Characterisation of the effects of the food borne toxicant furan on *Caenorhabditis elegans*

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All Ding' sind Gift und nichts ohn' Gift; allein die Dosis macht,
das ein Ding kein Gift ist.

All substances are poisons; there is none which is not a poison.
The right dose differentiates a poison and a remedy.

Paracelsus (1493-1541)

Abstract

Furan is a toxic organic compound which is usually used in industry where it helps in the production of nylon, lacquers, insecticides, or pharmaceuticals. But it has also been found in certain foods, mainly those that undergo heat treatment, such as tinned and jarred food including sauces, vegetables, and baby food (European Food Safety Authority, 2004; Jestoi *et al.*, 2009; U.S. Food and Drug Administration, 2004). Furan has been shown to induce liver tumors and cholangiocarcinomas (Burka *et al.*, 1991). As of 1995 furan is classified as "possibly carcinogenic to humans (Croup 2B)" by the International Agency for Research on Cancer (IARC). However, the mechanisms of furan toxicity are still unknown. Studies have yielded different results as to whether or not it shows genotoxic activity. It is also suspected that the genotoxic effects shown by some studies were induced by the toxic metabolite *cis*-2-butene-1,4-dial (Burka *et al.*, 1991; Peterson *et al.*, 2006). Genotoxic carcinogens can cause cancer in very low doses, as even a single DNA adduct may lead to a mutation that causes cancer. For risk assessment purposes it is therefore highly important to know what mechanisms of action are involved in furan carcinogenesis.

In this work I was trying to characterise the mechanisms of furan toxicity by identifying genes conferring furan resistance to previously generated *C. elegans* mutants. Furthermore, I looked at the visible effects of furan on *C. elegans* and the induction of genes known to be involved in stress response pathways.

Frozen stocks of mutant worms were thawed and L4 larvae incubated in furan for four days. The number of offspring was then counted and compared to N2 wild type worms. Several changes had to be made to the original protocol, but furan resistance could not be confirmed for any of the frozen stocks. It was found that 90 mM furan kills adult worms so the following experiments were done with concentrations ranging from 0 to 80

mM furan. To further characterise the toxic effects of furan on *C. elegans*, GFP reporter strains were used to monitor gene induction following incubation in furan. Several genes were assayed, which are known to be involved in *C. elegans* stress response. In addition, the effects of furan on growth and feeding were investigated. None of the genes tested in the GFP reporter assay showed induction after exposure of *C. elegans* to furan. However, it could be shown that furan affects *C. elegans* feeding and growth.

Since furan resistance could not be confirmed for any of the frozen stocks, SNP mapping and subsequent whole genome sequencing could not be done to identify the mutation. It could be shown that furan has an inhibitory effect on *C. elegans* feeding, egg laying, and growth. Feeding is inhibited in a dose-dependent manner with an EC_{50} of about 70 mM. *C. elegans* completely stop feeding at 80 mM. Similarly, egg laying is inhibited in a dose-dependent manner with an EC_{50} of about 20 mM. Growth seems to be affected by furan as well. However, these results could not be confirmed yet. Unfortunately, none of the genes tested in the GFP reporter assay was induced, so it was not possible to identify any proteins affected by furan toxicity.

Acknowledgements

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Abbreviations

Bp	Base pair
bwt	Body weight
C. elegans	*Caenorhabditis elegans*
CW	Continuous wave
cyp	Cytochrome P450
dAdo	Deoxyadenosine
dCyd	Deoxycytidine
DDT	Dichlorodiphenyltrichloroethane
DEB	Diepoxybutane
dGuo	Deoxyguanosine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
ER	Endoplasmic reticulum
g	Earth's gravitational acceleration
GC	Gas chromatography
GFP	Green fluorescent protein
Gly	Glycine
GST	Glutathione S-transferase
him	High incidence of males
IARC	International Agency for Research on Cancer
kB	Kilo bases
L1-L4	*C.elegans* larval stages
LB	Luria-Bertani (Medium)

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Abbreviations - continued from previous page

LH	Luteinising hormone/lutropin
Mb	Mega bases
mRNA	Messenger RNA
MS	Mass spectrometry
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
n.d.	No date
NGM	Nematode growth medium
NTG	Nitrosoguanidine
OD	Optical density
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SD	Standard deviation
SDR	Short chain dehydrogenase/reductase
SEM	Standard error of the mean
Ser	Serine
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
S. typhimurium	*Salmonella typhimurium*
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TF	Transcription factor
Tyr	Tyrosine
UHP	Ultra high purity
UV	Ultra violet
YT	Yeast-Tryptone (Medium)

1 Introduction and Literature Review

1.1 Introduction to Toxicology

The term "toxicology" was derived from the Greek words "toxicos" (poisonous) and "logos" (study). It was first used in the 17^{th} century and describes the study of harmful interactions between chemicals and biological systems. Toxic substances can be for example drugs, food additives, pesticides, industrial chemicals, environmental pollutants, natural toxins, or household poisons. Interactions are not only possible with animals or humans, but also with plants, microbes, and whole ecosystems. The toxic effect of certain plants was already known in China more than 3000 years ago. However, it was only in the 19^{th} century that Justus von Liebig (1803-1873) understood the importance of studying the relationship between cause and effect. In 1847 animal testing was introduced by Rudolf Buchheim. Since then the area of toxicology has developed rapidly (Reichl, 2009).

In 1973 the International Union of Pure and Applied Chemistry (IUPAC) (1993) established a Commission on Toxicology which later defined globally acceptable definitions of terms used in toxicology and ecotoxicology. They made up a glossary from which the following terms that are important for my work are derived:

Toxicity is defined as the capacity to cause injury or the adverse effects on a living organism. There should be a reference to the quantity of substance administered or absorbed, the way in which the substance is administered (e.g. inhalation, ingestion,

topical application, injection) and distributed in time (single or repeated doses), the type and severity of injury, the time needed to produce the injury, the nature of the organism(s) affected, and other relevant conditions. Therefore, further definitions are required.

There is a difference between **acute and chronic toxicity**. Acute toxicity comprises the adverse effects occurring within a short time (usually up to 14 days) after administration of a single dose or exposure to a given concentration of a test substance, or after multiple doses or exposures within 24 hours. In contrast, chronic toxicity are the adverse effects following continued exposures occurring over an extended period of time.

The **effective concentration (EC)** is the concentration of a substance that causes a defined magnitude of response in a given system, e.g. the EC_{50} is the median concentration that causes 50 % of maximal response. In a similar way, the **median lethal dose (LD_{50})** can be defined. These values can be used to compare the potency of two toxins.

The **lowest observed adverse effect level (LOAEL)** is the lowest concentration or amount of a substance, found by experiment or observation, which causes an adverse alteration of morphology, functional capacity, growth, development, or life span of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure. The **no observed adverse effect level (NOAEL)** therefore is the concentration of a substance at which there is no biologically or statistically significant increase in the frequency or severity of any adverse effects. The possibility that a harmful event (death, injury, or loss) arising from exposure to a chemical or physical agent may occur under specific conditions is referred to as **risk**. The identification and quantification of the risk, taking into account possible harmful effects on individual people or society of using the chemical or physical agent in the amount and manner proposed and all the possible routes of exposure, is

called **risk assessment**. For risk assessment purposes, it is important to define an **acceptable daily intake (ADI)**. This is an estimate of the amount of a substance in food or drinking water which can be ingested daily over a lifetime by humans without appreciable health risk. To calculate the ADI, the NOAEL is devided by a safety factor (usually 100) to account for differences between animals and humans (factor 10) and differences between humans (also factor 10). The ADI is generally expressed in mg/kg body weight (bwt). For calculation of the daily intake per person, a standard body mass of 60 kg is used.

1.2 *Caenorhabditis elegans*

C. elegans belongs to the phylum Nematoda (roundworms). Nematodes are a very diverse phylum with species ranging in length from less than 1 mm to over 35 cm. They are either free living or parasites of plants or animals, including humans, and can be found in almost all living environments. *C. elegans* usually lives in the pore water of soils and feeds mostly on bacteria. It has also been found on rotten fruit from compost samples, presumably because they contain high numbers of bacteria (Barrière & Félix, 2006).

Figure 1.1: Electron micrograph of *C. elegans*. The image shows an adult hermaphrodite lying on its right lateral side (http://www.wormatlas.org).

1.2.1 *C. elegans* anatomy

C. elegans is about 1 mm long, transparent, and has a cylindrical body shape with conical ends (Figure 1.1, page 11). The anatomy of *C. elegans* is very simple. The body is unsegmented and consists of two concentric tubes. The outer tube consists of the cuticle, hypodermis, excretory system, neurons, and muscles and the inner tube comprises the pharynx, intestine, and gonads (in adults only). The space between the tubes is called pseudocoelomic space (Strange, 2006; Wood, 1988; WormAtlas, 29/04/2010). The general nematode body plan is shown in Figure 1.2.

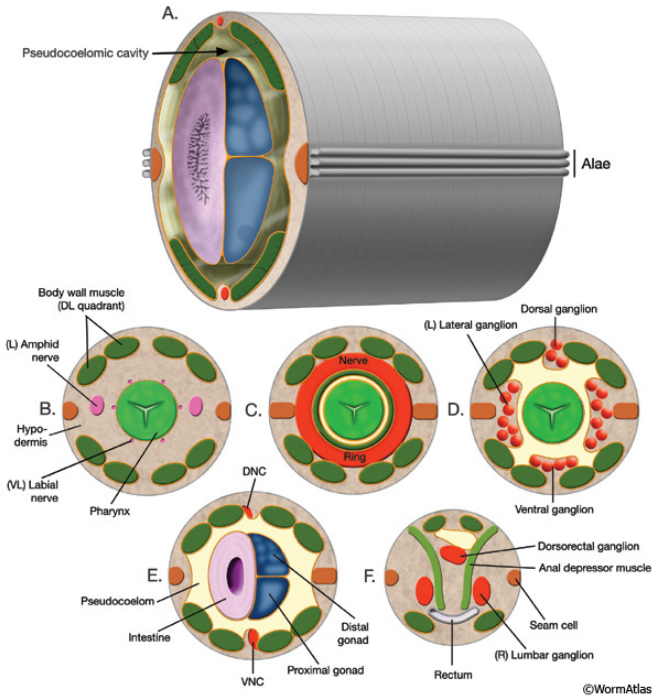

Figure 1.2: Body plan and cross sections of *C. elegans* from head to tail. A. Posterior body region. Body wall (outer tube) is separated from the inner tube (alimentary system, gonad) by a pseudocoelom. B. Anterior head. C. Middle of head. D. Posterior head. E. Posterior body. (DNC) Dorsal nerve cord; (VNC) ventral nerve cord. F. Tail, rectum area (http://www.wormatlas.org).

Contraction of the subventral muscles with simultaneous relaxation of the subdorsal muscles and vice versa results in sinusoidal movement (Riddle *et al.*, 1997). On an agar dish the worms move on either lateral side.

1.2.2 Life cycle of *C. elegans*

C. elegans has a relatively short life cycle under laboratory conditions (Figure 1.3). It consists of an embryonic stage, four larval stages (L1-L4), and adulthood which is reached after $3\frac{1}{2}$ days at 20 °C (Brenner, 1974). Eggs are roughly 30 x 50 µm in size. They develop internally for six hours before being laid. L1 larvae hatch after a further eight hours or so. During larval development the old cuticle is replaced by a stage-specific new one after each larval stage; this process is termed moulting. Adults produce eggs for several days and the average life span is about 2-3 weeks (Lewis & Fleming, 1995; Riddle *et al.*, 1997).

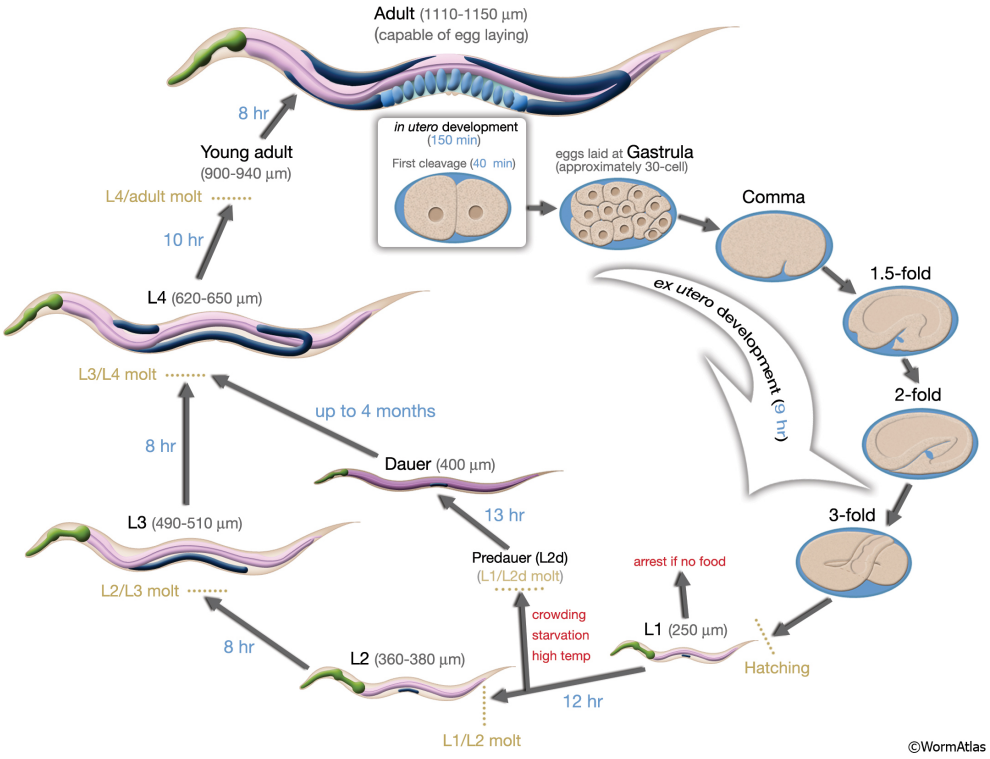

Figure 1.3: Life cycle of *C. elegans* at 25° C. (http://www.wormatlas.org)

1.2.3 The dauer stage

If the population density is too high and food is scarce, *C. elegans* larval development is arrested at the end of L1 /early L2 stage and the animals enter a "dauer" stage (Figure 1.3, page 13). Three environmental factors lead to the formation of dauer larvae. A pheromone produced by all *C. elegans* individuals serves as a measure of population density. A high population density and decreased availability of food as well as an increase in temperature result in an arrest in development and aging. Dauer larvae have distinguishable morphological and behavioral characteristics. Their energy metabolism is altered, they do not feed, and pharyngeal pumping is suppressed. The ability to move is maintained, but dauer larvae often lie motionless. A special cuticle is developed which gives resistance to detergent treatment (e.g. 1 % SDS) and dehydration. Dauer larvae are able to survive for several months by utilising food stored in intestinal granules. However, the dauer stage does not affect postdauer life span. In response to improved environmental conditions and an increase in food availability dauer larvae resume growth and normal development usually within an hour (Hu, 2007; Riddle, 1988).

1.2.4 *C. elegans* genetics

C. elegans is diploid and has five pairs of autosomes (I-V) and one pair of sex chromosomes (X). Adult *C. elegans* are hermaphrodite (referred to as XX) or male (referred to as X0) (Figure 1.4, page 15). However, males comprise only about 0.1 % of all individuals in the reference Bristol N2 strain and 1 % in the Hawaiian CB4856 strain. They arise because of X-chromosome nondisjunction. Reproduction occurs either by self-fertilisation in hermaphrodites, which produces about 300 progeny, or sexually by mating with males, the latter producing more than 1000 progeny. The hermaphrodite has 959 somatic cells whereas the male worm has 1031 somatic cells (Strange, 2006;

Wood, 1988). A number of *him* (high incidence of males) mutants showing an increased frequency of self-progeny males has been identified Hodgkin *et al.* (1979).

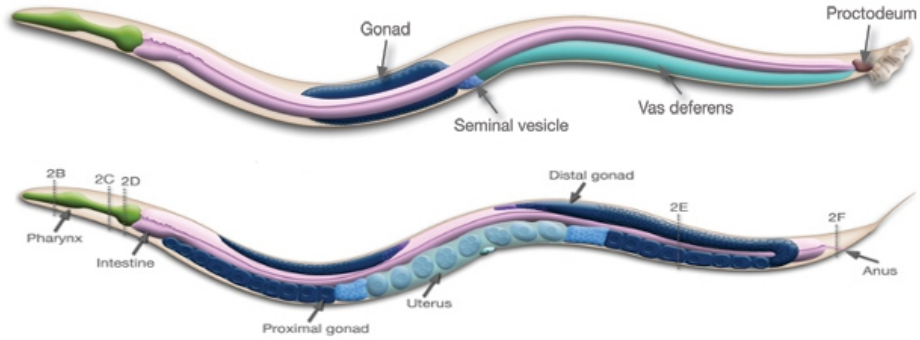

Figure 1.4: *C. elegans* male (top) and hermaphrodite (bottom). Males have a fan-shaped tail which makes it easy to distinguish them from hermaphrodites (modified from http://www.wormatlas.org).

The genome of *C. elegans* has a size of about 100 Mb. In 1998 *C. elegans* became the first multicellular organism for which the whole genome was sequenced. Genes make up about 40 % of the genome with a significant number arranged in operons; overall 25 % of the genome seems to be protein-coding (Hodgkin, 2005).

Normally, about 10^{-6} mutations per gene per generation occur. Mutations can be easily generated, e.g. with EMS (ethyl methanesulfonate) or other chemical mutagens such as nitrosoguanidine (NTG), diethyl sulfate, diepoxybutane (DEB) or formaldehyde. Exposure to gamma radiation, X-radiation or ultraviolet (UV) radiation also causes mutations. As only mutations in the germ line are passed on to the offspring, usually late L4 larvae or young adults are mutagenised (Anderson, 1995).

1.2.5 *C. elegans* as a model organism

Research into the biology of *C. elegans* began in 1963 when the molecular biologist Sydney Brenner started to look for a multicellular organism that could be used to study the development of the nervous system genetically. It has been used as a model

organism ever since. *C. elegans* has several advantages such as a short life cycle, small genome, constant somatic cell number, and large number of progeny. It can be grown on agar plates with *E. coli* as a food source which makes it easy to handle in the laboratory. Due to its transparency it can be easily looked at under a normal light microscope (Brenner, 1974; Wood, 1988). *C. elegans* is also a good model to investigate toxicity. Growth, size, number of offspring, behaviour, mortality, and changes in appearance or movement can all be monitored. A great number of toxicity assays have been developed, providing the basis for a number of studies on different toxins (Bishof *et al.*, 2006; Ura *et al.*, 2002). A variety of other tools are also available to look at changes at the genetic level, including transgenic reporter strains, microarrays, and RNA interference (Lindblom & Dodd, 2006).

