected.

The total well volume was tested between 200 μ l and 600 μ l with increments of 100 μ l. There was no difference observed between all volumes tested.

It was found that evaporation of water was a potentially important variable, (personal communication; David. R. Bell, University of Nottingham) and it was important to control this.

It was noted that after the assay plates were sealed with two layers of laboratory film, wrapped in a damp paper towel and enclosed in an air tight plastic container for incubation, results were more consistent. The damp paper towel provided humidity while the air tight plastic container reduced evaporation.

The egg isolation results were initially variable and conditions for isolation of eggs were optimised. A protocol was established which yielded robust and reliable isolation of viable L1 larvae.

3.3 Sodium azide positive control

Sodium azide was recommended as a toxin to use as a positive control for the *C.elegans* toxicity due to its well known toxic effects. Sodium azide is a highly toxic compound having chemical properties and biological effects in common with cyanide (Smith 1991). Sodium azide inhibits cytochrome oxidase, by binding irreversibly to the heme cofactor, and thereby inhibiting respiration.



3.3 Chemical structure of Sodium azide – taken from www.wikipedia.org

A positive control assay was set up with sodium azide along side all the toxicity assays. Sodium azide has been successful as a positive control, with consistent results observed each time for all assays.

The IC_{50} values of the L1 growth sodium azide controls for the 5 toxins were, 0.000989mM (95% CI = 0.000514 to 0.00764), 0.00160mM (95% = 0.000730 to 0.00350), 0.00318mM (95% CI = 0.00113 to 0.00895), 0.00214mM (95% CI = 0.000863 to 0.00512), and 0.00486mM (95% 0.00202 to 0.0116).

The IC_{50} of the brood size sodium azide control for the 5 toxins were 0.000129mM (95% CI = 0.0000752 to 0.000223), 0.000175mM (95% CI = 0.0000832 to 0.000369), 0.000147mM (95% CI = 0.0000794 to 0.000274), 0.000190mM (95% CI = 0.0000839 to 0.000430) and 0.000167mM (95% CI = 0.0000824 to 0.000340).

The IC_{50} values of the lethality assay sodium azide controls for the 4 toxins were 4.34mM (95% CI = 2.90 to 7.11), 4.11mM (95% CI = 1.82 to 8.26), 4.20mM (95% CI = 2.07 to 8.09), 4.17mM (95% CI = 1.73 to 6.21).

There is considerable overlapping of 95% confidence intervals, seen for all the IC_{50} values for all three assays, demonstrating the consistent inhibitory effect on growth, brood size and lethality and confirming that the assays produce reliable, consistent results.

The mean IC_{50} value for the L1 growth sodium azide controls was 0.00255 +/-0.00152.

The coefficient of variation is 0.6.

The mean IC_{50} value for the brood size sodium azide controls was 0.000162 +/-0.000024. The coefficient of variation is 0.15.

The mean IC_{50} value for the lethality sodium azide controls was 4.205 +/-0.0975.

The coefficient of variation is 0.02.

The mean IC_{50} values for the three assays are significantly different showing that sodium azide has different potencies at particular stages of the life cycle. It shows a higher potency to larval and embryonic stages than adults.

The coefficient of variation of the IC_{50} values for the three assays is small and shows that there is little variation, and therefore show that the assays give reproducible results and are a reliable measure of toxicity.

3.4 Quinine results

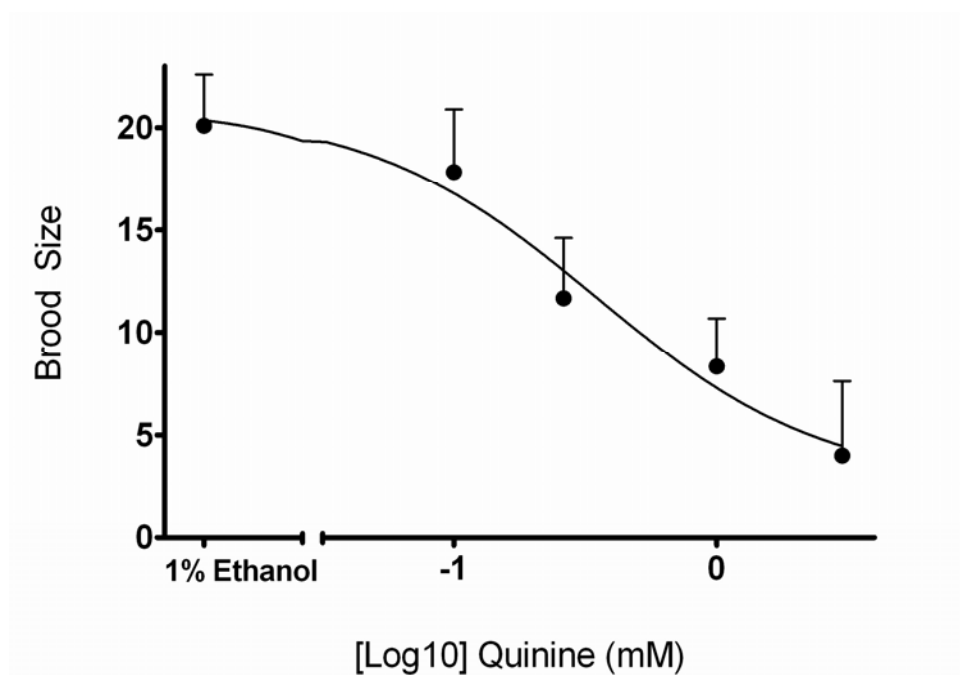


Figure 3.4.1 Effect of quinine on brood size. Individual L4 *C.elegans* N2 worms were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of quinine or vehicle control (1% Ethanol) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

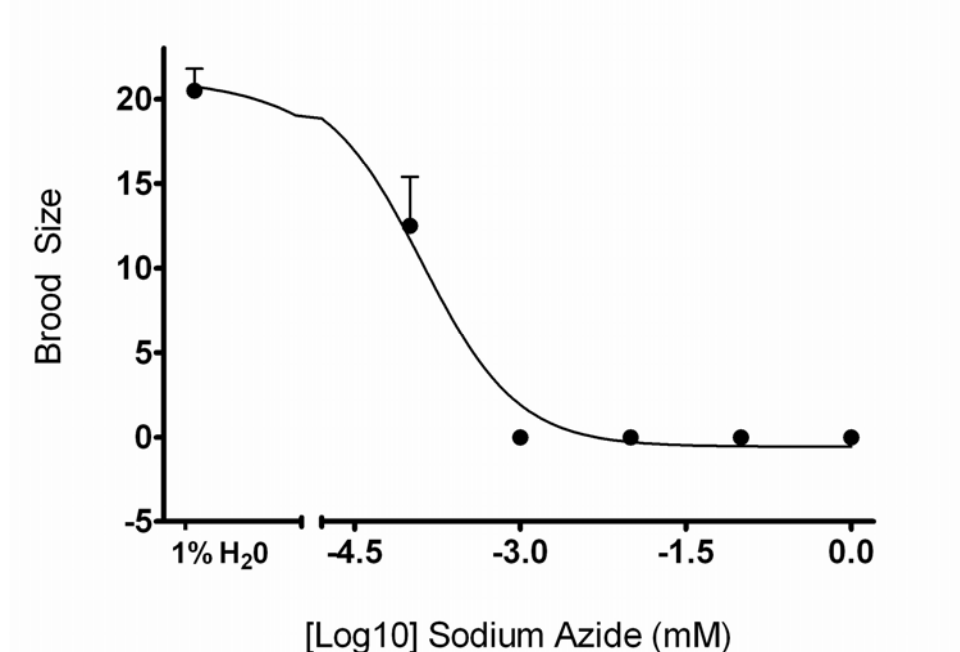


Figure 3.4.2 Effect of sodium azide on brood size. This experiment was set up and analysed as in figure 3.4.1 as a positive control.

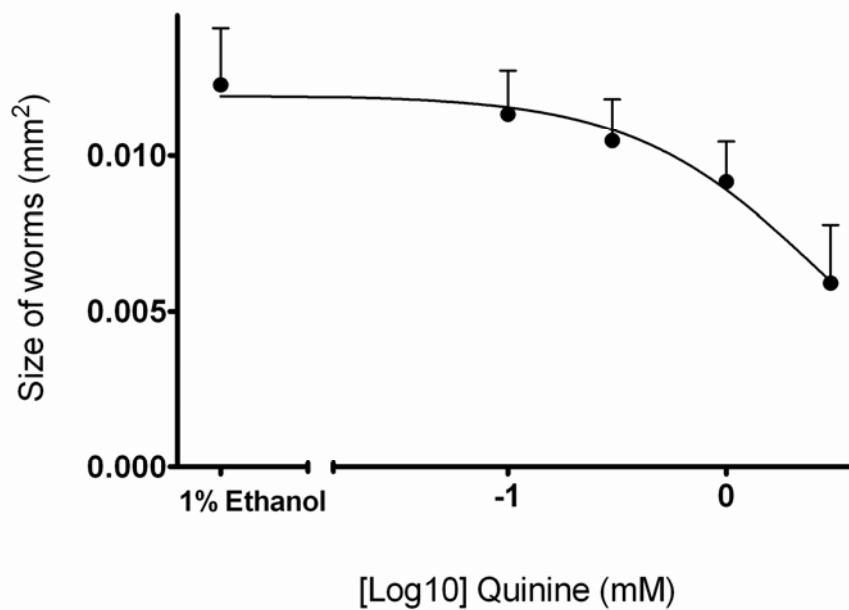


Figure 3.4.3 Effect of quinine on growth. 40 L1 worms per dose group were placed in 400µl of liquid medium (s-basal) with varying concentrations of quinine or vehicle control (1% ethanol) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

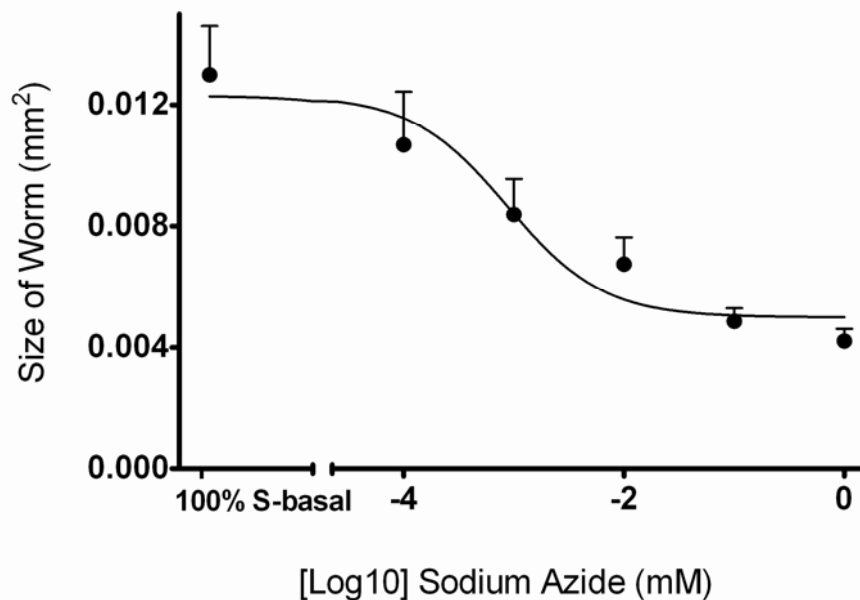


Figure 3.4.4 Effect of sodium azide on growth. This experiment was set up and analysed as in figure 3.4.3 as a positive control.

The effect of quinine on brood size was tested. Individual L4 animals were exposed to quinine at concentrations up to 3mM for 3 days. Figure 3.4.1 shows the results from this quinine brood size assay with the mean brood size for three replicates and standard deviations plot against the concentrations of quinine tested. A t-test was carried out to test if the difference between the treated and control values were statistically significant. The brood size was inhibited in a dose-dependent manner. The mean brood size was 20 +/- 2.5 for the control (1% ethanol). The maximum inhibition was observed at 3mM. The IC_{50} was 0.568mM (95% CI = 0.347 to 0.929)

The effect of quinine on larval growth was tested. L1 larvae were exposed to quinine at concentrations up to 3mM for 3 days. The animals were then photographed and measured using Adobe Photoshop. Figure 3.4.3 shows the results from this L1 growth assay with the mean size and standard deviations plot against the concentrations of quinine tested, for $n \geq 30$.

Growth inhibition was also affected in dose-dependent manner. The L1 *C.elegans* were $0.0042\text{mm}^2 \pm 0.0004$ when placed in the wells. The maximum growth recorded was 0.0123 ± 0.0018 for the control (1% ethanol). The maximum inhibition of growth was observed at 3mM. The IC_{50} was 0.711mM (95% CI = 0.318 to 1.59). Growth was significantly inhibited at 0.3mM and above $t = 0.086$. There was no significant effect on growth at 0.1mM $t = 0.99$. *C.elegans* from both the brood size and growth size assay remained viable at all concentrations tested.

It is possible that the brood size and growth size could be inhibited further with higher concentrations of quinine, but it was not possible to determine toxicity at concentrations above 3mM due to crystallisation of the quinine. An effect of lethality could not be determined as a concentration above 3mM is required to cause lethality.

The sodium azide positive control results had an IC_{50} of 0.000989mM (95% 0.000514 to 0.00764) and 0.000129mM (95% CI = 0.0000752 to 0.000223) for the L1 growth and

brood size assays respectively. These values are comparable with previous results, and validates that the assays are working properly and the worms are behaving as normal.

The results show that quinine does have an effect on *C.elegans*, and inhibits larval growth and brood size.

3.5 Chloroquine Results

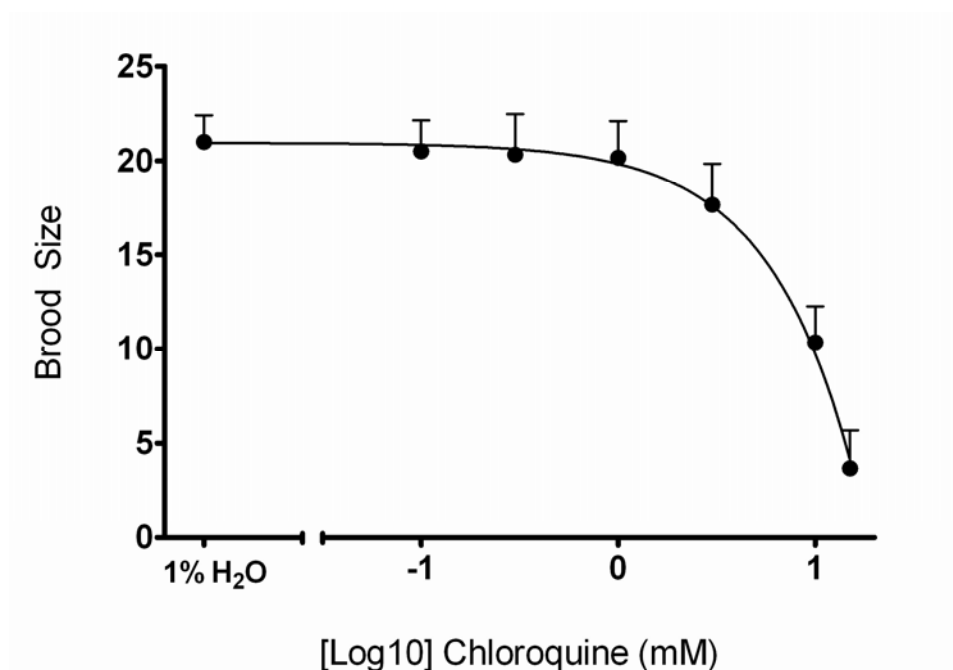


Figure 3.5.1 Effect of chloroquine on brood size. Individual L4 *C.elegans* N2 worms were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of chloroquine or vehicle control (1% H₂O) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism. This experiment was repeated with similar results.

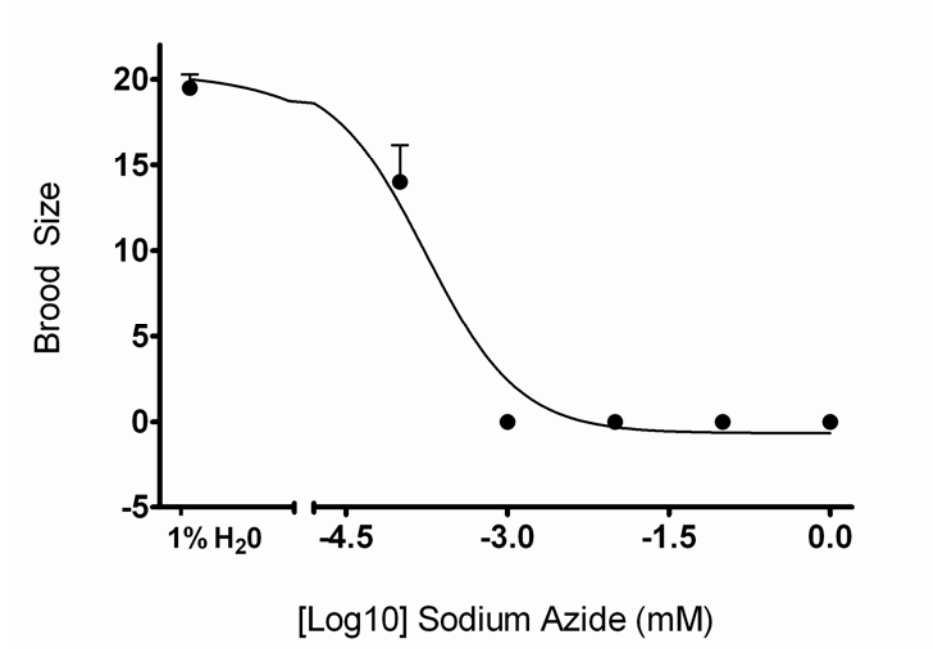


Figure 3.5.2 Effect of sodium azide on brood size. This experiment was set up and analysed as in figure 3.5.1 as a positive control.

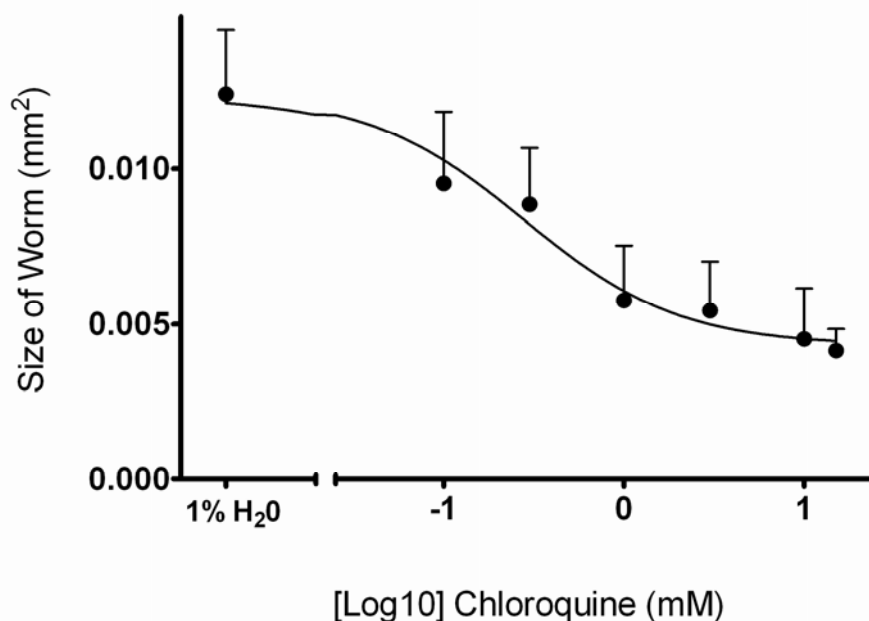


Figure 3.5.3 Effect of chloroquine on growth. 40 L1 worms per dose group were placed in 400µl of liquid medium (s-basal) with varying concentrations of chloroquine or vehicle control (1% H₂O) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results

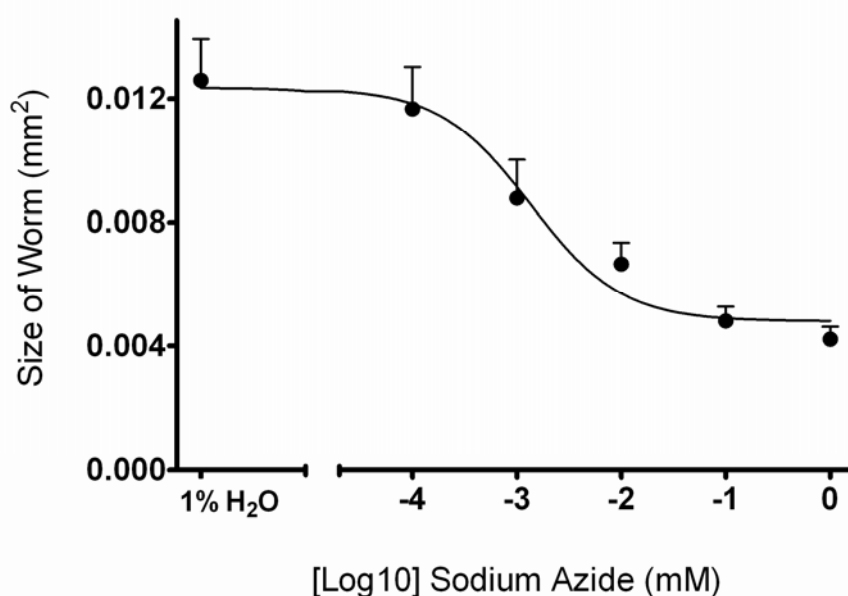


Figure 3.5.4 Effect of sodium azide on growth. This experiment was set up and analysed as in figure 3.5.3 as a positive control.