Sydney Brenner shared the 2002 Nobel Prize in Physiology or Medicine with J. Sulston and H.R. Horvitz "for their discoveries concerning 'genetic regulation of organ development and programmed cell death' " (Nobel Foundation, 2002).

GFP reporter strains

The green fluorescent protein (GFP) is a small protein that was originally isolated from the jellyfish *Aequorea victoria*. The 27 kDa protein is composed of a central α-helix containing the chromophore surrounded by an eleven stranded cylinder of antiparallel β-sheets. The chromophore is formed from residues 65-67 (Ser-Tyr-Gly) in the native protein. GFP absorbs UV light with a major excitation peak at 395 nm and blue light with a minor excitation peak at 475 nm, and it emits green light (509 nm). In addition to the wild type protein, several mutants of GFP have been engineered which have improved properties such as increased fluorescence or shifted absorbance and emission spectra. It does not need any enzymatic substrates, which reduces the impact on the experiment in which it is used. The GFP gene is often fused to target genes to monitor

gene expression or to locate proteins in cells (Tsien, 1998).

Table 1.1 shows the GFP reporter strains that were used in this project.

Table 1.1: List of candidate genes for the GFP reporter assay. The information is summarised from WormBase (29/04/2010).

Strain	Gene	Description
JR2474	*cep*-1	cep-1 encodes an ortholog of the human tumor suppressor p53 that promotes DNA damage-induced apoptosis and is required for normal meiotic segregation in the germ line. It also affects sensitivity to hypoxia-induced lethality and longevity in response to starvation. CEP-1 appears to be ubiquitous throughout embryonic development. After hatching, expression is restricted to a subset of pharynx cells.
BC20334	*cyp*-29A2	*cyp*-29A2 encodes a cytochrome P450. Semi-quantitative RT-PCR showed the elevated gene expression level after PCB52 treatment. *cyp*-29A2 is expressed in pharynx, intestine, body wall muscle, hypodermis, nervous system, head and tail neurons.
BC20306	*cyp*-34A9	*cyp*-34A9 encodes a cytochrome P450. *cyp*-34A9 is expressed in the intestine.
pPD97 87-35A2prIII-GFP	*cyp*-35A2	*cyp*-35A2 encodes a cytochrome P450. *cyp*-35A2 mRNA is upregulated in response to treatment with xenobiotics, such as PCBs or PAHs . Loss of *cyp*-35A/C gene family activity in the presence of xenobiotics can diminish the reproductive decline seen in wild type worms treated with the same compounds. *cyp*-35A(RNAi) also results in a reduction of fat content. *cyp*-35A2 reporter gene fusions are strongly expressed in the intestine following treatment with xenobiotics. The basal level of expression, if any, was below the sensitivity of the fluorescence microscopy method.
BC20305	C11E4.1 (GPB)	C11E4.1 is a gene with a glutathione peroxidase domain. It is expressed in pharynx, intestine, rectal gland cells, hypodermis, and unidentified cells in the head.
BC20316	*gst*-1	*gst*-1 encodes a putative glutathione S-transferase. GST-1 is required for sperm to normally migrate towards a PUFA-based signal exuded by oocytes. *gst*-1(RNAi) hermaphrodites are infertile, both through aberrant loss of their own sperm and through failure of males to effectively inseminate them.

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Table 1.1 – continued from previous page

Strain	Gene	Description
BC20330	*gst*-4	*gst*-4 encodes a putative glutathione-requiring prostaglandin D synthase. GST-4 is required for sperm to normally migrate towards a PUFA-based signal exuded by oocytes. *gst*-4(RNAi) hermaphrodites are infertile, both through aberrant loss of their own sperm and through failure of males to effectively inseminate them. Larval Expression: pharynx, nervous System, head neurons; Adult Expression: pharynx, reproductive system, vulval muscle, nervous system, head neurons. Accumulation in adults increases in response to paraquat.The transgene Ce-GST-p24 seems to be stage-specifically expressed, since the GFP expression was stronger in larvae than in adults and totally lacking in embryos.
BC20308	*hsp*-3	*hsp*-3 encodes a heat shock response 70 (hsp70) protein orthologous to human glucose regulated protein 78. HSP-3 likely functions as a molecular chaperone, and is expressed constitutively throughout development with greatest abundance during the L1 larval stage. Larval Expression: pharyngeal gland cells, intestine, stomato-intestinal muscle, nervous system, head and tail neurons, and unidentified cells in tail. Adult Expression: pharyngeal gland cells, intestine, stomato-intestinal muscle, coelomocytes, nervous system, head and tail neurons, and unidentified cells in tail. HSP-3 contains a long hydrophobic amino terminus and a carboxyl terminal KDEL sequence suggesting that it may be retained in the endoplasmic reticulum.
CL2050	*hsp*-16.2	*hsp*-16.2 encodes a 16-kD heat shock protein (HSP) that is a member of the hsp16/hsp20/alphaB-crystallin (HSP16) family of heat shock proteins. *hsp*-16.2 expression is induced in response to heat shock or other environmental stresses. HSP-16.2 is likely to function in temporarily preventing unfolded proteins from aggregating. It is ubiquitously expressed throughout most somatic tissues of larvae after heat shock, and the intensity gradually decreases with age. HSP-16 labelling is prominent in spermathecae and in the vulval region in L4 and adult hermaphrodites. *hsp*-16.2 expression is strongest in intestine and pharynx.
BC20350	*sod*-1	*sod*-1 encodes the Cu^{2+}/Zn^{2+} SOD, which protects cells from oxidative damage. sod-1 activity has been implicated in the increased life-span of dauer larvae where this enzyme demonstrates the highest activity compared to other life-stages. Unlike other eukaryotic SODs, sod-1 does not require the copper chaperone CCS for its activity and instead uses a glutathione pathway for acquiring copper. The protein is ubiquitously expressed throughout most tissues of the wild-type animals.

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Table 1.1 – continued from previous page

Strain	Gene	Description
CF1553	*sod*-3	*sod*-3 encodes a Fe^{2+}/Mn^{2+} SOD, predicted to be mitochondrial, that might defend against oxidative stress and promote normal lifespan. sod-3 mRNA levels are diminished by mutation of daf-16 and chromatin immunoprecipitation (ChIP) studies demonstrate that DAF-16 can directly bind the sod-3 promoter. In wild-type animals, the SOD-3::GFP protein is expressed constitutively in pharyngeal and intestinal cells. SOD-3::GFP was observed to be expressed mainly in the head and tail of *C. elegans* SOD-3::GFP distributed in a punctuate pattern, which is typical of localization in the mitochondria.
BC20333	*sod*-4	*sod*-4 encodes an extracellular Cu^{2+}/Zn^{2+} SOD. Genetic analyses indicates that sod-4 is required for redox regulation of a number of processes. Large-scale expression studies indicate that sod-4 is expressed in the nervous system, intestine, and rectal gland cells; sod-4 transcripts are significantly upregulated in dauers.

1.2.6 *C. elegans* on the internet

A lot of *C. elegans* data is available on the internet free of charge. These online resources not only provide a general introduction into nematode biology, but also enable researchers to share information and data.

WormBase is the database of the model organism *C. elegans* and related nematodes and can be used to access comprehensive gene information. Wormbase is an ongoing project which is updated every two weeks. Each release is assigned a new version number. In this work the latest information was obtained from release WS213. Datasets are frozen at regular intervals to enable researchers to work with the same set of data for a longer period of time. The Ensemble genome browser also uses these frozen datasets. WormBook is a collection of original, peer-reviewed articles on the biology of *C. elegans* and other nematodes. It includes protocols for nematode research (WormMethods) and the "Worm Breeder's Gazette", a biannual newsletter. WormAtlas provides an anatomical database with descriptions, schemes, and an electron micrograph database. The *C. el-*

egans Gene Knockout Consortium produces deletion alleles for gene targets requested by any researcher or laboratory. Strains produced by the consortium are freely available to anyone and they are usually distributed by the Caenorhabditis Genetics Center (CGC). Information generated by the Consortium is entered into Wormbase and can be accessed from there.

1.3 Metabolism of Xenobiotics

Xenobiotics are substances that are not normally present in an organism. These can include drugs or natural compounds that were taken up from the environment, but they do not necessarily have an adverse effect. Xenobiotics are metabolised by a variety of enzymes, dependent on their location in the body and on their chemical properties. The main purpose of metabolism of xenobiotic substances is to eliminate them from the body.

Due to their natural living environment, *C. elegans* and other free living nematodes are under high selective pressure for detoxification mechanisms. In addition to behavioral patterns such as sensing the presence of certain chemicals or pathogenic bacteria, a variety of pathways have been developed to metabolise different chemicals. *C. elegans* has a large number of genes encoding detoxification enzymes such as cytochromes P450, dehydrogenases, glutathione S-transferases, as well as other transferases that might also be involved in detoxification processes (Lindblom & Dodd, 2006). This makes *C. elegans* an ideal model organism to study the effects and the metabolism of xenobiotics. Chemicals might have effects on growth, size, number of offspring, behaviour, mortality, appearance, or movement of *C. elegans*. By using microarrays or GFP reporter strains the up- and downregulation of genes in response to different concentrations of chemicals can be monitored (de Pomerai *et al.*, 2010). Both approaches can give an insight into how a chemical acts and how it is metabolised in the body.

Xenobiotic metabolism can be divided in two phases: phase I and phase II. Some of the most important enzymes involved in phase I and phase II metabolism are listed below.

1.3.1 Phase I metabolism

In phase I metabolism chemicals are altered in order to either eliminate them directly or prepare them for phase II metabolism. Two classes of enzymes mainly catalyse the reactions of phase I metabolism, the cytochromes P450 and the short chain dehydrogenases/reductases (Lindblom & Dodd, 2006).

Cytochromes P450

Xenobiotics that are lipophilic and chemically inert usually have to be oxidised to a more polar form before they can be eliminated. Cytochromes P450 are the most common and most important of all metabolic enzymes involved in oxidation. They comprise a superfamily of heme-containing NADPH-dependent monoxygenases. In humans, cytochromes P450 are found in many different tissues but the highest concentrations are in the liver. Cytochromes P450 are membrane bound and located in the ER.
In its oxidised form cytochrome binds the substrate; Fe^{3+} is reduced to Fe^{2+} which then binds oxygen. The reaction cycle is shown in Figure 1.5 (page 22). To complete this cycle a cytochrome P450 reductase is needed (Reichl, 2009; Uetrecht & Trager, 2007).
WormBase lists 81 *C. elegans* genes in the *cyp* gene class (WormBase, 29/04/2010). Menzel *et al.* (2001, 2005) studied the effects of a range of cytochrome P450 inducers, including benzo[*a*]pyrene, benzene, rifampicin, and phenobarbital, using mRNA expression. They found that the subfamilies CYP35A, 35C, 31A, and 29A were most inducible.

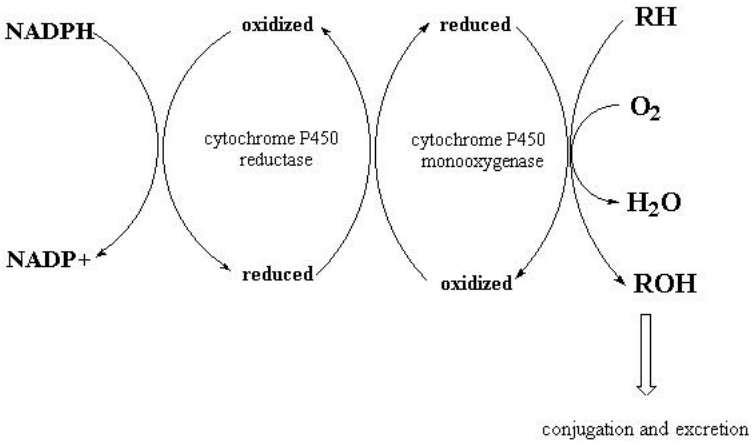

Figure 1.5: The P450 reaction cycle. (The University of Scranton, n.d.)

Short chain dehydrogenases/reductases (SDRs)

Short chain dehydrogenases/reductases can be found in the smooth ER and in the cytosol. They catalyse the reduction of carbonyl groups in aldehydes and ketones.
In *C. elegans* SDRs are encoded by the *dhs* gene class which comprises 30 genes (WormBase, 29/04/2010).

1.3.2 Phase II metabolism

Phase II metabolism is the actual detoxification of xenobiotics. The major aim is to prepare them for elimination from the body via the urine or in *C. elegans* most probably via the gut and defecation.

Glutathione S-transferases (GSTs)

The tripeptide glutathione (GSH) is a protective compound required for the removal of potentially toxic electrophilic compounds. This is particularly important, as many drugs are metabolised to strong electrophiles by phase I metabolism. The compounds are conjugated to GSH by glutathione S-transferases (GSTs). Substrates include various drugs, carcinogens, and pesticides. They are generally hydrophobic and elec-

trophilic and react non-enzymatically with the nucleophilic GSH to a certain extent. The induction of GSTs is an evolutionarily conserved response to oxidative stress in animal and plant kingdoms. There are three classes in humans: cytosolic, mitochondrial and microsomal GSTs. They are mainly found in liver, kidney, gut, testis, and adrenal glands. GSTs undergo induction by certain xenobiotics, e.g. 3-methylcholanthrene or TCDD and by the inorganic heavy metal Pb (Gibson & Skett, 1994; Hayes *et al.*, 2005; Timbrell, 2000).

In *C. elegans* 44 *gst* genes have been identified that encode GST proteins (WormBase, 29/04/2010). *gst* genes are upregulated in *C. elegans* following exposure to e.g. acrylamide (Hasegawa *et al.*, 2008). They also play a role in the protection against reactive oxygen species (ROS). *gst*-p24 is upregulated following exposure to ROS-generating chemicals (Leiers *et al.*, 2003).

Metallothioneins

Metallothioneins are a family of relatively small, cysteine-rich proteins that have the ability to bind heavy metals. They are involved in the transport of metals such as zinc in the body. Other metals, e.g. cadmium, may also bind to metallothioneins. A chronic increase in cadmium concentrations induces metallothionein expression. The protein therefore has a protective function (Timbrell, 2000).

In *C. elegans* there are two metallothionein genes (*mtl*). In addition to metal detoxification and homeostasis they function in stress adaptation, regulating growth and fertility. Their expression is also induced after exposure to heat (WormBase, 29/04/2010).

Heat shock proteins (HSPs)

The expression of heat shock proteins (HSPs) is increased when cells are exposed to elevated temperatures or other stresses. These could include infection or inflammation or exposure of the cell to toxins such as ethanol and trace metals. UV light, starvation,

hypoxia, or water deprivation can also increase *hsp* gene expression. HSPs are divided into different families according to their size (Santoro, 2000).

WormBase lists more than 20 *C. elegans* genes in the *hsp* gene class (WormBase, 29/04/2010).

1.4 Furan

1.4.1 Chemical and physical properties

Furan (also known as furane or furfuran, divinylene oxide, oxacyclopentadiene, or oxole) has the chemical formula C_4H_4O, a molecular weight (MW) of 68.08 g/mol and a density of 0.936 g/ml. It is a toxic organic compound. The chemical structure of furan is shown in Figure 1.6. Furan is a clear, colourless liquid with a characteristic odour which turns brown upon standing. The melting/freezing point of furan is at -85.6 °C and its boiling point at 31.3 °C. Thus, furan quickly vaporises and is highly flammable. Its solubility in water is only 1 %, but it is miscible with ethanol.

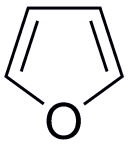

Figure 1.6: Chemical structure of furan. (own illustration)

Furan is irritating to the skin and eyes and must therefore be handled with care (Pohanish, 2002).

1.4.2 Occurrence and use of furan

Furan is ususally used in industry where it is both a solvent and an intermediate in the production of nylon, resins, lacquers, insecticides, or pharmaceuticals. It has also been found in a variety of foods, mainly those that undergo heat treatment such as bread,

coffee, tinned and bottled food including sauces, soups, meat, fish, canned fruit and vegetables, and baby food (Jestoi *et al.*, 2009; U.S. Food and Drug Administration, 2004). It is therefore one of the so called "food borne toxicants". Concentrations are especially high when food is heated in a closed system. The furan that is generated cannot leave the system and stays in the food (Kuballa, 2007). Smog and cigarette smoke also contain furan. As furan was found to be carcinogenic (Section 1.4.3), its occurrence in food has been a concern for several years now. Research is ongoing in order to determine the danger of furan in food and to find out more about its metabolism in the human body.

1.4.2.1 Mechanisms of furan formation in food

Several mechanisms have been proposed which explain the formation of furan in food (Crews & Castle, 2007; Vranová & Ciesarová, 2009; Yaylayan, 2006). The first one is the formation of furan from **carbohydrates**, also called the "Maillard reaction". This is also the main reaction responsible for the browning of foods (caramelisation). The carbohydrates react with amino acids in an aldol condensation reaction and the resulting aldotreose intermediate then undergoes cyclisation to form furan. A second mechanism is the formation of furan from **polyunsaturated fatty acids (PUFAs)** and **carotenoids**. This process involves lipid oxidation of PUFAs and the formation of lipid peroxides. Homolytic cleavage results in the formation of highly toxic 2-alkenals which are converted to furan by cyclisation and subsequent dehydration. The third mechanism is furan formation from **ascorbic acids** (vitamin C). Different pathways have been proposed depending on the reaction conditions. As ascorbic acid produces acetaldehyde and glycolaldehyde upon heating, furan can be produced through aldol condensation. In addition, similar to carbohydrate degradation, four carbon precursors could be formed and subsequently converted into furan. Furan generation from ascorbic acids is dependent on the reaction conditions (e.g. headspace, amount of starch present)

and seems to be less efficient than the generation from PUFAs (Owczarek-Fendor *et al.*, 2010). A summary of the proposed mechanisms of furan generation is shown in Figure 1.7.

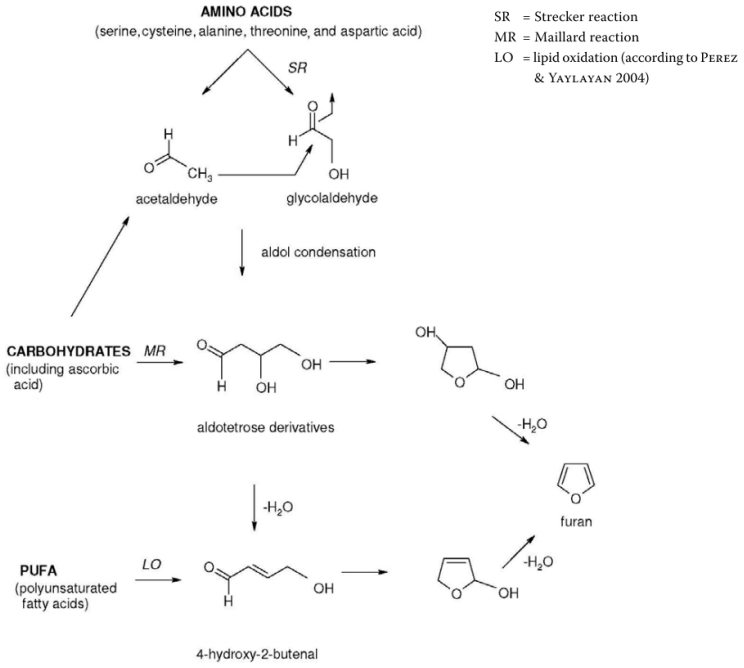

Figure 1.7: Proposed mechanisms of furan generation. Furan is generated from carbohydrates, polyunsaturated fatty acids, or via aldol condensation (Vranová & Ciesarová, 2009).

1.4.2.2 Detection and quantification of furan in food

There are two approaches for the detection of furan in food samples. The first is based on direct headspace-gas chromatography/mass spectrometry (GC/MS). Due to the high volatility of furan, this is the obvious method. Samples are usually chilled at ∼4 °C and homogenised. Sufficient cold water must be present for the the sample to be mobile. Partitioning of furan from the sample into the headspace is affected by time, temperature, and the mobility of the sample. For headspace analysis the samples are usually heated. However, for furan this is unnecessary. Also for some samples it might cause the formation of additional furan. The second method that was developed

for furan detection is based on solid phase microextraction-GC/MS (SPME-GC/MS), which is more sensitive. In both procedures furan is identified by GC, looking at the retention time and the correct ratio of the molecular ion at m/z 68 to the fragment ion at m/z 39. Quantification can be done by adding a deuterium-labelled internal standard (Crews & Castle, 2007; Yaylayan, 2006).

1.4.3 Furan toxicity and carcinogenesis

Furan is hepatotoxic and hepatocarcinogenic both *in vitro* and *in vivo*. It can induce cytolethality in freshly isolated F-334 rat hepatocytes in suspension. This effect is dose- and time-dependent (Carfagna *et al.*, 1993). *In vivo* studies have shown that furan is a rodent carcinogen. In a 2-year bioassay in F344 rats, furan induced liver tumors and cholangiocarcinomas (cancer of the bile ducts) (Burka *et al.*, 1991).

The National Toxicology Program (NTP) conducted several studies on furan toxicity and carcinogenesis in 1993. Doses up to 160 mg/kg body weight furan in corn oil were given by gavage to F344/N rats and B6C3F$_1$ mice of each sex for 16 days, 13 weeks and 2 years. After 16 days, mottled and enlarged livers were observed at necropsy in rats that were given 20-160 mg/kg. Mortality was increased in both species. After 13 weeks, increased liver weights were observed in all mice and rats that were given furan. There was also an increase in kidney weights in female rats and a decrease in thymus weights in all rats. Toxic lesions of the liver could be seen in all rats and mice that received furan and these lesions increased with dose. Rats that received 30 or 60 mg/kg showed kidney lesions; mice that received the same doses showed bile duct hyperplasia and cholangiofibrosis. Rats that received 60 mg/kg showed thymic atrophy and testicular or ovarian atrophy. After two years during which rats were given 2, 4, or 8 mg/kg five days per week, hepatocellular adenomas or carcinomas were significantly increased. The incidence of non-neoplastic liver lesions was also increased. Survival was significantly decreased in rats that received 8 mg/kg. Mice dosed with 8 or 15

mg/kg five days per week over two years showed decreased survival and various non-neoplastic hepatocellular lesions as well as an increase in hepatocellular adenomas and carcinomas (National Toxicology Program, 1993).

In a comprehensive study over three months, male Sprague Dawley rats were given daily doses of furan by gavage. They developed liver changes as early as 8 hours after dosage. Histological and immunocytochemical investigations revealed that repair processes following early liver injury caused by furan eventually led to cholangiofibrosis (Hickling *et al.*, 2010).

Since 1995 furan has been classified as "possibly carcinogenic to humans (Group 2B)" (International Agency for Research on Cancer (IARC), 1995).

Only recently it was found that subchronic furan treatment has an impact on the reproductive system of the male rat. Rats that were given 2, 4, and 8 mg furan/kg bwt daily over 90 days were examined. The number of red blood cells, thrombocyte numbers and hemoglobin concentrations were increased. No differences in sperm counts were observed, but furan caused apoptosis in germ cells and Leydig cells, impaired spermatogenesis and decreased luteinising hormone (LH) and testosterone levels (Karacaoğlu & Selmanoğlu, 2010).

The highest level of furan in food was 6.9 mg/kg, detected in roasted ground coffee (European Food Safety Authority, 2010). It is therefore worth noting that the lowest tested dose in all these studies was 2 mg/kg bwt per day. For an adult of 60 kg, this would be equal to 20.7 kg ground coffee per day. Mean levels of furan in coffee were generally less than 100 µg/kg.

Mechanism of furan toxicity

The mechanisms of furan toxicity are still unknown. Studies have yielded different results as to whether or not it shows genotoxic activity.

Reynolds *et al.* (1987) found a different pattern of mutations in *H-ras* genes of furan treated animals from the one observed in untreated controls, even though the overall frequency was similar. Spontaneous mutations occurred at codon 61 which resulted in activation of *H-ras* genes, whereas 60 % of the furan-induced mutations were either in *K-ras* genes or at positions other than codon 61. They concluded that there were genotoxic effects of furan, as they could not find cytotoxic effects that would have suggested an alternative mechanism. Unfortunately, it is unclear what doses caused the effects described in this study. Johansson *et al.* (1997) reached a similar result. They reported an increased incidence and multiplicity of liver tumors in $B6C3F_1$ mice treated with six doses of 200 mg/kg bwt furan. They also found a numerical but not statistically significant increase in tumor incidence and multiplicity following a single dose of 400 mg/kg bwt furan. Interestingly, they report an 82 % relative activation frequency of *Hras1* in tumors from the 400 mg/kg bwt group but only a 32 % relative activation frequency in the 6 x 200 mg/kg bwt group. These findings suggest that there may be a different mechanism of tumor induction at low and high doses. The authors conclude that the induction of liver tumors by furan is at least partially attributable to a genotoxic mode of action.

Furan induced trifluorothymidine (TFT) resistance in mouse lymphoma cells. Furthermore, higher frequencies of sister chromatid exchanges (SCE) and chromosomal aberrations were observed in bone marrow cells of male $B6C3F_1$ mice treated with furan compared to controls. The same was found in Chinese hamster ovary (CHO) cells (National Toxicology Program, 1993). These results are also indicative of genotoxicity.

Other NTP experiments of the 1993 study, however, did not indicate genotoxicity. Furan was tested for mutagenicity in *Salmonella typhimurium* (strains TA100, TA1535, TA1537, and TA98 with and without induced S9). Concentrations up to 10,000 µg/plate

were tested. None of the strains showed any mutagenic activity. The sex-linked recessive lethal (SLRL) test in male *Drosophila* also failed to show any increase in mutations after administration of furan in feed or by injection (National Toxicology Program, 1993). These results correspond with the findings of Burka *et al.* (1991). They studied male rats in a 2-year bioassay with [^{14}C]furan and found an increase in cholangiocarcinomas. After showing that most of the furan-derived radioactivity could be found in the liver, they could not detect any binding of furan to DNA. Furan metabolites could be found in the urine, therefore it was suspected that furan is metabolised in the liver with reactive intermediates binding to protein. These metabolites may also be responsible for carcinogenesis. Using a flow cytometer-based micronucleus assay looking at mouse erythrocytes and human lymphocytes, Durling *et al.* (2007) also found no evidence of genotoxicity *in vivo* or *in vitro*. In studies over three weeks and two years Moser *et al.* (2009) could show that furan induces hepatic cytotoxicity and compensatory cell proliferation in mice. A threshold of 4.0 mg furan/kg bwt for furan-induced liver tumors was found. Lower doses did not induce hepatocarcinogenesis. Again, these results indicate that furan is not genotoxic.

Pre-treatment of cells with the cytochrome P450 ligand and inhibitor 1-phenyl-imidazole inhibited furan-induced cytolethality and GSH-depletion *in vitro* and prevented increases in state 4 respiration and ATPase activity caused by furan in F-344 rats (Carfagna *et al.*, 1993; Mugford *et al.*, 1997). In contrast cytolethality and GSH-depletion were amplified by pre-treatment of rats with acetone, which induces cytochrome P450 2E1 (Carfagna *et al.*, 1993). Furan biotransformation in F-344 rats could be inhibited by pre-treatment with aminobenzotriazole, which is an inhibitor of cytochromes P450 (Kedderis *et al.*, 1993). These results suggest that cytochrome P450 is required for furan metabolism and that it is a metabolite that causes toxicity.

Peterson *et al.* (2006) conducted LC/MS/MS experiments on the urine of rats treated

with radioactively labeled furan. They detected the monoGSH conjugate of *cis*-2-butene-1,4-dial (Figure 1.8). This indicates that furan is mainly metabolised to *cis*-2-butene-1,4-dial which in turn undergoes GSH conjugation. It is suspected that the genotoxic effects shown by some studies were also induced by *cis*-2-butene-1,4-dial and not by furan itself (Durling *et al.*, 2007; Fransson-Steen *et al.*, 1997).

Figure 1.8: Chemical structure of *Cis*-2-butene-1,4-dial. (own illustration)

Peterson *et al.* (2000) found that *cis*-2-butene-1,4-dial is mutagenic in the Ames assay in *Salmonella typhimurium* TA104 but not in TA97, TA98, TA100, and TA102. These results indicate that *cis*-2-butene-1,4-dial induces specific mutations in bacteria and could therefore be an important genotoxic and toxic intermediate in furan-induced hepatocarcinogenicity. Byrns *et al.* (2006) developed an assay for the detection of *cis*-2-butene-1,4-dial-derived DNA adducts. They discovered that *cis*-2-butene-1,4-dial forms adducts with deoxycytidine (dCyd), deoxyadenosine (dAdo) and deoxyguanosine (dGuo) *in vitro*. They also detected dCyd and dAdo adducts in isolated DNA from *S. typhimurium* TA104 treated with *cis*-2-butene-1,4-dial.

Kellert *et al.* (2008) looked at the effect of *cis*-2-butene-1,4-dial on L5178Y $tk^{+/-}$ mouse lymphoma cells. They report an increase in DNA migration in the comet assay and an increase in the frequency of mutants in the mouse lymphoma $tk^{+/-}$ assay. However, they did not find any effect of furan in the micronucleus assay.

The discussion about the possible genotoxicity of furan is ongoing and the results remain inconclusive. Nevertheless, it seems likely that furan is metabolised by a cytochrome P450 in the liver with *cis*-2-butene-1,4-dial as the main metabolite, which appears to be responsible for the toxic effects seen in some studies.

1.4.4 Risk assessment

Assuming a NOAEL of 2 mg/kg bwt per day, Kuballa (2007) applied a safety factor of 1000. The ADI would therefore be 2 µg/kg bwt. For the average adult with 60 kg body weight, this means an ADI of 120 µg. The European Food Safety Authority (2010) reports the analysis of 1322 baby food samples between 2004 and 2009, detecting concentrations up to 224 µg/kg. Assuming an intake of commercial baby food in glass jars of 234 g food per day, this would lead to an exposure of up to 52 µg furan per day. Given that a baby has a relatively small body weight and jarred baby food might be the only diet for many infants, the acceptable daily intake would be reached easily.

For risk assessment purposes it is very important to know what mechanisms of action are involved in furan carcinogenesis. Non-genotoxic carcinogens usually activate receptors. Receptor activation follows non-linear kinetics, thus the dose response relationship has a no effect level. This means that below a certain threshold no effect can be seen (NOAEL). In contrast, genotoxic carcinogens cause cancer even in very low doses. One single DNA adduct may theoretically lead to a mutation that causes cancer. Figure 1.9 (page 33) shows the dose response curves for two different toxicants.

1.5 Aims of the project

The fact that the mechanism of furan toxicity and carcinogenesis is still unknown warrants further research into the problem. The aim of this project is to contribute to the understanding of furan toxicity. This is important as it might have implications on risk assessment. Current experiments described in the literature concentrate on the effects of furan in mice and rats or *in vitro* studies. The use of *C. elegans* molecular biology is a novel approach to the problem. As major pathways and mechanisms are often highly conserved between species, this could give further insight into the toxicity

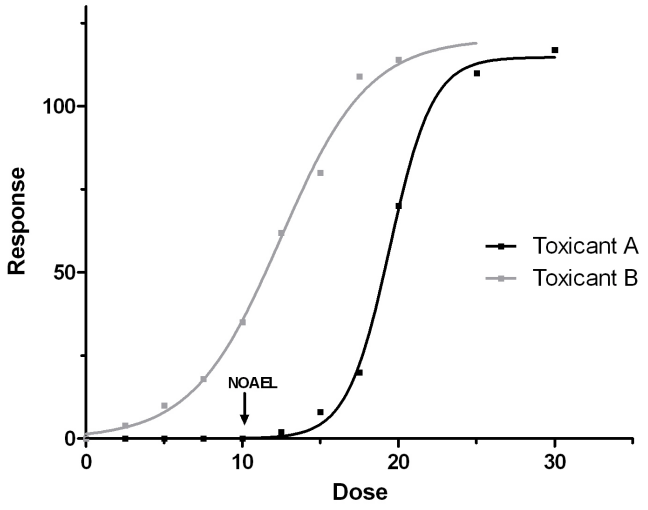

Figure 1.9: Comparison of the dose-response relationships for two different toxicants A and B. For toxicant B there is a response at any dose with no threshold. For toxicant A there is a threshold level below which there is "no observed adverse effect" (NOAEL). For compounds such as B there is no safe dose. (own illustration)

of furan. The following approaches were chosen to characterise the mode of action of furan in *C. elegans*:

- Furan inhibits growth, reduces brood size and increases lethality in C. elegans (Budd, 2008). In earlier projects EMS-derived mutants had been isolated that were apparently resistant to furan (Cai, 2009).

  The first aim of this project is to identify the gene affected. This is approached by selecting furan resistant mutants in a brood size assay. The next step is to map the mutation to a relatively small area of the genome using SNP mapping techniques and subsequent whole genome sequencing.

- Toxicity assays are used to characterise the effect of different furan concentrations on growth and feeding.

- Changes in the expression of several genes known to be involved in *C. elegans* stress response are monitored using GFP reporter strains.

2 Materials and Methods

2.1 Chemicals

The hazard and risk assessments of all substances used in my work were read and understood in advance of carrying out the work and the recommended precautions were adhered to.

Table 2.1 lists all chemicals that were used in these experiments.

Table 2.1: List of chemicals and suppliers

Chemical	**CAS-No.**	**Supplier**
Agar	n/a	Melford Laboratory Ltd.
$CaCl_2$	10043-52-4	Sigma-Aldrich
Cholesterol	57-88-5	Sigma-Aldrich
Citric acid ($C_6H_8O_7$)	77-92-9	B.D.H. Ltd.
$CuSO_4 \cdot 5\ H_2O$	7758-99-8	Fisher Scientific UK Ltd.
Ethanol absolut (C_2H_5OH)	64-17-5	Sigma-Aldrich
$FeSO_4 \cdot 7\ H_2O$	7782-63-0	Sigma-Aldrich
Furan C_4H_4O	111-00-9	Sigma-Aldrich
Glycerol $C_3H_5(OH)_3$	56-81-5	Duchefa Farma B.V.
KCl	7447-40-7	B.D.H. Ltd.
KH_2PO_4	7778-77-0	Fisher Scientific UK Ltd.

Continued on next page

Table 2.1 – continued from previous page

Chemical	CAS-No.	Supplier
K_2HPO_4	7758-11-4	Fisher Scientific UK Ltd.
LB Agar	n/a	Melford Laboratory Ltd.
LB Broth	n/a	Melford Laboratory Ltd.
$MgSO_4$	1003-99-8	Fisher Scientific UK Ltd.
$MnCl_2 \cdot 4\ H_2O$	13446-34-9	Sigma-Aldrich
NaCl	7647-14-5	Fisher Scientific UK Ltd.
Na_2EDTA	139-33-3	Sigma-Aldrich
NaOCl	7681-52-9	Sigma-Aldrich
NaOH	1310-73-2	Fisher Scientific UK Ltd.
Peptone	n/a	Sigma-Aldrich
Potassium citrate ($C_6H_5K_3O_7$)	866-84-2	Fisher Scientific UK Ltd.
Sodium azide (NaN_3)	26628-22-8	Sigma-Aldrich
Sucrose ($C_{12}H_{22}O_{11}$)	57-50-1	Fisher Scientific UK Ltd.
Tryptone	n/a	Melford Laboratory Ltd.
Yeast extract	n/a	Melford Laboratory Ltd.
$ZnCl_2$	7646-85-7	Sigma-Aldrich
$ZnSO_4 \cdot 7\ H_2O$	7446-20-0	Sigma-Aldrich

2.2 Buffers and Media

Solutions were made up to 1 l with UHP water, autoclaved, and stored at room temperature unless otherwise stated.

Citrate buffer (1 M, pH 6.0)

1 M citric acid

1 M potassium citrate

500 ml of citric acid and potassium citrate were prepared and autoclaved. The citric acid was then titrated with potassium citrate until pH 6.0 was reached.

Egg isolation bleach solution

6 ml 1 M NaOCl (available chlorine 10-15 %, stored at 4 °C)

12 ml 10 M NaOH

30 ml H_2O

The NaOCl should not be older than 6 months to prevent degradation. The bleach solution can be stored at 4 °C for several weeks.

Freezing Down Solution

300 g/l Glycerol

5.85 g/l NaCl

50 mM Potassium phosphate buffer (pH 6.0)

After autoclaving, $MgSO_4$ was added to a final concentration of 0.3 mM. Storage was at 4 °C.

K-Medium

32 mM KCl

53 mM NaCl

Luria Bertani (LB) agar

10 g/l NaCl

5 g/l Tryptone

5 g/l Yeast Extract

17 g/l Agar

LB agar was bought as a ready-made mix from Melford Laboratory Ltd. and made up according to the manufacturers instructions.

Luria Bertani (LB) broth

10 g/l NaCl

10 g/l Tryptone

5 g/l Yeast Extract

LB broth was bought as a ready-made mix from Melford Laboratory Ltd. and made up according to the manufacturers instructions.