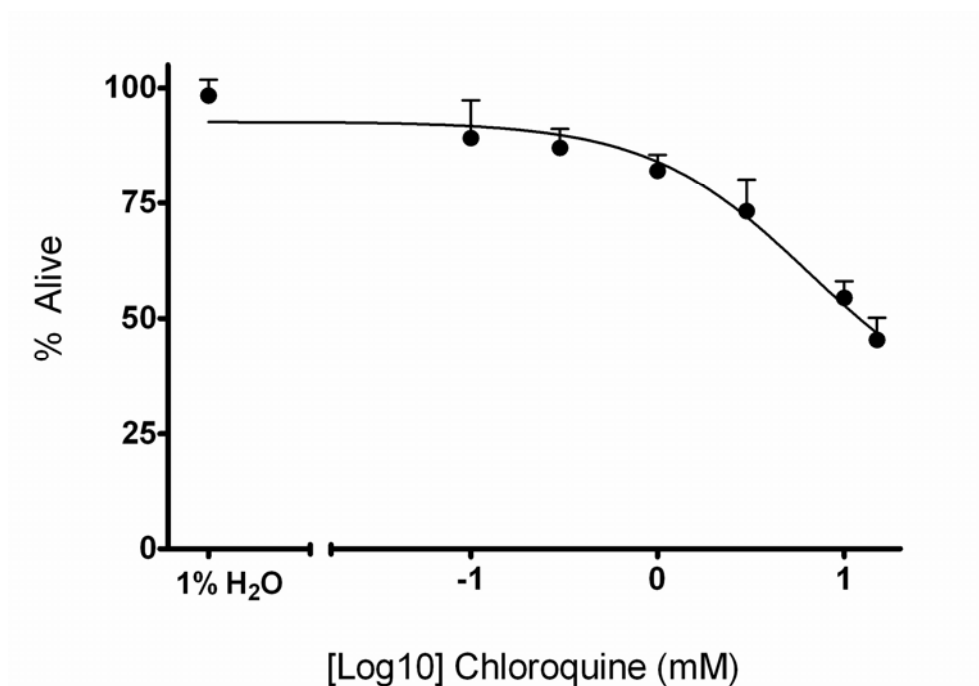


Figure 3.5.5 Effect of chloroquine on lethality. 50 L4 *C.elegans* N2 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of chloroquine or vehicle control (1% H₂O) and incubated for 3 days at 15°C, as described in section 2.4.3. After incubation the percentage of worms alive was determined by counting the number of viable and dead worms in each well based on movement. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean percentage of viable worms (represented by the dots) where n=150. The data was fitted to a non linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

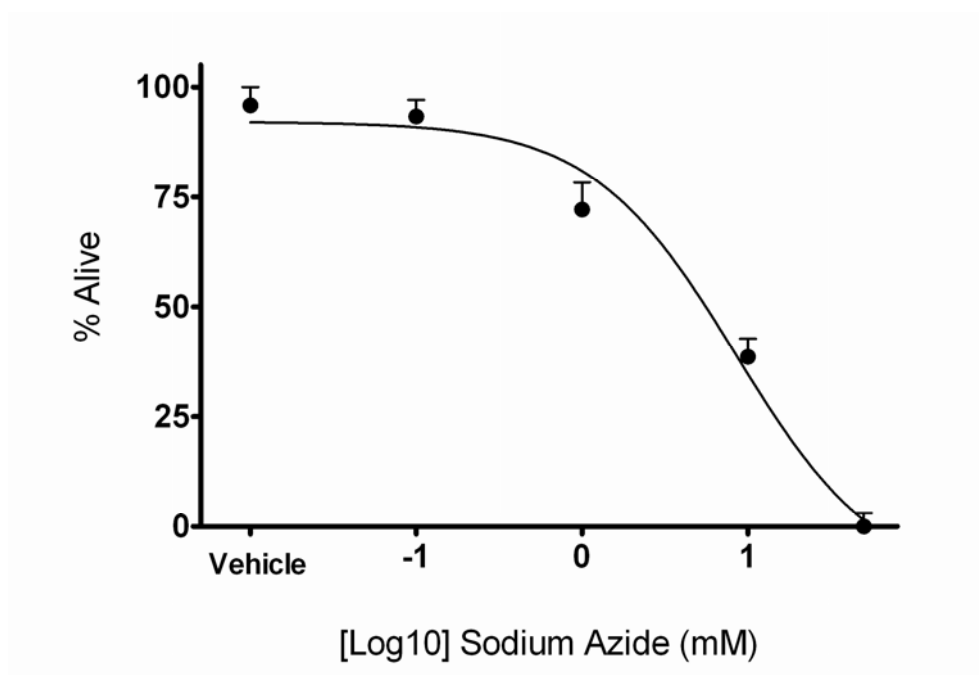


Figure 3.5.6 Effect of sodium azide on lethality. This assay was set up and analysed as described for figure 3.5.5 as a positive control.

The effect of chloroquine on brood size, growth and lethality of *C.elegans* was tested.

Chloroquine had a dose-dependent effect on all three assays. A t-test was carried out to test if differences between the treated and the control values were statistically significant. Individual L4 animals were exposed to concentrations of chloroquine up to 15mM for 3 days. Figure 3.5.1 shows the results from this chloroquine brood size assay, with the mean brood size of three replicates and standard deviations plot against the concentrations of chloroquine tested. There was an inhibitory effect on brood size observed with increasing concentrations of chloroquine. Inhibition was greatest at 15mM, the highest concentration tested. The mean brood size for the control (1% H₂O) was 21 +/- 1.414. The IC₅₀ was 8.39mM (95% CI = 6.64 to 10.6). At 0.1mM, 0.3mM and 1mM there was no significant effect on brood size compared to the control; t = 0.4, t = 0.47 and t = 0.6 respectively.

L1 larvae were exposed to concentrations of chloroquine up to 15mM. After 3 days, they were photographed and measured using Adobe Photoshop. Figure 3.5.3 shows the results from this L1 growth assays with the mean size and standard deviations plot against the concentrations of quinine tested for n≥30.

Chloroquine had an inhibitory effect on growth, of L1 *C.elegans*. The *C.elegans* had a mean size of 0.004mm² +/- 0.0025 when they were placed in the wells. The greatest inhibition was observed at 15mM, where the mean size was 0.0041mm² +/- 0.0007; there was no significant difference from the start size; t = 0.0357. The mean size recorded for the control (1%H₂O) was 0.0124mm² +/- 0.0004. The IC₅₀ was 0.162mM (95% CI = 0.0834 to 0.316).

L4 *C.elegans* were exposed to concentrations of chloroquine up to 15mM. After 3 days the worms were scored as dead or alive based on movement and the % alive was determined. Figure 3.5.5 shows the results from this lethality assay with the mean % viability of three replicates and standard deviations plot against the concentrations of

chloroquine tested. Chloroquine did have an effect on the lethal dose assay but 100% kill was not achieved for the concentrations tested. The maximum effect on lethality was observed at 15mM where the % alive was 45.39 +/- 4.73. The IC_{50} was 10.45mM (95% CI = 6.58 to 16.6). It was not possible to test above 15mM of chloroquine, as the compound was not soluble above this concentration.

The sodium azide positive control results had an IC_{50} of 0.00160mM (95% 0.000730 to 0.00350), 0.000175 (95% CI = 0.0000832 to 0.000369) and 4.34 (2.90 to 7.11) for the L1 growth, brood size, and lethality assays respectively. These values are comparable with previous results, and validates that the assays are working properly and the worms are behaving as normal.

The results show that chloroquine does have an effect on *C.elegans* and inhibits larval growth brood size and lethality.

3.6 Furan Results

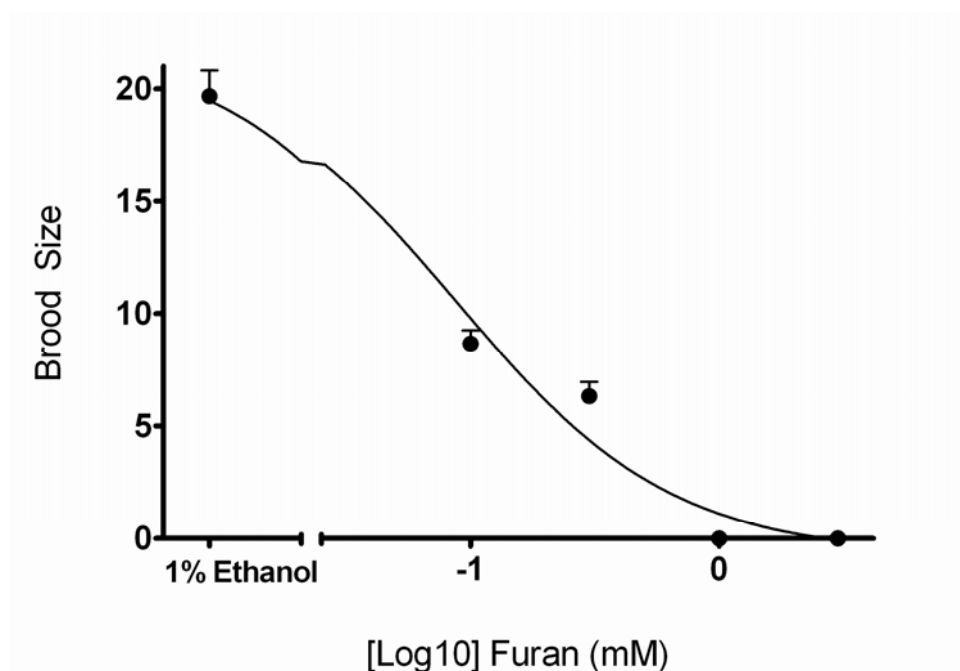


Figure 3.6.1 Effect of furan on brood size. Individual L4 *C.elegans* N2 worms were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of furan or vehicle control (1% Ethanol) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

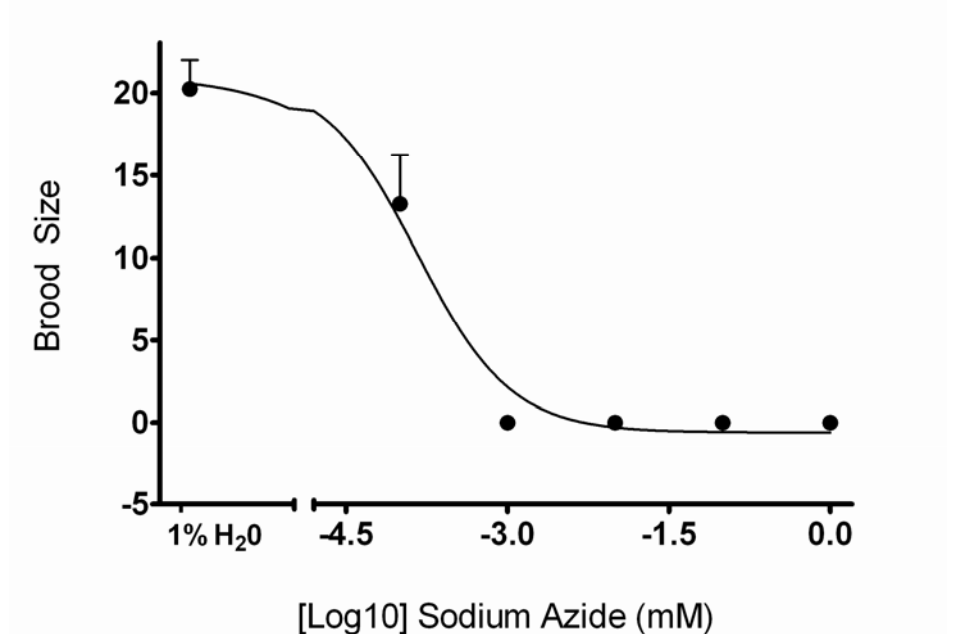


Figure 3.6.2 Effect of sodium azide on brood size. This experiment was set up and analysed as in figure 3.6.1 as a positive control.

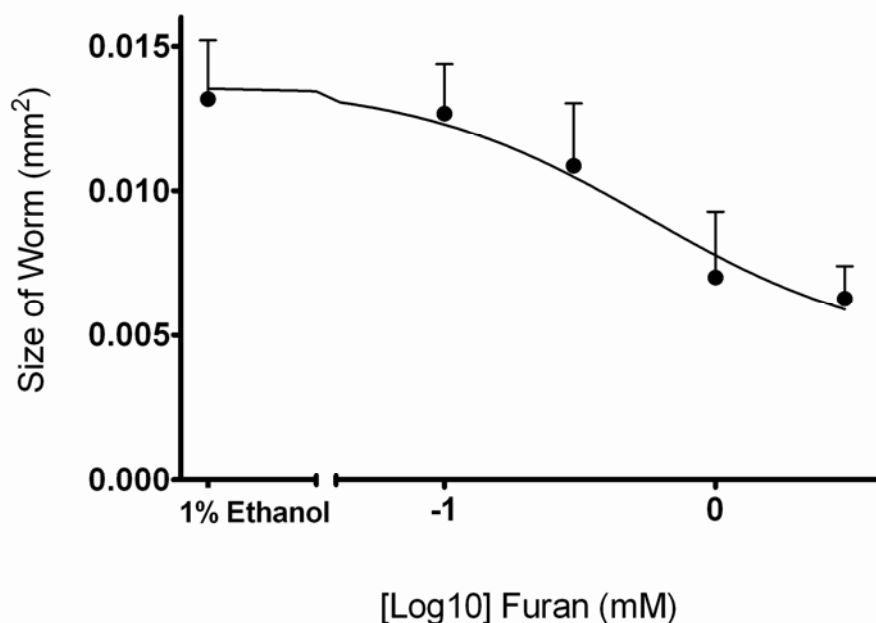


Figure 3.6.3 Effect of furan on growth. 40 L1 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of furan or vehicle control (1% ethanol) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

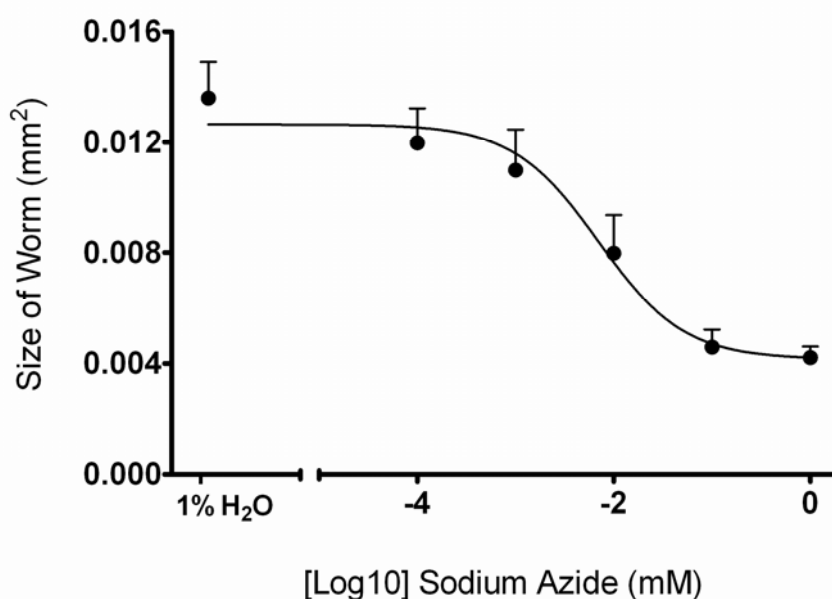


Figure 3.6.4 Effect of sodium azide on growth size. This experiment was set up and analysed as in figure 3.6.3 as a positive control

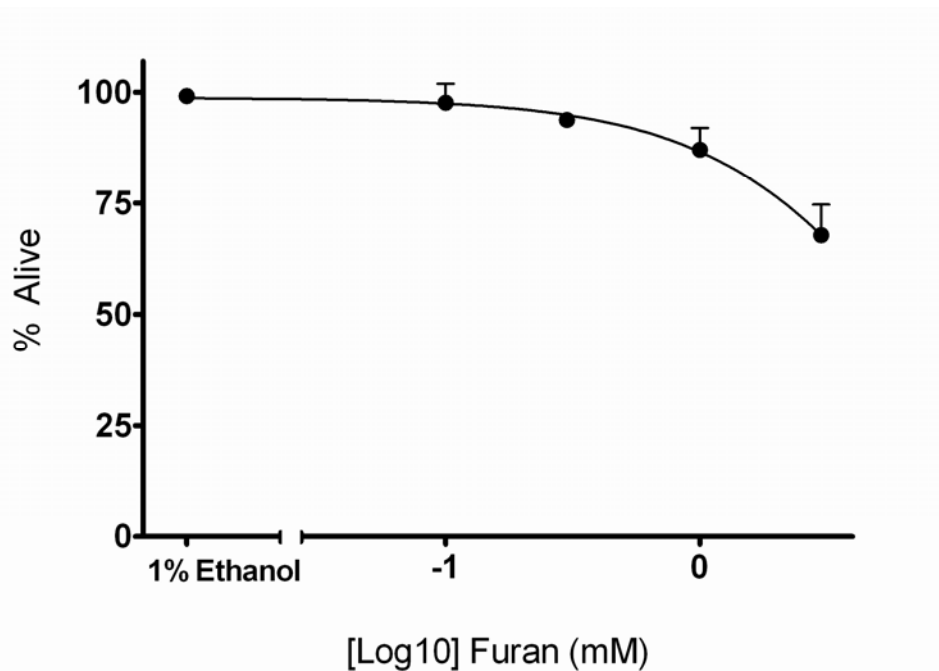


Figure 3.6.5 Effect of furan on lethality. 50 L4 *C.elegans* N2 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of furan or vehicle control (1% ethanol) and incubated for 3 days at 15°C, as described in 2.4.3. After incubation the percentage of worms alive was determined by counting the number of viable and dead worms in each well based on movement. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean percentage of viable worms (represented by the dots) where n=150. The data was fitted to a non linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

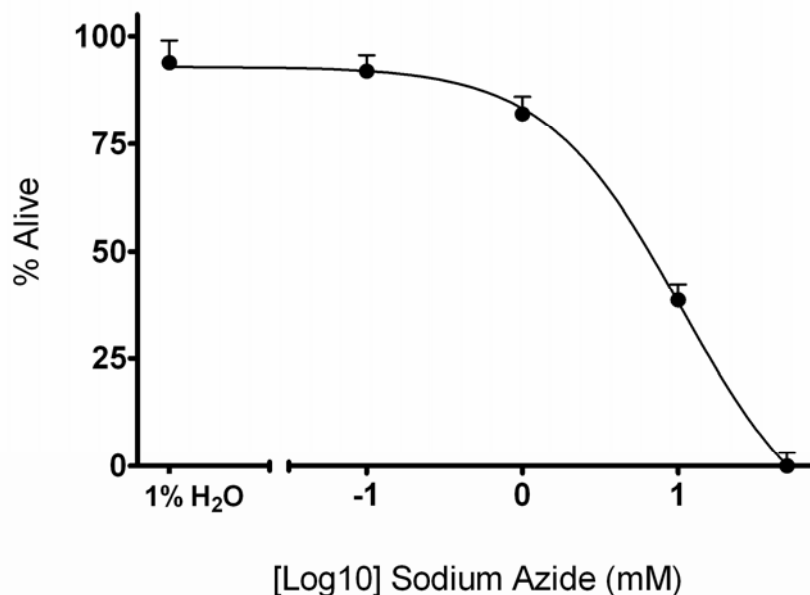


Figure 3.6.6 Effect of sodium azide on lethality. This assay was set up and analysed as described for figure 3.6.5 as a positive control.