Nematode Growth medium (NGM) agar

3 g/l NaCl

17 g/l Agar

2.5 g/l Peptone

5 mg/l Cholesterol

Cholesterol was prepared as a 5 mg/ml stock in ethanol and kept at 4 °C. The stock solution was added before autoclaving. After autoclaving the NGM agar was allowed to cool to 60 °C. The following solutions were heated to 60 °C in a water bath and added to the agar to give a final concentration of:

1 mM $CaCl_2$

1 mM $MgSO_4$

40 mM Potassium phosphate buffer (pH 6.0)

Potassium phosphate buffer (pH 6.0)

1 M KH_2PO_4

1 M K_2HPO_4

1 M KH_2PO_4 was titrated with 1 M K_2HPO_4 until pH 6.0 was reached.

S-Basal

5.84 g/l NaCl

50 mM Potassium phosphate buffer (pH 6.0)

S-Medium

The following substances were added to S-Basal to get S-Medium:

10 ml/l Cholesterol

2 mM Citrate buffer (pH 6.0)

0.3 mM $CaCl_2$

0.3 mM $MgSO_4$

0.05 % Trace metal solution

Trace Metal Solution

2.5 mM $FeSO_4 \cdot 7\ H_2O$

5 mM Na_2EDTA

1 mM $MnCl_2 \cdot 4\ H_2O$

1 mM $ZnSO_4 \cdot 7\ H_2O$

0.1 mM $CuSO_4 \cdot 5\ H_2O$

The trace metal solution was stored in the dark.

2.3 *C. elegans* - General methods

The reference Bristol N2 strain was used in previous studies to generate furan resistant mutants of *C. elegans* (Cai, 2009). Mutant stocks had been frozen and were used in these experiments for further analysis. They were labeled 8R1, 13R1, 13R2, 16R2, and 16R6. CB1480 *him*-7 and CB1467 *him*-5 strains that show 3 % and 20 % male (XO) self-progeny respectively, were used in additional experiments (WormBase, 29/04/2010). *C. elegans* were kept on Nematode Growth Medium (NGM) agar plates with *E. coli* OP50. They were handled according to established protocols (Stiernagle, 1999). Worms were usually kept at 15 °C until otherwise stated.

2.3.1 Microscopy of *C. elegans*

C. elegans were looked at under a Wild M3 stereo microscope at 6.4x, 16x and 40x magnification. Worms from the L1 growth assay were imaged on microscope slides using a Zeiss Axiovert 135TV microscope at 20x magnification, and photographed using a Scion 1310M CCD camera and the MicroManager 1.3 software. The worm images were saved as .tif files and processed in Adobe Photoshop CS3.

Preparation of agarose pads

For the agarose pads, a 2 % (w/v) agarose solution was used. Figure 2.1 illustrates the preparation process. Tape was stuck lengthwise along two slides. This provided a spacer for the agarose pad. A clean slide was placed between the two taped slides. Two drops of melted agarose were placed on the slide and another slide was quickly placed on top at right angles. After a few minutes the agarose was set. The top slide was removed immediately before using the pad in order to prevent the agarose from drying out.

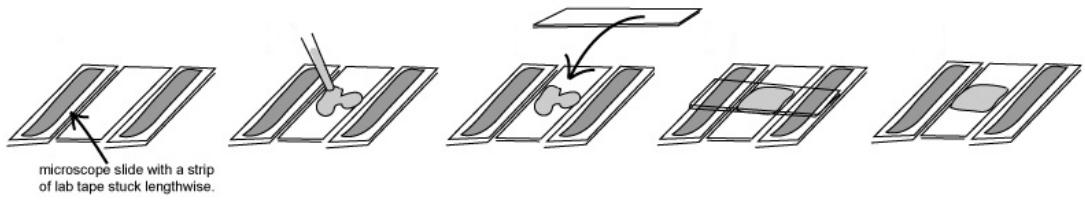

Figure 2.1: Preparation of agarose pads on microscope slides (Hyman, 2010).

2.3.2 Preparation of agar plates

NGM agar was prepared as explained in Section 2.2. The following steps were carried out under a class II hood:

For 3 cm plates, exactly 4 ml of agar was poured into each plate with a syringe. 9 cm and 14 cm plates were poured by hand. The plates were left to dry and *E. coli* culture was added. *E. coli* OP50 is dependent on uracil, which results in a thin bacterial lawn on the plates. This is important to enable *C. elegans* movement. After drying, the plates were incubated overnight at 37 °C and then kept in a closed plastic container at room temperature until use.

2.3.3 Transfer of worms

A wormpick was used to transfer single worms from one plate to another. A wormpick is a small piece of platinum wire sealed to a glass pasteur pipette. When plates were almost starved, worms were transferred to new plates by "chunking": a small piece of agar was cut out with a sterile scalpel and transferred to a new plate. To transfer all worms from one plate, e.g. to a centrifuge tube, worms were washed off with several millilitres of K-Medium or S-Basal and transferred with a pipette.

2.3.4 Egg isolation

The isolation of eggs from a plate is referred to as "bleaching". It is done to synchronise cultures, and to remove contamination such as mould or bacteria other than *E. coli*. For synchronisation, 3 cm plates containing mostly gravid adult worms were used. The worms were washed off the plate with 2 ml S-Basal and 3 x 0.5 ml were transferred to three 1.5 ml Eppendorf tubes. The same amount of bleach solution was added and the tubes were immediately vortexed for three minutes. The tubes were then centrifuged for two minutes at 500 g and 4 °C. The supernatant was discarded and the pellets were each washed with 1 ml of S-Basal. Centrifugation and washing were repeated three times. The pellets were then resuspended in 3 x 0.5 ml of S-Basal. The eggs were transferred to a six well plate (0.5 ml per well) which was sealed with parafilm and incubated at 15 °C over night to allow L1 worms to hatch. The worms were then transferred to fresh 3 cm NGM plates containing *E. coli* OP50 with a pipette.

2.3.5 Freezing and Thawing

Worm stocks were frozen at -80 °C for future use. To prepare worms for freezing, an egg isolation was done and the L1 worms were transferred to a bacteria-free 9 cm plate. The plate was incubated at 20 °C until the worms were starved. They were then washed off with 3 ml K-medium. The K-Medium was transferred to a 15 ml tube and

3 ml of freezing down solution were added. The tube was vortexed and 1 ml aliquots were transferred to Eppendorf tubes. The tubes were labelled with the name of the strain and the date and stored at -80 °C.

To check if freezing was successful one aliquot was thawed at room temperature the next day and the contents transferred to a 9 cm agar plate containing *E. coli* OP50. The plate was incubated at 20 °C over night to see if worms were alive.

2.3.6 *C. elegans* liquid culture

- Single colonies of *E. coli* OP50 were incubated in 5 ml LB medium at 240 rpm, 37 °C overnight.
- 5 ml of OP50 overnight culture were added to 500 ml of LB medium in a 1 l flask and incubated in a shaking incubator at 240 rpm, 37 °C overnight.
- 500 ml OP50 overnight culture were decanted into two 500 ml centrifuge bottles and centrifuged at 11,000 g for 20 minutes.
- The pellet was resuspended in K-Medium and diluted to a OD_{600} of 1.5-1.7. The OD was measured in a Cecil Instruments Super Aquarius CE 9500 spectrophotometer.
- A 14 cm plate of starved worms was washed off with K-Medium.
- The worms were left on ice for 30 minutes to settle and the supernatant was discarded. The worms were then resuspended in 10 ml of K-Medium. This washing was repeated three times.
- The worms were then added to 500 ml of the bacterial culture in a 1 l flask.
- The liquid culture was incubated at 20 °C in a shaking incubator at 240 rpm for 5-6 days.

2.3.7 Synchronising liquid cultures

- The liquid culture containing large numbers of gravid adults was set on ice for 30 minutes for the worms to settle.
- The supernatant was removed until about 100 ml were left. The remaining liquid containing the adult worms was transferred to two 50 ml centrifuge tubes and set on ice again for 30 minutes.
- As much of the supernatant as possible was removed.
- An equal amount of bleach solution was added to the remaining liquid.
- The tubes were immediately vortexed for five minutes. The tubes were then centrifuged for two minutes at 500 g and 4 °C. The supernatant was discarded and the pellet was washed with S-Basal. Centrifugation and washing were repeated three times. The pellet was then resuspended in S-Basal.
- The isolated eggs were transferred to six well plates (1 ml per well) which were sealed with parafilm and incubated at 15 °C overnight to allow L1 worms to hatch. The worms were then transferred to fresh NGM plates with a pipette.

2.4 Toxicity Assays

Appropriate precautions were observed when handling furan.

2.4.1 Furan brood size assay

Because of the low solubility of furan in water, the assay was initially set up using stock solutions of furan in 100 % ethanol (Table 2.2, page 44). The assay ran over eight days:

- **Day 1:** 3 cm plates containing adult worms were bleached as described in section 2.3.4.

Table 2.2: Furan stock solutions in ethanol.

Stock concentration	Furan	Ethanol
200 mM	14.5 µl	985.5 µl
400 mM	29 µl	971 µl
800 mM	58 µl	942 µl
2,000 mM	145.4 µl	854.6 µl
4,000 mM	290.8 µl	709.2 µl
8,000 mM	581.6 µl	418.4 µl
12,000 mM	872.4 µl	127.6 µl

- **Day 2:** L1 worms were transferred to 3 cm NGM plates and incubated at 15 °C for three days.

- **Day 5:** 10 ml of *E. coli* OP50 liquid culture were centrifuged at 2,000 g for five minutes. The supernatant was discarded and the pellet resuspended in 10 ml S-Basal. The absorbance was adjusted to OD_{600} 0.7 against S-Basal. The assay was then set up in 48 well plates. Concentrations were tested in triplicate. Each well contained 40 µl of bacterial culture, a single L4 worm, toxin and S-Basal to a final volume of 400 µl (Table 2.3). The toxin was added last to prevent evaporation.

Table 2.3: Furan brood size assay with furan dissolved in ethanol. This is the initial protocol as carried out by Cai (2009).

Furan	OP50	L4 worms	S-Basal	Furan stock solution
0 mM	40 µl	1	359 µl	1 µl of ethanol
0.5 mM	40 µl	1	359 µl	1 µl of 200 mM
1 mM	40 µl	1	359 µl	1 µl of 400 mM
2 mM	40 µl	1	359 µl	1 µl of 800 mM
5 mM	40 µl	1	359 µl	1 µl of 2,000 mM
10 mM	40 µl	1	359 µl	1 µl of 4,000 mM
20 mM	40 µl	1	359 µl	1 µl of 8,000 mM
30 mM	40 µl	1	359 µl	1 µl of 12,000 mM

The plates were immediately sealed with two layers of parafilm and placed in a plastic container together with a damp paper towel to provide humidity. The

plates were incubated at 15 °C for three days.

- **Day 8:** The number of progeny in each well was counted. Note that this is only a partial brood size.

The concentrated furan solutions were found to dissolve the pipette tips. Therefore, tenfold dilutions of all stocks were prepared in S-Basal and 350 µl S-Basal plus 10 µl of the diluted stocks were used in the assay. This also improved the accuracy, as 1 µl is very hard to pipette. However, the results of the brood size assay were very inconsistent, so changes to the protocol were made to reduce variability.
As up to 1 % furan is soluble in water it was decided not to dissolve it in ethanol. This should reduce the confounding effects caused by chemicals other than furan. It also allowed higher concentrations of furan to be tested. The incubation time was extended to four days, because the offspring were very tiny and hard to count after three days.

The modified assay was set up over nine days:

- **Day 1:** 3 cm plates containing adult worms were bleached as described in section 2.3.4.

- **Day 2:** L1 worms were transferred to 3 cm NGM plates and incubated at 15 °C for three days.

- **Day 5:** 50 ml of bacterial culture were centrifuged at 2,000 g for five minutes. The supernatant was discarded and the pellet resuspended in 50 ml S-Basal. The absorbance was adjusted to OD_{600} 0.7 against S-Basal. The assay was then set up in a 48 well plate. Concentrations of 0, 10, 30, 60, and 90 mM furan were tested and each concentration was tested in triplicate. Each well contained 40 µl of bacterial culture, a single L4 worm, toxin and S-Basal to a final volume of 400 µl. The furan was dissolved in S-Basal (65.4 µl in 9 ml) and vortexed for

three minutes. It was added last to prevent evaporation (Table 2.4). The plates were sealed and incubated in a plastic box with damp paper towels at 15 °C for four days.

Table 2.4: Modified furan brood size assay without ethanol. 65.4 µl of furan were dissolved in 9 ml of S-Basal to give the furan solution used in this experiment.

Furan	**OP50**	**L4 worms**	**S-Basal**	**Furan solution**
0 mM	40 µl	1	360 µl	—
10 mM	40 µl	1	320 µl	40 µl
30 mM	40 µl	1	240 µl	120 µl
60 mM	40 µl	1	120 µl	240 µl
90 mM	40 µl	1	—	360 µl

- **Day 9:** The number of progeny in each well was counted. Again this was only a partial brood size.

2.4.2 L1 growth assay

The L1 growth assay measures the effect of furan on *C. elegans* growth and development. It is set up and run over five days.

- **Day 1:** 5 ml of LB broth were inoculated with a single colony of *E. coli* OP50 and incubated over night at 37 °C, 240 rpm. A plate of gravid hermaphrodite worms was bleached as described in Section 2.3.4.

- **Day 2:** 1 ml of the bacterial culture was centrifuged for five minutes at 2,000 g. The supernatant was discarded and the pellet resuspended in S-Basal.

- L1 worms were centrifuged for one minute at 500 g. The supernatant was discarded and the worms were resuspended in S-Basal to give a final concentration of 30-40 worms per 5 µl.

- The OD_{600} of the bacterial culture was adjusted to 0.7 with S-Basal.

- The assay was then set up in a 24 well plate with four wells for each concentration. Each well contained 5 µl of L1 worms, 40 µl of *E. coli* OP50 solution, toxin and S-Basal to a final volume of 400 µl. Again, the furan was dissolved in S-Basal (58.2 µl in 9 ml) and vortexed for three minutes. It was added last to prevent evaporation (Table 2.5).

Table 2.5: L1 growth assay. 58.2 µl of furan were dissolved in 9 ml of S-Basal to give the furan solution used in this experiment.

Final conc.	**OP50**	**L1 worms**	**S-Basal**	**Furan solution**
0 mM	40 µl	5 µl	355 µl	—
10 mM	40 µl	5 µl	310 µl	45 µl
30 mM	40 µl	5 µl	220 µl	135 µl
45 mM	40 µl	5 µl	152.5 µl	202.5 µl
60 mM	40 µl	5 µl	90 µl	270 µl
80 mM	40 µl	5 µl	—	360 µl

- The plates were sealed and incubated in a plastic box with damp paper towels at 15 °C for three days.
- **Day 5:** 15 µl of 1 M sodium azide were added to each well to immobilise the worms.
- The contents of each well were transferred to 1.5 ml centrifuge tubes. The lids of the tubes were removed before so that the pellet is not disturbed and centrifuged for one minute at 500 g. The supernatant was removed, leaving approximately 10-20 µl. The pellet was resuspended in the remaining liquid by briefly vortexing.
- Agarose pads were prepared as described in Section 2.3.1. Each slide was labelled with a concentration.
- The worms were gently mixed and 10 µl were carefully pipetted onto the agarose pads. A coverslip was placed on top.
- The worms were imaged under 20x magnification and photographed.

- The worm size (area) was measured in pixels using Adobe Photoshop CS3 and the magnetic lasso tool (Figure 2.2).
- The worm sizes at different concentrations were recorded as "percent of control".

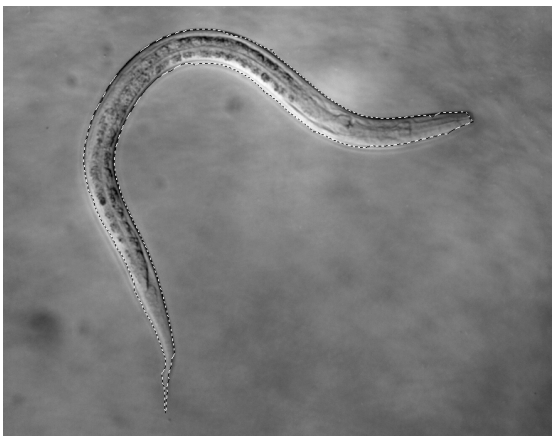

Figure 2.2: Measurement of *C. elegans* size. The worms were imaged at 20x magnification, photographed and their size (area) measured in Adobe Photoshop CS3 using the magnetic lasso tool.

2.4.3 Furan feeding inhibition assay

For the feeding inhibition assay worms grown in liquid culture were used.

- The worms were set on ice for 30 minutes to settle down. Then the supernatant was discarded and the worms washed with K-Medium. This washing procedure was repeated several times to remove all bacteria.
- The worms were resuspended in a small volume of K-Medium, transferred to a small beaker, and stirred.
- Six well plates were labelled with the concentrations.
- 50 µl of worms were added to each well. The worms were stirred during dispensing to make sure equal numbers were added to each well.

- 1,500 µl of *E. coli* OP50 (OD_{550} 1.0) containing the furan were added to each well. Table 2.6 shows the amount of furan dissolved per milliliter OP50 suspension for this experiment. The solution was vortexed for three minutes before dispensing.

Table 2.6: Furan solutions used for feeding inhibition assay. The furan was dissolved directly in OP50 suspension. This was done immediately before use to prevent evaporation of the furan.

Final concentration of furan	**Volume furan per ml bacterial suspension**
10 mM	0.73 µl
20 mM	1.45 µl
30 mM	2.18 µl
45 mM	3.27 µl
60 mM	4.36 µl
90 mM	6.54 µl

- The plates were immediately covered with two layers of parafilm and incubated in a plastic box for 24 hours at 15 °C.
- The next day the worms were transferred to Eppendorf tubes and left on ice for 30 minutes to settle.
- The OD of the supernatant was measured at 550 nm.

2.4.4 GFP reporter assay

In this assay GFP reporter strains of *C. elegans* were used to measure the expression levels of several genes involved in stress response pathways (de Pomerai *et al.*, 2010). The following strains were obtained from the Baillie Genome GFP Project (Simon Fraser University, Burnaby, Vancouver, Canada) as integrated promoter::GFP fusions: BC17553 (T09A12.2, designated GPA::GFP), BC20305 (C11E4.1, designated GPB::GFP), BC-20306 (cyp-34A9::GFP), BC20308 (hsp-3::GFP), BC20316 (gst-1::GFP), BC20330 (gst-4::GFP), BC20333 (sod-4::GFP), BC20334 (cyp-29A2::GFP), and BC20350

(sod-1: :GFP). Thanks also to Cynthia Kenyon for CF1553 (sod-3::GFP), Chris Link for CL2050 (hsp-16.2::GFP), Joel Rothman for JR2474 (cep-1::GFP), and Ralph Menzel for a strain with pPD97 87-35A2prIII-GFP (cyp-35A2::GFP). A description of the genes and their functions can be found in Table 1.1 (page 17).

- Two 14 cm plates containing worms of all stages were washed off with K-Medium and set on ice for 30 minutes to settle down. Then the supernatant was discarded and the worms resuspended in K-Medium. This washing procedure was repeated several times to remove all bacteria.

- The worms were resuspended in a small volume of K-Medium, transferred to a small beaker, and stirred.

- 24 well plates were labelled with the concentrations. Individual plates were used for different concentrations. Because of the high volatility of furan, individual plates were also used for the measurements at different time points so the plates did not have to be opened during the course of the experiment.

- 150 µl of worms were added to each well. The worms were stirred during dispensing to make sure equal numbers were added to each well.

- 150 µl of 2x concentrated furan in K-Medium were added to each well. The furan solutions were vortexed for three minutes before distribution. Because of the low solubility of furan only concentrations up to 60 mM could be tested.

- The plates were covered with two layers of parafilm and incubated in a plastic box at 15 °C.

- Measurements were taken after 4, 8, 16, and 24 hours in order to see early, intermediate, and late induction. The contents of the wells were transferred to black, non-fluorescent 96 well plates and the levels of GFP fluorescence measured.

This was done in a Perkin-Elmer Victor 1420 plate fluorometer with the Wallac 1420 Manager software. Each well was measured four times for one second at a time, using a "F485" CW (continuous wave) excitation filter and a "F535" emission filter.

2.5 Data presentation and statistics

All graphs and statistical analyses were done using GraphPad Prism 5.

Statistical analysis

Statistical analysis was carried out to determine if the difference between groups of worms was statistically significant ($P<\alpha$, $\alpha=0.05$). The null hypothesis (H_0) was that there is no difference between the groups. If $P<0.05$, H_0 was rejected and the difference was statistically significant. If $P>0.05$, H_0 could not be rejected.
For the brood size assay and the feeding inhibition assay, one-way ANOVA was used which is based on several assumptions:

- The data must be from a population that is normally distributed (or approximately normally distributed).
- The samples must be independent.
- The variances of the samples must be equal. This was proven by Bartlett's test for equal variances.

Dunnett's multiple comparison test was done to look at differences between group means. This test compares groups against a reference. The null hypothesis is that no group has a mean that is significantly different from the mean of the reference group. For the brood size assay, all mutant strains and the two *him* strains were compared to N2 wild type. For the feeding inhibition assay, the mean OD_{550} of the samples that did not contain furan was used as reference.

3 Results

3.1 Thawing of frozen mutant strains

Stocks of furan resistant mutant worms had been frozen and were thawed to identify the mutation. The stocks were labelled 8R1, 13R1, 13R2, 16R2, and 16R6. Two aliquots of 1 ml were available for each strain. Unfortunately, it was not possible to find out which of these strains corresponded to the "resistant lines" mentioned by Cai (2009). Therefore, all strains were thawed and analysed. The aliquots were thawed at room temperature and the contents of the tubes transferred to 9 cm agar plates containing *E. coli* OP50. The plates were incubated at 20 °C over night.

As can be seen in Table 3.1, all stocks only contained very small numbers of worms (<10 in each aliquot). The 13R1 stocks did not contain any living worms and no further analyses could be done for this strain. The first aliquot of 16R6 worms only contained one living worm. This was found to be male so the second aliquot had to be thawed as well.

Table 3.1: Thawing of frozen mutant worms. This table shows the number of living worms obtained from each frozen sample of 1 ml.

strain	**1st aliquot**	**2nd aliquot**
8R1	7	not thawed
13R1	0	0
13R2	4	not thawed
16R2	7	not thawed
16R6	1	2

The strains 8R1, 13R2, 16R2, and 16R6 were grown on agar plates and tested for furan resistance in the brood size assay.

3.2 Furan brood size assay

The brood size assay was done to identify furan resistant *C. elegans* mutants. The N2 wild type worms were expected to be sensitive to 30 mM furan (Cai, 2009) and should not give any offspring. Furan resistant worms should give offspring in sublethal concentrations of furan. The experiment was also used to identify the selection concentration (if different from 30 mM) to distinguish between resistant and non-resistant worms in the following experiments.

In a 48 well plate single L4 worms were incubated in increasing concentrations of furan for three days. After incubation, the number of offspring was counted. Initially the brood size assay was done according to the method of Cai (2009). Only N2 wild type worms were tested to see whether the assay was working. The furan was dissolved in ethanol with final concentrations from 0 mM to 30 mM furan.

The results were inconsistent, highly variable and no reduction in brood size could be seen (Figure 3.1, page 55).

3.2.1 Adjustment of experimental conditions

To locate possible errors, new N2 worms were taken from frozen stocks, all solutions were freshly prepared, and a new bottle of furan was purchased. However, the results could not be improved (data not shown).

Effect of temperature changes

The temperature of the incubator was found to be slightly too high (17 °C instead of 15 °C, data not shown) and varying within the incubator from 10.8 °C on the bottom

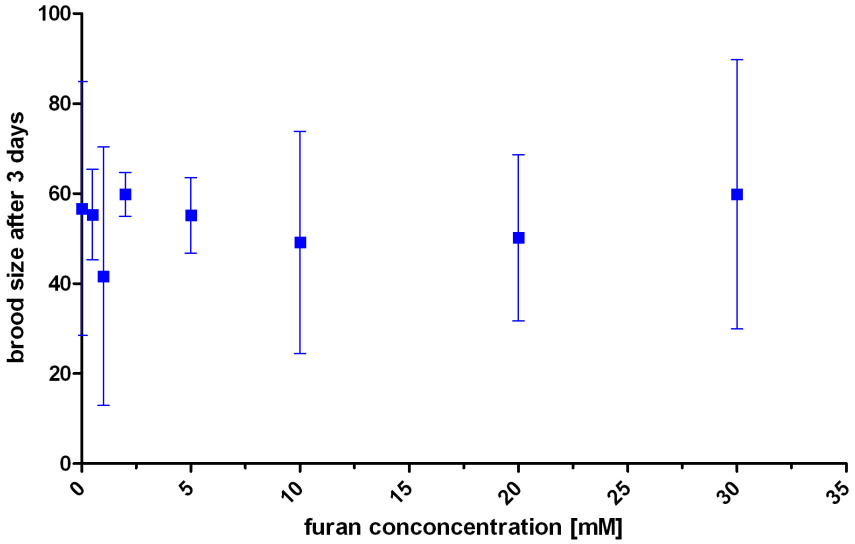

Figure 3.1: Results of furan brood size assay for N2 (mean and SD). Individual L4 worms were incubated in furan for four days and the number of offspring was counted. Each concentration was tested in triplicate. This graph shows the result of a single experiment. Similar results were obtained in three independent experiments.

shelf to 17.8 °C on the top shelf. Because higher temperatures might increase the volatility of furan, the assay was incubated in a different incubator and a data logger was put in the plastic box to monitor temperature changes. No significant difference was seen between the two incubators (data not shown). Even though the results could not be improved, the assay was from then on only incubated at the same place in the new incubator to reduce variations in experimental conditions to a minimum.

Effect of ethanol as solvent for furan on *C. elegans* brood size

A literature research showed that ethanol, which was used as solvent for furan, has an effect on movement and egg-laying in *C. elegans* (Davies *et al.*, 2003, 2004). Ethanol increases the activity of SLO-1 in neurons, which is a subunit of Ca^{2+}-activated K^+ channels (BK channels). As a result, SLO-1 inhibits neurotransmitter release which causes the effect seen in *C. elegans* (Crowder, 2004). To eliminate the effects of ethanol

on brood size, the furan was from then on dissolved directly in S-Basal.

The maximum solubility of furan in H_2O is 1 % which allowed testing up to a final concentrations of 90 mM. In addition, the incubation time was extended to four days because the offspring were very small and hard to count in the 48 well plates after three days.

Lethality

Again, a dose response experiment for N2 worms in increasing furan concentrations (0-90 mM) was done. After four days, most of the adult worms were dead even at concentrations lower than 30 mM (data not shown). This was surprising, as 30 mM was used as selection concentration by (Cai, 2009) and was not considered to be lethal to adult worms.

It was suspected that the reason for the lethality might be the high volatility of furan. Different concentrations were subsequently incubated in individual 48 well plates to prevent the furan from spreading to different wells and thus altering the concentration. This approach considerably reduced the variability within the triplicates and increased the survival of adult worms.

Final results

The results of the improved brood size assay are shown in Figure 3.2 (page 57). They indicate that furan decreases the brood size of wild type *C. elegans* in a dose-dependent manner. The brood size assay in its modified form gave robust and reproducible results. To calculate the EC_{50}, a sigmoidal curve was fitted plotting the log(agonist) vs. response. In this case, the agonist was the furan concentration and the response was the brood size. The EC_{50} for this experiment was 19.28 mM. 90 mM furan killed all adult worms and no offspring were found in these wells. Concentrations lower than 90 mM are considered sublethal. At 60 mM furan, N2 worms only gave very few offspring.

Therefore, this concentration was chosen as selection concentration to test the frozen mutant strains for furan resistance.

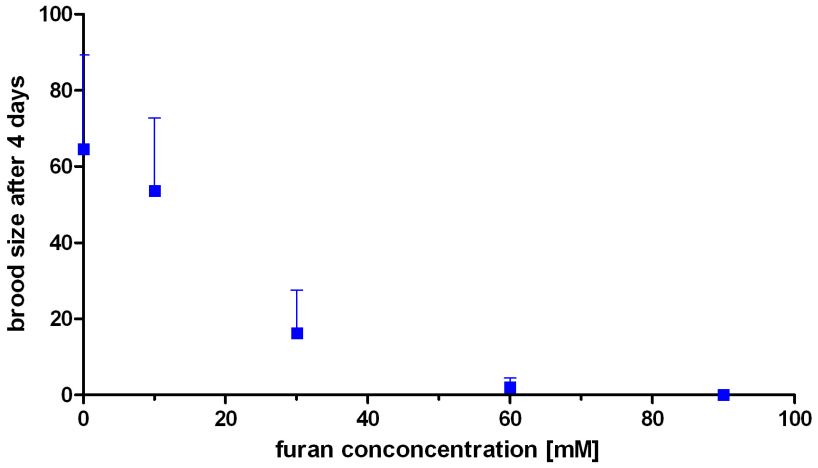

Figure 3.2: Results of furan brood size assay for N2 (mean and SD). Individual L4 worms were incubated in furan for four days and the number of offspring was counted. Each concentration was tested in triplicate. This graph shows the results of three independent experiments. Furan decreases *C. elegans* brood size in a dose-dependent manner. The EC_{50} is 19.28 mM. 90 mM furan killed all adult worms and therefore no offspring could be found. At 60 mM furan, N2 worms only gave 1-2 offspring. This concentrations was chosen as selection concentration for subsequent experiments.

3.2.2 Comparison of mutant strains to wild type

To test the mutant strains for furan resistance, a brood size assay with the new conditions was performed with worms from the frozen strains 8R1, 13R2, 16R2, and 16R6 and the number of offspring compared to N2. In addition, the two *him*-strains CB1467 and CB1480 were tested. This was done, because an increased incidence of males was observed for the mutant strains in earlier experiments and it was suspected that the mutation conferring resistance to furan might be in a *him*-gene (Cai, 2009). It was expected that at 60 mM furan, resistant worms would give significantly higher numbers of offspring than N2 worms. The results of these assays are shown in Figures 3.3 and

3.4 (pages 58 and 60).

Furan resistance of strains 8R1, 16R2, and 16R6

As can be seen in Figure 3.3, the brood sizes of N2, 8R1, 16R2, and 16R6 without furan are very similar. All brood sizes are clearly decreased at 60 mM. A one-way ANOVA (α=0.05) was carried out to determine if any of the differences between the brood sizes were statistically significant (P<α). Bartlett's test for equal variances gave a P value

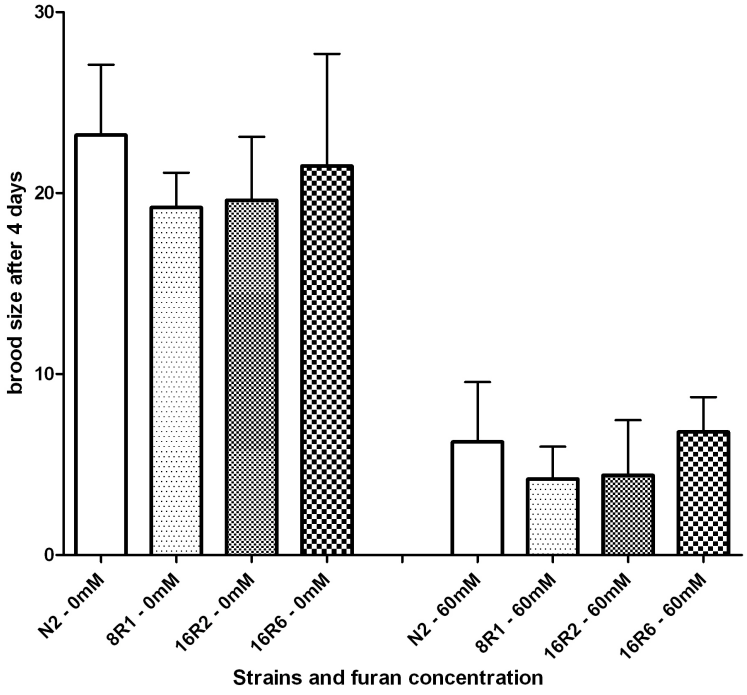

Figure 3.3: Results of furan brood size assay for strains N2 (control), 8R1, 16R2, and 16R6 (mean and SD). L4 worms were incubated in furan for four days and the number of offspring counted. Concentrations of 0 mM (left) and 60 mM (right) were tested in triplicate. The graph shows the results of three individual experiments.

of 0.3469, which shows that the variances of the samples are equal. Dunnett's Multiple Comparison Test showed that without furan, the mean brood size of 8R1, 16R2, and 16R6 are not significantly different from the mean brood size of N2 (Table 3.2, page 59).

Table 3.2: Dunnett's Multiple Comparison Test (GraphPad Prism 5). N2 worms without furan were used as control group against which all other groups were compared.

N2 (0 mM furan) vs	**Mean Difference**	**Significant? P<0.05?**
8R1 - 0 mM	4.000	No
16R2 - 0 mM	3.600	No
16R6 - 0 mM	1.800	No
N2 - 60 mM	17.00	Yes
8R1 - 60 mM	19.00	Yes
16R2 - 60 mM	18.80	Yes
16R6 - 60 mM	16.40	Yes

The brood sizes of N2, 8R1, 16R2 and 16R6 at 60 mM furan, however, are different from the mean brood size of N2 without furan. This means that the reduction in brood size seen at 60 mM furan is significant for all strains.

Table 3.3 shows that there is no difference between the brood sizes of N2 and 8R1, 16R2, and 16R6 at 60 mM furan. This means that none of the three mutant strains is significantly more resistant to furan than N2 wild type worms.

Table 3.3: Dunnett's Multiple Comparison Test (GraphPad Prism 5). N2 worms in 60 mM furan were used as control group against which all other groups were compared.

N2 (60 mM furan) vs	**Mean Difference**	**Significant? P<0.05?**
N2 - 0 mM	-17.00	Yes
8R1 - 0 mM	-13.00	Yes
16R2 - 0 mM	-13.40	Yes
16R6 - 0 mM	-15.20	Yes
8R1 - 60 mM	2.000	No
16R2 - 60 mM	1.800	No
16R6 - 60 mM	-0.600	No

Furan resistance of strains 13R2, CB1467, and CB1480

Figure 3.4 (page 60) shows that without furan, there seems to be no difference between the brood sizes of N2, 13R2, CB1467, and CB1480. Bartlett's test for equal variances gave a P value of 0.4848, which means that the variances are equal. Therefore, a

one-way ANOVA could be done here as well.

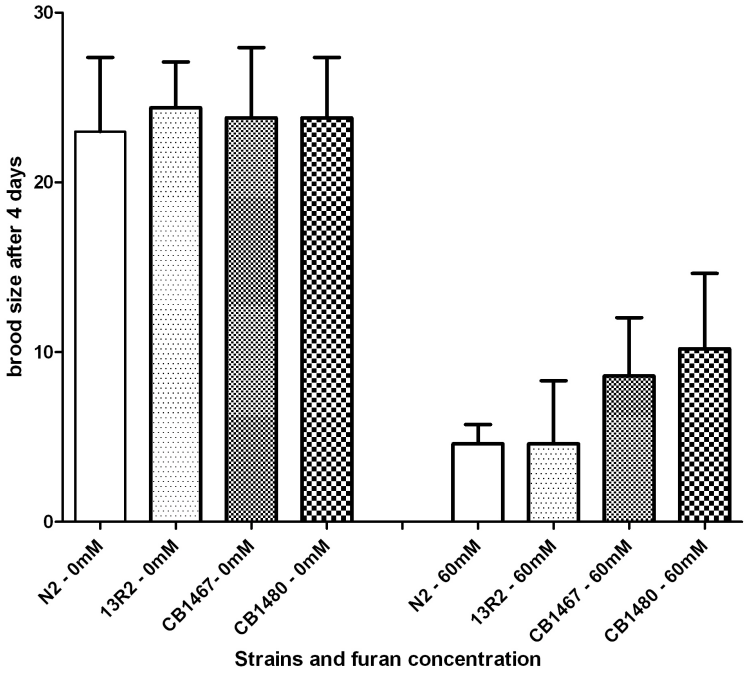

Figure 3.4: Results of furan brood size assay for strains N2 (control), 13R2, CB1467, and CB1480 (mean and SD). L4 worms were incubated in furan for four days and the number of offspring counted. Concentrations of 0 mM (left) and 60 mM (right) were tested in triplicate. The graph shows the results of three individual experiments. There is no significant difference between brood sizes of N2 and 13R2.

There is no difference between the brood sizes of N2 and 13R2, CB1467 and CB1480 without furan (Table 3.4, page 61). All brood sizes are decreased at 60 mM furan. It seems as if the brood sizes of CB1467 and CB1480 at 60 mM are different from the brood size of N2. However, Dunnett's multiple comparison test showed that under these conditions, the difference is not significant (Table 3.5, page 61).

Table 3.5 also shows that there is no significant difference between the brood sizes of N2 and 13R2. Thus, there is no significant increase in furan resistance in 13R2, CB1467 and CB1480 compared to N2 wild type.

Table 3.4: Dunnett's Multiple Comparison Test (GraphPad Prism 5). N2 worms without furan were used as control group against which all other groups were compared.

N2 - 0 mM furan vs	**Mean Difference**	**Significant? P<0.05?**
13R2 - 0 mM	-1.400	No
CB1467 - 0 mM	-0.800	No
CB1480 - 0 mM	-0.800	No
N2 - 60 mM	18.40	Yes
13R2 - 60 mM	18.40	Yes
CB1467 - 60 mM	14.40	Yes
CB1480 - 60 mM	12.80	Yes

Table 3.5: Dunnett's Multiple Comparison Test (GraphPad Prism 5). N2 worms in 60 mM furan were used as control group against which all other groups were compared.