The effect of furan on larval growth, brood size and lethality was tested. Furan had a dose-dependent effect on the brood size, growth and lethality of *C.elegans*. A t-test was carried out to test if differences between the treated and the control values were statistically significant.

Individual L4 animals were exposed to concentrations of furan up to 3mM for 3 days and the brood size was scored. Figure 3.6.1 shows the results from this brood size assays with the mean brood size of three replicates and standard deviations plot against the concentrations of furan tested.

The brood size of *C.elegans* was greatly inhibited by increasing levels of furan. The mean brood size recorded for the control (1% ethanol) was 19.6 +/- 1.15. The brood size was inhibited by 100% at 1mM and above. The IC₅₀ was 0.102mM (95% CI = 0.0454 to 0.231). The *C.elegans* remained viable at all the concentrations tested but those exposed to 3mM and 1mM exhibited very little movement.

L1 *C.elegans* were exposed to concentrations of furan up to 3mM. After 3 days the worms were photographed and measured using Adobe Photoshop. Figure 3.6.3 shows the results from this L1 growth assay with the mean size and standard deviations plot against the concentrations of furan tested for n≥30.

Furan had an inhibitory effect on the growth of L1 *C.elegans*. The L1 *C.elegans* were 0.0041mm² +/- 0.0003 when placed in the wells. The mean size was 0.0132mm² +/- 0.002 for the control (1% ethanol). The maximum inhibition of growth was observed at 3mM where the mean size was 0.0063mm² +/- 0.001. The IC₅₀ was 0.407mM (95% CI = 0.370 to 0.447). At 0.1mM there was no significant effect on growth, compared to the control size; t = 0.33. The *C.elegans* remained viable at all the concentrations tested.

L4 animals were exposed to concentrations of furan up to 3mM. After 3 days the worms were scored as dead or alive based on movement to determine the % viability. Figure

3.6.5 shows the results from this lethality assay with the mean % viability of three replicates and standard deviations plot against the concentrations of furan tested.

Furan had an inhibitory effect on lethality although a 100% kill was not achieved with the concentrations tested. The maximum effect was observed at 3mM where the % viable was 67.8 +/- 6.86. At 0.1mM there is no significant effect on lethality compared to the control value; $t = 0.58$. The IC_{50} was 6.74mM (95% CI = 4.95 to 9.17) It was not possible to test concentrations above 3mM without the concentration of ethanol having a significant effect on the results.

The sodium azide positive control results had an IC_{50} of 0.00318mM (95% 0.00113 to 0.00895), 0.000147 (95% CI = 0.0000794 to 0.000274) and 4.11 (1.82 to 8.26) for the L1 growth, brood size, and lethality assays respectively. These values are comparable with previous results, and validates that the assays are working properly and the worms are behaving as normal.

The results show that furan does have effect on *C.elegans* and inhibits larval growth, brood size and lethality.

3.7 DDT Results

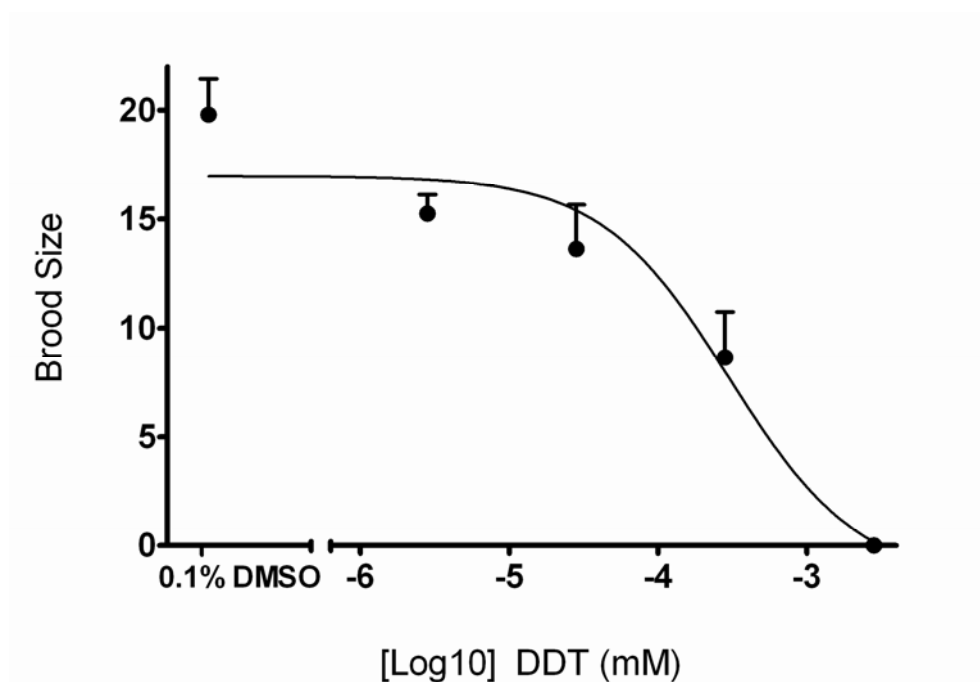


Figure 3.7.1 Effect of DDT on brood size. Individual L4 *C.elegans* N2 worms were placed in 400µl of liquid medium (s-basal) with varying concentrations of DDT or vehicle control (0.1% DMSO) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

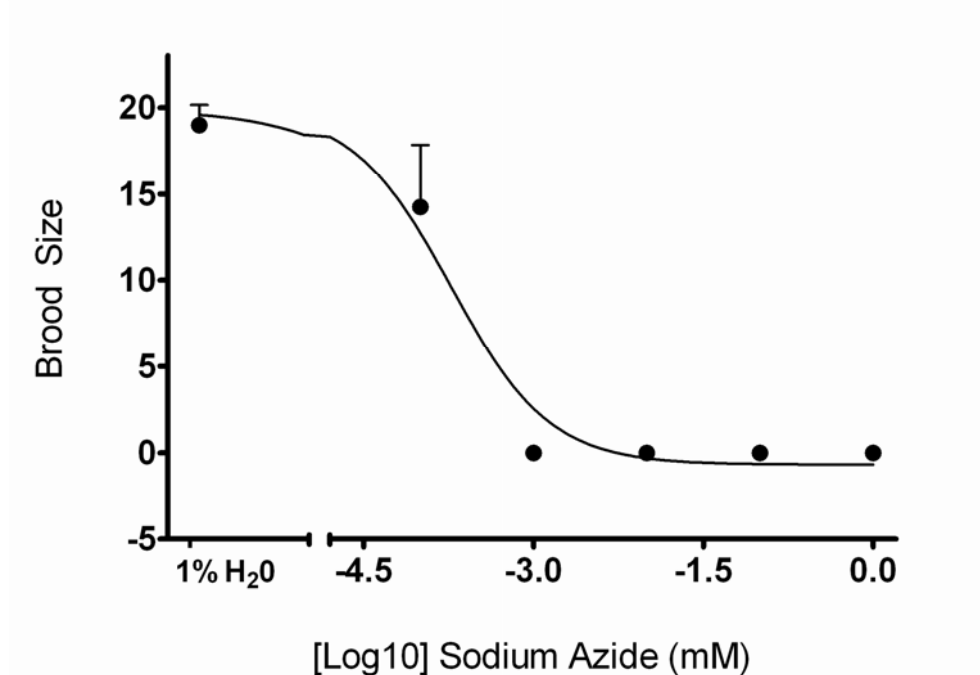


Figure 3.7.2 Effect of sodium azide on brood size. This experiment was set up and analysed as in figure 3.7.1 as a positive control

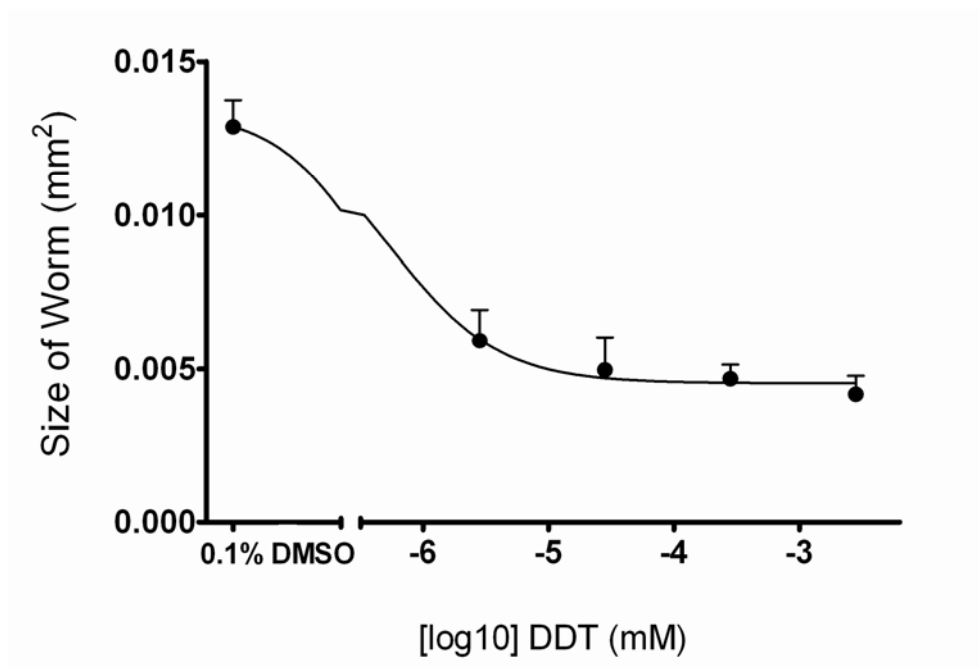


Figure 3.7.3 Effect of DDT on growth. 40 L1 worms per dose group were placed in 400µl of liquid medium (s-basal) with varying concentrations of DDT or vehicle control (0.1% DMSO) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

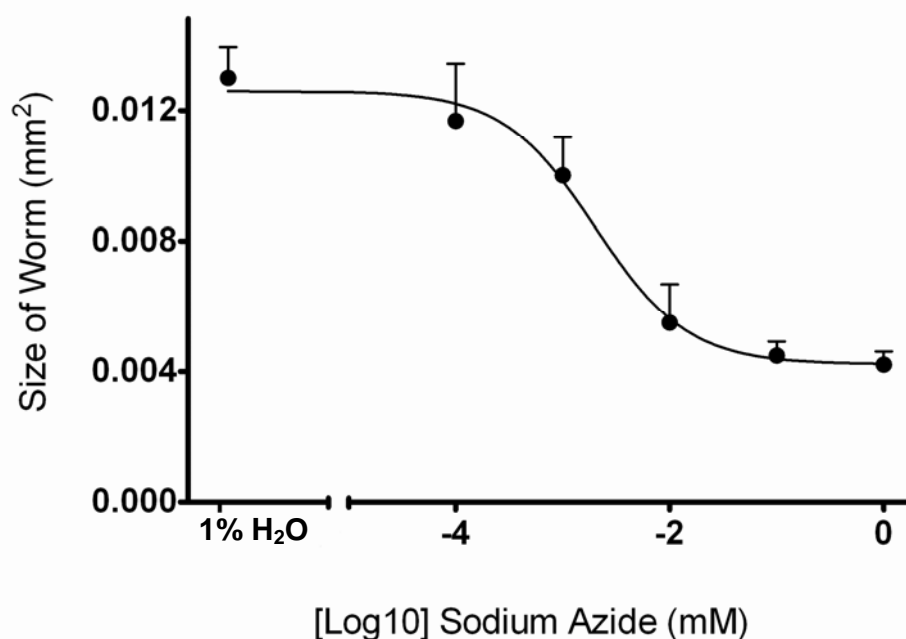


Figure 3.7.4 Effect of sodium azide on growth. This experiment was set up and analysed as in figure 3.7.3 as a positive control.

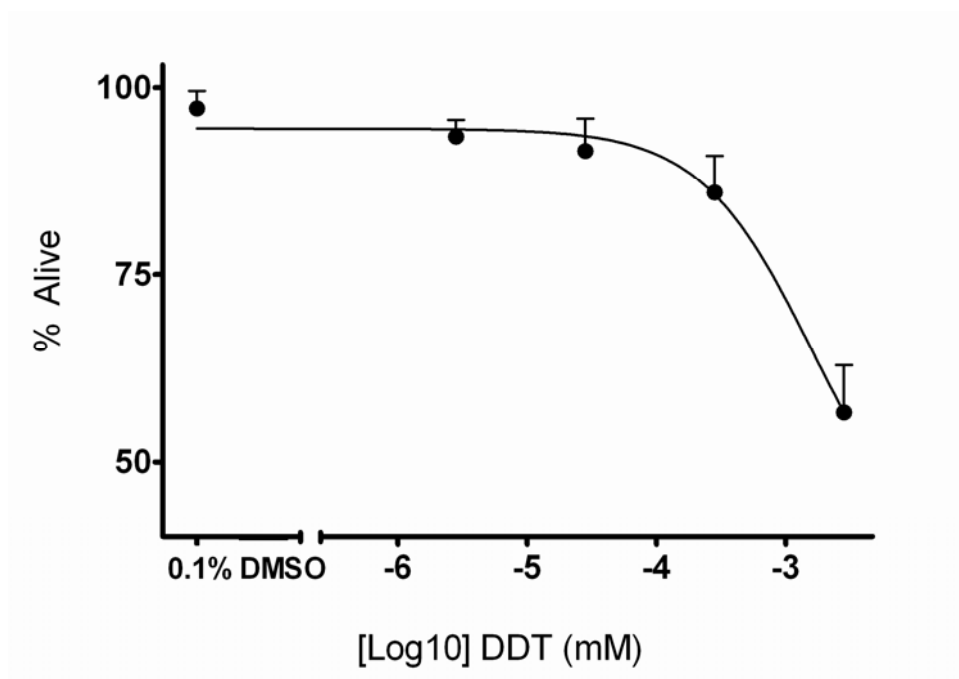


Figure 3.7.5 Effect of DDT on lethality. 50 L4 *C.elegans* N2 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of DDT or vehicle control (0.1% DMSO) and incubated for 3 days at 15°C, as described in section 2.4.3. After incubation the percentage of worms alive was determined by counting the number of viable and dead worms in each well based on movement. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean percentage of viable worms (represented by the dots) where $n=150$. The data was fitted to a non linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

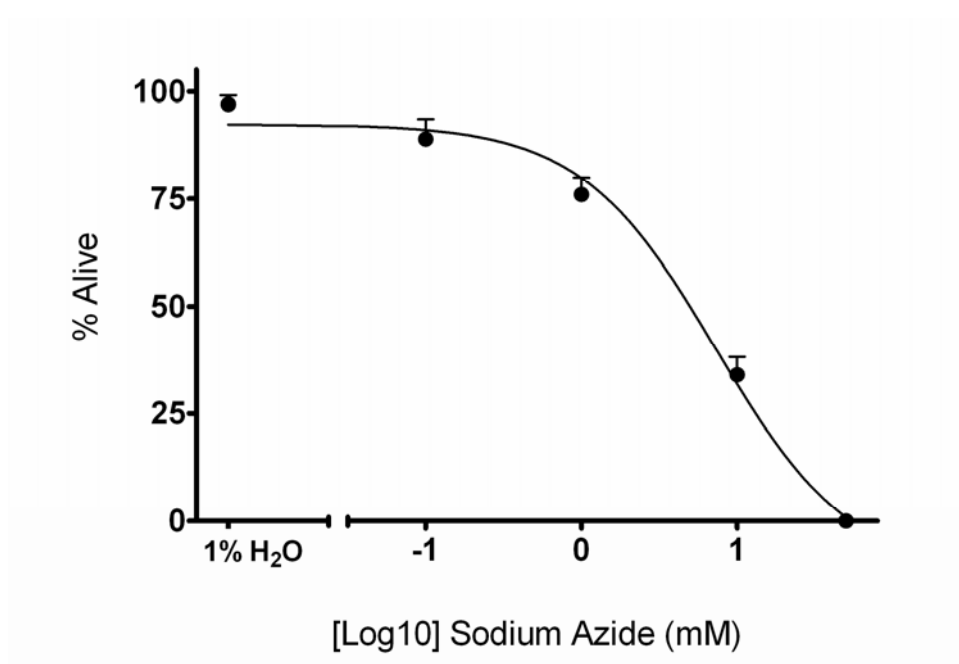


Figure 3.7.6. Effect of sodium azide on lethality. This assay was set up and analysed as described for figure 3.7.5 as a positive control.

The effect of DDT on larval growth, brood size and lethality was tested.

DDT had a dose-dependent inhibitory effect on the brood size, growth and lethality of *C.elegans*. A t-test was carried out to test if differences between the treated and the control values were statistically significant.

Individual L4 *C.elegans* were exposed to concentrations of DDT up to 0.00282mM. After 3 days the brood size was scored. Figure 3.7.1 shows the results from this assay with the mean brood size of three replicates and standard deviations plot against the concentrations of DDT tested.

There was an inhibitory effect was seen in the brood size assay. The brood size was inhibited by 100%, at 0.00282mM DDT. The mean brood size recorded was 19.8 +/- 1.64 for the control (0.1% DMSO). The IC_{50} was 0.0000946mM (95% CI = 0.0000160 to 0.000559). All the worms remained viable for all the concentrations tested.

L1 larvae were exposed to concentrations of DDT up to 0.00282mM. After 3 days the worms were photographed and measured using Adobe Photoshop. Figure 3.7.3 shows the results from this L1 growth assays with the mean growth and standard deviations plot against the concentrations of DDT tested for $n \geq 30$.

A significant inhibitory effect was observed in the L1 growth assay. The worms were $0.0041\text{mm}^2 \pm 0.0001$ when they were placed in the wells. The mean size recorded was $0.0129\text{mm}^2 \pm 0.00086$ for the control (0.1% DMSO). The maximum inhibition of growth was observed at 0.00282mM DDT where the growth size recorded was $0.0041\text{mm}^2 \pm 0.0604$, therefore there was no growth at all since the worms were placed in the wells; $t = 0.002$. The IC_{50} was 0.000000843mM (95% CI = 0.000000162 to 0.00000439). There was very little growth with all the concentrations tested, with only a 0.0018mm^2 increase in size between the highest and lowest concentrations of DDT tested, therefore the range of concentrations tested was too high. All the worms remained viable for the concentrations tested.

L4 animals were exposed to concentrations of DDT up to 0.00282mM. After 3 days the worms were scored as dead or alive based on movement to determine the % viability. Figure 3.7.5 shows the results from this lethality assay with the mean % viability of 3 replicates and standard deviations plot against the concentrations of DDT tested.

DDT had a significant effect on lethality, but 100% kill was not achieved with the concentrations tested. The greatest inhibitory effect observed was at 0.00282mM DDT, where viability was only 56.6% +/- 6.29. At 0.00000282mM and 0.0000282mM there was no significant effect on the lethality of worms compared to the control; $t = 1.99$ and $t = 2.00$ respectively. The IC_{50} was 0.00338mM (95% CI = 0.00173 to 0.00658).