N2 - 60 mM furan vs	**Mean Difference**	**Significant? P<0.05?**
N2 - 0 mM	-18.40	Yes
13R2 - 0 mM	-19.80	Yes
CB1467 - 0 mM	-19.20	Yes
CB1480 - 0 mM	-19.20	Yes
13R2 - 60 mM	0.000	No
CB1467 - 60 mM	-4.000	No
CB1480 - 60 mM	-5.600	No

3.2.3 Summary

Together, these results clearly show that furan has an effect on *C. elegans* brood size. Furan inhibits *C. elegans* egg laying with an EC_{50} of about 20 mM. A decrease in the number of offspring at 60 mM furan was observed not only in N2 wild type worms but also in the mutant strains 8R1, 13R2, 16R2, and 16R6 as well as the *him*-strains CB1467 and CB1480. The furan resistance of the mutant strains generated by Cai (2009) could not be confirmed. Therefore SNP mapping and subsequent whole genome sequencing could not be done for any of the strains as originally planned.

It could be argued that an intermediate dose of furan (e.g. 30 mM or 45 mM) might reveal some significant differences between N2 and the mutant strains. These concentrations were not tested on the mutant strains, because a selection concentration was

needed for SNP mapping. Lower concentrations would not have decreased the brood size of wild type worms enough to identify resistant worms.
It is also possible that with more data (and narrower error bars) the difference between the brood sizes of CB1467 and CB1480 compared to N2 at 60 mM will be significant. This was not further investigated as the mutation in these strains is known. Therefore SNP mapping and whole genome sequencing with these strains would not have given any new information.

3.3 L1 growth assay

To measure the effect of furan on *C. elegans* growth, L1 worms were incubated in different concentrations of furan. After three days, the worms were imaged at 20x magnification and photographs were taken. The size of the worms was measured and compared to controls without furan.

3.3.1 Experimental conditions and problems

The measurements of *C. elegans* size were done using Adobe Photoshop and the magnetic lasso tool to determine the area of the worm. The size was then compared to 0 mM furan control. Initial measurements were very inaccurate and the results were inconsistent between experiments. The contrast of the worm images was not high enough for the magnetic lasso tool to find the exact outline of the worms. Some of the worms were not lying straight, therefore it was impossible to measure the length of the worms.
No effect of furan on *C. elegans* size could be seen (Figure 3.5, page 63). To improve the results of the measurements, more sodium azide was added for the worms to completely "relax". This gave better data in Adobe Photoshop. Also, more worms were photographed.

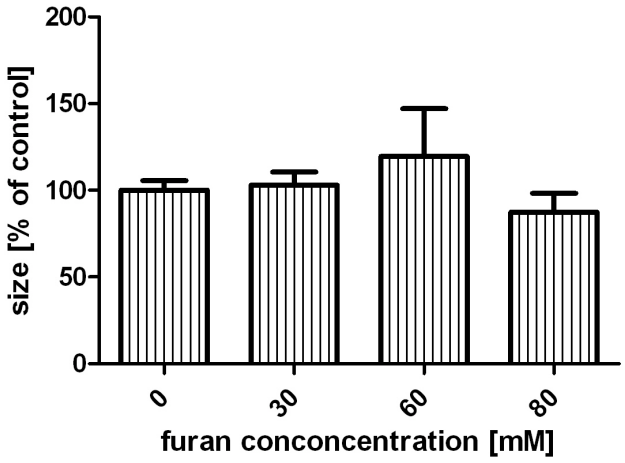

Figure 3.5: Initial results of L1 growth assay with N2 (mean and SEM). L1 worms were incubated in furan for 3 days. 10 worms were photographed at 20x magnification and the area measured in Adobe Photoshop. The measurements were inaccurate and the results were not consistent between experiments. No effect of furan on *C. elegans* growth could be seen.

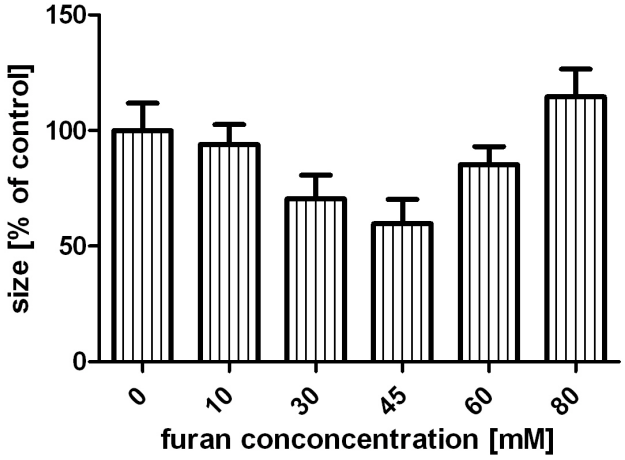

Figure 3.6: Results of the modified L1 growth assay with N2 (mean and SEM). L1 worms were incubated in furan for three days. 20 worms were photographed at 20x magnification and the size measured in Adobe Photoshop. A reduction of *C. elegans* size can be seen for furan concentrations from 10 mM to 45 mM, although this effect is not statistically significant.

3.3.2 Effect of furan on *C. elegans* growth

The effect of furan on C. elegans growth can be seen in Figure 3.6 (page 63). Growth seems to be inhibited at concentrations ranging from 10 mM to 45 mM furan. Interestingly, this is not true for 60 and 80 mM. At 80 mM *C. elegans* are even larger than without furan. These worms do not have any obvious phenotype, e.g. they are not dumpy, which would increase the area in the photographs and therefore influence the measurements. However, a Dunnett's test showed that these differences in size are not statistically significant (Table 3.6). So far, these results have not been confirmed.

Table 3.6: Dunnett's Multiple Comparison Test (GraphPad Prism 5). N2 worms incubated without furan were used as control group against which all other groups were compared.

0 mM furan vs	**Mean Difference**	**Significant? P<0.05?**
10 mM	1850 px	No
30 mM	8966 px	No
45 mM	12223 px	No
60 mM	4491 px	No
80 mM	-4415 px	No

3.3.3 Summary

Growth seems to be inhibited by up to 45 mM furan in a dose-dependent manner. At higher concentrations furan seem to enhance growth. However, this effect is not statistically significant in this experiment. Further experiments have to be done to see if these anomalous results prove to be true.

3.4 Furan feeding inhibition assay

The feeding inhibition assay is a very sensitive assay that can be used to monitor the effect of a toxin on *C. elegans*. If wild type *C. elegans* feeding is inhibited by furan, this could explain the effects seen in the brood size and growth assays.

N2 wild type worms were incubated for 24 hours in *E. coli* OP50 with increasing concentrations of furan. The OD_{550} of the *E. coli* solution was measured before and after the experiment to monitor changes in feeding.

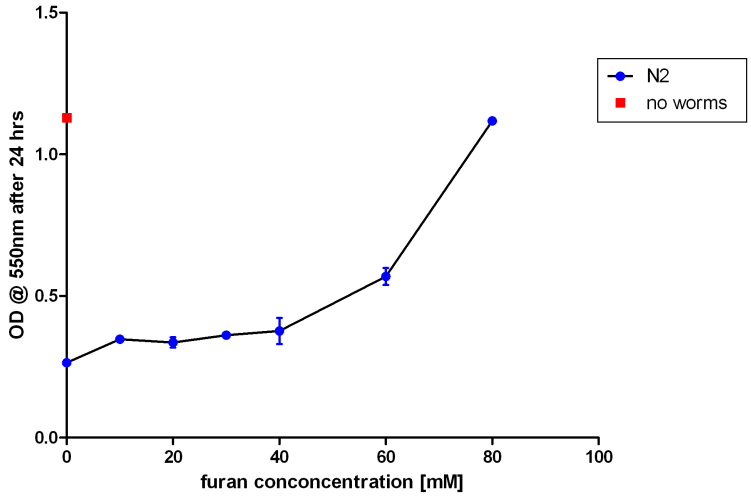

Figure 3.7: Results of feeding inhibition assay with N2. A mixture of worms in all developmental stages from liquid culture were incubated in a bacterial solution containing furan. After 24 hours the OD of the bacteria was measured. The red data point shows the control which did not contain any worms. Furan has a dose-dependent effect on *C. elegans* feeding, which is inhibited at concentrations as low as 10 mM. This graph shows the result of a single experiment with three biological replicates. Three independent experiments gave similar results.

The results of the feeding inhibition assay are shown in Figure 3.7. Furan inhibits feeding of *C. elegans* in a dose-dependent manner. Feeding is only slightly inhibited at concentrations ranging from 10 mM to 40 mM. Dunnett's multiple comparison test showed that the effect is statistically significant ($P<0.05$) for all concentrations tested (Table 3.7, page 66). However, it is also possible that there is some lethality at lower concentrations, e.g. affecting the larval stages. This would influence the result and would indicate feeding inhibition where there is none. It is more likely that there is no inhibition at concentrations ranging from 0 mM to 30 mM. Furan seems to inhibit feeding with a NOAEL of 30-40 mM.

Table 3.7: Dunnett's Multiple Comparison Test (GraphPad Prism 5). N2 worms without furan were used as control group against which all other groups were compared.

N2 - 0 mM furan vs	**Mean Difference**	**Significant? P<0.05?**
10 mM	4.760	Yes
20 mM	4.099	Yes
30 mM	5.593	Yes
40 mM	6.445	Yes
60 mM	17.48	Yes
80 mM	49.04	Yes

At 80 mM furan, the OD_{550} reaches the same value as the control which did not contain any worms. This means that the worms completely stop feeding at this concentration. To calculate the EC_{50}, mean values were normalised and the control value (bacteria without worms) was set as top plateau of the curve. A sigmoidal curve was fitted plotting the log(agonist) vs. response. In this case, the agonist was the furan concentration and the response was the OD_{550}. The EC_{50} is 69.9 mM.

Summary

Furan inhibits *C. elegans* feeding in a dose-dependent manner with an EC_{50} of about 70 mM. The maximum effect is reached at 80 mM where the worms completely stop feeding.

3.5 GFP reporter assay

GFP reporter strains were used to measure expression levels of several genes involved in stress response pathways in *C. elegans*. The worms were incubated in increasing concentrations of furan and the fluorescence was measured. Measurements were taken at early (4 hours), intermediate (8 hours and 16 hours) and late time points (24 hours). Strong upregulation (2-4x) was seen for the genes used in these experiments following incubation with other agents, e.g.:

- *cep*-1, *cyp*-29A2, *cyp*-35A2, and *gst*-1: Zn^{2+}
- *cyp*-34A9, *hsp*-3: dichlorvos
- C11E4.1, *hsp*-16.2, and *sod*-4: Fe^{3+}
- *gst*-4 and *sod*-1: Cr^{3+}
- *hsp*-3, *sod*-3: Cu^{2+}
- *sod*-4: Cd^{2+}

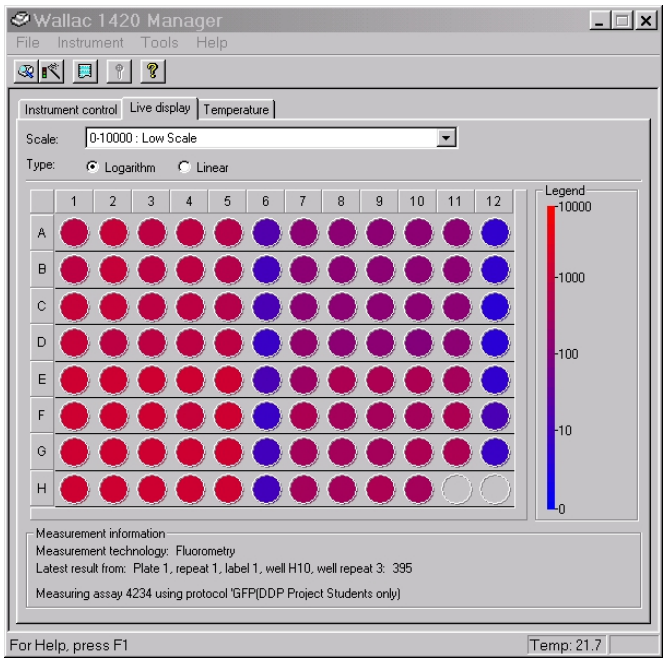

Figure 3.8: Live output of GFP reporter assay from the Wallac 1420 Manager software.

3.5.1 Considerations

As the worms are incubated without a food source, they are under mild stress even without the addition of any toxicant. This explains why for some strains the basal fluorescence changes over time. The flourescene at a given furan concentration must

therefore be compared to the value at 0 mM furan at the same time point rather than the value for the same concentration at an earlier time point.

Usually, the measurements at different time points are done with the same set of worms. Because of the high volatility of furan, a separate plate was used for each measurement. This method possibly increases the variability between results, as the number of worms in each well might vary slightly. However, transferring worms between plates results in the loss of a certain number of worms which remain in the pipette tip and in the wells. If measurements are taken at several time points, the number of worms might decrease significantly with each measurement. Using individual plates for each time point therefore decreases the possibility of artefacts.

3.5.2 Cytochrome P450 induction

cyp-29A2, *cyp*-34A9, and *cyp*-35A2 encode cytochrome P450 enzymes. It is known that furan is metabolised by a cytochrome P450 in humans, therefore one would expect to find one of the *cyp*-genes to be induced by furan. Menzel *et al.* (2001) found that CYP35 genes are strongly inducible by different xenobiotics. It was therefore expected to be induced by furan as well.

Figures 3.9 to 3.11 (pages 69 and 70) show the results for the GFP reporter assays for the three *cyp*-genes. There is no induction at any concentration or time point for these three genes.

3.5.3 Glutathione S-transferase induction

GST-1 and GST-4 are glutathione S-transferases. GSTs catalyse the conjugation of GSH to usually hydrophobic and electrophilic compounds. Furan is thought to be activated to an electrophilic intermediate by a cytochrome P450. The intermediate would then be conjugated to GSH (Carfagna *et al.*, 1993) which would result in an induction of *gst*-genes.

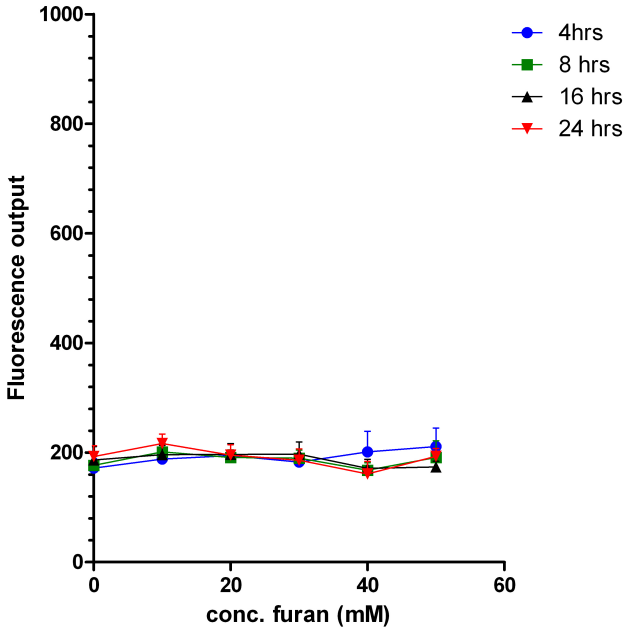

Figure 3.9: Results of GFP reporter assay for gene *cyp*-29A2 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

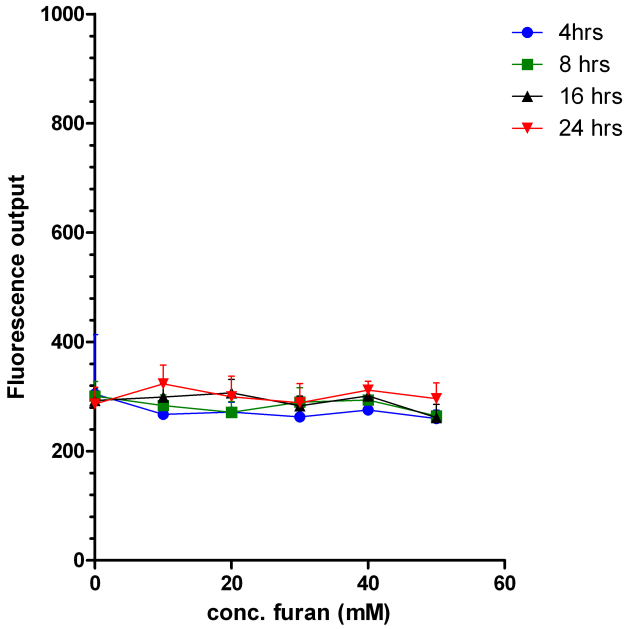

Figure 3.10: Results of GFP reporter assay for gene *cyp*-34A9 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

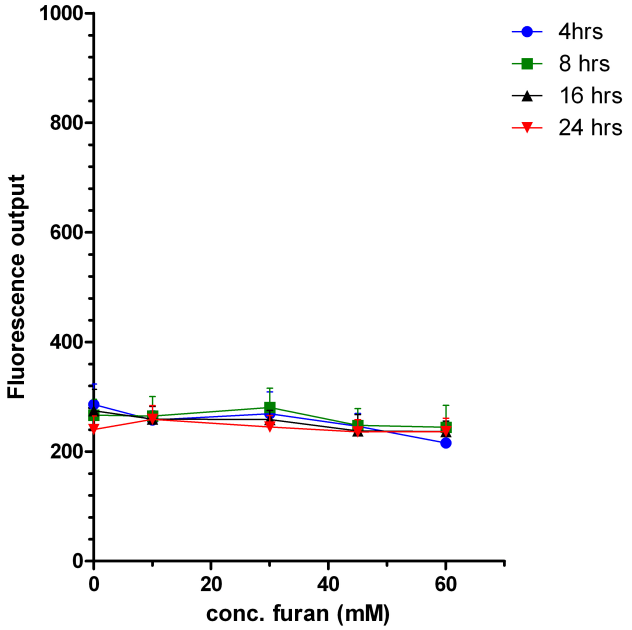

Figure 3.11: Results of GFP reporter assay for gene *cyp-35A2* (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

Figures 3.12 and 3.13 (page 71) show the results of the GFP reporter assay for the genes *gst*-1 and *gst*-4. *gst*-1 basal expression slightly increases over time. However, neither gene is upregulated following exposure of *C. elegans* to furan over 24 hours.

3.5.4 P53 induction

In *C. elegans* *cep*-1 (*C. elegans* p53-like-1) encodes an ortholog of the human tumor suppressor p53. P53 is also called the "guardian of the genome". It promotes DNA damage-induced apoptosis. *cep*-1 seems to be the only p53 in *C. elegans* (Derry *et al.*, 2001; WormBase, 29/04/2010). If *cep*-1 were to be induced by furan, this might be evidence for a genotoxic mode of action.

The result of this assay is shown in Figure 3.14 (page 72). There is no induction at any concentration or time point.

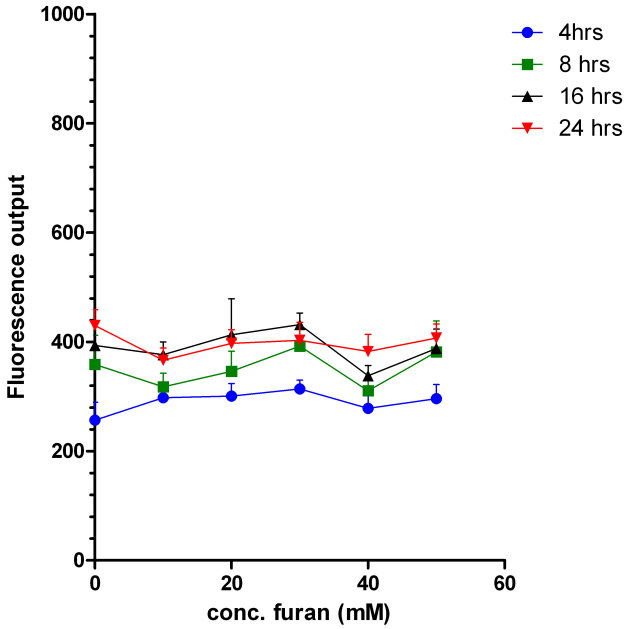

Figure 3.12: Results of GFP reporter assay for gene *gst*-1 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours. The basal expression increases slightly over time, but no effect of furan could be observed.

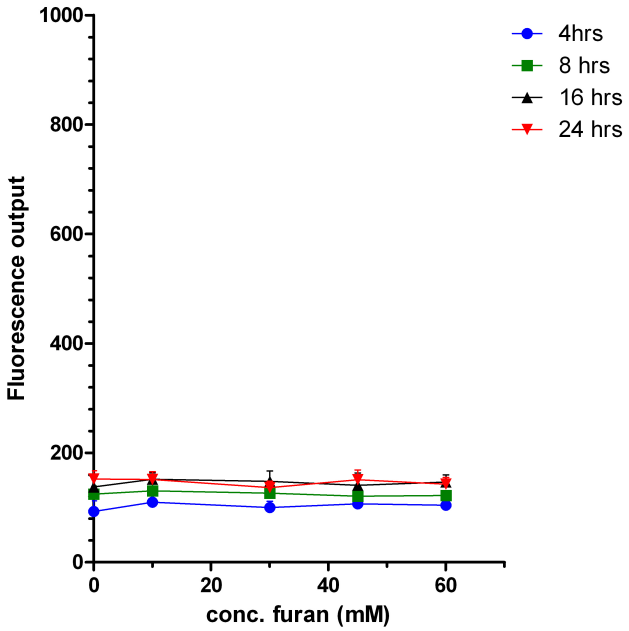

Figure 3.13: Results of GFP reporter assay for gene *gst-4* (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

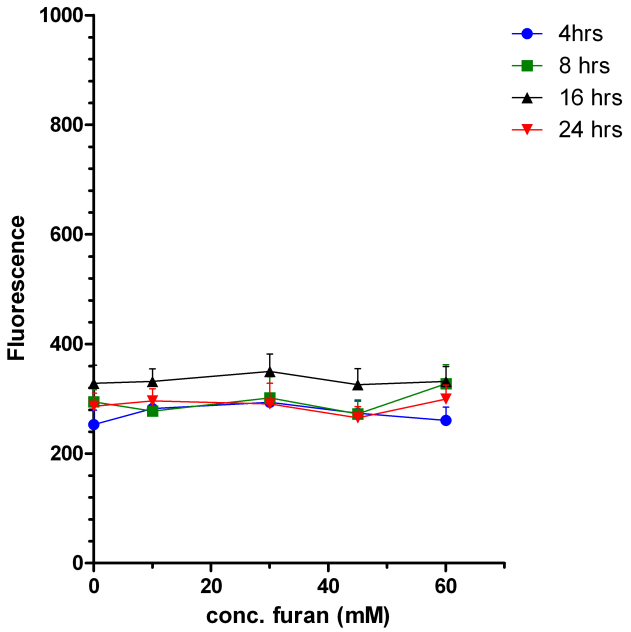

Figure 3.14: Results of GFP reporter assay for gene *cep-1* (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

3.5.5 Induction of heat shock proteins

Heat shock proteins (HSP) are proteins whose expression is increased when cells are exposed to increased temperatures or other stresses. Here, the induction of *hsp*-3 and *hsp*-16.2 was examined.

The results are shown in Figures 3.15 and 3.16 (page 73). *hsp*-3 shows a slight decrease in its basal expression over time. The expression of both genes, however, is not affected by furan.

3.5.6 Superoxide dismutase (SOD) induction

Superoxide is a reactive oxygen species in the cell. Superoxide dismutases (SODs) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Therefore, they are important antioxidants. In *C. elegans sod* genes seem to be involved in a number of processes. The three *sod* genes tested were *sod*-1, *sod*-3, and

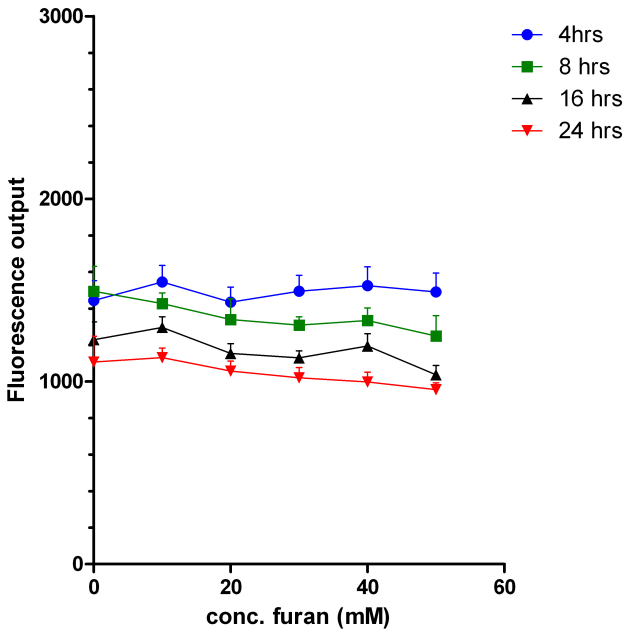

Figure 3.15: Results of GFP reporter assay for gene *hsp*-3 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours. The expression of *hsp*-3 increases slightly over time, but no effect of furan could be observed.

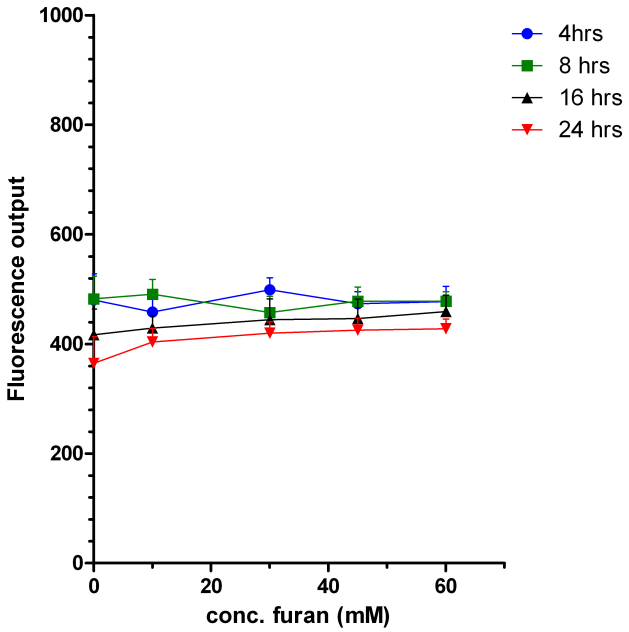

Figure 3.16: Results of GFP reporter assay for gene *hsp*-16.2 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

sod-4. Mn-SOD enzymes such as SOD-3 are mitochondrial, whereas Cu^{2+}/Zn^{2+}-SOD enzymes are cytosolic. In *C. elegans*, SOD-4 appears to be extracellular. (WormBase, 29/04/2010).

None of the three genes tested was induced by furan (Figures 3.17 to 3.19, pages 74 to 75).

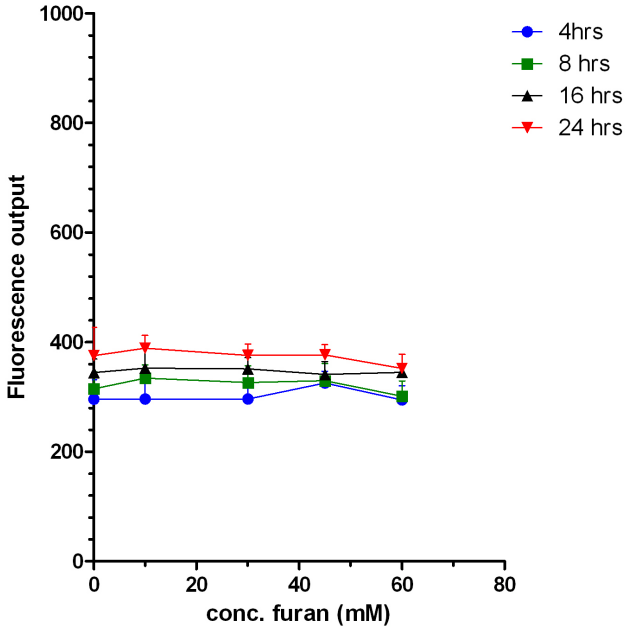

Figure 3.17: Results of GFP reporter assay for gene *sod*-1 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

3.5.7 Glutathione peroxidase induction

Another enzyme frequently induced during oxidative stress is glutathione peroxidase. Of at least twelve such genes in *C. elegans* (so far uncurated), the C11E4.1 gene (designated GPB) is expressed mainly in the intestine and is strongly induced by may stressors (de Pomerai *et al.*, unpublished results).

As can be seen in Figure 3.20 (page 76), GPB was not induced by furan.

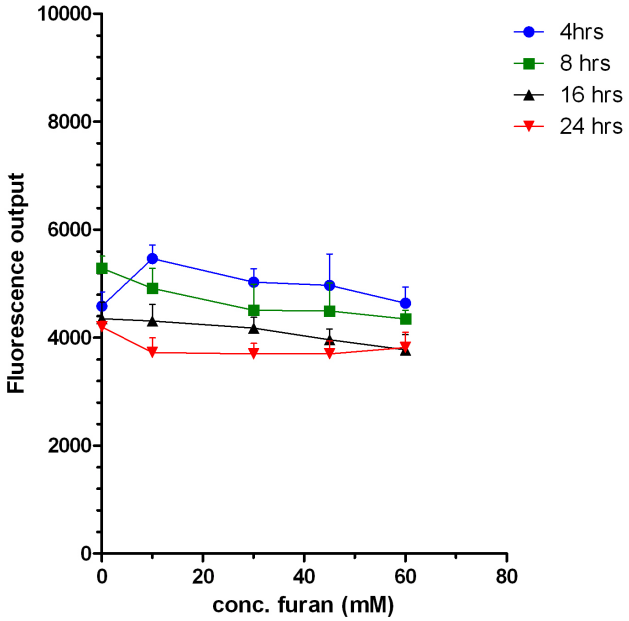

Figure 3.18: Results of GFP reporter assay for gene *sod*-3 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

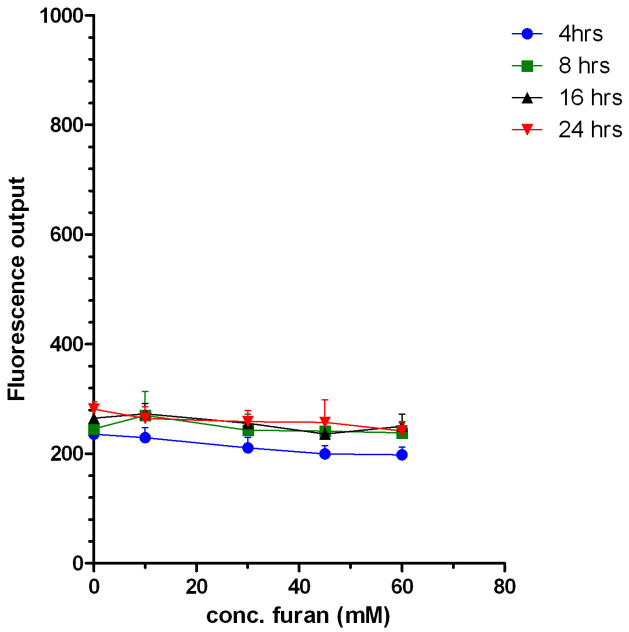

Figure 3.19: Results of GFP reporter assay for gene *sod*-4 (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

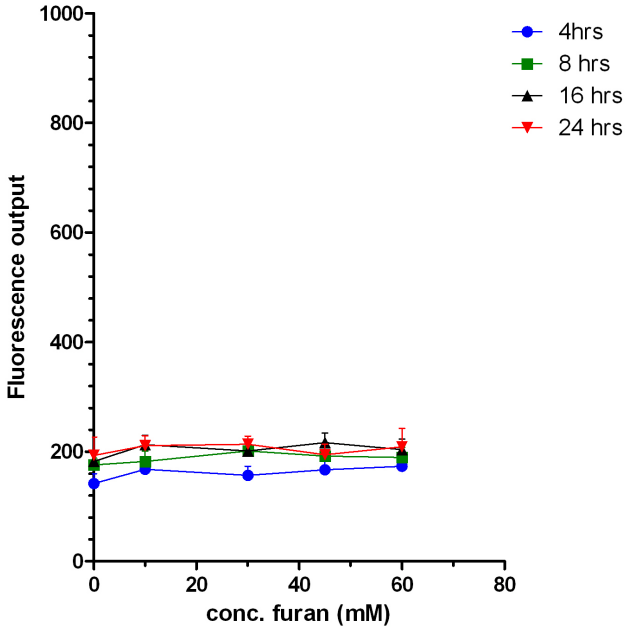

Figure 3.20: Results of GFP reporter assay for gene GPB (mean and SD). The fluorescence was measured after 4, 8, 16, and 24 hours.

3.5.8 Summary

The GFP reporter assay is inexpensive and easy to perform but did not yield a positive result in the case of furan, since none of the genes tested was induced after exposure of *C. elegans* to furan.

4 Discussion

4.1 *C. elegans* as a model organism to study the toxicity of furan

The choice to use *C. elegans* as a model organism to investigate the mechanism of furan toxicity was rational since this model is now widely used in toxicity testing and drug screening (Artal-Sanz *et al.*, 2006; Boyd *et al.*, 2010a,b; Ura *et al.*, 2002). Being simple enough to be studied in great detail, *C. elegans* is nevertheless a multicellular eukaryotic organism. Many of its genes are highly conserved and findings can therefore be extrapolated (with caution) to other organisms, including humans.

4.2 Optimisation of protocols

Optimising protocols is a very time-consuming process which usually takes longer than the actual experiment. It was difficult to establish a working protocol for the brood size assay. Initial results were highly variable and no reduction in brood size could be seen. It was not possible to confirm the results of earlier experiments done by Budd (2008) and Cai (2009). A reason for these differences in the results had to be found and a series of assays was run to find the optimal conditions. The high volatility of furan made it necessary to apply extra precautions. An increase in furan concentration and the use of individual plates for each test concentration finally led to reliable and

reproducible results. Similar problems were encountered with the L1 growth assay. These optimisation experiments took a good deal of the time available for the project.

4.3 Identification of genes conferring resistance to furan

The reported furan resistance of the *C. elegans* mutants generated by Cai (2009) could not be confirmed for any of the four strains (Section 3.2). One possible reason for this might be the use of ethanol as solvent for furan. Ethanol itself has an effect on brood size (Davies *et al.*, 2003) and there might be an additive or synergic effect with furan. Notably, as indicated in Table 2.2 (page 44), the different furan test concentrations using Cai's original procedure each involved a different furan:ethanol ratio, making the results difficult to interpret. It is also possible that the mutation caused a change which made the worms sensitive to freezing. This would also explain the low recovery rates from frozen stocks (Section 3.1).

As the mutant strains were shown not to be any more resistant to furan than N2 wild-type worms, they could not be used to map the mutations and identify any of the genes possibly involved in furan metabolism in *C. elegans*. Therefore, no more work could be done on this part of the project.

As can be seen from the dose response experiment in Figure 3.2 (page 57), there clearly is a toxic effect of furan on *C. elegans*. It was therefore decided to change the initial objectives of the project and to further characterise this effect using different assays to measure stress response in *C. elegans*.

4.4 Subcellular effects of furan on *C. elegans*

GFP reporter assays offer an inexpensive alternative to gene arrays. However, they have some limitations as only a small number of genes can be monitored. The range

of GFP reporter genes currently available largely reflects those known to be strongly inducible under a range of test conditions (e.g. the *cyp* genes identified as xenobiotic inducible by Menzel *et al.* (2001)).

Leiers *et al.* (2003) found that the RNAi silencing of *Ce-GST-p24* was not compensated for by other GSTs. This shows that there is a high degree of specialization of the individual enzymes within the GST superfamily in *C. elegans*. As there are 44 genes coding for GST proteins, it is not surprising that no upregulation could be found in any of the two tested *gst*-genes. The same applies to the three P450 genes used in this experiment. None of them is induced after incubation of the worms in furan. However, there are over 80 P450 genes in *C. elegans* WormBase (29/04/2010). It would therefore be worth looking at more of the P450 genes. It is very likely that one or more of them are involved in furan metabolism in *C. elegans*.

cep-1 was also not induced after exposure of *C. elegans* to furan. As *cep*-1 is the only known p53 gene in *C. elegans*, this might suggest that furan is not genotoxic. Since furan was only used at sublethal concentrations, DNA repair might be induced if furan had any genotoxic effect.

4.5 Visible and behavioural effects of furan on *C. elegans*

It could be shown that furan inhibits wild type *C. elegans* feeding and egg laying. Feeding is reduced at concentrations as low as 10 mM and completely inhibited at 80 mM, with an EC_{50} of about 70 mM. Egg laying is greatly inhibited at 60 mM with an EC_{50} of about 20 mM. Thus, egg laying appears to be more sensitive to furan than feeding. Given that feeding was greatly inhibited at higher furan concentrations, one would expect a similar effect on *C. elegans* growth. Interestingly, this effect was not statistically significant. However, this result has not been confirmed and further experiments are required.

4.6 Comparison to published data

There are several issues that has to be taken into account when comparing the data presented in this work to data that has been published in the literature.

- All furan concentrations given in the literature are instantaneous concentrations which will rapidly reduce in vivo in mice and rats.
- It is not known if furan is effectively absorbed in *C. elegans* so the concentrations used in this work are also only instantaneous.
- The high volatility of furan leads to a certain loss over time.

The precise amount of furan in rodents and worms obviously affects the comparison. Therefore, only a rough estimation can be made. In addition, experiments in rodents can be done over several weeks or months, the NTP study even includes an experiment over two years (National Toxicology Program, 1993). *C. elegans* average life span is about 2-3 weeks, so long term effects can not be monitored.