The sodium azide positive control results had an IC_{50} of 0.00214mM (95% 0.000863 to 0.00512), 0.000190 (95% CI = 0.0000839 to 0.000430) and 4.20 (2.07 to 8.09) for the L1 growth, brood size, and lethality assays respectively. These values are comparable with previous results, and validates that the assays are working properly and the worms are behaving as normal.

The results show that DDT does have an effect on *C.elegans* and inhibits larval growth, brood size and lethality.

3.8 Deltamethrin Results

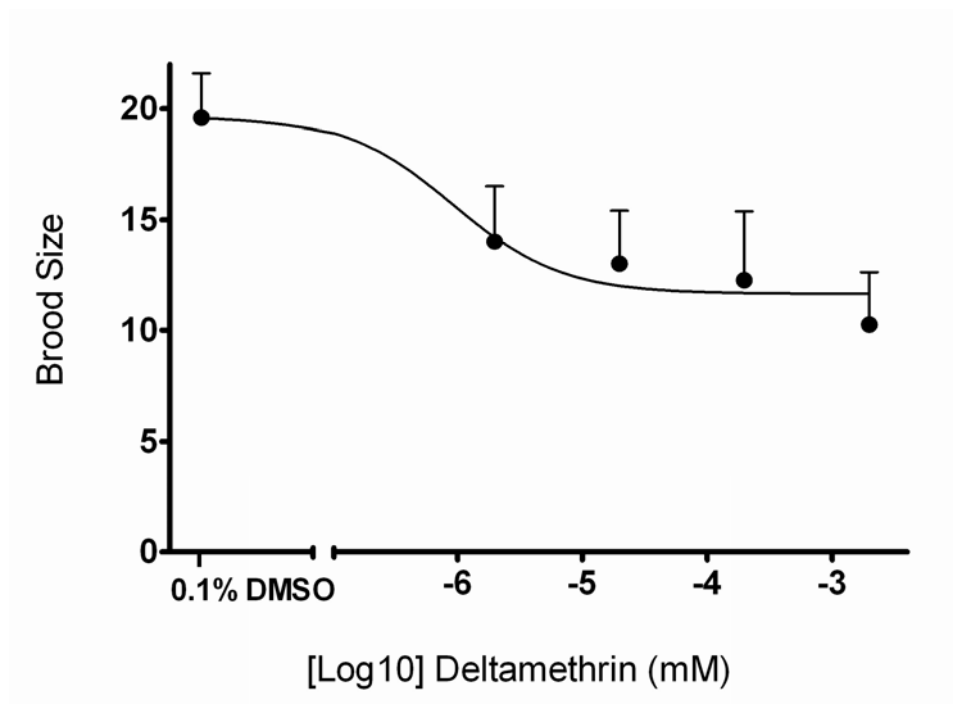


Figure 3.8.1 Effect of deltamethrin on brood size. Individual L4 *C.elegans* N2 worms were placed in 400µl of liquid medium (s-basal) with varying concentrations of deltamethrin or vehicle control (0.1% DMSO) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

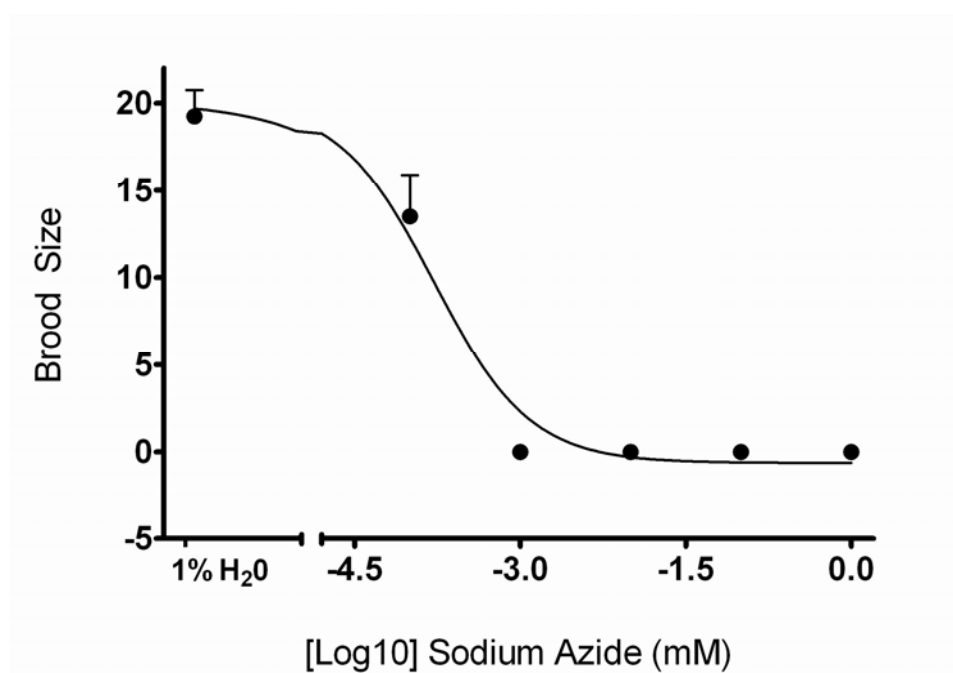


Figure 3.8.2 Effect of sodium azide on brood size. This experiment was set up and analysed as in figure 3.8.1 as a positive control

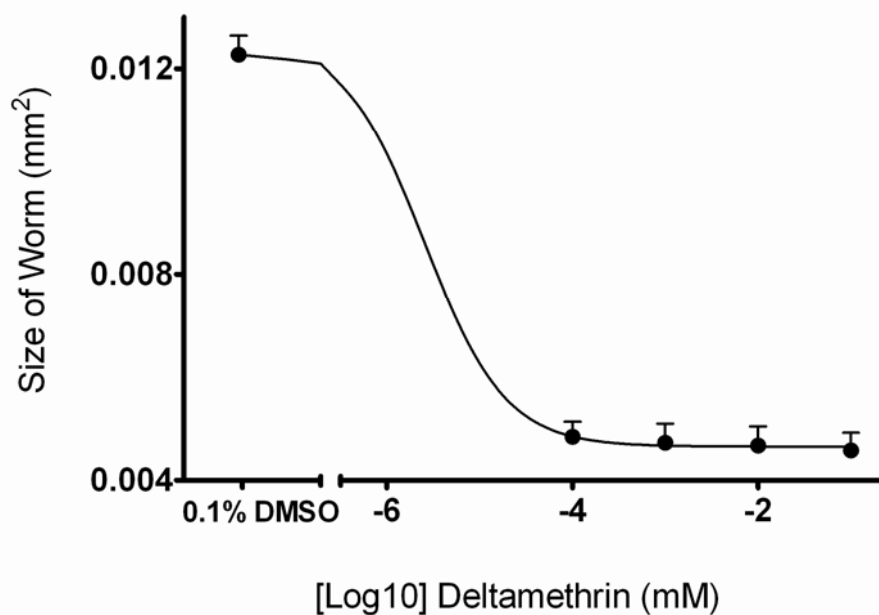


Figure 3.8.3 Effect of deltamethrin on growth. 40 L1 worms per dose group were placed in 400µl of liquid medium (s-basal) with varying concentrations of deltamethrin or vehicle control (0.1% DMSO) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

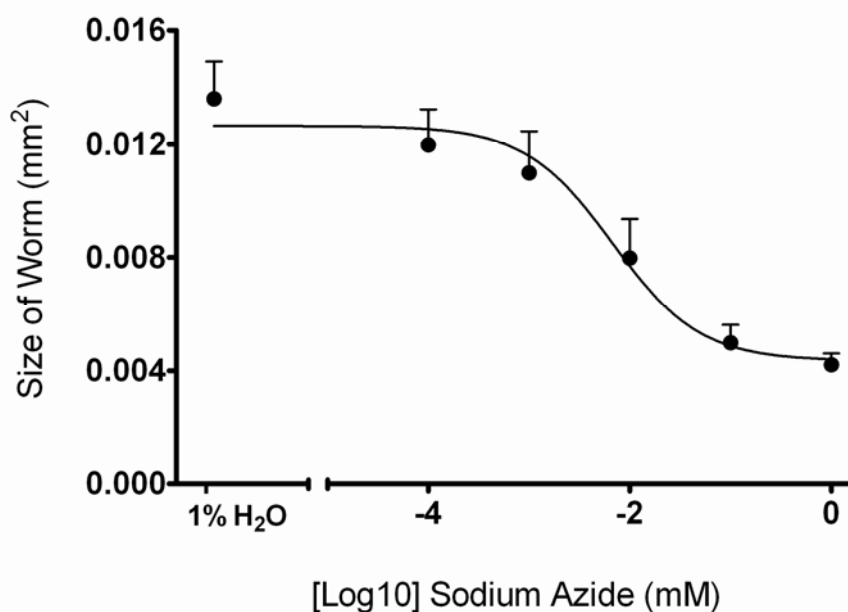


Figure 3.8.4 Effect of sodium azide on growth. This experiment was set up and analysed as in figure 3.8.3 as a positive control.

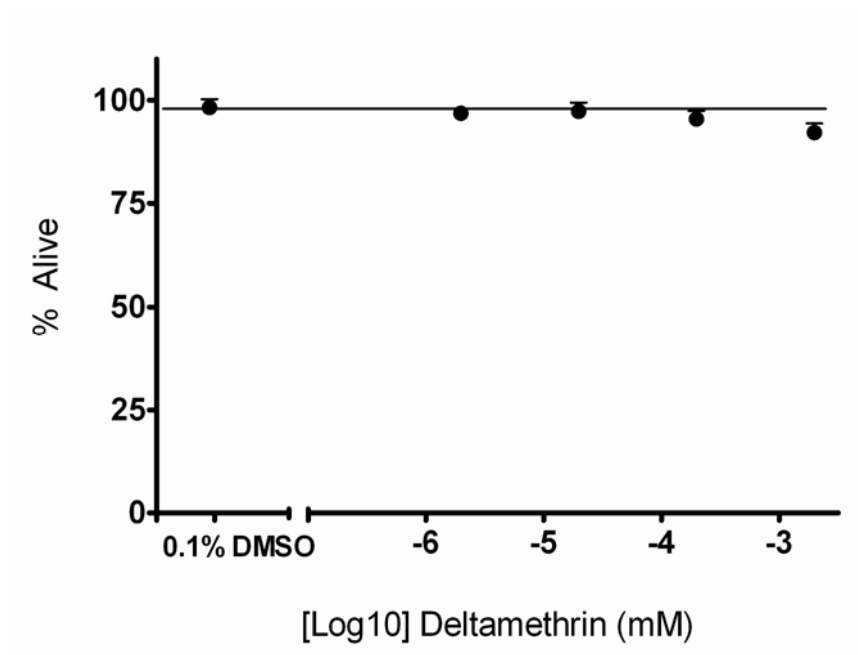


Figure 3.8.5 Effect of deltamethrin on lethality. 50 L4 *C.elegans* N2 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of deltamethrin or vehicle control (0.1% DMSO) and incubated for 3 days at 15°C, as described in section 2.4.3. After incubation the percentage of worms alive was determined by counting the number of viable and dead worms in each well based on movement. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean percentage of viable worms (represented by the dots) where n=150. The data was fitted to a non linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

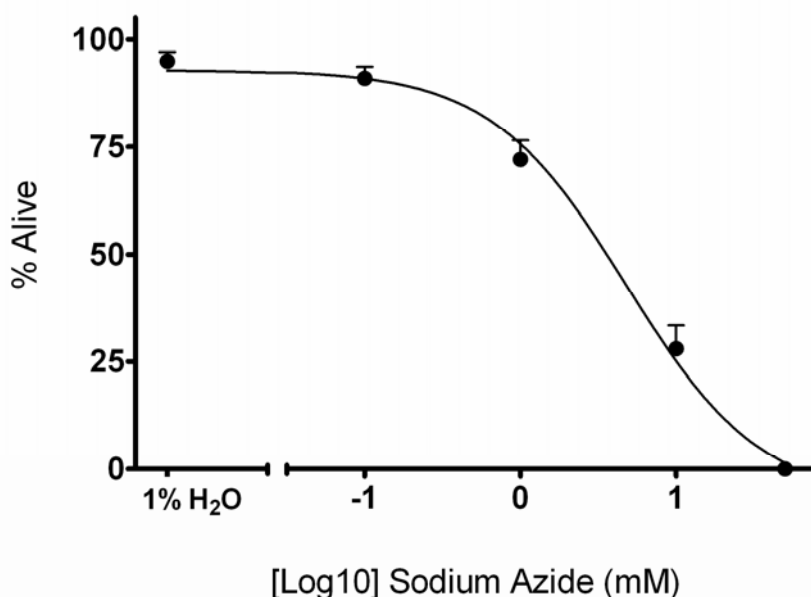


Figure 3.8.6 Effect of sodium azide on lethality. This assay was set up and analysed as described for figure 3.8.5 as a positive control.

Deltamethrin had a dose-dependent effect on lethality and brood size of *C.elegans*; however the effects were only slight. A t-test was carried out to test if differences between the treated and the control values were statistically significant.

Individual L4 *C.elegans* were exposed to concentrations of deltamethrin up to 0.00198mM. The brood size was scored after 3 days. Figure 3.8.1 shows the results from this brood size assay with the mean brood size of three replicates and standard deviations plot against the concentrations of deltamethrin tested.

The maximum brood size recorded was 19.6 +/- 2 for the control (0.1% DMSO). The greatest inhibition of brood size was at 0.00198mM deltamethrin where the mean brood size recorded was 10.25 +/-2.36. There was no significant difference in brood size seen for the concentrations of deltamethrin tested with only an increase of 3.75 between the highest and lowest concentrations tested $t = 2.02$. All the worms remained viable for all the concentrations tested. The IC_{50} was 0.00195mM (95% CI = 0.0000289 to 0.131). There is difficulty interpreting this experiment as there is no obvious dose-response effect.

L1 larvae were exposed to concentrations of deltamethrin up to 0.00198mM. After 3 days the worms were photographed and measured using Adobe Photoshop. Figure 3.8.3 shows the results from the L1 growth assay with the mean size and standard deviations plot against the concentrations of deltamethrin tested for $n \geq 30$.

Deltamethrin had a significant inhibitory effect on the growth of *C.elegans*. The *C.elegans* had a mean size of $0.0042\text{mm}^2 \pm 0.0004$, when they were placed in the wells. The maximum size recorded was $0.012\text{mm}^2 \pm 0.0004$ for the control (0.1% DMSO). The maximum inhibition of growth was recorded at 0.00198mM, 0.000198mM, 0.0000198 and 0.00000198 where the mean size recorded was $0.0046\text{mm}^2 \pm 0.000292$, $0.00468\text{mm}^2 \pm 0.000366$, $0.0047\text{mm}^2 \pm 0.000367$, and $0.00474\text{mm}^2 \pm 0.000337$, respectively. There was no significant difference between the measurements for all the

concentrations tested; $t = 1.01$. There was no significant difference between the start measurement and maximum measurement recorded for exposure to deltamethrin $t = 2.22$, with only 0.0002mm^2 between the two measurements, indicating very early arrest of development. All the worms remained viable for all the concentrations tested. The IC_{50} was 0.00000504mM (95% CI = 0.00000180 to 0.0000141).

L4 animals were exposed to concentrations of deltamethrin up to 0.00198mM . After 3 days the worms were scored as dead or alive based on movement to determine the % viability. Figure 3.8.5 shows the results from this lethality assay with the mean % viability of 3 replicates and standard deviations plot against the concentrations of deltamethrin tested.

Deltamethrin had a slight effect on the lethality of *C.elegans*, but 100% was not achieved for the concentrations tested. The maximum effect was seen at 0.00198mM deltamethrin where the percentage viable was $92\% \pm 2.21$. There was no significant effect on lethality between the control and highest concentration of deltamethrin tested with less than a 6% difference in viability; $t = 3.54$. There was also no significant effect on lethality between the concentrations of deltamethrin tested with less than a 5% difference between the highest and lowest concentrations tested $t = 2.88$. The IC_{50} was 0.0220mM (95% CI = 0.00777 to 0.0621).

The sodium azide positive control results had an IC_{50} of 0.00486mM (95% 0.00202 to 0.0116), 0.000167mM (95% CI = 0.0000824 to 0.000340) and 4.17mM (95% CI = 1.73 to 6.21) for the L1 growth, brood size, and lethality assays respectively. These values are comparable with previous results, and validates that the assays are working properly and the worms are behaving as normal.

The results show that deltamethrin does have an effect on *C.elegans* and inhibits larval growth and brood size.

3.9 Vehicle control assays

The vehicles used in this study were ethanol as a solvent for quinine and furan, and DMSO as a solvent for DDT and deltamethrin. Acetone was also tested as a vehicle but was not used as solvent in the assays. These vehicles were tested at various concentrations. The concentration that had no significant effect on the *C.elegans* was used in the toxicity assays. DMSO and Acetone was tested at 0.1%, 0.5% and 1%. Ethanol was tested at 10%, 6%, 5%, 4%, 3%, 2% and 1%. These were tested along side 100% S-basal as a negative control.

3.9.1 DMSO as a vehicle/solvent.

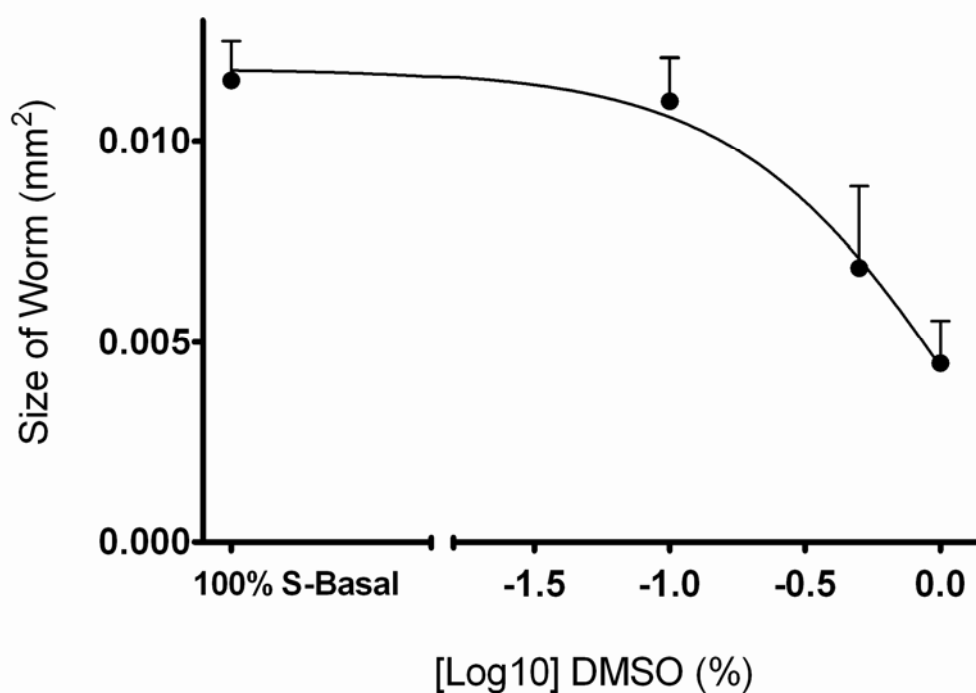


Figure 3.9.1 DMSO vehicle control for growth assay. 40 L1 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of DMSO or vehicle control (100% S-Basal) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was done as a vehicle control to determine if the *C.elegans* could handle this solvent and if it had an effect on growth.

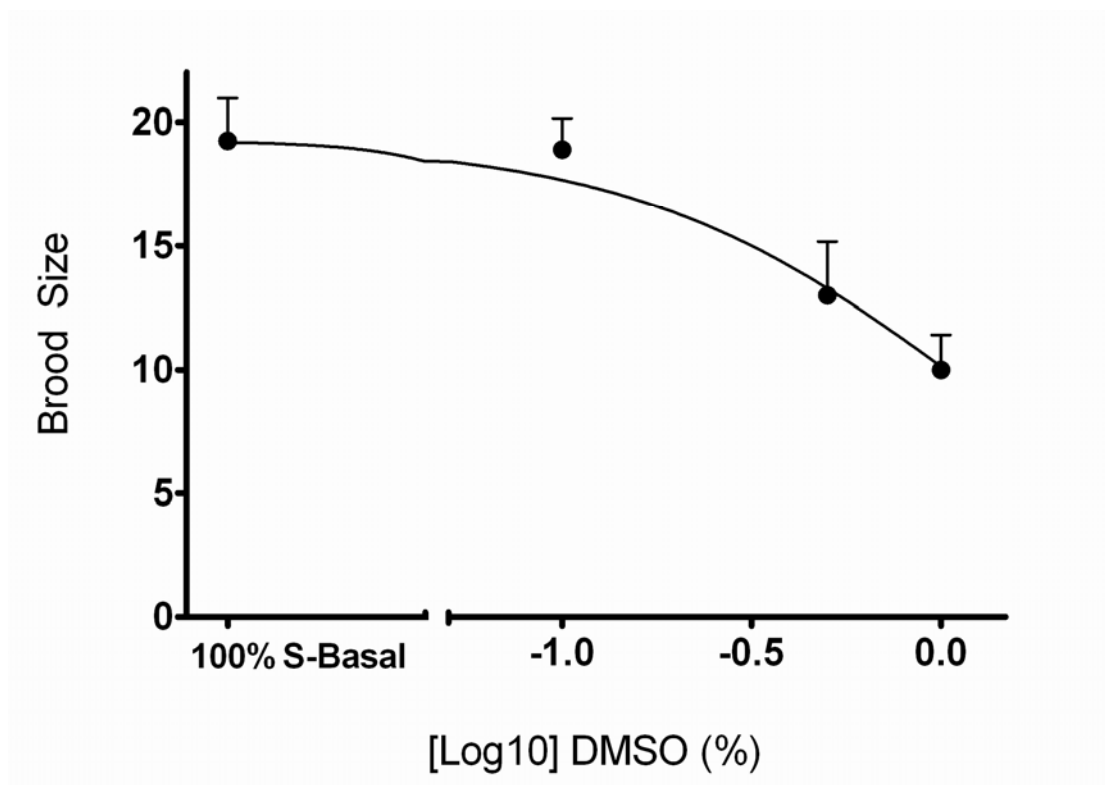


Figure 3.9.2 DMSO vehicle control for brood size assay. Individual L4 *C.elegans* N2 worms were placed in 400µl of liquid medium (s-basal) with varying concentrations of DMSO or vehicle control (100% s-basal) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

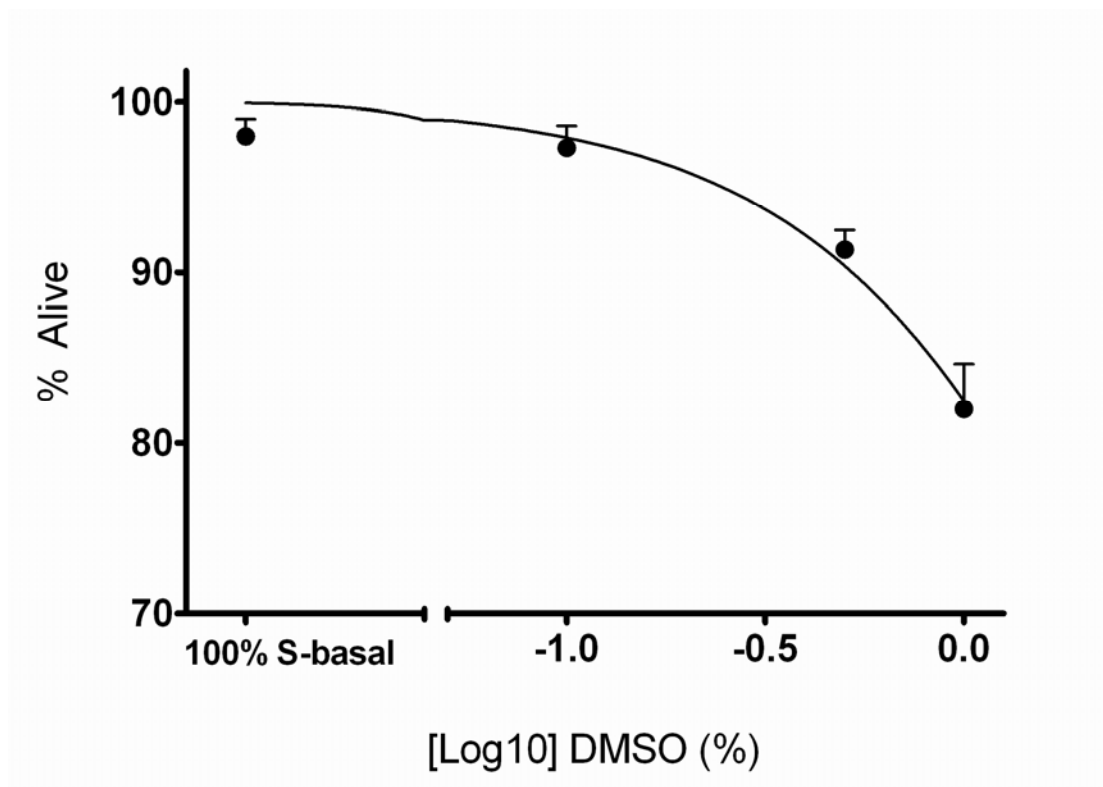


Figure 3.9.3 DMSO vehicle control for lethality assay. 50 L4 *C.elegans* N2 worms per dose group were placed in 400µl of liquid medium (s-basal) with varying concentrations of DMSO or vehicle control (100%) and incubated for 3 days at 15°C, as described in section 2.4.3. After incubation the percentage of worms alive was determined by counting the number of viable and dead worms in each well based on movement. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean percentage of viable worms (represented by the dots) where n=150. The data was fitted to a non linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

The effect of DMSO on brood size, growth and lethality was tested as a vehicle control.

There was a dose-dependent effect seen across all three assays. A t-test was carried out to test if differences between the treated and the control values were statistically significant.

DMSO had a significant inhibitory effect on the brood size of *C.elegans*. The greatest inhibition was at 1% where the mean brood size was 10 ± 1.14. There no significant effect on the brood size at 0.1% compared to the brood size; $t=0.29$. The IC_{50} was 1.113% (95% CI = 0.7759 to 1.597).

Of all the assays, the greatest inhibitory effect was seen for the L1 growth assay. The worms were $0.0042\text{mm}^2 \pm 0.00003$ when they were placed in the wells, and the

maximum growth recorded was $0.0115\text{mm}^2 \pm 0.00097$ for the control (100% S-basal). The greatest inhibition of growth was seen in L1 *C.elegans* exposed to 1% DMSO, where the mean size was $0.0044\text{mm}^2 \pm 0.00006$. There was no significant growth at this concentration; $t = 0.55$, indicating very early cessation of growth. There was no significant difference between the control and lowest concentration of DMSO tested; $t = 1.15$, with only a 0.0005mm^2 difference between the measurements recorded. The IC_{50} was 1.33% (95% CI = 0.0577 to 1.47)

DMSO showed only a slight effect on the lethality of *C.elegans* with only an 18% kill at the highest concentration tested (1% DMSO). There is no significant effect on lethality of *C.elegans* at 0.1% DMSO $t = 0.75$, with less than 0.7% difference from the control. The IC_{50} was 4.71% (95% CI = 3.68 to 6.03).

From these results it is clear that DMSO at 0.1% has no significant effect on *C.elegans*, growth, brood size and lethality and is suitable to use as a vehicle/solvent.

3.9.2 Acetone as vehicle/solvent.

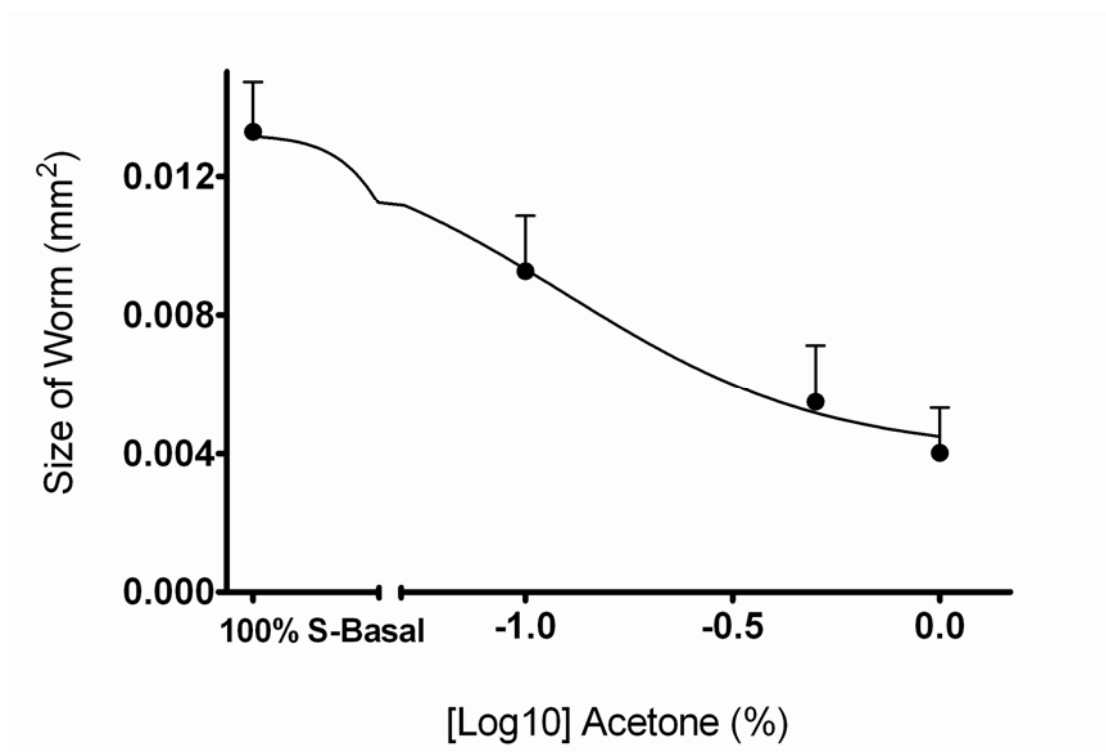
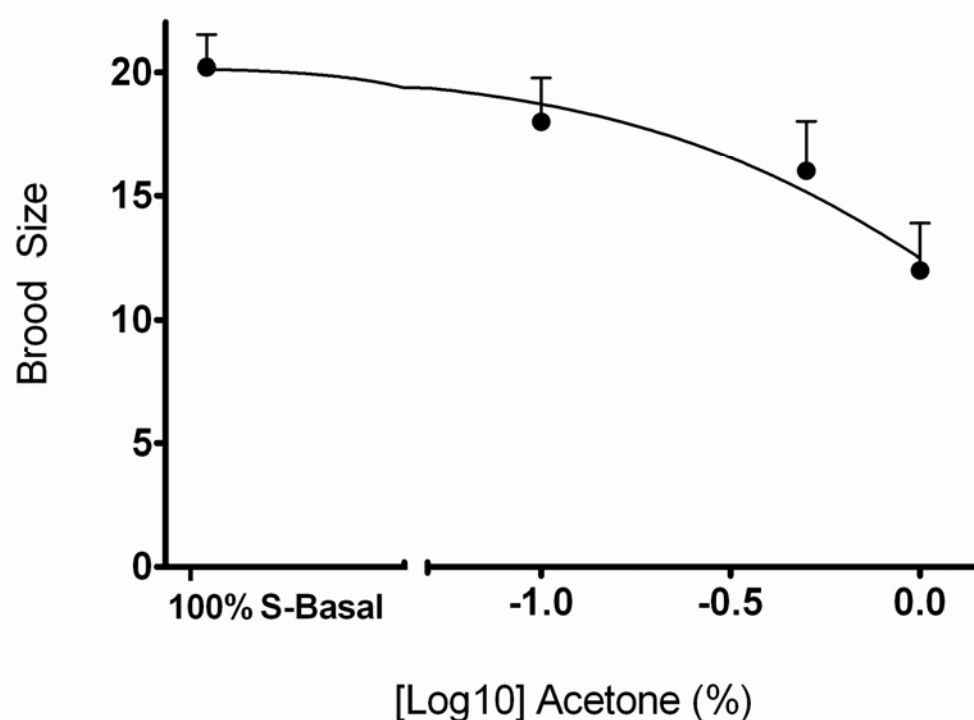


Figure 3.9.4 Effect of Acetone on growth. 40 L1 worms per dose group were placed in 400µl of liquid medium (s-basal) with varying concentrations of acetone or vehicle control (100% S-Basal) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was done as a vehicle control to determine if the *C.elegans* could handle this solvent and if it had an effect on growth.



3.9.5 Acetone vehicle control for brood size assay. Individual L4 *C.elegans* N2 worms were placed in 400µl of liquid medium (s-basal) with varying concentrations of acetone or vehicle control (100% s-basal) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

The effects of acetone on *C.elegans* brood size and growth was tested as a vehicle control. Acetone showed a dose-dependent effect on the growth and brood size of *C.elegans*. A t-test was carried out to test if differences between the treated and the control values were statistically significant.

Acetone had a significant inhibitory effect on the growth of L1 *C.elegans*. The L1 *C.elegans* had mean size of $0.004\text{mm}^2 \pm 0.0002$ when they were placed in the wells. At 1% acetone, the highest concentration tested, the mean size recorded was 0.0041mm^2 , ± 0.0013 , there was no significant growth; ($t=0.13$) since they were placed in the well

and suggests immediate cessation of growth. At 0.1% acetone (the lowest concentration tested) there was a significant inhibition of growth compared to the control size $t = 3.29$. The IC_{50} was 0.125% (95% CI = 0.0747 to 0.210).

Acetone had an inhibitory effect on the brood size of *C.elegans* with almost a 33% reduction in brood size compared to the control, recorded at 1%. At 0.1% acetone, (the lowest concentration tested) there was a significant reduction in brood size compared the control $t = 1.73$. The IC_{50} was 1.71% (95% CI = 0.477 to 6.13). All the *C.elegans* remained viable at all the concentrations tested for these two assays.

The effect of acetone on lethality was not tested due to the results seen from these two assays showing a significant effect on the growth and brood size of *C.elegans* even at 0.1%, it thus not as suitable as DMSO to use as a solvent.

3.9.3 Ethanol as a vehicle/solvent

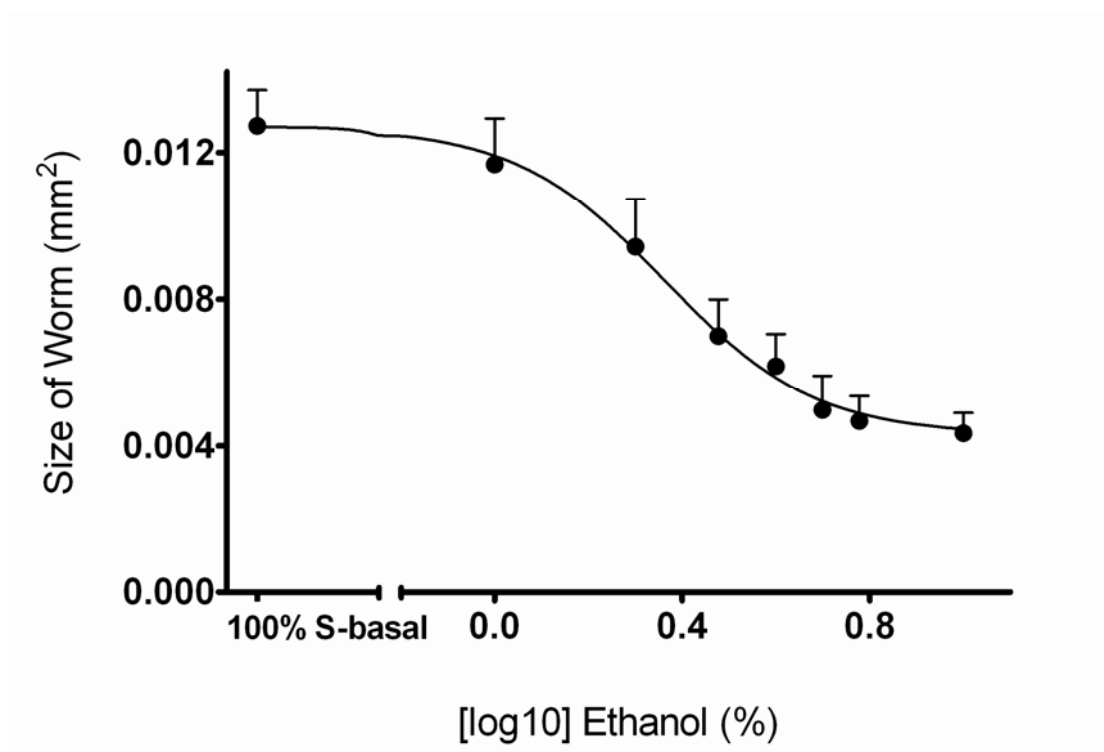


Figure 3.9.6 Ethanol vehicle control for growth assay. 40 L1 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of ethanol or vehicle control (100% S-Basal) and incubated for 3 days at 15°C, as described in section 2.4.1. After incubation the worms were imaged with a 10X objective, using Visicapture and a Scion 1310M CCD camera, and the size of the worms was measured using Adobe Photoshop CS3. A minimum of 30 worms were measured for each concentration. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean worm size (represented by the dots), where $n \geq 30$. The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was done as a vehicle control to determine if this solvent and if it had an effect on growth.

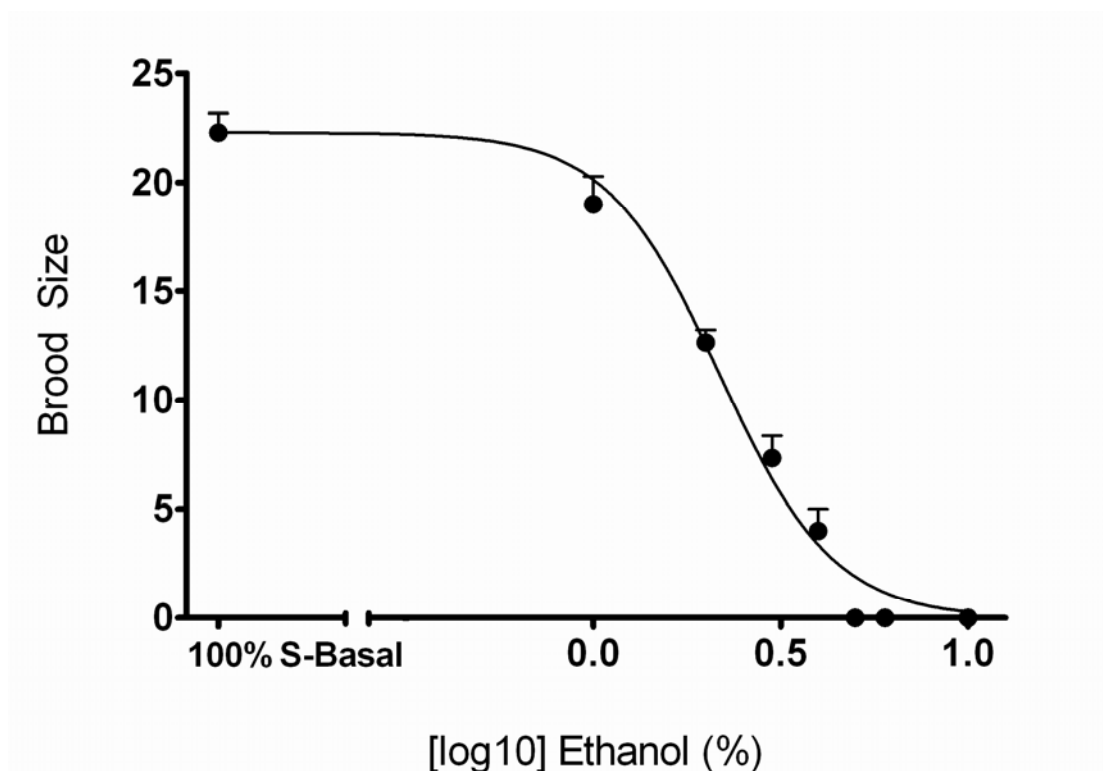


Figure 3.9.7 Ethanol vehicle control for brood assay. Individual L4 *C.elegans* N2 worms were placed in 400µl of liquid medium (s-basal) with varying concentrations of ethanol or vehicle control (100% s-basal) and incubated for 3 days at 15°C, as described in section 2.4.2. After incubation the brood size was determined by counting the number of offspring in each well. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean brood size (represented by the dots). The data was fitted to a non-linear regression dose response curve (log [agonist] vs. response) using GraphPad prism 5. This experiment was repeated giving similar results.