In all rodent experiments, doses were in the range of 0 to 200 mg/kg bwt per day. For example, in the NTP study (National Toxicology Program, 1993) pathological changes in rats and mice could be seen with 20-160 mg/kg bwt per day for 16 days. Doses as low as 2-8 mg/kg bwt led to carcinomas when given over two years.
Furan has a molecular weight of 68.08 g/mol, so 100 mM furan in my experiments equals 6.8 g furan per kg body weight in rodents. The lethal dose of 90 mM furan found in my experiments roughly equals 6.12 g/kg bwt in rodents. This means that the doses effective in the *C. elegans* experiments were ten to thousand times higher than those used in mice and rats.
It is possible that *C. elegans* are less sensitive to furan, e.g. because the uptake of furan from the environment is low. Another reason for these differences might be that there

are special detoxification mechanisms that do not exist in other animals (Lindblom & Dodd, 2006).

It is important to note that the experiments done in this project did not aim to find an EC or LOAEL that can be applied to rodents or even humans but to identify the pathways of furan metabolism and toxicity. Therefore, direct comparability in this case is not necessary.

5 Future Work

5.1 SNP mapping

The findings of Cai (2009) that 30 mM furan can be used as a selection concentration for furan-resistant mutants could not be confirmed. Unfortunately, the supposedly resistant strains did not show any increase in furan resistance compared to N2 wild type worms. However, the identification of genes conferring resistance to furan could give further insight into the mechanism of furan toxicity. Therefore, new mutant strains should be generated and screened for furan resistance. A selection concentration between 60 mM and 90 mM is recommended for the brood size assay, although a concentration that does not kill adult worms, but reduces brood size in N2 wild-type worms to 0 has not yet been determined.

If mutants could be generated that show furan resistance, these mutations could be mapped to determine the identity of the gene. Single nucleotide polymorphism (SNP) mapping would be the technique of choice to identify the chromosome and the rough position of the gene affected. SNPs are in fact single nucleotide changes, usually "C to T" or "A to G". However changes such as an insertion or deletion of a nucleotide are also common. SNP mapping was first done in 2001 (Wicks *et al.*, 2001). DNA polymorphisms between the N2 wild type and a closely related strain such as the Hawaiian CB4856 are used as genetic markers. They are approximately as dense as

genes and do not have an associated phenotype (Davis *et al.*, 2005). Initially, at least three SNPs from each chromosome would be used, one from each arm and one from the centre. Once the position of the mutation has been mapped to one arm of a chromosome, more markers can be used to narrow down the region. In a final step, whole genome sequencing of both the N2 reference strain and the mutant would make it possible to identify the mutated gene. Identifying this gene could give an insight into furan metabolism in *C. elegans* and possibly in humans.

5.2 DNA microarrays

The results from feeding inhibition and GFP reporter assays show that there is an effect of furan on *C. elegans*. It is therefore worth investigating the effect of furan on more genes known to be involved in stress response in *C. elegans*. Due to time limitations only a small number of genes could be tested in the GFP reporter assay. To enable the screening of more genes at a time, a DNA microarray would be the best choice. Rather than doing a genome wide screen, research should initially focus on P450 genes, glutathione transferase genes and genes involved in DNA repair. This would essentially be a stress response gene chip for *C. elegans* which could also be used to look at other stressors. Looking at these genes in *C. elegans*, the proposed mechanism of furan metabolism could be confirmed. In a second step, a genome wide screen could reveal unknown pathways of furan toxicity.

Bibliography

Anderson, P. 1995. Mutagenesis. *Pages 31–58 of:* Epstein, Henry F., & Shakes, Diane C. (eds), *Caenorhabditis elegans: Modern Biological Analysis of an Organism.* Methods in Cell Biology, vol. 48. Academic Press.

Artal-Sanz, M., de Jong, L., & Tavernarakis, N. 2006. *Caenorhabditis elegans*: A versatile platform for drug discovery. *Biotechnology Journal*, **1**, 1405–1418.

Barrière, A., & Félix, M-A. 2006. Isolation of *C. elegans* and related nematodes. *In:* The *C. elegans* Research Community (ed), *WormBook*. URL `http://www.wormbook.org/chapters/www_nematodeisolation/nematodeisolation.pdf`.

Bishof, L.J., Huffman, D.L., & Aroian, R.V. 2006. *C. elegans - Methods and Applications.* Humana Press. Chap. Assays for Toxicity studies in *C. elegans* With Bt Crystal Proteins, pages 139–154.

Boyd, W.A., McBride, S.J., Rice, J.R., Snyder, D.W., & Freedman, J.H. 2010a. A high-throughput method for assessing chemical toxicity using a *Caenorhabditis elegans* reproduction assay. *Toxicology and Applied Pharmacology*, **245**, 153–159.

Boyd, W.A., Smith, M.V., Kissling, G.E., & Freedman, J.H. 2010b. Medium- and high-throughput screening of neurotoxicants using *C. elegans*. *Neurotoxicology and Teratology*, **32**, 68–73.

Brenner, S. 1974. Genetics of Caenorhabditis elegans. *Genetics*, **77**(1), 71–94.

Budd, C.D. 2008. *Toxicological Studies in C. elegans.* Masters thesis, The University of Nottingham.

Burka, L.T., Washburn, K.D., & Irwin, R.D. 1991. Disposition of [C14]Furan in the male F344 rat. *Journal of Toxicology and Environmental Health*, **34**(2), 245–257.

Byrns, M.C., Vu, C.C., Neidigh, J.W., Abad, J.L., Jones, R.A., & Peterson, L.A. 2006. Detection of DNA adducts derived from the reactive metabolite of furan, cis-2-butene-1,4-dial. *Chemical Research in Toxicology*, **19**(3), 414–420.

Cai, N. 2009. *Identification Of Genes Conferring Resistance To Furan In Caenorhabditis Elegans.* Masters thesis, The University of Nottingham.

Carfagna, M.A., Held, S.D., & Kedderis, G.L. 1993. Furan-Induced Cytolethality in Isolated Rat Hepatocytes: Correspondence with *In Vivo* Dosimetry. *Toxicology and Applied Pharmacology*, **123**, 264–273.

Crews, C., & Castle, L. 2007. A review of the occurence, formation and analysis of furan in heat-processed foods. *Trends in Food Science & Technology*, **18**, 365–372.

Crowder, C.M. 2004. Ethanol targets: a BK channel cocktail in *C. elegans*. *Trends in Neurosciences*, **27**(10).

Davies, A.G., Pierce-Shimomura, J.T., Kim, H., VanHoven, M.K., Thiele, T.R., Bonci, A., Bargmann, C.I., & McIntire, S.L. 2003. A Central Role of the BK Potassium Channel in Behavioral Responses to Ethanol in *C. elegans*. *Cell*, **115**, 655–666.

Davies, A.G., Bettinger, J.C., Thiele, T.R., Judy, M.E., & McIntire, S.L. 2004. Natural Variation in the *npr*-1 Gene Modifies Ethanol Responses of Wild Strains of *C. elegans*. *Neuron*, **42**, 731–743.

Davis, M.W., Hammarlund, M., Harrach, T., Hullett, P., Olsen, S., & Jorgensen, E.M. 2005. Rapid single nucleotide polymorphism mapping in *C. elegans*. *BMC Genomics*, **6**, 11.

de Pomerai, D., Anbalagan, C., Lafayette, I., Rajagopalan, D., Loose, M., Haque, M., & Kind, J. 2010. *Environmental Toxicology III*. Vol. 132. Wessex Institute of Technology, UK. Chap. High-Throughput Analysis of multiple stress pathways using GFP reporters in C. elegans, pages 177–187.

Derry, W.B., Putzke, A.P, & Rothman, J.H. 2001. *Caenorhabditis elegans* p53: Role in Apoptosis, Meiosis, and Stress Resistance. *Science*, **294**, 591–595.

Durling, L.J.K., Svensson, K., & Abramsson-Zetterberg, L. 2007. Furan is not genotoxic in the micronucleus assay in vivo or in vitro. *Toxicology Letters*, **169**(1), 43–50.

European Food Safety Authority. 2004. Report of the Scientific Panel on Contaminants in the Food Chain on provisional findings on furan in food. *The EFSA Journal*, **137**, 1–20. URL `http://www.efsa.europa.eu/en/scdocs/scdoc/137.htm`.

European Food Safety Authority. 2010. Update of results on the monitoring of furan levels in food. *The EFSA Journal*, **8**(7), 1702. URL `http://www.efsa.europa.eu/en/scdocs/doc/1702.pdf`.

Fransson-Steen, R., Goldsworthy, T.L., Kedderis, G.L., & Maronpot, R.R. 1997. Furan-induced liver cell proliferation and apoptosis in female B6C3F1 mice. *Toxicology*, **118**(2-3), 195–204.

Gibson, G. Gordon, & Skett, Paul. 1994. *Introduction to Drug Metabolism*. 2 edn. Blackie Academic & Professional.

Hasegawa, K., Miwa, S., Isomura, K., Tsutsumiuchi, K., Taniguchi, H., & Miwa, J. 2008. Acrylamide-Responsive Genes in the Nematode Caenorhabditis elegans. *Toxicological Sciences*, **101**, 215–225.

Hayes, J.D., Flanagan, J.U., & Jowsey, I.R. 2005. Glutathion transferases. *Annual Review of Pharmacology and Toxicology*, **45**, 51–88.

Hickling, K.C., Hitchcock, J.M., Chipman, J.K., Hammond, T.G., & Evans, J.G. 2010. Induction and Progression of Cholangiofibrosis in Rat Liver Injured by Oral Administration of Furan. *Toxicologic Pathology*, **38**(2), 213–229.

Hodgkin, J. 1999. *C. elegans - A practical approach*. Oxford University Press. Chap. Conventional Genetics, pages 245–270.

Hodgkin, J. 2005. Introduction to genetics and genomics. *In:* The *C. elegans* Research Community (ed), *WormBook*. URL `http://www.wormbook.org/chapters/www_introgeneticsgenomics/IntroGenetGenom.pdf`.

Hodgkin, J., Horvitz, H.R., & Brenner, S. 1979. Nondisjunction Mutants of the Nematode Caenorhabditis elegans. *Genetics*, **91**(1), 67–94.

Hu, P.J. 2007. Dauer. *In:* The *C. elegans* Research Community (ed), *WormBook*. URL `http://www.wormbook.org/chapters/www_dauer/dauer.pdf`.

Hyman, A. 2010. *Max Planck Institute of Molecular Cell Biology and Genetics*, URL `http://hymanlab.mpi-cbg.de/hyman_lab/methods/c_elegans/agarose_pads.shtml`.

International Agency for Research on Cancer (IARC). 1995. Dry Cleaning, Some Chlorinated Solvents and Other Industrial Chemicals. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **63**, 393–407.

International Union of Pure and Applied Chemistry (IUPAC). 1993. *Glossary for Chemists of Terms used in Toxicology*, URL `http://sis.nlm.nih.gov/enviro/glossarymain.html`.

Jestoi, M., Järvinen, T., Järvenpää, E., Tapanainen, H., Virtanen, S., & Peltonen, K. 2009. Furan in the baby-food samples purchased from the Finnish markets – Determination with SPME–GC–MS. *Food Chemistry*, **117**, 522–528.

Johansson, E., Reynolds, S., Anderson, M., & Maronpot, R. 1997. Frequency of Ha-*ras*-1 Gene Muatations Inversely Correlated With Furan Dose in Mouse Liver Tumors. *Molecular Carcinogenesis*, **18**, 199–205.

Karacaoğlu, E., & Selmanoğlu, G. 2010. Effects of heat induced food contaminant furan on reproductive system of male rats from weaning through postpuberty. *Food and Chemical Toxicology*, **48**, 1293–1301.

Kedderis, G.L., Carfagna, M.A., Held, S.D., R.Batra, Murphy, J.E., & Gargas, M.L. 1993. Kinetic Analysis of Furan Biotransformation by F-344 Rats *in Vivo* and *in Vitro*. *Toxicology and Applied Pharmacology*, **123**, 274–282.

Kellert, M., Brink, A., Richter, I., Schlatter, J., & Lutz, W.K. 2008. Tests for genotoxicity and mutagenicity of furan and its metabolite *cis*-2-butene-1,4-dial in L5178Y $tk^{+/-}$ mouse lymphoma cells. *Mutation Research/Genetix Toxicology and Envirmonmental Mutagenesis*, **657**, 127–132.

Kuballa, T. 2007. Furan in Kaffee und anderen Lebensmitteln. *Journal füer Verbraucherschutz und Lebensmittelsicherheit*, **2**, 429–433.

Leiers, B., Kampkötter, A., Grevelding, C.G., Link, C.D., Johnson, T.E., & Henkle-Dührsen, K. 2003. A stress-responsive glutathion S-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*. *Free Radical Biology & Medicine*, **34**(11), 1405–1415.

Leopardi, P., Cordelli, E., Villani, P., Cremona, T.P., Conti, L., Luca, G. De, & Crebelli, R. 2010. Assessment of *in vivo* genotoxicity of the rodent carcinogen furan: evaluation of DNA damage and induction of micronuclei in mouse splenocytes. *Mutagenesis*, **25**(1), 57–62.

Lewis, J.A., & Fleming, J.T. 1995. *Methods in Cell Biology*. Vol. 48 - *Caenorhabditis elegans*: Modern Biological Analysis of an Organism. Academic Press Inc. Chap. Basic Culture Methods, pages 3–29.

Lindblom, T.H., & Dodd, A.K. 2006. Xenobiotic Detoxification in the Nematode *Caenorhabditis elegans*. *Journal of Experimental Zoology*, **305A**, 720–730.

Menzel, R., Bogaert, T., & Achazi, R. 2001. A Systematic Gene Expression Screen of *Caenorhabditis elegans* Cytochrome P450 Genes Reveals CYP35 as Strongly Xenobiotic Inducible. *Archives of Biochemistry and Biophysics*, **395**(2), 158–168.

Menzel, R., Rödel, M., Kulas, J., & Steinberg, C.E.W. 2005. CYP35: Xenobiotically induced gene expression in the nematode *Caenorhabditis elegans*. *Archives of Biochemistry and Biophysics*, **438**, 93–102.

Moser, G.J., Foley, J., Burnett, M., Goldsworthy, T.L., & Maronpot, R. 2009. Furan-induced dose-response relationships for liver cytotoxicity, cell proliferation, and tumorigenicity (furan-induced liver tumorigenicity). *Experimental and Toxicologic Pathology*, **61**(2), 101–111.

Mugford, C.A., Carfagna, M.A., & Kedderis, G.L. 1997. Furan-Mediated Uncoupling of Hepatic Oxidative Phosphorylation in Fischer-344 Rats: An Early Event in Cell Death. *Toxicology and Applied Pharmacology*, **144**, 1–11.

National Toxicology Program. 1993. *Toxicology and Carcinogenesis Studies of Furan (CAS No. 110-00-9) in F344/N Rats and B6C3F1 Mice (Gavage Studies).* Tech. rept. NTP TR #402.

Nobel Foundation. 2002.

Owczarek-Fendor, A., Meulenaer, B. De, Scholl, G., Adams, A., Lancker, F. Van, Yogendrarajah, P., Pauw, G. Eppeand E. De, Scippo, M., & Kimpe, N. De. 2010. Furan formation from vitamin C in a starch-based model system: Influence of the reaction conditions. *Food Chemistry*, **121**, 1163–1170.

Peterson, L.A., Naruko, K.C., & Predecki, D.P. 2000. A Reactive Metabolite of Furan, *cis*-2-Butene-1,4-dial, Is Mutagenic in the Ames Assay. *Chemical Research in Toxicology*, **13**(7), 531–534.

Peterson, L.A., Cummings, M.E., Chan, J.Y., Vu, C.C., & Matter, B.A. 2006. Identification of a *cis*-2-butene-1,4-dial-derived Glutathione Conjugate in the Urine of Furan-Treated Rats. *Chemical Research in Toxicology*, **19**, 1138–1141.

Pohanish, R. P. 2002. *Sittig's Handbook of Toxic and Hazardous Chemicals and Carcinogens (4th Edition).* William Andrew Publishing.

Reichl, F.-X. 2009. *Taschenatlas Toxikologie.* Thieme.

Reynolds, S.H., Stowers, S.J., Patterson, R.M., Maronpot, R.R., Aaronson, S.A., & Anderson, M.W. 1987. Activated Oncogenes in B6C3F1 Mouse Liver Tumors: Implications for Risk Assessment. *Science*, **237**(4820), 1309–1316.

Riddle, D.L. 1988. The Dauer Larva. *Chap. 12 of:* Wood, William B. (ed), *The Nematode Caenorhabditis elegans.* Cold Spring Harbor.

Riddle, D.L., Blumenthal, T., Meyer, B.J., & Priess, J.R. 1997. *C. elegans II.* Cold Spring Harbor. Chap. Introduction to *C. elegans*, pages 1–22.

Santoro, M.G. 2000. Heat Shock Factors and the Control of Stress Response. *Biochemical Pharmacology*, **59**, 55–63.

Spieth, J., & Lawson, D. 2006. Overview of gene structure. *In:* The *C. elegans* Research Community (ed), *WormBook*. URL `http://www.wormbook.org/chapters/www_overviewgenestructure/overviewgenestructure.pdf`.

Stiernagle, T. 1999. Maintenance of C. elegans. *In:* Hope, Ian A. (ed), *C. elegans: A Practical Approach*. A Practical Approach Series. Oxford: Oxford University Press.

Strange, Kevin (ed). 2006. *An Overview of C. elegans Biology*. Methods in Molecular Biology, vol. 351. Totowa: Humana Press Inc.

The University of Scranton. URL `http://academic.scranton.edu/faculty/CANNM1/toxicologymodule.html`.

Timbrell, John. 2000. *Principles of Biochemical Toxicology*. Taylor & Francis Ltd.

Tsien, R.Y. 1998. The Green Fluorescent Protein. *Annual Review of Biochemistry*, **67**, 509–544.

Uetrecht, Jack P, & Trager, William. 2007. *Drug Metabolism - Chemical and Enzymatic Aspects*. informa healthcare.

Ura, K., Kai, T., Sakata, S., Iguchi, T., & Arizono, K. 2002. Aquatic Acute Toxicity Testing Using the Nematode *Caenorhabditis elegans*. *Journal of Health Science*, **48**(6), 583–586.

U.S. Food and Drug Administration. 2004. URL `http://www.fda.gov/Food/FoodSafety/FoodContaminantsAdulteration/ChemicalContaminants/Furan/default.htm`.

Vranová, J., & Ciesarová, Z. 2009. Furan in Food - a Review. *Czech Journal of Food Sciences*, **27**(1), 1–10.

Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H., & Plasterk, R.H.A. 2001. Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. *Nature Genetics*, **28**(2), 160–164.

Wood, W.B. 1988. Introduction to C. elegans Biology. *In:* Wood, W.B. (ed), *The Nematode Caenorhabditis elegans*. New York: Cold Spring Harbor.

WormAtlas. 29/04/2010. URL `http://www.wormatlas.org`.

WormBase. 29/04/2010. *release WS213*, URL `http://www.wormbase.org`.

Wypych, George. 2008. *Knovel Solvents - A Properties Database*. ChemTech Publishing.

Yaylayan, V.A. 2006. Precursors, Formation and Determination of Furan in Food. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, **1**, 5–9.