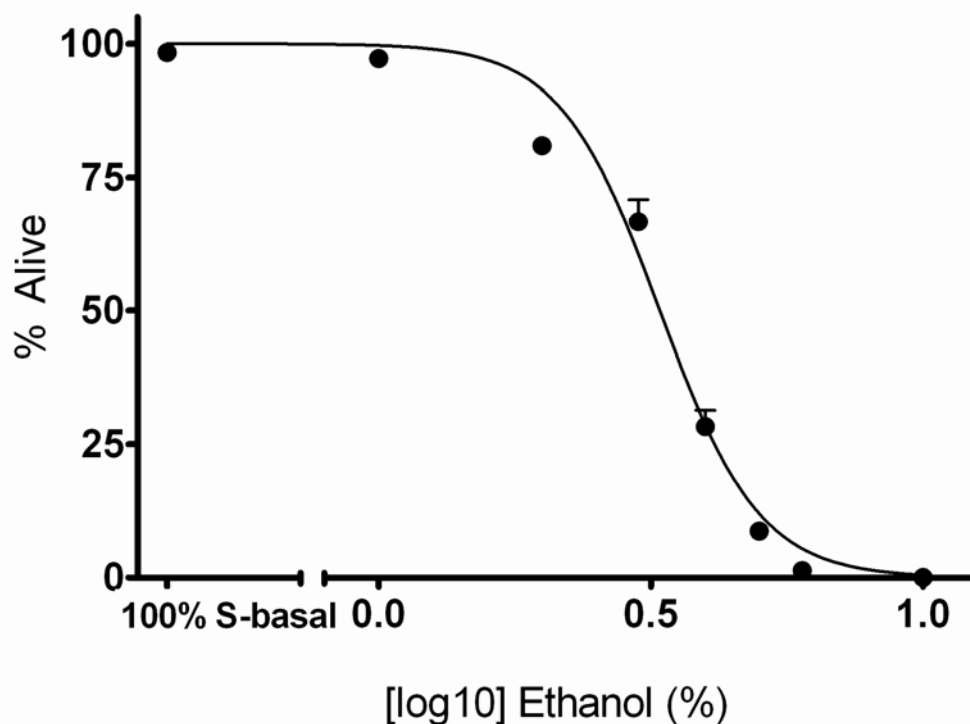


Figure 3.9.8 Ethanol vehicle control for lethality assay. 50 L4 *C.elegans* N2 worms per dose group were placed in 400 μ l of liquid medium (s-basal) with varying concentrations of ethanol or vehicle control (100%) and incubated for 3 days at 15°C, as described in section 2.4.3. After incubation the percentage of worms alive was determined by counting the number of viable and dead worms in each well based on movement. The graph represents a single experiment with each concentration tested in triplicate to produce standard deviations (represented by the bars) and mean percentage of viable worms (represented by the dots) where n=150. The data was fitted to a non linear regression dose response curve (log [agonist] vs. response) using GraphPad Prism 5. This experiment was repeated with similar results.

The effect of ethanol on growth, brood size and lethality of *C.elegans* was tested as a vehicle control. A dose-dependent effect was observed across all three assays. A t-test was carried out to test if differences between the treated and the control values were statistically significant.

Ethanol has significant inhibitory effect on the growth of L1 *C.elegans*. The *C.elegans* had a mean size of 0.0042mm² +/- 0.0002 when placed in the wells. The greatest inhibition of growth was seen at 10% ethanol (the highest concentration tested) where the mean size was 0.0043mm² +/- 0.0006, there was no significant growth at this concentration t = 0.27, indicating very early cessation of growth. The worms at 10% and

6% ethanol were dead, which would explain the lack of growth at these concentrations. At 1% ethanol (the lowest concentration tested) the mean size recorded was 0.0116mm^2 , there is no significant effect on growth compared to the control size $t = 1.15$. The IC_{50} was 2.31 % (95% CI = 2.16 to 2.46).

Ethanol had a significant inhibitory effect on the brood size, with a 100% reduction in brood size recorded for the top three concentrations tested (10%, 6% and 5%). The parental hermaphrodite at 10% and 6% ethanol was dead, but was viable at 5% with very little movement seen. The mean brood size was $22.3\text{mm}^2 \pm 0.87$ for the control (100% s-basal). At 1% ethanol (the lowest concentration tested) the brood size recorded was 19.0 ± 1.28 , there is no significant effect on brood size compared to the control; $t = 3.67$. The IC_{50} was 2.18% (95% CI = 1.92 to 2.47).

Ethanol had a significant effect on the lethality of *C.elegans*, with 100% kill recorded for the two highest concentrations tested (10% and 6%). At 1% ethanol (the lowest concentration tested) there was no significant effect on lethality compared to the control; $t = 1.53$. The IC_{50} was 3.28% (95% CI = 3.02 to 3.57)

From the results of these assays, it can be concluded that ethanol had an effect on growth brood size and lethality at all the concentrations tested; however the effect seen for ethanol at 1% was not significant, as demonstrated by the t-test values; and is therefore a suitable solvent at this concentration.

Ethanol at 1% was used as a solvent and vehicle for furan and quinine toxicity assays.

3.10 PCR

In order to confirm the genotype of the offspring of the F2 to F6 generation the parental hermaphrodite for each generation was genotyped. Genotyping was done by setting up PCR reactions using Lat-1 primers and genomic DNA of the parental hermaphrodite as described in section 2.6.

3.10.1 PCR optimisation

PCR conditions were optimised by varying the concentrations of primers and genomic DNA, number of cycles and annealing temperatures. PCR products were run on a 12% polyacrylamide gel electrophoresis to confirm amplification. The primer concentrations tested were 20pm and 100pm, results showed brighter bands with 100pm for lat-1 null PCR product. The primer concentration did not seem to affect the wild-type PCR product as an equally bright band was seen with 20pm and 100pm. The concentration of the genomic DNA was varied by carrying out worm lysis in 10µl and 30µl of lysis buffer. The results showed a brighter band for the wild-type PCR product for worm lysis in 10µl, but did not show any difference for the lat-1 PCR product. The annealing temperatures tested ranged between 51°C and 66°C increasing by 3°C. Wild-type PCR products were seen at 54°C and 57 °C and lat-1 PCR products were seen at 54 °C, 57 °C and 60 °C. 54 °C annealing temperature showed clear bright bands for both lat-1 and wild-type PCR products. The number of cycles for the PCR was varied; 26, 30 and 35 cycles. The results showed a bright band at 26, 30 and 35 cycles for the lat-1 PCR product, but only 35 cycles for the wild-type PCR product showed any band. The PCR protocol was adjusted to suit the above conditions and run as described in section 2.6

3.11 Breeding results

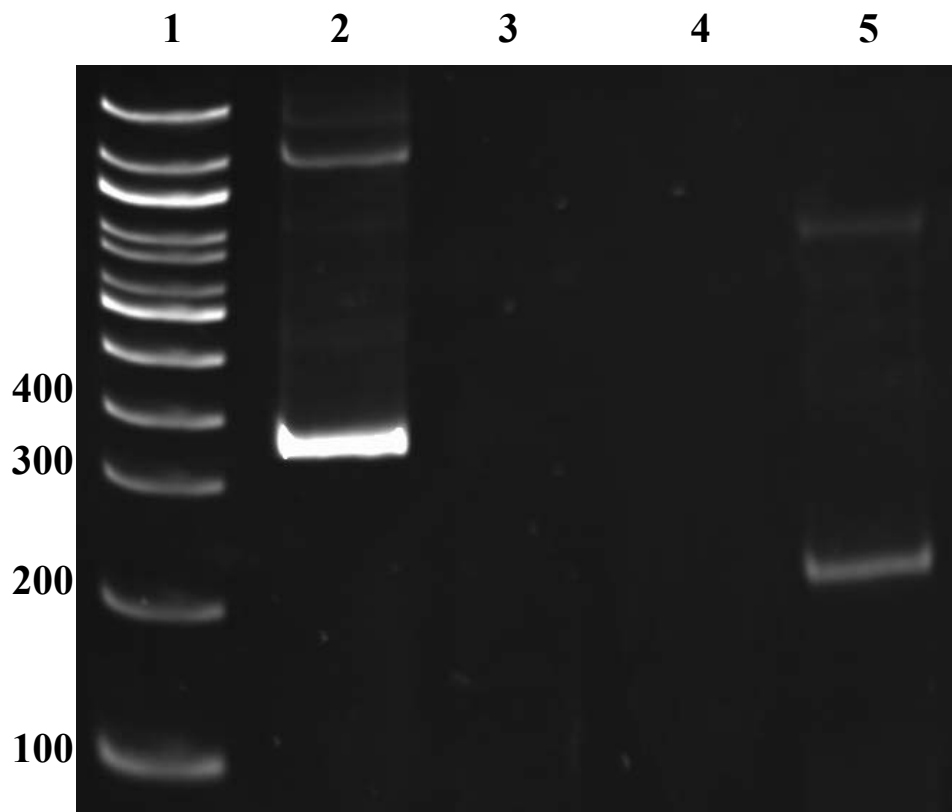


Figure 3.11.1 Acrylamide gel showing specificity of primers. Primers were designed to establish the presence of the *lat-1* deletion; the size of the PCR fragment amplified by these primers is 214bp. Another set of primers were designed to confirm the presence of the wild-type gene; the size PCR fragment amplified by these primers is 320bp. 30µl PCR reactions were set up with either set of primers, *C.elegans* genomic DNA, and master mix, and run for 35 cycles; 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute, as described in section 2.6. The PCR reaction was mixed with loading buffer and run alongside a positive and negative control, on 12% polyacrylamide gel electrophoresis at 100V for 1 hour. The gel was visualised under a UV trans-illuminator, and photographed using Bio Rad Gel-Doc system and the Quantity One software. A 100bp DNA ladder was used and sizes up to 400bp are labelled. Every next band represents 100bp larger from 400bp. Lane 1 has 100bp DNA ladder. Lane 2 has CB4856 *C.elegans* DNA with wild-type primers. L3 has OK1465 *C.elegans* DNA with wild-type primers, a negative control for lane 2. Lane 3 has CB4856 *C.elegans* DNA with Lat1 primers, a negative control for lane 4. Lane 4 has OK1465 *C.elegans* DNA with Lat1 primers.

The Lat-1 ok1465 *C.elegans* have a deletion in the *lat-1* gene and primers were designed to establish the presence of this *lat-1* deletion, which is located between the coordinates 18185bp and 20395bp. These primers were designed to bind just outside the deletion; the 5'primer binds at 18011-18030bp, and the 3'primer binds at 20410-20432bp. When the *lat-1* deletion is present the PCR fragment amplified by these primers is 214bp long. The PCR fragment is 2421bp long in the case of the wild-type gene. Another set of primers

was designed for wild-type *C.elegans* to amplify the region of the sequence deleted in the lat-1 (ok1465) *C.elegans*. The 5' primer binds at 18701-18720 and the 3' primer binds at 19001-19020bp. The PCR fragment amplified by these primers is 320bp long. PCR reactions were set up with the wild-type primers and wild-type genomic DNA, and lat-1 (ok1465) genomic DNA as a negative control. These reactions were loaded onto lanes 2 and 3 of the gel. PCR reactions with these primers produced a fragment about 310bp in size for the reaction with the wild-type genomic DNA and no fragment for the reaction with lat-1 (ok1465) genomic DNA, thus confirming specificity of the primers showing that the wild-type primers only amplify wild-type DNA. The specificity of the lat-1 primers was confirmed in the same way. PCR reactions were set up and loaded onto lanes 4 and 5 of the gel. Lane 4 was loaded with a PCR reaction set up with the lat-1 primers and wild-type genomic DNA and lane 5 was loaded with a PCR reaction set up with lat-1 (ok1465) genomic DNA. A PCR fragment of about 210bp in size was produced by these primers in lane 5, for the reaction with lat-1 (ok1465) DNA, no fragment was produced for the reaction with wild-type DNA in lane 4, which confirms the specificity of the lat-1 primers to only amplify DNA in a lat-1 genome.

Figure 3.11.1 confirms the specificity of the primers, with positive and negative controls as described above, which verifies that the PCR products were genuine; however these bands were not confirmed by sequencing for further verification.

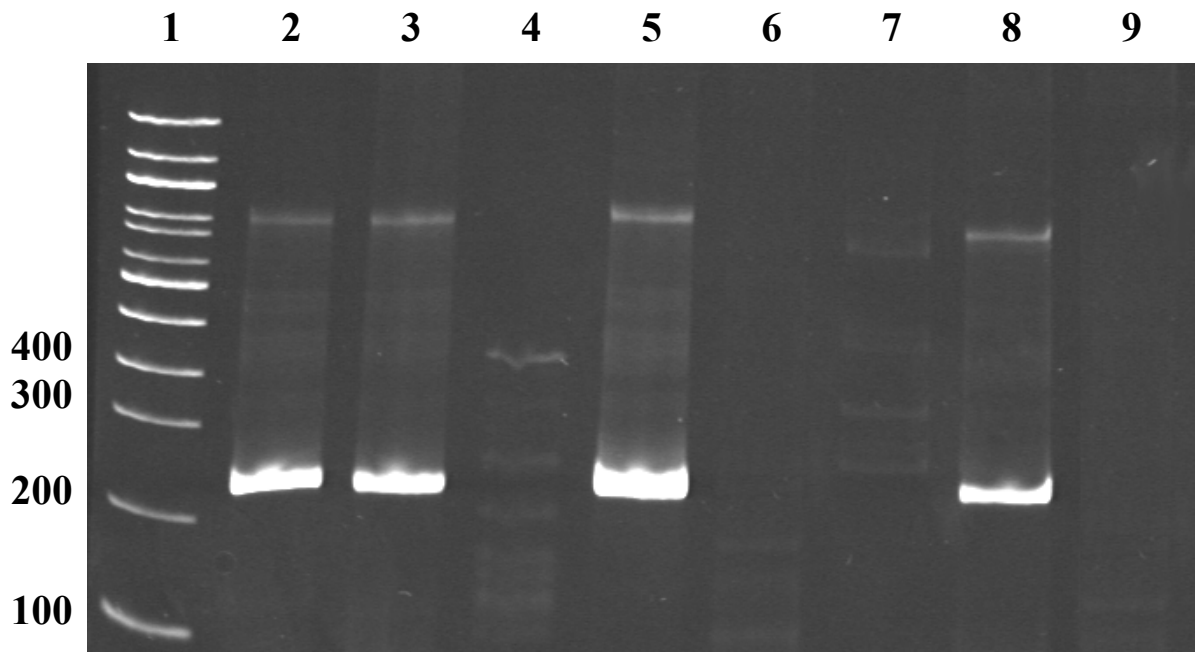


Figure 3.11.2 Acrylamide gel showing genotype of hermaphrodite from breeding. Breeding was carried out between a homozygous *lat-1* (-/-) hermaphrodite and a homozygous wild-type (+/+) CB4856 male. In order to identify the genotype of the F3 offspring the parental F2 hermaphrodite was genotyped. The parental hermaphrodite could be either wild-type homozygous or heterozygous. The hermaphrodite was added to 10 μ l of lysis buffer and frozen for 1 hour at -80°C and then thawed and heated for 6 hours at 65°C and 1 hour at 94°C to isolate the genomic DNA. A 30 μ l PCR reaction was set up, run on a gel and analysed as described for figure 3.11.1. A 100bp DNA ladder was used and sizes up to 400bp are labelled. Every next band represents 100bp larger from 400bp. Lane 1 has 100bp DNA ladder. Lane 2-7 has DNA from different hermaphrodites in the same generation with *Lat-1* primers. Lane 8 is *Lat ok1465* DNA with *Lat-1* primers and is a positive control for lanes 2-7. Lane 9 is wild-type N2 DNA with *Lat-1* primers and is a negative control for lanes 2-7.

A (*ok1465*) homozygous *lat-1* (-/-) *C.elegans* hermaphrodite was crossed with a homozygous wild-type CB4856 (+/+) *C.elegans* male for 6 generations. This cross produced only heterozygous (+/-) offspring in the F1 generation. F1 hermaphrodites were crossed again with a CB4856 male to produce the F2 generation. This was repeated until the F6 generation.

The genotype of the offspring for the F2 to F6 generation depended on the genotype of the parental hermaphrodite. A homozygous hermaphrodite (+/+) only produced homozygous wild-type offspring, while a heterozygous hermaphrodite (+/-) produced a mixture of homozygous wild-type and heterozygous offspring at 1:1 ratio. The genotype of the offspring from each generation was determined by genotyping the parental hermaphrodite. The parental hermaphrodite was either heterozygous (+/-) or wild-type

homozygous (+/+). PCR reactions were set up with genomic DNA of the parental hermaphrodite and lat-1 primers. Positive and negative control reactions were set up with lat-1 primers and lat-1 DNA and wild-type DNA. These PCR reactions were loaded onto lanes 2-7 of the gel in figure 3.11.2. The positive and negative control was loaded onto lanes 8 and 9 respectively. PCR fragments of about 210bp is seen in lanes 2, 3 and 5. An identical band is seen in lane 8, the lat-1 positive control. This confirms the genotype of the parental hermaphrodites from lanes 2, 3 and 5 as having a lat-1 heterozygous genotype. The absence of a PCR fragment in lane 8 confirms the specificity and sensitivity of the lat-1 primers and also confirms that the absence of a PCR fragment in lanes 4, 6 and 7 is due a homozygous wild-type genotype. The results shown in figure 3.11.2 are for 6 parental hermaphrodites, although at least 8 were genotyped for each generation. We are interested in the offspring from a heterozygous animal where the offspring from a breed will be 50% lat-1 heterozygous and 50% wild-type homozygous. Picking and breeding 8 hermaphrodites from each generation gives a 1/128 chance that a heterozygous animal will be picked.

At each generation, at least two of the hermaphrodites genotyped were heterozygous for the lat-1 deletion. A successful breed is confirmed by a 1:1 male to female ratio observed in the offspring. However in the first generation, a successful breed was also confirmed by observation of more viable offspring, if the breed was unsuccessful there would be only 3-4 viable offspring due to the high incidence of embryonic and larval lethality seen in ok1465 lat-1 C.elegans. For all subsequent generations a breed was deemed successful if there was more than 10 male C.elegans in the offspring.

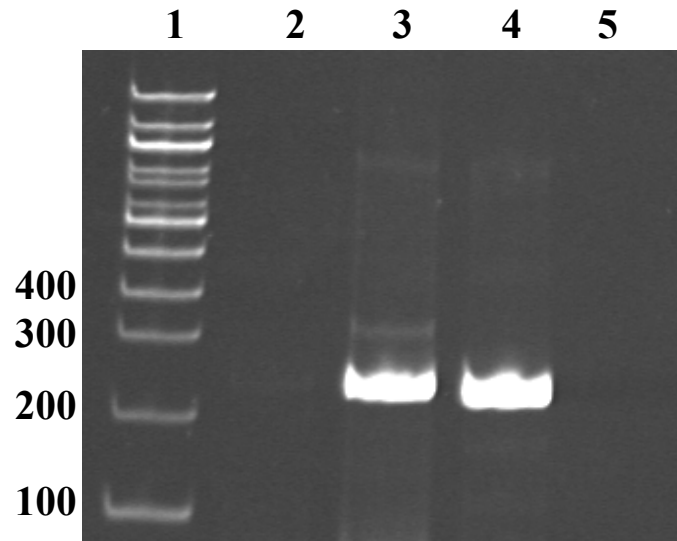


Figure 3.11.3 Acrylamide gel showing genotype of F6 Lat *C.elegans*. Breeding was carried out between a homozygous *lat-1* (*ok1465*) hermaphrodite *C.elegans* and a homozygous wild-type CB4856 male *C.elegans*. Breeding was carried out for 6 generations as described in section 2.6. A 100bp DNA ladder was used and sizes up to 400bp are labelled. Every next band represents 100bp larger from 400bp. Lane 1 has 100bp DNA ladder. Lane 2 has F6 DNA with wild-type primers. Lane 3 has F6 DNA with *lat* primers (fragment size 220) Lane 4 has *Lat1 ok1465* DNA with *Lat1* primers and is a positive control. Lane 5 has wild-type DNA with *Lat1* primers and is a negative control.

After 6 generations of breeding the F6 offspring were grown to L4. 15 F6 hermaphrodites were picked and selfed on separate NGM plates for two days. The 15 parental hermaphrodites were genotyped and analysed as described for figure 3.10.2 on the third day. 3 of the 15 hermaphrodites genotyped were heterozygous. The offspring from heterozygous animals were each transferred to an individual NGM plates and allowed to self for a week. 360 animals were transferred in total. These plates were examined to identify a *lat-1* homozygous animal. Only 1 plate was found to have homozygous *lat-1* animals, and had approximately 7 L4 animals and many unhatched embryos. Plates with more than 30 viable animals were discarded. To confirm the genotype of these *C.elegans*, a hermaphrodite from this plate was genotyped as described for figure 3.11.2. Figure 3.11.3 shows the results from this genotyping. Lane 2 shows the result from a PCR reaction set up with F6 DNA and wild-type primers, where there is no PCR fragment. Lane 3 shows the result from a PCR reaction set up with F6 DNA and

lat-1 primers, which shows a PCR fragment of about 210bp in size. Lane 4 shows a PCR fragment of identical size, for a PCR reaction set up as a positive control with lat-1 DNA and lat-1 primers. Lane 5 shows the result from a PCR reaction set up with wild-type DNA and lat-1 primers. The presence of a PCR fragment in lane 3 which is of identical size to the positive control, confirms the presence of the lat-1 deletion. The absence of a PCR fragment in lane 2 confirms that the genotype is lat-1 homozygous and not lat-1 heterozygous which would produce a PCR fragment of 320bp with wild-type primers. The absence of a PCR fragment in lane 5 confirms the sensitivity and specificity of the lat-1 primers as well as being a negative control. Thus these results demonstrate that the lat-1 (ok1465) allele has been successfully bred onto the Hawaiian (CB4856) background through 6 generations.

4. Discussion

The *C.elegans* has played an invaluable role in understanding a wide range of biological processes and has proven itself as a model for biological studies relevant to higher animals in areas such as cell death, genetics, neuroscience, development and aging (Nguyen 1995). Therefore it is suggested that toxicological results in *C.elegans* are likely to be relevant to higher animals. In this study *C.elegans* are used as a model system in which to examine the possible mechanisms of toxicity caused in humans by the anti-malarial drugs quinine and chloroquine, as well as toxicity caused in rodents by furan and investigating the pharmacology of the insecticides DDT and deltamethrin and how they affect invertebrates. The results from the toxicity assays for these compounds have identified visible phenotypes which can be screened for, to identify resistant mutants.

Genetic screens in *C.elegans* allows for molecular insight into both the mechanism of action of a toxin and how *C.elegans* may respond to protect itself from the toxin. Identification of genes responsible for causing toxicity in *C.elegans* could potentially help identify the genes causing toxicity in higher animals if the gene is conserved.

The *lat-1* (ok1465) *C.elegans* was crossed with the CB4856 wild-type strain of *C.elegans* through 6 generations and allowed to self to produce a homozygous *lat-1* *C.elegans* in a wild-type background. Because of the 97% lethality seen in this strain, it would be relatively easy to screen for resistant animals that produced a normal number of offspring.

4.1 Reliability of methods and results.

Sodium azide was used as a positive control, as it is a known toxin, and its mechanism of action (inhibition of the mitochondrial electron transport chain) will affect all developmental stages of *C.elegans*. Initial results were highly variable, and so it was

necessary to optimise conditions. The volume and OD of the bacterial culture, the total well volume and different types of vehicle medium were all tested to make the toxicity assay methods as robust as possible. Using the optimised protocol, the results from the sodium azide positive control assays show that the results from these assays are reproducible. Specifically, the coefficient of variation was less than 0.7, showing that the estimates of potency showed low variability.

In order to enhance the reliability of measurements, all toxicity assays and control assays were repeated at least once to ensure results were consistent. When analysing brood and lethality assays, worms in each well were counted at least twice. Furan assays were set up on ice, and each of the wells was covered with laboratory film to prevent the furan from vaporising. However due to its volatility the final concentrations in the wells may not have been accurate. As a key variable, the temperature in the incubator was monitored using a data logger and shown to be stable.

In order to further enhance the statistical power of analysis, the concentrations of chemical tested used semi-log dilutions (i.e. dilution by three-fold, as opposed to 10-fold) for quinine, chloroquine and furan; this provides a more robust characterisation of the dose-response curve, and hence estimation of EC_{50} . Deltamethrin and DDT were tested using 10-fold dilutions to identify the concentrations at which there is an effect; however even these wide concentration ranges have not yet identified the concentration at which DDT and deltamethrin have an onset of effect. Further characterisation of these compounds would use a three-fold dilution series to estimate potency of these agents.

The results of the breeding were confirmed by the acrylamide gels which showed genotypes of both homozygous and heterozygous animals. The results seen on the acrylamide gels were confirmed when the subsequent generation was genotyped, as a mixture of homozygous and heterozygous genotypes will be seen. The PCR reactions

were very sensitive to contamination, and contamination was encountered on two occasions. An increase in the number of cycles also affected contamination with PCR fragments seen for negative controls at 35 cycles but not at 26 cycles; however this did not occur when using fresh primers. Primers were aliquot and a fresh aliquot was used for each set of PCR reactions set up to minimise cross contamination. It was observed that no contamination occurred after the PCR reactions were set up under a PCR hood using filter tips.

8 hermaphrodites were picked at each generation for the next round of breeding; to increase the possibility of picking at least one heterozygous animal. The probability of all 8 hermaphrodites picked being homozygous is $1/256$. On one occasion during breeding two of these hermaphrodites died when being transferred, leaving only 6 hermaphrodites and increasing the probability to $1/64$ that all the hermaphrodites would be homozygous wild-type. To account for such occurrences at least 12 hermaphrodites should have been picked at each generation, which would reduce the probability of picking all homozygous animals to $1/4096$. It was also important that the hermaphrodites picked for breeding were virgins. To ensure this, only L4 hermaphrodites were selected for breeding. It is vital that the hermaphrodite is at the L4 stage and the vulva has not yet formed, in order to allow time for mating and ensure self-fertilization of the eggs does not occur. An L4 hermaphrodite can be recognised by a half moon shape in the middle of the body which is the developing vulva.

4.2 Vehicles

Ethanol, H₂O and DMSO were used as vehicles in this study. Ethanol at 1% was used as a vehicle for Furan and Quinine, H₂O was used as a vehicle for chloroquine and sodium azide and DMSO at 0.1% was used as a vehicle for DDT and deltamethrin. The vehicle

used in these assays is very important as they can have a significant effect on the *C.elegans* and affect the results of the assay. It is important that the vehicle used has no significant effect on the characteristic being tested, to ensure the results show a response that is due to the effects of the toxin and not a combined effect of the vehicle and toxin. Where possible H₂O should be used as a vehicle, as it has no effect on *C.elegans*, however this is not always possible due to the low solubility of some compounds in H₂O. The effects of Ethanol and DMSO at different concentrations were tested in all three assays with a negative control of 100% s-basal. 1% ethanol and 0.1% DMSO showed minimal or no significant effect on the characteristic being tested and was used in the assays as a vehicle. The results from the toxicity assays of Furan, Quinine, DDT and deltamethrin should be compared with the results from the vehicle control assays to determine the effects of the toxin from the effects of the vehicle. This comparison highlighted the inhibitory effects of ethanol on growth and brood size as the control values of growth and brood size in the quinine and furan assays (1% ethanol) was slightly lower than the control values recorded in the absence of ethanol (100% s-basal) for the ethanol vehicle control assays, however these differences were not significant. Ethanol at 1% had no significant effect on the lethality of *C.elegans* which was confirmed by similar control values between the ethanol vehicle control assay and furan lethality assay. Comparison of DDT and deltamethrin results to DMSO vehicle control results confirmed that DMSO at 0.1% had no effect on the growth, brood size or lethality of *C.elegans* with similar control values recorded between the DMSO vehicle control assays and all the DDT and deltamethrin assays. These results confirm the suitability of DMSO at 0.1% as vehicle for these studies in *C.elegans*. Due to effects of ethanol observed even at 1% (although not significant), DMSO should have been used instead of ethanol as a vehicle for furan and quinine.

4.3 Quinine

The effect of quinine on *C.elegans* at different stages of its life cycle was tested between 0.1mM and 3mM using toxicity assays as described in section 2.4.1-2.4.2. Quinine had a dose-dependent inhibitory effect on brood size and growth of *C.elegans*. There was no significant effect on brood size and larval growth at 0.1mM and quinine causes toxicity in *C.elegans* at 0.3mM and above. The IC_{50} for the quinine brood size assay was 0.568mM (95% CI = 0.347 to 0.928) and 0.711mM (95% CI = 0.318 to 1.59) for the L1 growth assay. This shows that the quinine was slightly more toxic to larval growth than brood size. The concentration at which quinine causes toxicity in *C.elegans* is comparable to the concentration causing toxicity in humans.

4.4 Chloroquine

The effect of chloroquine on *C.elegans* at different stages of its life cycle was tested between 0.1mM and 15mM using toxicity assays as described in section 2.4.1-2.4.3. Chloroquine had a dose-dependent inhibitory effect on the brood size, larval growth and lethality of *C.elegans*. There was no significant effect on brood size compared to the control below 1mM. The IC_{50} was 8.40mM (95% CI = 6.635 to 10.62). Larval growth was significantly inhibited at all concentrations tested. The IC_{50} was 0.162mM (95% CI = 0.0834 to 0.316). There was no significant effect on lethality of *C.elegans* below 0.3mM. The IC_{50} was 10.45mM (95% CI = 6.58 to 16.6). The IC_{50} values for these three assays show that chloroquine was significantly more toxic to larval growth than brood size and lethality. The IC_{50} for the lethality assay is considerably higher than those for the growth and brood size assays and shows that there is a considerable difference in toxicity between these three assays. The concentration at which chloroquine causes toxicity in *C.elegans* is comparable to the concentration causing toxicity in humans.

4.5 Furan

The effect of furan on *C.elegans* at different stages of its life cycle was tested between 0.1mM and 3mM using toxicity assays as described in section 2.4.1-2.4.3. Furan had a dose-dependent inhibitory effect on the brood size, larval growth and lethality of *C.elegans*. Brood size was significantly affected at all the concentrations tested, and was inhibited by 100% at 1mM and above. The IC_{50} was 0.1023mM (95% CI = 0.0454 to 0.231). There was no significant effect on larval growth below 0.3mM. The IC_{50} was 0.407mM (95% CI = 0.370 to 0.447). There was no significant effect on the lethality of *C.elegans* below 0.3mM. The IC_{50} was 6.74mM (95% CI = 4.95 to 9.17). The IC_{50} values show that furan was significantly more toxic to brood size than for larval growth and lethality. The IC_{50} for the lethality assay is considerably higher than those for the other assays and shows that the brood size and larval growth are more sensitive to the effects of furan. The results show that furan causes toxicity at 0.3mM and above in the growth and lethality assays whereas in the brood size assay toxicity occurs even at concentrations below 0.1mM. For comparison, furan causes hepatic toxicity in rats after a dose of 8 mg/ kg/ day, which would be an instantaneous concentration of ~0.1mM. The comparison is inexact, since this estimate takes no account of distribution/ metabolism, repeat dosing, or potential loss of furan by volatilisation in our study. Thus furan is acting as a potent toxin at doses comparable to those where it induces toxicity in rodents, suggesting that there may be a common mechanism of action.

The considerably higher toxicity of furan on embryos over adult and larval stages is interesting and could be due to a metabolic pathway that is not yet developed in embryonic stages but is functional at larval and adult stages which decreases the toxicity of this drug at these stages. It may also be due to a receptor which is only active during embryogenesis.

4.7 DDT

The effect of DDT on *C.elegans* at different stages of its life cycle was tested between 0.00000282mM and 0.00282mM using toxicity assays as described in section 2.4.1-2.4.3. DDT had a dose-dependent effect on brood size and lethality of *C.elegans*. There was a significant inhibitory effect on brood size at all the concentrations tested, with a 100% reduction in brood size at 3mM. The IC_{50} was 0.0000946mM (95% CI = 0.0000160 to 0.000559).

There was a significant inhibitory effect on larval growth compared to the control at all the concentrations tested. There was no significant growth at all the concentrations tested since the worms were placed in the wells, showing that the range of concentrations tested was too high. The IC_{50} was 0.000000843mM (95% CI = 0.000000162 to 0.00000439). There was no significant effect on lethality of *C.elegans* at 0.0000282mM and below. The IC_{50} was 0.003375mM (95% CI = 0.001732 to 0.006575). The IC_{50} values show that DDT has different considerably different toxicities at different stages and is significantly more potent at larval stages than on brood size and L4 *C.elegans*.

4.8 Deltamethrin

The effect of deltamethrin on *C.elegans* at different stages of its life cycle was tested between 0.00000198mM and 0.00198mM using toxicity assays as described in section 2.4.1-2.4.3. There was a significant difference in brood size between the control and treated. However, there was no significant difference in brood size between all the concentrations tested. There is difficulty interpreting the results of this experiment and although the reduction in brood size between the control and treated is significant, I do not think these results show a dose-response relationship between deltamethrin and effect on brood size over 3 logs of concentration. This experiment was repeated giving similar results. The IC_{50} was 0.00195mM (95% CI = 0.0000290 to 0.131). There was a

significant inhibitory effect seen on larval growth compared to the control at all concentrations tested. There was no significant growth at all concentrations compared to the start size, and no significant difference in size between the concentrations tested. The concentrations of deltamethrin tested were too high to see a dose-dependent effect. The IC_{50} was 0.00000504mM (95% CI = 0.00000180 to 0.0000141). Deltamethrin had no significant effect on the lethality of *C.elegans*. There was no significant difference between the control and treated and significant difference between the concentrations tested. The IC_{50} was 0.0220mM (95% CI = 0.00776 to 0.0621). The IC_{50} values show that deltamethrin is significantly more potent to larval growth than brood size and L4 *C.elegans* and shows stage-specific toxicity. This stage specific toxicity is interesting as it is not seen in other toxins. This toxicity could be due to deltamethrin binding to a receptor that is that is only present during larval growth. Adult *C.elegans* were not affected by deltamethrin, which may be due to the cuticle having a decreased permeability to the drug at the adult stage.

4.8 Screening and mutagenesis.

In order to identify genes that are responsible for drug toxicity a genetic screen needs to be carried out. The *C.elegans* would be screened for mutants that were resistant to the effects of the drug tested. Resistance is defined as the ability to grow and reproduce at concentrations that prevent wild-type animals. This would involve identifying mutants where either the brood size or growth size was no longer inhibited by exposure to the compound or where exposure no longer had a lethal effect. A simple F_2 screen would initially identify these mutants. During an F_2 screen genes in the germ cells are randomly mutated by exposing a population of wild-type F_0 hermaphrodites to a mutagen, such as ethyl methane sulphonate (EMS). A particular mutation in an egg or sperm would result in a heterozygous F_1 individual (for that mutation) when fertilization occurs (Jorgensen

2002). This mutation would be transmitted to the F₂ generation as the F₁ individual would produce eggs and sperm carrying this particular mutation. Self fertilisation of the F₁ individual would result in a F₂ generation where 25% of the progeny would be homozygous for the mutation. In order to identify if this mutation is involved in the mechanism of toxicity for a particular toxin, the F₂ generation would have to be screened to identify individuals that were no longer affected by the toxin. This would be done by exposing the F₂ generation to the toxin as described for the three assays in section 2.4.1 – 2.4.3. If such individuals are found, they would be transferred to a fresh agar plate and left for 3 day to self-fertilise producing the F₃ generation. In order to determine whether the mutant phenotype breeds true and is passed through to the next generation, the F₃ progeny are exposed to a particular toxin to identify animals that are no longer affected by the toxin just as described for the F₂ generation. Using this method one null mutation at a particular locus is recovered for every 2,000 copies of a gene analysed in a screen. 12,000 copies of a gene can be assayed in a typical screen of haploid genomes, thus we would expect to recover six mutations in a particular gene (Jorgensen 2002).

In the case of lat-1 (ok1465) *C.elegans*, which as described previously were crossed with the CB4856 strain to generate a homozygous lat-1 strain in a wild-type background, mutagenesis, would be carried out in the same way however screening would be to identify mutant individuals that no longer showed lethality. This would be relatively easy as the majority of animals would only produce 2/3 viable offspring per generation and a resistant mutant would produce 300+ viable offspring.

5. Conclusion and further studies.

In this study, toxicity assays were used to determine the effects of chloroquine, quinine, furan, DDT and deltamethrin on the growth and development, brood size and lethality in the model organism *Caenorhabditis elegans*.

For each of the toxins tested, a visible phenotype was identified for all three toxicity assays, which can be used to screen for resistant mutants.

It was found that quinine had a dose-dependent inhibitory effect on larval growth and brood size. Chloroquine, furan and DDT all had an inhibitory effect on growth, brood size and lethality of *C.elegans*. Chloroquine and DDT showed a greater potency on larval growth while furan showed a higher potency on brood size. Deltamethrin showed inhibitory effects on larval growth but showed no significant effect to the brood size and lethality of *C.elegans*. Deltamethrin showed stage-specific toxicity, as larval growth is significantly inhibited and effects on brood size and lethality were insignificant.

Breeding was carried out between the Lat-1 (ok1465) strain and the Hawaiian CB4856 strain to generate a homozygous strain carrying the lat-1 deletion in a Hawaiian (CB4856) background.

5.1 Further Studies

C.elegans from these toxicity assays could be monitored to determine if there are any visible physiological affects that can be seen which may help explain how the toxins tested inhibit growth and development, brood size or lethality.

5.2 Genetic Mapping

The goal of genome mapping is to generate high resolution maps of chromosomes which are useful for investigating genes and their functions. A mutation must be mapped to determine the identity of the gene (Swan 2002).

Since its introduction in 2001, single nucleotide polymorphism (SNP) mapping has transformed genetic linkage studies in *C.elegans*, and has made SNP the technique of choice for many *C.elegans* researchers. In SNP mapping, a genome map is constructed using SNPs as genetic markers; it is the easiest and most reliable way to map genes in *C.elegans*. DNA markers are molecular markers, DNA regions in the genome that differ sufficiently between individual strains of *C.elegans* so that they can be detected by molecular analysis of DNA. Using SNPs as genetic markers for mapping is advantageous and favoured, due to their relative abundance and because they have no associated phenotype unlike conventional marker mutations used in traditional methods of linkage mapping of mutations which rely on the use of markers that cause a visible phenotype. Some mutant phenotypes such as those with subtle behavioural defects that would normally be masked by conventional marker mutations can be mapped using SNPs. Using SNP mapping it is possible to narrow down the location of a mutation to a region smaller than a single cosmid and theoretically to a single gene (wormbook).

In SNP mapping DNA sequence polymorphisms between the N2 wild-type *C.elegans* strain and the closely related CB4856 *C.elegans* strain are used as genetic markers. The CB4856 *C.elegans* show a uniformly high density of polymorphisms in comparison to the N2 wild-type. On average differences in the genomic sequence of CB4856 and N2 occur every 1000bp. Single nucleotide polymorphisms are in fact single nucleotide changes in the nucleotide sequence. These are normally changes from a C to T or an A to a G; however changes such as an insertion or deletion of a nucleotide are also common.

A listing of the SNPs that exist between CB4856 and N2, and their genetic position on the chromosome is found in the SNP database. The SNP database is organised according to the physical map by chromosomes, chromosomal segments and cosmids (worm book).

For standard SNP mapping, the aim is to map mutations relative to SNP markers in CB4856. First the linkage relationship between resistance causing mutations and SNP markers in CB4856 must be established. To do this we must obtain a homozygous resistant mutant with CB4856 SNP markers. Mutant animals are generated by chemical mutagenesis and resistant mutants are identified by screening as described in section 4.8. A population of resistant mutants was generated by allowing resistant animals to self-fertilize. CB4856 males would then be crossed into a hermaphrodite resistant mutant, producing a heterozygous cross progeny which are left to grow and self fertilize, which would regenerate homozygous resistant mutants (wormbook). In the case of the homozygous *lat-1* resistant mutants which would be identified as described in section 2.6, these mutants would be crossed with males from the F6 generation of breeding rather than CB4856 males. A few animals from this F2 generation are randomly picked into tubes containing worm lysis buffer and lysed as described in section 2.7. PCR reactions are then set up using the lysate as described in section 2.6 but with primers designed to amplify the region of DNA containing the SNP being tested. After amplification the PCR products would be digested with a restriction enzyme specific to the SNP being tested for. These digestion reactions would then be loaded and run on an agarose gel.

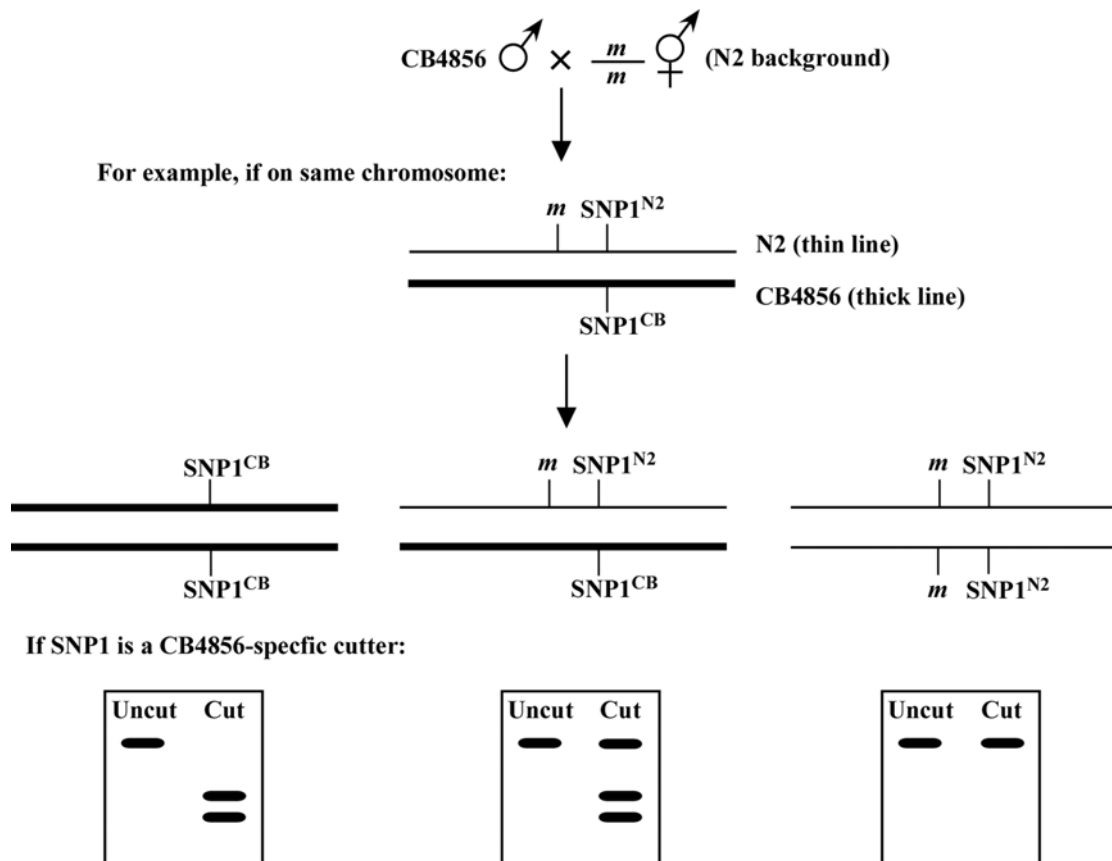


Figure 5.2 Two point SNP mapping.

This figure shows the basic scheme for two-point SNP mapping, showing that the closer a mutation lies to the SNP being tested the less likely a homozygous mutant will carry an allele of that polymorphism. This figure shows the linkage between the homozygous mutant and an SNP used to position the mutant on the chromosome relative to the SNP

Figure 5.2 shows a diagrammatic representation of the procedure described above, of two-point SNP mapping. If the mutation and the SNP being mapped and tested are on the same chromosome it is very unlikely that the homozygous mutant will harbour a CB4856 allele of that polymorphism.

Mutant strains that show drug resistance are generally resistant through one of three mechanisms.

- Mutations that alter the target site for the drug, rendering it dysfunctional, or less sensitive.

- Mutations that affect the metabolism of the drug or alter permeability thereby decreasing the concentration at which the drug is effective at the sensitive site.
- Mutations that lead to cellular or metabolic alterations that can bypass or compensate for the action of the toxin (Nguyen 1995).

6. Appendix

A1. Chloroquine brood assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Chloroquine (μl)
15	40	320	40 of 150mM
10	40	328	32 of 125mM
3	40	352	8 of 150mM
1	40	356	4 of 100mM
0.3	40	356	4 of 30mM
0.1	40	356	4 of 10mM
0	40	356	4 of H ₂ O

A2. Chloroquine brood assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Chloroquine(μl)	L1 Worms (μl)
15	40	315	40 of 150mM	5
10	40	323	32 of 125mM	5
3	40	347	8 of 150mM	5
1	40	351	4 of 100mM	5
0.3	40	351	4 of 30mM	5
0.1	40	351	4 of 10mM	5
0	40	351	4 of H ₂ O	5

A3. Chloroquine lethality assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Chloroquine(μl)	L4 Worms (μl)
15	40	315	40 of 150mM	5
10	40	323	32 of 125mM	5
3	40	347	8 of 150mM	5
1	40	351	4 of 100mM	5
0.3	40	351	4 of 30mM	5
0.1	40	351	4 of 10mM	5
0	40	351	4 of H ₂ O	5

A4. Quinine brood assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Quinine (μl)
3	40	355.2	4.8 of 250mM
1	40	356	4 of 100mM
0.3	40	356	4 of 30mM
0.1	40	356	4 of 10mM
0	40	356	4 of ethanol

A5. Quinine growth assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Quinine (μl)	L1 Worms (μl)
3	40	350.2	4.8 of 250mM	5
1	40	351	4 of 100mM	5
0.3	40	351	4 of 30mM	5
0.1	40	351	4 of 10mM	5
0	40	351	4 of ethanol	5

A6. Furan brood assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Furan (μl)
3	40	352	8 of 150mM
1	40	356	4 of 100mM
0.3	40	356	4 of 30mM
0.1	40	356	4 of 10mM
0	40	356	4 of ethanol

A7. Furan growth assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Furan (μl)	L1 Worms (μl)
3	40	347	8 of 150mM	5
1	40	351	4 of 100mM	5
0.3	40	351	4 of 30mM	5
0.1	40	351	4 of 10mM	5
0	40	351	4 of ethanol	5

A8. Furan lethality assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Furan (μl)	L4 Worms (μl)
3	40	347	8 of 150mM	5
1	40	351	4 of 100mM	5
0.3	40	351	4 of 30mM	5
0.1	40	351	4 of 10mM	5
0	40	351	4 of ethanol	5

A9. DDT brood assay.

% DDT	OP50 (μl)	S-Basal (μl)	DDT (μl)
0.001	40	359.6	0.4 of 2.82mM
0.0001	40	359.6	0.4 of 0.282mM
0.00001	40	359.6	0.4of 0.0282mM
0.000001	40	359.6	0.4of 0.00282mM
0	40	359.6	0.4 of 0.1% DMSO

A10. DDT growth assay.

% DDT	OP50 (μl)	S-Basal (μl)	DDT (μl)	L1 Worms (μl)
0.001	40	354.6	0.4 of 2.82mM	5
0.0001	40	354.6	0.4 of 0.282mM	5
0.00001	40	354.6	0.4of 0.0282mM	5
0.000001	40	354.6	0.4of 0.00282mM	5
0	40	354.6	0.4 of 0.1% DMSO	5

A11. DDT lethality assay.

% DDT	OP50 (μl)	S-Basal (μl)	DDT (μl)	L4 Worms (μl)
0.001	40	354.6	0.4 of 2.82mM	5
0.0001	40	354.6	0.4 of 0.282mM	5
0.00001	40	354.6	0.4of 0.0282mM	5
0.000001	40	354.6	0.4of 0.00282mM	5
0	40	354.6	0.4 of 0.1% DMSO	5

A12 Deltamethrin brood assay.

% Deltamethrin	OP50 (μl)	S-Basal (μl)	Deltamethrin (μl)
0.001	40	359.6	0.4 of 1.98mM
0.0001	40	359.6	0.4 of 0.198mM
0.00001	40	359.6	0.4of 0.0198mM
0.000001	40	359.6	0.4of 0.00198mM
0	40	359.6	0.4 of 0.1% DMSO

A13. Deltamethrin growth assay.

Deltamethrin (%)	OP50 (μl)	S-Basal (μl)	DM (μl)	L1 Worms (μl)
0.001	40	359.6	0.4 of 1.98mM	5
0.0001	40	359.6	0.4 of 0.198mM	5
0.00001	40	359.6	0.4of 0.0198mM	5
0.000001	40	359.6	0.4of 0.00198mM	5
0	40	359.6	0.4 of 0.1%DMSO	5

A14. Delthamethrin lethality assay.

Deltamethrin (%)	OP50 (μl)	S-Basal (μl)	DM (μl)	L4 Worms (μl)
0.001	40	359.6	0.4 of 1.98mM	5
0.0001	40	359.6	0.4 of 0.198mM	5
0.00001	40	359.6	0.4of 0.0198mM	5
0.000001	40	359.6	0.4of 0.00198mM	5
0	40	359.6	0.4 of 0.1%DMSO	5

A15. Control ethanol brood assay.

Ethanol (%)	OP50 (μl)	S-Basal (μl)	Ethanol (μl)
10	40	320	40
6	40	336	24
5	40	340	20
4	40	344	16
3	40	348	12
2	40	352	8
1	40	356	4
0	40	360	0

A16. Control ethanol growth assay.

Ethanol (%)	OP50(μl)	S-Basal(μl)	Ethanol(μl)	L1 Worms(μl)
10	40	315	40	5
6	40	331	24	5
5	40	335	20	5
4	40	339	16	5
3	40	343	12	5
2	40	347	8	5
1	40	351	4	5
0	40	355	0	5

A17. Control ethanol lethality assay.

Ethanol (%)	OP50(μl)	S-Basal(μl)	Ethanol(μl)	L4 Worms(μl)
10	40	315	40	5
6	40	331	24	5
5	40	335	20	5
4	40	339	16	5
3	40	343	12	5
2	40	347	8	5
1	40	351	4	5
0	40	355	0	5

A18. Control sodium azide brood assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Sodium Azide (μl)
1	40	356	4 of 100mM
0.1	40	356	4 of 10mM
0.01	40	356	4 of 1mM
0.001	40	356	4 of 0.1mM
0.0001	40	356	4 of 0.01
0	40	356	4 of H ₂ O

A19. Control sodium azide growth assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Sodium Azide (μl)	L1 Worms (μl)
1	40	351	4 of 100mM	5
0.1	40	351	4 of 10mM	5
0.01	40	351	4 of 1mM	5
0.001	40	351	4 of 0.1mM	5
0.0001	40	351	4 of 0.01mM	5
0	40	351	4 of H ₂ O	5

A20. Control sodium azide lethality assay.

Concentration (mM)	OP50 (μl)	S-Basal (μl)	Sodium Azide (μl)	L4 Worms (μl)
1	40	351	4 of 100mM	5
0.1	40	351	4 of 10mM	5
0.01	40	351	4 of 1mM	5
0.001	40	351	4 of 0.1mM	5
0.0001	40	351	4 of 0.01mM	5
0	40	351	4 of H ₂ O	5

7. References

- Adenle, A. (2008). Genetic analysis of latrophilin in the toxicity of combined latrotoxin for *C.elegans*. School of Biology. Nottingham, University of Nottingham. **PhD**: 173.
- AlKadi.O.Hussain (2007). "Anti-malarial drug toxicity: A review." Chemotherapy **53**: 385-391.
- Badiou, A., and L.P Belzunces (2008). "Is acetylcholinesterase a pertinent biomarker to detect exposure of pyrethroids? A study case with deltamethrin." Chem-bio interactions **In press**.
- Ballesterio, J. A., et al (2005). "Effects of Quinine, Quinidine, and Chloroquine on $\alpha 9 \alpha 10$ Nicotinic Cholinergic Receptors." Molecular pharmacology **28**(3): 822-829.
- Bischof, L. J, et al (2006) *C.elegans: Methods and applications*. Methods in Molecular Biology. K. Strange, Humana Press Inc. Pg 139, Chapter 11, **351**.
- Bruinsma, J. J., et al. (2008). "Identification of Mutations in *Caenorhabditis elegans* That Cause Resistance to High Levels of Dietary Zinc and Analysis Using a Genomewide Map of Single Nucleotide Polymorphisms Scored by Pyrosequencing." Genetics **179**: 811-829.
- Clark. MJ and S.B Symington (2007). "Pyrethroid action on calcium channels: neurotoxicological implications." Invert Neuroscience **7**: 3-16.
- Cole, R. D., G.L.Anderson, P.L.Williams (2004). "The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity." Toxicology and applied pharmacology **194**: 248-256.
- Daaboub, J., et al (2008). "Resistance to pyrethroid insecticides in *Culex pipiens pipiens*." Acta Tropica **107**: 30-36.
- Davies E, et al, (2008). "Knockdown resistance to DDT and pyrethroids: from target-site mutations to molecular modelling." Pest Management Science.
- Davis, M. W., et al. (2005). "Rapid single nucleotide polymorphism mapping in *C.elegans*." BMC Genomics **6**(118).
- Dawe, A. S. (2004). Investigation of the biological effects of radiofrequency radiation using the nematode *caenorhabditis elegans*. Nottingham, University of Nottingham. **PhD**: 237.
- Dent, J. A., et al (2000). "The genetics of ivermectin resistance in *Caenorhabditis elegans*." PNAS **97**(6): 2674-2679.
- Durling, L. J., K. Svensson, L. Abramsson-Zetterberg (2007). "Furan is not genotoxic in the micronucleus assay in vivo or in vitro." Toxicology letters **169**: 43-50.
- Fitch, C. D., and N.D.Russel (2006). "Accelerated Denaturation of Haemoglobin and the Antimalarial Action of Chloroquine." Antimicrobial agents and chemotherapy **50**(7): 2415-2419.
- Flanagan K.L, et al. (2006). "Quinine levels revisited: the value of routine drug level monitoring for those on parenteral therapy." Acta Tropica **97**: 233-237.
- Foster, S. P., et al (2003). "Analogous pleiotropic effects of insecticide resistance genotypes in peach–potato aphids and houseflies." Heredity **91**: 98-106.
- Fuhrman, L. E., K. V. Shianna, A. Aballay (2008). "High-Throughput Isolation and Mapping of *C. elegans* Mutants Susceptible to Pathogen Infection." PLoS One **3**(8).
- Guest, M., et. al. (2007). "The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic,

- emodepside, in *Caenorhabditis elegans*." The international journal for parasitology **37**: 1577-1588.
- Hemingway, J., et al (2004). "The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem.*" Molecular Biology **34**: 653-665.
- Heppner, C. W., and J.R. Schlatter (2007). "Data requirements for risk assessment of furan in food." Food additives and contaminants **24**(1): 114-121.
- Howard, M. A., et al (2003). "Quinine allergy causing acute severe systemic illness: report of 4 patients manifesting multiple hematologic, renal, and hepatic abnormalities." BUMC proceedings **16**: 21-26.
- Huston, M., and M. Levinson (2006). "Are one or two dangerous? Quinine and quinidine exposure in toddlers." The Journal of emergency medicine. **31**(4): 395-401.
- Jorgensen, M. E., and Susan. E.Mango (2002). "The art and design of genetic screens: *Caenorhabditis Elegans*." Nature **3**: 356-367.
- Kedderis, G. L., and S.D. Held (1996). "Prediction of Furan Pharmacokinetics from Hepatocyte Studies: Comparison of Bioactivation and Hepatic Dosimetry in Rats, Mice, and Humans." Toxicology and applied pharmacology **140**: 124-130.
- Kenneth, G. M., et al (1996). "A genetic selection for *Caenorhabditis elegans* synaptic transmission mutants." Neurobiology **93**: 12593-12598.
- Laloo, D. G., et al (2007). "UK malaria treatment guidelines." Journal of infection **54**: 111-121.
- Massie, M. R., et.al (2003). "Exposure to the metabolic inhibitor sodium azide induces stress protein expression and thermotolerance in the nematode *Caenorhabditis elegans*." Cell stress and chaperones **8**(1): 1-7.
- Mee, C. (2002). Molecular Analysis of the latrophilin receptor. Latrophilin in the nematode *Caenorhabditis elegans*. Nottingham, University of Nottingham. **PhD**.
- Mee, C. J., et al (2004). "Latrophilin is required for toxicity of black widow spider venom in *Caenorhabditis elegans*." Biochem. J **378**: 185-191.
- Nguyen, M., et al. (1995). "*Caenorhabditis elegans* mutation resistant to inhibition of acetylcholinesterase." Genetics **140**: 527-535.
- NTP, National Toxicology Program. (1993). "Toxicology and carcinogenesis studies of furan (CAS no. 110-00-9) in F344/N rats and B6C3F1 mice (gavage studies)."
- O'Reilly, A. O., et al (2006). "Modelling insecticide-binding sites in the voltage-gated sodium channel." Biochem. J **396**: 255-263.
- Perera M.D, B., et al (2008). "Multiple insecticide resistance mechanisms involving metabolic changes and insensitive target sites selected in anopheline vectors of malaria in Sri Lanka." Malaria Journal **7**.
- Peterson, L. A. (2006). "Electrophilic Intermediates Produced by Bioactivation of Furan " Drug metabolism reviews **38**(4): 615-626.
- Popham, J. D., and J.M. Webster (1979). "Cadmium Toxicity in the Free-Living Nematode, *Caenorhabditis elegans*." Environmental research **20**: 183-191.
- Riddle, D., T. Blumenthal, B. Meyer, J. Priess (1997). C. Elegans II. New York, Cold Spring Harbor Laboratory Press.
- Sanchez, C., S.Wünsch, M. Lanzer. (1997). "Identification of a Chloroquine Importer in *Plasmodium falciparum*." The Journal of Biological Chemistry. **272**(5): 2652-2658.

- Smith, R. P., et al (1991). "Acute neurotoxicity of Sodium azide and Nitric Oxide." Fundamental and Applied toxicology **17**: 120.
- Swan, K et al (2002). "High Throughput Gene Mapping in *Caenorhabditis elegans*." Genome Research: 1100-1105.
- Taylor, W., and N. White (2004). "Antimalarial Drug Toxicity." Drug safety **27**(1): 25-61.
- Thompson, A. J., and Lummis, S.C.R (2008). "Antimalarial drugs inhibit human 5-HT₃ and GABA-A but not GABA-C receptors." British journal of pharmacology **153**: 1686-1696.
- Wicks, S. R., et al (2001). "Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map." Nature genetics **28**: 160-164.
- Wilson, J., et al. (2004). "Latrotoxin receptor signalling engages the UNC-13-dependent vesicle priming pathway in *C.elegans*." Current Biology **14**: 1374-1379.